Liquid Biopsy: A New, Non-Invasive Early Diagnostic and Prognostic Tool in Oncology

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Abstract: Cancer is essentially a genetic disease. Neoplastic progression consists of a subsequent series of genetic alterations that cumulate. In the bloodstream of an affected subject, circulating tumor cells (CTC) and/or small deoxy-ribonucleic acid (DNA) fragments, known as "circulating tumor DNA" (ctDNA), can be found as a consequence of cancer cells' death. Cell-free circulating DNA (cfDNA) consists of small fragments of DNA that are found free in plasma or serum, but also in other body fluids. The term "liquid biopsy" (LB) describes a highly sensitive method (based on a simple sampling of peripheral blood) for the isolation and analysis of cfDNA, which can also contain ctDNA and CTC. Its purpose is to look for cancer cells or portions of their DNA that are circulating in the blood. LB can be used to help find cancer in an early stage. It also has the additional advantage of being largely non-invasive and, therefore, being done more frequently, allowing better tumor and genetic mutations tracking. It can also be used to validate the efficacy of a drug for cancer treatment by taking multiple samples of LB within a few weeks. This technology can also be beneficial for patients after treatment to control relapse. The aim of this work is to give an overview of this technique, from its history, state-of-the-art, and methodology of execution, to its applications in oncology and with a hint to the gynecological field.

Keywords: Liquid biopsy, cancer, gynecological oncology, cell-free DNA, circulating tumor cells, precision oncology, early diagnosis.

INTRODUCTION

Cancer

Cancer is a pathology due to genetic alterations in which the cellular component does not respond correctly to factors that normally control its proliferation. Cancer cells are characterized by the presence of mutations capable of predisposing and/or guiding their uncontrolled growth. For this reason, precision medicine (also known as "personalized medicine"), aimed indeed at finding the primary alteration of disease, uses a therapeutic approach with the goal of altering cancer cells' deoxy-ribonucleic acid (DNA), trying to contrast them and/or cure them. In this context, sequencing methods of DNA or ribonucleic acid (RNA) of tumor origin represent a very promising tool not only to deepen mechanisms underlying the pathogenesis of various cancer forms, but also for early diagnosis and monitoring of disease, using tumor genetic material as biomarker of cancer presence and extension. Most cancers are related in literature to the presence of somatic gene mutations [1,2]. These somatic mutations develop spontaneously potentially in any type of cell. These DNA alterations can result from random errors during replication, or from exposure to accidental, professional, or lifestyle-dependent mutagenic environmental factors. Unlike heritable pathogenetic variants (germline mutations) that are present in the germ line, somatic mutations are not transmissible to progeny. It has now been widely demonstrated that early diagnosis (secondary prevention) of cancer correlates, in most cases, with better prognosis. Cancer diagnosis requires a series of analyses, among which tissue biopsy is the gold standard. Therapeutic strategy against cancer, and control of therapeutic response, are conventionally decided through an analytical approach that associates diagnostic imaging with pathological characterization of tissue biopsy. In the past, biopsy for taking a tissue sample, on which to study cancer molecular responses, was possible only by invasive methods, such as needle aspiration or intraoperative sampling. Invasive procedures often represented a source of comorbidity for patients. The cost of performing sequential biopsies, for the dynamic evaluation of residual disease and changes in cancer genomic composition during and after therapy [3,4], was often prohibitive. A limitation of tissue biopsy was also represented by the fact that this option could only be considered when primary tumor sites, or metastases, were known and these were accessible.

Liquid Biopsy

It is now possible to find both cells and tumor DNA in circulating blood through equipment, technologies,

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and scientific know-how. A new method of analyzing somatic mutations, called generically "liquid biopsy" (LB), also known as "fluid biopsy" or "fluid phase biopsy", studies cell-free DNA (cfDNA), more specifically circulating tumor DNA (ctDNA), contained in a peripheral blood sample. Sample study supports, and replaces, in the sequential monitoring phase of cancer genomic heterogeneity, traditional direct study of tumor tissue sample taken by invasive methods, and today represents a technology considered highly sensitive and specific towards somatic mutations in various types of cancer [5,6]. In fact, ctDNA carries within it the pathological characteristics of the original cancer such as genetic mutations. Hence, ctDNA isolated from plasma with non-invasive techniques could significantly improve current cancer diagnosis system or even be used to identify early stage cancers. LB is useful to broaden knowledge, compared to traditional methods, of genomic heterogeneity of cancer tissue. DNA analysis performed on a blood sample is useful for therapeutic choice and for monitoring cancer during treatment, progression, or remission phase [7,8].

Oncosomes

All cells, including cancer cells, spread DNA in the circulatory system. Currently, blood-based LB measurements focus on the evaluation of certain types of biomarkers, including ctDNA, circulating tumor cells (CTC), extracellular vesicles (oncosomes), and tumor-educated platelets (TEP). In patients with cancer, but also with other pathological conditions such as renal failure and myocardial infarction, higher levels of cfDNA than in healthy patients are often found [9]. Mechanisms by which cancer cells release DNA into blood have not yet been fully elucidated. ctDNA can enter the bloodstream through direct secretion of viable cancer cells, such as cfDNA, as vesicles called "exosomes", or following apoptosis or necrosis processes of cancer cells [10-13]. In the bloodstream, DNA persists only for a short period of time (t½ of about 2 hours) [14]. Most cfDNA and ctDNA fragments are between 180 and 200 base pairs (bp) in size [15-18]. In case of solid tumors, ctDNA can be distinguished from the whole cfDNA by the presence of somatic mutations, even if it represents only a small fraction of it (often <1%) [19]. What described in general is not valid also for hematological malignancies (for example, leukemias) where blood contains higher percentages of ctDNA. ctDNA fraction, compared to total circulating cfDNA, increases proportionally to disease progression and detectable levels in the samples can vary considerably among patients [20]. Extracellular vesicles are membrane-limited structures derived from the cell membrane. They contain specific molecules including proteins, DNA, messenger RNA (mRNA), microRNA (miRNA), and non-coding RNA (ncRNA) that are secreted in the extracellular space. Extracellular vesicles can be classified according to their size and/or origin into micro-vesicles (150-1000 nm) and exosomes (30-200 nm). Micro-vesicles are released by budding from the plasmatic membrane, whereas exosomes are released via the endocytic pathway by fusion of multivesicular bodies with the plasmatic membrane. This endosomal origin means that exosomes contain an abundance of cell-specific biomolecules which may act as a "fingerprint" of the cell of origin. However, their intrinsically small size leads to great challenges to isolate them from complex body fluids with high productivity and high purity. When detergents and phospholipid membranes are dispersed in aqueous solutions, they tend to self-assemble into vesicles of various shapes and sizes by virtue of their hydrophobic and hydrophilic segments. A clearer understanding of such vesiculation processes holds promise for better elucidation of human physiology and pathology, and paves the way to improved diagnostics, drug development, and drug delivery. The energetics and thermodynamics of vesiculation stand on non-linear elasticity and large deformation that may arise during the vesiculation process. The effects of membrane size, spontaneous curvature, and membrane stiffness on vesiculation and vesicle size distribution and the critical size for vesicle formation are found to compare favorably with available experimental evidence. The critical membrane size for spontaneous vesiculation is correlated with membrane thickness, and the combined effects of membrane thickness and physical properties influence the size, shape, and distribution of vesicles. These findings shed light on the formation of physiological extracellular vesicles, such as exosomes. The findings also suggest pathways for manipulating the size, shape, distribution, and physical properties of synthetic vesicles, with potential applications in vesicle physiology, the pathobiology of cancer and other diseases, diagnostics using *in vivo* LB, and drug delivery methods [21].

Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) endows epithelial cells with enhanced motility and invasiveness, allowing them to participate in many physiological and pathological processes. EMT contributes to the generation of CTC in epithelial cancers because it

increases cancer cell invasiveness, promotes cancer cell intravasation, and ensures cancer cell survival in the peripheral system. Although EMT contribution to cancer cell invasiveness has been confirmed, the role EMT transition plays in metastasis remains debated. As a favorable material for LB, CTC have been shown to have promising values in the clinical management of cancer. Furthermore, an increasing number of studies have begun to explore the value of CTC-related biomarkers, and some studies have found that the expression of EMT and stemness markers in CTC, in addition to CTC detection, can provide more information on cancer diagnosis, treatment, prognosis, and research [22]. CTC are very attractive surrogate markers for systemic cancer. Currently, major efforts are being made to use these rare cells in the sense of LB to gain molecular information for rational therapeutic decision-making. Advances in molecular analyses of CTC down to the single-cell level have been significant in recent years and some applications are ready to be used in clinical studies. A major challenge for translating such molecular CTC-based assays into the clinical setting is the extremely low frequency of CTC and the associated problems of their reliable detection and isolation. A potential solution to overcome the low CTC frequency is the recently introduced diagnostic leukapheresis that permits screening of liters of blood [23].

Comparison between Liquid and Traditional Biopsy

LB is becoming a very popular sampling procedure, replacing invasive methods for diagnostic protocols. Advantages of this method include the possibility to isolate cell-free nucleic acids (cfNA) for diagnostic or screening purposes. A comprehensive review combining all current and perspective applications of cfNA is missing. Published articles are dealing with one type of cfNA, or discuss them from the perspective of a single disorder. Beyond doubt, cfNA will have a tremendous impact in future screening, diagnosis, prognosis, follow-up, and treatment of cardiovascular diseases (CVD), cancer, diabetes, and other diseases [24,25]. One of the advantages of LB, which we should call much more appropriately “molecular profiles of circulating nucleic acids” (in blood and other biological fluids) is precisely the allowing of early diagnosis by detecting cells and molecules of cancer origin in the bloodstream, even before pathology’s frank manifestation, or relapses resumption, or metastasis appearance. Obviously, in order to be effective, the used technologies must be highly sensitive from an analytical point of view. Genetic material (DNA and RNA), thanks to its nature and to the possibility of amplifying it with increasingly sophisticated techniques, is therefore an ideal candidate for this purpose. Finally, unlike traditional biopsy sampling, this investigation is characterized by low invasiveness for recovering the sample; think, for example, of a simple collection of venous blood for the research of ctDNA present in the bloodstream instead of tissue biopsy. This characteristic makes it a very useful tool in monitoring cancer reduction/progression and in addressing therapeutic choices. Thanks to this, LB has been considered the “Holy Grail” in the goal of effective cancer management, the most recent and extraordinary innovation of molecular biology applied to oncology, with blood tests that offer a minimally invasive, safe, and sensitive alternative or a complementary approach for tissue biopsies. Despite the potential of individual techniques, each has its advantages and disadvantages. LB offers an alternative tool to traditional biopsy with histological evaluation since, although it is still recommended for the initial assessment of the disease to support both

Table 1: Advantages of Liquid Biopsy vs Tissue Biopsy

Tissue biopsy	Liquid biopsy
Invasive and expensive	Non-invasive with good cost/benefit ratio
Strongly affected by cancer site: specific when cancer site is located	Independent of cancer site
Evaluation limited by heterogeneity	It also detects heterogeneity
Biopsy collection sometimes difficult	Blood sampling simple and always accessible
Not utilizable if primary tumor has been removed	Accessible in the absence of perceptible primary tumor or metastasis
Not applicable as follow-up after surgery	Applicable as follow-up after surgery for dynamic monitoring of any residual disease
Biopsy sample repetition is not well tolerated	Blood sampling repetition is tolerated
	Dynamic monitoring of response to treatment
	Dynamic monitoring of resistance development
	Useful tool for early diagnosis of disease recovery

techniques (for dynamic monitoring and control of the progress of therapy and tumor response), the use of LB is confirmed to be advantageous compared to traditional techniques (Table **1**).

For the definition of the optimal therapy to be adopted for the patient, the characterization of the primary tumor mutation profile is normally carried out through traditional biopsy, however, the quantity and quality of the material does not always allow correct genotyping, also in consideration of its heterogeneity. ctDNA, on the other hand, represents cancer in all its heterogeneity, is easily obtainable from a blood sample, and is repeatable, allowing monitoring over time of the disease status. ctDNA can also provide a picture of cancer evolution to identify the genetic changes that may occur, responsible for relapses, progression, and development of drug resistance. Furthermore, ctDNA is able to offer not only information related to the genetic profile of the primary lesion, as occurs with traditional biopsy (tissue DNA), but also of metastases. In light of these advantages, considering the correlation between mutations in tumor tissue and ctDNA presence, as reported by multiple studies, LB can represent, in the clinical setting, an excellent diagnostic and prognostic tool for the study of solid tumors [26]. In the literature, it is confirmed that cancer is genomically heterogeneous [27-32] and traditional tissue biopsy can only provide a “snapshot” of a portion of it. Blood sampling is simple and accessible, and ctDNA contained therein derives from all cancer sites, providing the necessary potential to monitor the disease and its progression in real time. DNA is also detectable in other body fluids such as urine, cerebrospinal fluid, and saliva, and ctDNA analysis may in the future be performed using these sources [33].

LB can be performed either from a blood sample or from a urine sample in dedicated test tubes; the combined approach to the test (urine + blood) has a markedly superior performance compared to the individually performed tests. LB from a urine and/or blood sample is a useful test to diagnose, quantify, and monitor driver mutations of oncological pathologies. Many oncological pathologies have known genetic bases that guide their development and progression (driver mutations). Therefore, monitoring mutations allows to customize therapy and let the oncologist know, in advance of computed tomography (CT) or magnetic resonance imaging (MRI), the pathologies’ evolution, the effectiveness of the treatment in progress, and the possibility of making pharmacological changes. The test is aimed at anyone with a cancer (late stage or metastatic) who has shown on histological examination the presence of a driver mutation or in the monitoring of mutations onset in patients with cancer without driver mutations. The test, in addition to having European Conformity (CE)-*in vitro* diagnostic (IVD) certifications for its use for diagnostic purposes, is a clinically validated test: that is, the usefulness and effectiveness of the test has been compared to real results, in terms of survival, of LB advantages. LB focuses on mutations that, if present, determine personalized therapeutic protocols. The used exclusive technology has an analytical sensitivity of 0.006%; in this way, therapeutic decisions are anticipated. The test quantifies in absolute terms ctDNA concentration. This information is extremely useful for the clinician in order to modulate therapy and monitor it. The test can be run and rerun simply without having to resort to tissue biopsy. These potentialities provide valuable and not otherwise obtainable diagnostic opportunities. Early diagnosis, pharmacological monitoring, monitoring of the adequacy of the used therapy, and early identification of drug resistance are all information obtainable with accuracy and simplicity. Thanks to LB, it is possible to have, at any time and in a non-invasive way, cancer genetic typing and its charge, contributing substantially to the choice of the therapeutic plan. LB is currently used in excellence oncology centers, to verify the appropriateness of treatments in case of lung, colon, pancreas, gastrointestinal stromal tumors (GIST), and leukemias but, in the near future, its use will also be extended to other forms of cancer such as breast, ovarian, and brain tumors.

The sampling can be performed before, after, and during drug therapy. Through this technique, it is therefore possible to identify targeted and personalized therapies and to detect earlier the onset of resistance-related mutations by allowing pharmacological switch. In case of impossibility to perform biopsy samples, LB biopsy can be used for cancer genetic typing. Investigated genes are all those that have clinical utility and a rationale of literature or ongoing clinical trials: Kirsten rat sarcoma (KRAS), murine sarcoma viral oncogene homolog B (BRAF), neuroblastoma RAS (NRAS), anaplastic lymphoma kinase (ALK), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor alpha (PDGFRA), tyrosine-protein kinase (KIT), erythroblastic oncogene B 2 (ERBB2), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), ERBB3, RAF1, and estrogen receptor 1 (ESR1). The used technique

allows to obtain the greatest number of "clinical" information in a single sampling. Compared to other technologies, LB investigates the greatest number of so-called "actionable" variants or variants for which drugs or other therapeutic behaviors are available: KRAS, NRAS, BRAF, and ALK. ALK is a receptor tyrosine kinase (RTK) that is altered in a number of cancers. Activating mutations in the full-length ALK have been identified in neuroblastomas whereas structural alterations consisting of the ALK-TK domain fused to a separate gene product have been identified in colorectal, ovarian, and non-small cell lung cancer (NSCLC), where ALK fusions comprise approximately 3-5% of lung cancers. The majority of ALK fusion variants have been identified to contain portions of echinoderm microtubule-associated protein-like 4 (EML4) gene fused to ALK-TK. Although ALK fusion products have been shown to confer sensitivity to ALK-TK inhibitors (TKI), emergence of mutations in the ALK-TK domain have been reported to confer resistance to treatment.

LB applies the latest discoveries in the field of genetics for personalized preventive medicine: deepening the knowledge of one's genetic heritage is fundamental both for prevention of pathologies transmitted in a hereditary way and for the setting of lifestyle, correct food, or behavioral rules. LB uses the latest discoveries in the field of genetics and in the following sectors of use:

1. nutrigenetics: analysis of metabolism to advise patient on the optimal nutritional choice: slim panels, intolerances, and well-being;
2. CVD predisposition;
3. cancer prevention: identification of the presence of genes predisposing to various types of tumors and planning of an effective prevention and diagnosis program: colorectal, breast, ovary, and prostate cancers;
4. identification of drugs compatible with one's DNA;
5. pre-conceptional genetics;
6. non-invasive prenatal diagnosis (NIPD); and
7. sport performances: analysis of the athlete's genes in order to improve performance and reduce the risk of injuries, identifying a personalized training in addition to a more suitable diet.

In summary, LB consists of a simple blood sample through which to search and analyze traces of genetic material, CTC, or fragments of nucleic acid (DNA or RNA), released into the circulation by cancer itself. Compared to traditional biopsy, from which effectiveness in the search for cancer tissues is mutual, the technique is less invasive in the search for solid tumors, since there is no removal of cancer tissues or aspiration of cells through needle aspiration and painful operations, and traditional biopsy is not always able to provide tissues that fully reflect disease severity. This examination provides indications on cancer with the same effectiveness as classic biopsy, indeed, with optimal aspects that would improve investigation effectiveness. In fact, first of all, since it is not an invasive technique, it can be performed more frequently, so as to keep disease evolution under control. Furthermore, it would be more reliable than classic biopsy, as the latter provides limited data at the point of sampling and at the precise moment in which it is performed; conversely, the new technique also provides data concerning the processes of all those cancer or necrotic cells that leave traces in the bloodstream, thus providing information on cancer stage and its prognosis. LB allows to anticipate cancer diagnosis, predict a recurrence, and correct an ongoing therapy; for example, by comparing LB result with the finding of a spiral CT scan in lung cancer, it was possible to diagnose it two years earlier. LB is able to identify the patients most likely to respond to the action of a drug that targets particular cancer genetic variations. The test, in addition to this, also allows to understand if therapy is working or not and to monitor the development of resistance to certain drugs by cancer. Although minimal invasiveness and temporal resolution are interesting features of LB, the limited amount of ctDNA in plasma poses some problems. Recent developments in digital polymerase chain reaction (PCR) and next generation sequencing (NGS) technology have improved LB accuracy. In particular, molecular barcode technology in NGS-based methods, i.e. molecular barcodes coding in ctDNA before amplification, reduces technical errors by validating consensus on sequences from a single molecule, leading to a slight improvement of accuracy and reduction of detection limits. However, substitutions caused by DNA damage and somatic mutations in normal cells are still obstacles to sensitivity in the detection of mutations on ctDNA. Since nowadays there have been only a few clinical applications, a deeper understanding of cfDNA biology and more advanced analytical technology are needed for LB practical application.

HISTORY AND STATE-OF-THE-ART OF LIQUID BIOPSY IN ONCOLOGY

Definition

Although the term "LB" is rather new, the real essence of its meaning has already been part of the world of Laboratory Medicine for many years. In fact, blood count, which consists of the research and study of blood cells present in the bloodstream, represents one of the milestones of hematology and onco-hematology. This allows to take a "photograph" of the health state of blood cells (for example in anemias) and to detect any fundamental alteration also in the field of onco-hematology (for example in leukemias). In more recent years, the possibility of studying genetic material (nucleic acids, DNA and RNA) in qualitative and quantitative terms, has made it possible to significantly improve the diagnostic possibilities at the blood level in terms above all of analytical sensitivity. Nowadays, the study of minimum amounts of nucleic acids allows to obtain multiple useful information for the management of patients suffering from leukemia or lymphoma, changing, in fact, the onco-hematological approach in many respects and so much to also reconsider the classification of onco-hematological diseases on the basis of the qualitative-quantitative content of DNA and RNA.

The possibility of cancer dissemination through the blood system has been known for years. Already in 1997, even before the introduction of the captivating term "LB", the presence of RNA sequences of cancer origin in the bloodstream of patients suffering from solid tumors had been demonstrated. The term "LB" was introduced a few years later, to describe an examination performed on a blood sample looking for cells, subcellular elements, or genetic material released into the circulation following necrosis of cancer cells belonging to solid tumors localized in various organs and tissues. Given the enormous potential of these investigations, it is no wonder that the tests performed on genetic material, under the exclusive competence of "clinical laboratory technicians", were immediately integrated into the panel of tests carried out by anatomic-pathologists, at the forefront in the fight against solid tumors. We think that, regardless of what "professional label" is, it is the skill and knowledge of the operator/physician to ensure that the benefits of the so-called "LB" are fully used. The high levels of analytical sensitivity required for the search for elements of cancer origin (and not only) within body fluids (mostly blood, but also saliva, breast milk, and others), have led to the activation of many clinical studies focused on the use of increasingly refined technologies (such as NGS and other high productivity "omic" analyses) [34].

Salivary Biomarkers

The analysis of saliva as a diagnostic approach for systemic diseases was proposed just two decades ago, but recently great interest in the field has emerged because of its revolutionary potential as LB and its usefulness as a non-invasive sampling method. Multiple molecules isolated in saliva have been proposed as cancer biomarkers for diagnosis, prognosis, drug monitoring, and pharmacogenetic studies [35]. Salivary biomarkers for disease detection and diagnostic and prognostic assessments have become increasingly well established in recent years. The current leading technology that has been used to characterize salivary ncRNA from the extracellular RNA (exRNA) fraction is RNA sequencing. Therefore, there are two main sections regarding the type of the constructed library (small and long ncRNA libraries), from saliva collection and RNA extraction and quantification to cDNA library generation and corresponding quality controls. Using these invaluable technical tools, thousands of ncRNA species in saliva can be identified. These methods indicate that salivary exRNA provides an efficient medium for biomarker discovery of oral and systemic diseases [36]. Circulating miRNA have emerged as excellent candidates for cancer biomarkers. Several recent studies have highlighted the potential use of saliva for the identification of miRNA as novel biomarkers, which represents a great opportunity to improve diagnosis and to monitor general health and disease. Normalizing the levels of specific miRNA present in saliva is a critical step in their analysis, and the challenge to validate salivary miRNA is a reality to manage cancer patients [37].

Circulating Tumor DNA

Blood is an important resource that allows to obtain information on molecular changes for prognostic and predictive purposes in response to treatments. Over the years, studies have been conducted to isolate CTC or cfDNA in order to analyze the amount of ctDNA and perform molecular analyses through a simple blood sampling. In fact, it is believed that ctDNA derives largely from necrotic or apoptotic cancer cells. In recent years, the need for the correct identification of all patients with EGFR mutation, even when biological material, intended for mutational analysis, was characterized by low percentage of neoplastic cells,

Table 2: Frequency of Somatic Mutations by Gene and Tumor Type*

Tumor type	Gene	Frequency of somatic mutations
Breast	PIK3CA	26%
	TP53	23%
Colorectal	BRAF	11%
	KRAS	36%
	NRAS	5%
	PIK3CA	14%
	TP53	45%
Endometrium	KRAS	14%
	PIK3CA	21%
	TP53	17%
Ovary	BRAF	7%
	FOXL2	18%
	KRAS	12%
	PIK3CA	9%
	TP53	46%
Granulosa cell	FOXL2	97%
Head & neck	EGFR	2%
	PIK3CA	7%
	TP53	38%
Kidney	TP53	5%
Lung	BRAF	1-4%
	EGFR	1% in non-small cell lung cancer (NSCLC)
	KRAS	29%
	PIK3CA	17%
	TP53	4%
		34%
Melanoma	BRAF	45%
	NRAS	18%
	TP53	12%
Pancreas	BRAF	2%
	KRAS	57%
	PIK3CA	2%
	TP53	36%
Prostate	BRAF	1%
	EGFR	3%
	KRAS	4%
	PIK3CA	2%
	TP53	14%
Testicle	BRAF	2%
	FOXL2	2%
	KRAS	4%
	NRAS	2%
	TP53	5%
Thyroid	BRAF	41%
	GNAS	3%
	KRAS	2%
	NRAS	7%
	PIK3CA	3%
	TP53	6%

*Catalogue of Somatic Mutations in Cancer (COSMIC) database. Available from: http://cancer.sanger.ac.uk/cosmic. [Last accessed on 7 Apr 2020].

has determined the identification of alternative methods towards the use of analysis procedures. In fact, it is not always possible to perform biopsy or cytological sampling and collect the appropriate material for molecular analysis. Thousands of somatic mutations, which can influence cancer onset, development of metastases, or response/resistance to treatment, have been catalogued on international databases (Table **2**). These genes include BRAF, the RAS gene family, EGFR, PIK3CA, fork-head box protein L2 (FOXL2), and tumor protein 53 (TP53). Somatic mutations of BRAF gene are commonly associated with melanoma, non-Hodgkin's lymphoma, colorectal cancer, papillary thyroid carcinoma, NSCLC, and lung adenocarcinoma, while somatic mutations in EGFR gene have been observed in lung cancer [38]. Mutations of PIK3CA gene are more frequent in breast and colorectal cancer [39]. Mutations of FOXL2 gene have been observed in granulosa tumors and mutations of TP53 gene are detected in almost all types of cancer. The identification and understanding of these DNA alterations can be crucial in diagnosing cancer and planning its treatment, from monitoring response to therapy to early identification of recovery. Furthermore, during cancer progression, the tissue continues to develop further new mutations and the latter can influence the response to therapeutic agents by triggering resistance mechanisms. Observing hotspot mutations can help the oncologist recommend a personalized treatment plan during which to monitor disease response and potential drug resistance development. For example, in patients with metastatic melanoma, if a specific somatic mutation of BRAF gene (V600E) is present, treatment with BRAF inhibitors such as dabrafenib, trametinib, and vemurafenib is often indicated, individually or in combination [40]. In addition, EGFR inhibitors cetuximab and panitumumab have been shown to be more useful in patients with lung cancer in whom there are no mutations of KRAS gene (wild type) and in which EGFR is expressed. Several major clinical studies have shown that EGFR-TKI, afatinib and erlotinib, are only useful for the treatment of patients whose cancers have mutations that activate in the TK domain of EGFR gene [41].

Recent studies have shown the possibility of using LB to monitor tumor dynamics. Several projects have shown that results relating to somatic mutations, identified through LB, agree with those obtained by traditional techniques on the same patients [42,43]. ctDNA data correlated with clinical and radiological results also seem useful for predictive purposes for patient survival [44]. It has also been shown that the reappearance, or increasing levels, of ctDNA can be observed months before disease resumption. Therefore, serial reassessment of ctDNA has been shown to be useful as a monitoring of disease progression, and the appearance of new somatic mutations in the treatment phase may be associated with the development of drug resistance in different types of cancer [45]. The test was designed for patients who have already been diagnosed with cancer, in order to:

1. provide tumor profiling for the correct application of precision medicine: the test can provide the oncologist with information useful for creating a personalized treatment plan;

2. monitor recommended therapy effectiveness by detecting the presence of mutations before and during treatment;

3. monitor residual disease and/or the presence of recurrence in patients with known mutations in the primary tumor early, especially in cases where patients have undergone a tumor resection and/or are in a remission period;

4. assist the oncologist in choosing new treatment options when the patient develops resistance to ongoing therapy;

5. provide an alternative biopsy method when tissue is difficult or impossible to reach, or when unknown sites may be present in addition to the primary site of metastatic disease, or when the amount of tissue obtained by traditional biopsy sampling is insufficient for molecular genotyping;

6. provide prognostic information; and

7. support the insertion of a patient in a clinical trial: this is an additional function of the test, to correctly profile the patient and his disease in order to identify a possible ongoing clinical study for which this patient falls within the eligibility criteria.

The main field of application of LB to date is represented by the identification of predictive factors in patients with advanced disease. However, it is possible that in the near future these indications will be extended, with particular regard to the identification of minimum residual disease in patients undergoing radical surgery. For this latter application, however, further studies and technical and standardization improvements are needed, before it can be directly

Table 3: Investigated Genes, Main Types of Associated Cancer, and Target Therapies

Gene	Sensibility	Resistance	Role	Tissue
AKT1	AKT inhibitors Triciribine MK-2206 Ritonavir		Oncogene	Breast, Lung, Colorectal*
ALK	Alectinib Crizotinib Ceritinib LDK378 X-396	Alectinib Crizotinib Ceritinib		Lung, Neuroblastoma, Rhabdomyosarcoma
APC	LGK974 WNT pathway inhibitors			Colorectal, Breast
AR				Prostate
ATM				Colorectal, Breast
BRAF	Dabrafenib Vemurafenib Trametinib MEK & RAS inhibitors	Dabrafenib Vemurafenib	Oncogene	Melanoma*, Colorectal*, Lung, Ovary, Stomach, Glioma, Thyroid, Pancreas, Prostate
CDH1				Colorectal, Breast
CDKN2A	Palbociclib Dinaciclib			Breast, Pancreas
CTNNB1	Nutlin 3a			Prostate, Ovary, Colorectal
EGFR	Afatinib Axitinib Cetuximab Erlotinib Gefitinib Lapatinib Linifatinib Motesanib Neratinib Panitumumab Pelitinib Ponatinib Sorafenib Sunitinib Tivozanib	Afatinib Tyrosin kinase inhibitors Gefitinib Erlotinib azd9291	Oncogene	Lung*, Head & neck, Prostate, Breast, Ovary
ERBB2	Afatinib AMG 386 Ganestespib Kadcyla Lapatinib LJM 716 MGAH22 MM-302 Neratiinib Trastuzumab		Oncogene	Breast, Lung, Ovary
ERBB3				Breast
ERBB4				Endometrium, Colorectal, Stomach

(Table 3). Continued.

Gene	Sensibility	Resistance	Role	Tissue
ESR1				Breast
EZH2				Prostate, Breast
FBXW7				Breast, Colorectal, Ovary
FGFR1	Dovotinib			Oral squamous cell carcinoma, Breast, Esophagus, Ovary, Bladder, Prostate, Lung
FGFR2	FGFR inhibitors and antibodies			Stomach
FGFR3	ENMD-2076			Bladder
FOXL2			Oncogene	Ovary
GNA11			Oncogene	Melanoma
GNAQ			Oncogene	Melanoma
GNAS				Kidney, Thyroid, Pituitary, Leydig cells, Adrenocortical, Colorectal
HNF1A				Kidney
HRAS	Trametinib MEK & RAS inhibitors			Bladder
IDH1				Glioma
KDR	VEGF inhibitors & antibodies			Infantile capillary hemangioma
KIT	Amuvatinib Axitinib Cabozantinib Dasatinib Imatinib Linifanib Masitinib Motesanib Pazopanib Sorafenib Sunitinib	Imatinib Sunitinib	Oncogene	Stomach, Melanoma*, Timo, Breast
KRAS	Trametinib MEK & RAS inhibitors		Oncogene	Colorectal*, Stomach, Lung*, Ovary, Thyroid, Endometrium, Pancreas, Prostate
MAP2K1				Melanoma, Lung, Ovary, Colorectal
MET	Crizotinib Cabozantinib		Oncogene	Lung*, Colorectal, Stomach
MLH1				Colorectal
MPL	JAK-STAT inhibitors			Myelofibrosis with myeloid metaplasia
NOTCH1	BMS-906024 RO4929097			Hematopoietic cancers, Esophagus, Colorectal
NRAS	Trametinib MEK & RAS inhibitors		Oncogene	Colorectal*, Lung, Melanoma, Thyroid

(Table 3). Continued.

Gene	Sensibility	Resistance	Role	Tissue
PDGFRA	Amuvatinib Apatinib Imatinib Masitinib Pazopanib Ponatinib Sorafenib Sunitinib	Imatinib Sunitinib	Oncogene	Stomach, Melanoma
PIK3CA	Alpelisib (in study phase) AMG479 BEZ235 BKM120 BYL719 Buparlisib CC-122 CC-223 Everolimus GDC-0032 GDC-0941 GDC-0980 MK-2206 Temsirolimus		Oncogene	Lung, Breast, Prostate, Colorectal, Ovary, Head & neck, Pancreas, Thyroid, Endometrium, Stomach
PTEN	GSK2636771 MK-2206 PIK3 inhibitors			Breast, Lung, Prostate, Colorectal
PTPN11				Leukemia, Colorectal, Endometrium
RB1				Urinary tract, Eye, Endometrium, Colorectal, Breast
RET	Cabozantinib Motesanib Sunitinib		Oncogene	Lung*, Thyroid
ROS1				Lung
SF3B1				Breast
SMAD4	Fresolimumab			Colorectal, Pancreas
SMARCB1				Rhabdoid tumors
SMO	GDC-0449 LDE225 Hedgehog inhibitors			Basal cell carcinoma, Glioblastoma, Medulloblastoma, Rhabdomyosarcoma
SRC	Saracatanib Dasatinib			Colorectal
STK11				Gastrointestinal cancers, Lung, Skin, Colorectal
TP53			Onco-suppressor	Lung, Melanoma, Ovary, Colorectal, Breast, Endometrium, Head & neck, Kidney, Pancreas, Prostate, Thyroid
VHL				Kidney, Hemangioblastoma, Cerebellum, Colorectal, Kidney

*National Comprehensive Cancer Network (NCCN) guidelines by type of tumor.

imported into clinical practice. Growing evidence in the field of LB suggests that this kind of analysis can be applied to patients with the most diverse types of cancer [46-56]. Many clinical studies currently use LB as a tool for diagnostic, predictive, prognostic evaluation, and cancer response to treatment. The test was designed for the detection of somatic hotspot mutations in genes involved in breast cancer (alpha serine/threonine-protein kinase 1 [AKT1], EGFR, ERBB2, ERBB3, ESR1, F-box and WD repeat domain containing 7 [FBXW7], KRAS, PIK3CA, splicing factor 3b subunit 1 [SF3B1], and TP53) and in several other cancers (Table **3**). Gene selection was carried out starting from the scientific consensus attributed to genes inserted in the panel by organizations such as the National Comprehensive Cancer Network (NCCN) [57] and the European Society of Medical Oncology (ESMO) [58]. The panel includes genes, gene regions including single nucleotide variants (SNV), and insertions/deletions (indels) which have proven useful in the molecular study of cancer tissue. In 2014, the European Medicines Agency (EMA) approved the use of plasma to evaluate mutation status of EGFR for patients candidate for treatment with EGFR-TKI. With the arrival of osimertinib, LB is mainly used for the identification of threonine790methionine (T790M) mutation of EGFR gene in patients progressing to first- and second-generation EGFR inhibitors [59].

Lung Cancer

According to prospective studies, ctDNA analyses are at least as good as tissue-based genotyping to identify genomic alterations on which to act therapeutically in patients with NSCLC, which represents up to 90% of all malignant lung cancers and sees cigarette smoking as the main risk factor. The exam implementation times are also significantly shorter [60].

The recent discovery of genetic alterations that cause cancer progression in some cases has led to the formulation of molecular target therapies. All patients with advanced lung cancer, for example, could undergo diagnostic biopsy which also allows to obtain the molecular profile of their disease. However, there are two limitations of modern target therapy:

1. the relatively small proportion of patients who can benefit from it; and

2. the fact that cancer never reaches complete molecular stability: by adapting to the host organism, it could therefore cause resistance to target therapy.

In the event that disease progresses, it is very important to understand what happens in it through a new biopsy. Unfortunately, it is not always possible to perform it for a number of reasons (difficult access to the lesion, presence of other disabling pathologies, especially in elderly patients, refusal by the patient, etc.). In this context, LB may represent a solution. In patients with lung cancer, ctDNA can be an alternative to monitor response in cases with EGFR mutation and to identify earlier acquired resistance mechanisms, such as T790M mutation that appears in more than 40% of patients treated with specific EGFR inhibitors. LB is currently mainly used for mutational analysis of EGFR gene in patients with advanced NSCLC. In particular, there are two different scenarios in which EGFR LB test is currently indicated:

1. mutational analysis of EGFR at the time of diagnosis in naive patients with advanced NSCLC. Determination of mutational status of EGFR gene on tissue/cytological sample obtained by biopsy or surgery at the time of diagnosis is currently recommended in all patients with NSCLC, stage IIIB-C and IV, with the exception of smoking patients with squamous histotype, for the choice of the best therapeutic strategy. However, recent data suggest that around 25% of standard lung biopsies fail to provide an adequate quantity/quality of material for molecular analysis. Multiple studies and meta-analyses have assessed diagnostic accuracy of ctDNA analysis for the identification of the most frequent activating mutations of EGFR gene (exon 19 deletions and L858R of exon 21) in naive patients with advanced NSCLC. Overall, these studies have shown good specificity of EGFR test on plasma, generally higher than 90%. Sensitivity is instead lower, with oscillations between 50% and 80% depending on the used technology. Based on this evidence, evaluation of the mutational status of EGFR gene on LB is currently recommended as a possible alternative to the analysis of tumor tissue in patients with a new diagnosis of advanced NSCLC in which the quantity and/or quality of available tissue are not sufficient to perform the expected molecular analyses [61]; and

2. mutational analysis of EGFR gene at the time of disease progression to EGFR-TKI in patients with advanced NSCLC. The recent approval and reimbursement admission by the Italian Drugs

Agency (AIFA) of the first third generation EGFR inhibitor (Determines AIFA Official Gazette [GU] n. 184 of August 8th, 2017), for the treatment of patients with locally advanced or metastatic positive NSCLC for T790M mutation, makes it necessary to re-evaluate the mutational state of EGFR gene at the time of radiological progression after treatment with an EGFR-TKI, in order to define the best therapeutic strategy. Results of the AURA study showed that disease-free progression (DFP) of patients stratified by the presence of T790M mutation based on the test performed on tissue or on LB is superimposable, thus confirming the possibility of using the test on ctDNA. However, it should be considered that the sensitivity and specificity of T790M test appear generally lower than EGFR sensitizing mutations. Given its non-invasiveness and rapidity of execution, T790M test by LB is currently used in several centers as the first diagnostic approach in all patients with advanced EGFR-positive NSCLC progressing after treatment with EGFR-TKI. However, due to the risk of "false negatives" associated with this method, all patients in whom mutation analysis on cfDNA is "negative" must undergo the same analysis on cancer tissue taken by re-biopsy, so as to define the best therapeutic strategy. Before proceeding with tissue biopsy, repeat LB may be considered. To ensure a better interpretation of the test, in addition to T790M, the evaluation of the sensitizing mutation of origin must also be performed. In the absence of such a mutation, the test should be considered non-informative as the sample does not contain enough amounts of ctDNA. In this regard, multiple studies and a recent meta-analysis have clearly highlighted how the site of tumor metastases significantly affects diagnostic accuracy of EGFR gene mutational analysis performed on ctDNA. The sensitivity of this method in determining both activating mutations and T790M resistance mutation of EGFR gene can in fact vary from 80% in the presence of extra-thoracic metastases to 50% in the presence of exclusively intra-thoracic localizations [62]. In order to increase LB chances of success, the test should be performed at the time of disease evident progression, when the chances of cancer DNA being released into circulation are greater. Finally, there is currently no evidence from prospective studies on the advisability of stopping treatment with EGFR-TKI in the event of appearance of a T790M mutation in LB in the absence of disease progression.

Colorectal Cancer

The future perspectives concern the clinical application of LB in metastatic colorectal cancer. Numerous studies have demonstrated the feasibility of performing RAS test on LB as a potential substitute for the analysis of tumor tissue in metastatic colorectal cancer. The concordance between the two approaches is, in modern studies, ≥90% and, if on the one hand there is further room for improvement in terms of sensitivity, on the other hand it must be taken into account that:

1. tumor and peripheral blood are two distinct tissues and therefore perfect concordance cannot be expected; and

2. discrepancies observed in terms of specificity – taking cancer tissue as a reference – find justification in the fact that LB is able to overcome the spatial and temporal heterogeneity that limits tissue analysis.

Furthermore, LB offers the advantages of a relatively non-invasive and more ductile approach, both for the possibility of carrying out the determination of mutation status more easily based on the exact moment of therapeutic intervention with anti-EGFR, and for the reduced turnaround time. However, some important considerations limit the import *tout court* of LB in the clinical use as a substitute for mutational analysis of RAS in colorectal cancer for the purpose of exclusion from therapy with cetuximab and panitumumab. Firstly, it is difficult to interpret negative test results that could be conditioned by ctDNA levels and by the sensitivity of the methods and therefore be false negatives. In addition, this analysis is not yet controlled by external quality control programs such as those implemented for tissue analysis. Furthermore, there is not enough clinical evidence to establish in quantitative terms what is the percentage threshold of mutated RAS alleles determined on peripheral blood that confers resistance to anti-EGFR therapy, given that current knowledge is based on analyses performed on cancer tissue. Therefore, the analysis of tumor RAS status for therapeutic choice must be carried out as the gold standard on cancer tissue in all cases where this is possible and vicarious from analysis by LB only in the rare cases of insufficient/inaccessible tissue. Finally, as regards LB use to monitor response to anti-EGFR drugs, this must be considered today only as a

research investigation and does not find current application in clinical practice.

In general, the term "biomarker" refers to any biological signal related to a physiological process, a pathological condition, or response to therapy. It is in this sense that the meaning of LB, whose main use at the moment is linked to the analysis of mutations of EGFR gene in patients with NSCLC in an advanced stage, must be explained [63-76]. In the field of oncology, the shades of meaning and clinical use of a biomarker extend significantly and the connotation given to a new technique is likely to be misunderstood. It is therefore appropriate to understand well what LB is, before submitting it to its clinical applications. The term "LB", in some ways, is inappropriate because it suggests that it is an alternative in all respects to tissue biopsy. This is certainly not the case. This term, in fact, refers to the identification of biomarkers in biological fluids of oncological patients, in order to obtain diagnostic and prognostic information or to predict therapeutic response to well-defined anticancer drugs. Contrary to what is supposed, LB is not performed only on blood – which currently remains the most widely used analysis matrix – but also on other fluids such as urine, cerebrospinal fluid, saliva, or pleural and peritoneal effusions. The list of biomarkers that can be dosed in it is equally vast. In general, when we talk about LB we refer to ctDNA analysis which is a fraction of cfDNA extracted from peripheral blood, but by LB we mean also the search for proteins, other nucleic acids (RNA, miRNA), or extracellular vesicles, such as exosomes which in turn contain DNA, RNA, and proteins. The definition of LB therefore includes various materials on which to perform the dosage of an even wider range of biomarkers. It is a non-invasive technique, it can be easily repeated over time, and is able to offer a rather complete picture of the molecular heterogeneity of disease. However, LB alone cannot be used diagnostically. It can be used to determine biomarkers but not to diagnose cancer, which is why tissue biopsy remains indispensable. At the moment, LB cannot replace tissue biopsy because the data in our possession indicate that the fact of finding a certain mutation in the blood does not necessarily imply that the patient is suffering from cancer. However, we cannot rule out that in the future we can arrive to a diagnosis of cancer from LB, perhaps by combining different markers. Even the limited amount of ctDNA can be an obstacle from which false negative results can arise. This is a limiting factor that places LB in a context in which its usefulness is linked to verifying the level of some extremely valuable biomarkers for setting up and choosing a therapy.

Janku and coll. (2015) [44] tested 21 mutations on BRAF, EGFR, KRAS, and PIK3CA genes in 157 patients with advanced tumors (including melanoma, NSCLC, colorectal, appendicular, ovarian, and uterine cancer) capable of improving systemic treatment. The authors demonstrated concordance between mutations in archived tissue samples and those detected in ctDNA from blood samples. The same study shows that the mean survival of 41 patients with more than 1% mutated KRAS was shorter on average than that of 20 patients with ≤1% mutated KRAS (4.8 vs 7.3 months, $p = 0.008$). Similarly, 67 patients with >1% mutated cfDNA (BRAF, EGFR, KRAS, or PIK3CA) had a shorter mean survival than 33 patients with ≤1% mutated cfDNA (5.5 vs 9.8 months, $p = 0.001$). In a large study on different types of cancer [77], it was shown that a fraction of ctDNA is detectable in more than 75% of patients with metastatic disease (pancreas, ovaries, colorectal, bladder, stomach, breast, melanoma, liver, and head/neck) and in less than 50% of patients with primary stage disease (brain, kidney, prostate, and thyroid). In a separate group of 206 patients with metastatic colorectal cancer, the authors showed high sensitivity and specificity of ctDNA detection for clinically relevant KRAS mutations (87.2% and 99.2%, respectively). A recent study conducted by Perrone *et al*. (2014) showed promising results for the application of ctDNA analysis as a screening tool for individuals at high risk of developing colorectal cancer. Furthermore, the amount and type of ctDNA observed in the sample can be indicative of tumor stage and therefore potentially used for staging [78]. Another study [7] found somatic mutations in 68% of colorectal cancer patients (n = 38) who underwent LB test for a panel of 46 mutations including those related to BRAF, KRAS, EGFR, and PIK3CA genes. In this study, patients positive for somatic mutations were divided as follows: 54% in early stage (I-III) and 93% in advanced stage (Stage IV). As for mutations and genes involved, 50% of patients showed mutations of KRAS, 16% of PIK3CA, and 8% of BRAF. No EGFR mutation was detected in patients belonging to the study. The obtained data confirmed correlation between cancer and the associated mutations in the literature. In patients undergoing liver metastasis surgery, ctDNA levels have been shown to be more useful for early prediction of recurrence than imaging techniques or research of carcinoembryonic antigen (CEA). Furthermore, 4% of the healthy subjects (n = 47) presented ctDNA values at the limit of detection. In one of the largest multicenter clinical trials (CORRECT) [45], the effect of a TKI (regorafenib) was evaluated on ctDNA levels in patients with metastatic colorectal cancer. Mutation analysis was conducted on a total of

760 patients, which included 505 treated with the active substance and 255 treated with placebo. Comparison between archived solid tumor samples and fresh plasma samples showed 76-97% concordance of results for the three genes analyzed. Mutations of KRAS were identified in 69% of patients, mutations of PIK3CA in 84% of patients, and mutations of BRAF in 3% of patients. In the regorafenib group, patients with KRAS mutations showed reduction in the progression-free survival (PFS) period compared to patients treated with placebo. It is also interesting to note that in the group of placebo-treated patients, patients with the highest amount of ctDNA already had poor overall and PFS. The study therefore supports the use of ctDNA to establish cancer genotypes before choosing treatment. If targeted therapy is stopped, metastatic colon cancer lowers the defenses it had raised against drugs. Mutations of its DNA, which had allowed it to circumvent the effect of molecular target treatments, begin to decline. The discovery was obtained, with the support by the Italian Association for Cancer Research (AIRC), thanks to LB developed at the Scientific Hospitalization and Treatment Institute (IRCCS) of Candiolo (Turin), in collaboration with the Niguarda Cancer Center of Milan. In fact, the test allows, with a simple blood sampling, to examine DNA released into the bloodstream by cancer cells, thus avoiding the need to make a biopsy. With LB, it is possible to monitor disease progress during treatment, identifying earlier the moment when cancer learns to neutralize its effect. And now it is also possible to understand when, after discontinuing therapy, cancer "relaxes", becoming vulnerable again. The study was conducted in 100 patients treated with two molecular target drugs, cetuximab and panitumumab: the study has shown for the first time that, in some cases, treatment stopping also corresponds to a reduction of mutated DNA in the circulation. Technically, we still don't have proof that the number of cancer cells that over time have developed mechanisms of treatment resistance has also decreased, but that's likely to be the case. Research shows that the evolution of resistant cell clones does not proceed linearly and irreversibly, but is dynamic and affected by exposure to drugs. These exert a selective pressure that favors some cells to the detriment of others. In particular, KRAS gene mutation levels, which make treatments ineffective, fluctuate in relation to the presence of the drugs cetuximab and panitumumab. Being able to identify in time, thanks to this test, the "window" in which cells are most susceptible, will allow us to understand what is the best time to resume therapies, thus improving therapeutic strategy [79-81].

Pancreatic Cancer

Pancreatic cancer is the fourth most common cause of death due to cancer in the world. It is known to have a poor prognosis, mostly because early stages of the disease are generally asymptomatic. Progress in pancreatic cancer research has been slow, leaving several fundamental questions pertaining to diagnosis and treatment unanswered. Recent studies highlight the putative utility of tissue-specific vesicles (i.e. extracellular vesicles) in the diagnosis of disease onset and treatment monitoring in pancreatic cancer. As exosomes contain cellular proteins and RNA molecules in a cell type-specific manner, they may provide extensive information about the signature of the tumor and pancreatic cancer progression [82]. Zill *et al*. [43], in a 2015 study, searched for mutations in a set of 54 genes, finding KRAS and TP53 as the most commonly mutated genes, followed by adenomatous polyposis coli (APC), mothers against decapentaplegic homolog 4 (SMAD4), guanine nucleotide binding protein (G protein) alpha stimulating activity polypeptide 1 (GNAS), FBXW7, and BRAF, also recurrently mutated. During the study of these five genes (KRAS, TP53, APC, FBXW7, and SMAD4), the average sensitivity was 92.3%, specificity 100%, and the average diagnostic accuracy 97.7%. The authors also identified mutations detectable during patients' follow-up, otherwise unobservable due to the inherent limitations of primary tumor tissue biopsy method.

Optimism came from the American Society of Clinical Oncology (ASCO) Annual Conference that was held in Chicago in 2018: LB confirmed an interesting perspective to get to diagnose certain types of cancer in the future before patients show their symptoms. It was the subject of study of hundreds of active researches all over the world and the eyes of the experts, gathered at the major annual oncology appointment, were focused on the potential of this strategy. Of course, caution is needed for now because there is still no test capable of making this type of early diagnosis, but LB is already useful on several fronts in those who are affected by cancer. At the Chicago conference, a new study was presented by scientists from the Taussig Cancer Institute of Cleveland Clinic and Stanford University: the research examined cases of over 1,600 people, 749 of whom were healthy and 878 who had been diagnosed with cancer. The test was more accurate in detecting cancer of the pancreas, ovaries, liver, and gallbladder, properly detecting diseases in at least four out of five patients. Blood test then revealed lymphoma and myeloma with slightly

lower accuracy, 77% and 73%, and intestinal cancer in two out of three patients. Lung cancer was detected in 59% of patients and head and neck cancer in 56% [83]. In 2019, Cristiano and collaborators developed a new analysis to evaluate fragmentation of cfDNA released by cells in normal processes such as apoptosis or by cancer cells that circulate in the bloodstream. For this, blood samples were taken where blood plasma was isolated from 236 patients with 7 different types of cancer, including breast, colorectal, lung, ovarian, pancreatic, gastric, and bile duct cancer. Researchers compared fragmentation profiles obtained through genome-wide association studies (GWAS) and compared them among cancer patients against DNA fragmentation profiles of 245 healthy patients. To observe the difference between fragments size and to decide which fragments corresponded to cancer patients or healthy patients, ***DNA*** evaluation of fragments for early interception (DELFI) was developed. DELFI is an evaluation method that is based on a type of artificial intelligence (AI) called "machine learning". Specifically, to distinguish between patients using these fragmentation profiles, the authors used the "stochastic gradient increase model" (or gradient boosting model [GBM]), which is defined as serially constructed algorithmic models, thus each model can "learn from the previous one". Therefore, GBM seeks to reduce errors in algorithmic models that are created sequentially. Typically, decision trees are used for these learning models. Finally, this gradient tree impulse machine learning was used to examine whether cfDNA could be classified as having characteristics of a cancer patient or a healthy individual. Using this method, through LB, earlier diagnosis of cancer patients could be improved. However, DELFI requires additional validation in other studies [84]. On large numbers, in fact, confirmation is needed and the test must be refined: we are still far from the goal of the use of this biopsy on the general population (i.e. all healthy people) and more research is needed, but one day not far it could be used on adults over 40 years of age, to know if they have the first signs of cancer. Microsoft's founder Bill Gates and Amazon's chief executive officer (CEO) Jeff Bezos have recently invested in a biotech company that is developing these tests with two longitudinal observational studies: the Circulating Cell-free Genome Atlas (CCGA) which plans to enroll more than 15,000 people with and without cancer and STRIVE (in progress) who tries to enroll 120,000 women at the time of mammography and to track any asymptomatic breast cancer for five years [85,86].

Prostate Cancer

To date, the most promising results of LB have been obtained in prostate cancer: some studies seem to emerge a role of CTC in defining not only the prognosis, but also the sensitivity of prostate malignant cells to chemotherapy or new generation hormonal therapies [87,88]. The technique is being studied, quite advanced, in breast and colon cancers or cutaneous melanoma, in an attempt to identify new predictive factors for the efficacy of new drugs or for early disease recurrence. The risk of developing prostate adenocarcinoma is very low in patients under 40 years of age, but increases progressively with age. However, the age at diagnosis has gradually decreased since the beginning of the 1990s thanks to the introduction of prostate specific antigen (PSA). This marker's spread has led the prevalence of metastatic disease at diagnosis from 45% of the 1980s to less than 4% of the present day, and has therefore allowed neoplasms to be "discovered" in increasingly early stages (from 37% to 91%). This early diagnosis has contributed to a significant increase in new diagnosed cases of prostate cancer, with mortality that has, however, significantly reduced. In fact, there has been an increase in cancer-specific survival, which now exceeds 80% at 20 years in organ-confined disease treated early. This important prognostic improvement was largely due to the updating of therapeutic strategies in all risk classes. The definitive diagnosis of prostate cancer is, however, still placed today on an invasive biopsy basis, therefore it is important to identify new bio-markers to be added to existing ones to increase the probability of early and, above all, non-invasive diagnosis. In recent years, it has been observed that among ncRNA, miRNA can be used in the diagnosis and prognosis of different types of cancer. miRNA are small RNA molecules that regulate gene expression through the inhibition of translation or degradation of target molecule. By their nature, miRNA seem to remain rather intact and stable in biological fluids and can therefore exercise a potential diagnostic and prognostic role in the oncological field, even in the specific case of prostate cancer. In this regard, the research group headed by the University of Perugia Urological Oncology Clinic has shown that a family of miRNA could discriminate between patients with a clinical diagnosis of prostate cancer and patients with benign prostatic hypertrophy. In a recent study, in fact, the team evaluated with NSG all miRNA present in the urine after prostate massage. Among 62 miRNA, let-7 family paid particular attention, which highlighted a potential diagnostic role in prostate cancer. Finally, it should be noted that these data are

confirmatory of previous scientific work carried out by the same group in this field [89,90].

METHODOLOGY

Sample Collection

LB is performed starting from a blood sample (about 10 ml). The collected blood sample is centrifuged to separate the plasma component. Through complex laboratory analysis, cfDNA is isolated and amplified by PCR technique. Subsequently, through a process of DNA sequencing through the use of NGS techniques, gene regions are sequenced at high reading depth. Obtained gene sequences are subsequently analyzed through an advanced bioinformatics analysis to identify any somatic mutations in the genes under examination. Mutations are searched through the Catalog Of Somatic Mutation In Cancer (COSMIC) database, capable of associating pathological mutations using the data present in scientific publications [91,92]. cfDNA can be extracted from various biological fluids. However, the most standardized procedures in clinical practice concern the isolation of cfDNA from peripheral blood. The amount of DNA that can be extracted from peripheral blood is often very limited, to the extent of a few ng/ml, of which ctDNA is only a fraction. In fact, the concentration of target DNA in the plasma depends on several factors, among which: the disease load, the expression levels of the mutation in primary tumor cells, the release rate of ctDNA in the circulatory stream, and DNA levels released by unprocessed cells (for example, as a consequence of inflammatory processes that are established in healthy tissue and surrounding cancer, or lysis of leukocytes after blood sampling). For these reasons, the pre-analytical phase must be carefully checked. A first problem that can affect sample quality is hemolysis that can occur during phlebotomy: therefore, blood sampling must be carried out by highly qualified personnel. cfDNA can be isolated from serum or plasma. However, several studies have shown that the use of plasma is preferable to serum; in the latter, in fact, coagulation process causes the release of genomic DNA deriving from leukocytes. At present, there are no conclusive indications on the amount of blood to be used for diagnostic purposes and in laboratory routine. However, many diagnostic kits indicate the minimum amount of plasma needed for analysis. The sample can be collected in standard K2- or K3-ethylenediaminetetra-acetic acid (EDTA) tubes or by using tubes containing special fixatives, capable of stabilizing blood and cfDNA for several days. If the sampling is carried out using standard tubes, two important factors must be taken into account:

1. cfDNA has a short half-life, estimated at around 2.5 hours; and

2. several studies have shown that after three hours from collection, the lysis of leukocytes can occur with the consequent release of germ DNA which determines a dilution of cancer DNA.

Preservation of blood at a temperature of 4°C does not prevent the lysis of leukocytes. In cases where it is not possible to process the sample within three hours from collection, the use of tubes containing specific preservatives capable of stabilizing both cfDNA and leukocytes is recommended. However, these tubes generally guarantee that the sampling will only be kept in a limited temperature range (16-24°C). Therefore, it is important to ensure that these temperatures are adhered to when transporting the sample.

For the elimination of cellular residues and to obtain a suitable sample for subsequent analyses, plasma must be isolated by centrifugation, making sure that leukocyte contaminant deriving from the buffy coat has been completely removed. There are several centrifugation protocols. It is advisable to carry out a first centrifugation at low speed (1200-1600 r) to avoid the lysis of leukocytes and a subsequent centrifugation of the supernatant at high speed (≥3000 r) to remove all contaminants. Centrifugations must be performed without brake. Use of a refrigerated centrifuge (4°C) is also recommended. The obtained plasma can be stored at -20°C for short periods (about one month) or, for longer periods, at -80°C, a temperature which guarantees greater DNA stability. However, as the storage period increases, the total amount of cfDNA that can be extracted decreases, especially if the sample undergoes freezing and thawing cycles.

Cell-Free DNA Extraction

There are many methods for cfDNA extraction, which include both the use of commercial kits and protocols developed by laboratories. Due to the small amount and very fragmented nature of cfDNA in plasma (<1,000 bp), validated extraction methods should not be used on tissue samples or other biological matrices. The extraction method must be very reliable and must generate as much DNA as possible of the sample in question, in order not to compromise the analysis result and generate false negative or positive results. Various commercial kits

dedicated to this specific use are now available for the extraction and purification of cfDNA from plasma. These kits are generally based on the use of columns equipped with silica membranes, in association with a vacuum pump, or on the use of magnetic beads, for nucleic acids capture. These systems are equipped with simple execution protocols and allow to extract from 1 to 24 samples, fresh or frozen, simultaneously, and to capture fragments of cfDNA from plasma starting from a minimum of 10 µl to a maximum of 10 ml of sample. In general, it is believed that 2 ml of plasma is the minimum necessary quantity capable of providing accurate results using different extraction methods. In addition, most of the above-mentioned kits contain reagents or, in general, devices, capable of concentrating the eluate in a flexible elution volume (20-150 µl). Once extracted, cfDNA must be subjected to quantification, in order to optimize the amplification process and to know if subsequent molecular analyses may be possible starting from the extracted cfDNA. Accuracy in the quantification phase can be obtained with capillary or fluorometric electrophoresis systems. In general, the above-mentioned extraction kits allow to obtain high quality cfDNA samples with a concentration higher than 5 ng/ml. However, the amount of extracted DNA is influenced by disease state and by collection time. Optimal cfDNA storage allows its use even after some time to be able to perform further molecular investigations, with the explicit informed consent of the patient. The process requires adequate equipment, including freezers at -20/-80°C, graphic temperature control devices, acoustic alarm systems, and quality controls of the preserved biological material. The analysis of point mutations or small indels on ctDNA can be carried out through the use of real time PCR technologies, often modified to increase test sensitivity. For example, there are commercially available kits for ctDNA based on Amplification Refractory Mutation System (ARMS)/Scorpion that detect mutations in exons 19, 20, and 21 of EGFR. These kits allow the co-amplification of one or more mutated alleles and an endogenous control gene. Furthermore, a specific mixture of control oligonucleotides allows the evaluation of quality and quantity of DNA present in the samples. The analysis with these kits allows to detect low percentages of mutated allele in the presence of high quantities of wild-type genomic DNA by amplification with sequence-specific probes marked with fluorescein amidite (FAM) and hexachloro (HEX), which can also reach a limit of detection (LOD) of 0.5%, with differences between the various identified mutations [93].

Digital Polymerase Chain Reaction

Digital PCR (dPCR) is the latest generation evolution of PCR of which there are two technological platforms:

1. “droplet dPCR” (ddPCR); and
2. “Beam, Emulsion, Amplification, and Magnetics dPCR” (BEAMing dPCR).

Both methods are based on the distribution of the sample into thousands of homogeneous “droplets” in an oil-water emulsion; the subsequent amplification of the emulsion containing DNA allows discrimination of the target DNA thanks to the use of fluorescent probes. These characteristics are of great relevance in the context of ctDNA analysis, where it is necessary to research and amplify rare tumor DNA molecules in the presence of a large excess of wildtype germinal DNA. In fact, the division of the sample into droplets has the function of reducing competition between mutated tumor DNA and wild-type DNA, increasing specificity and sensitivity of the analysis. In this way, the relative abundance of mutated target DNA compared to wild-type one is increased. The amount of DNA required for digital PCR amplification is 3 ng (with a range of 50-100 ng) and 3-30 ng for BEAMing dPCR. ddPCR is characterized by a single amplification phase of target DNA, which takes place within about 20,000 droplets, equal in size and volume, formed by the oil-water emulsion, and by the final processing of data through Poisson statistics. BEAMing dPCR, on the other hand, provides for a pre-amplification phase of cfDNA using conventional PCR to amplify the target of interest; subsequently, generated amplification products are divided into thousands of homogeneous droplets in an oil-water emulsion with the addition of magnetic microspheres to which PCR products remain physically bonded and then easily separated using a magnet. The final data processing takes place through flow cytometry. There are commercially available assays for determination of mutations in EGFR exons 18-21 by dPCR techniques. The sensitivity and specificity of the tests with ddPCR and BEAMing dPCR are 0.1% and 0.01%, respectively. Although the approach with BEAMing dPCR would seem to have higher sensitivity, a lower specificity is frequently reported in the literature compared to ddPCR (87% vs 97%, respectively). dPCR reactions must be set up under a laminar flow hood, in an environment other than that used for cfDNA extraction and the analysis of amplification products, using appropriate precautions to avoid contamination (dedicated gown, gloves, tips with filter, etc.). In any

case, an area dedicated to dPCR preparation procedures must be set up. ddPCR execution involves a first step in which the solution containing DNA, the master mix, and the probes is prepared. This solution is then transferred to a special cartridge, inside which the oil is dispensed to form the emulsion. The cartridge is then introduced into the appropriate droplet generator to form drops contained in the oil-water emulsion. The next step involves transferring the emulsion from the cartridge to the 96-well plate, then to proceed with the amplification reaction. For each analysis, a positive amplification control (for example, using a previously validated cfDNA sample) and a negative control (i.e. the reaction mixture without DNA template) must be provided. Each laboratory should validate the dPCR method in advance by using dilutions of mutated DNA in non-mutated DNA from cell lines whose EGFR mutational status is known. Alternatively, certified reference samples can be used which guarantee correct determination of sensitivity, specificity, and LOD. The analysis of results takes place thanks to the use of a reader connected to a computer in which a specific software is able to transform the signal from analog to digital and detect negative droplets (without target DNA and/or reference DNA) and positive (which contain target allele) in each sample thanks to the various detected fluorescence. In ddPCR, negative and positive droplets are counted individually and the fraction of positive droplets in a sample determines the concentration of target DNA expressed in copies/µl. The analysis software is able to give information about the total number of droplets generated (for a good reaction these should be not less than 11,000) considering the number of drops positive for mutation and positive for wild type allele. The results can be elaborated to provide mutation concentration as copies/ml, allelic fraction, relationship between alleles, and fractional abundance. Finally, factors such as ethanol or paraffin (such as that used in some tubes to preserve cell lysis) can interfere with the formation of the emulsion making the analysis not evaluable.

Next Generation Sequencing

The new sequencing methodologies defined as NGS are characterized by an enormous productivity that varies from a few giga-bases (gb) for benchtop sequencers to 6,000 gb for larger sequencers. With NGS, it is possible to simultaneously sequence millions of different DNA molecules, being able to identify mutations, variations in the number of gene copies, fusions, and gene expression in a single analysis. Furthermore, its flexibility allows to evaluate panels of genes simultaneously (from a few units to hundreds), the entire human exome (about 30 mb), the entire genome (about 3.3 gb), or the entire transcriptome. Molecular barcode marking allows simultaneous sequencing of samples from different patients. The use of NGS panels allows a better interpretation of possibly negative investigations for driver mutations of interest. In fact, the identification of at least one gene variant in LB represents the proof of the presence of ctDNA and allows to report with greater certainty a negative result for the mutation of interest. The two most popular technologies are produced by Illumina (San Diego, CA, USA) and Thermofisher (Ion Torrent, Waltham, MA, USA). The first involves the *in situ* clonal fixation and amplification of DNA molecules on thin vitreous support. In the second, DNA is separated and amplified in lipid droplets and then distributed in microscopic wells at the bottom of which there is a thin semiconductor chip. In both cases, the pool of individual DNA molecules to be sequenced is called “the library”. The library is generated by the selective amplification of target regions with multiple PCR reactions or with hybrid capture technology with DNA baits. The use of NGS for LB requires changes to the protocols normally used for the analysis on blood and tissues. The error rate of NGS varies from 0.1% to 1%, therefore, for example, a mutation with an allelic frequency below this threshold cannot be differentiated from the so-called “background noise”. Operating protocols dedicated to LB reduce the error rate. A classic example of this modification is the incorporation of a specific molecular identifier before DNA amplification phase. Ultrasensitive NGS techniques dedicated to ctDNA analysis have been developed. Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) has as its characterizing element a selector that identifies different classes of somatic mutations with sensitivity and specificity greater than 90%. Similar results in terms of sensitivity and specificity have been achieved with the Tagged-amplicon deep sequencing (Tam-Seq) and Safe-Sequencing System (Safe-SeqS) techniques. Thus, LB and detection of tumor-associated mutations in ctDNA often require the ability to identify single nucleotide variants at allelic frequencies below 0.1%. Standard sequencing protocols cannot achieve this level of sensitivity due to background noise caused by DNA damage and polymerase-induced errors. The addition of unique molecular identifiers allows the identification and removal of the errors responsible for this background noise. Theoretically, high-fidelity enzymes will also reduce error rates in the NGS barcode, but this has not been thoroughly studied. The impact of

polymerase fidelity on the amplitude of error reduction in the different phases of the NGS barcode library construction has been assessed. It was therefore discovered that the barcode itself has a greater impact on reducing errors, even with low-fidelity polymerases. The use of high-fidelity polymerases in the barcoding phase of the libraries' construction further suppresses the error in the NGS barcode and allows the detection of variant alleles below the allelic frequency of 0.1%. However, the improvement in error correction is modest and is not directly proportional to polymerase fidelity. Depending on the specific application, other polymerase characteristics such as multiplexing capacity, PCR efficiency, buffer requirement, and the ability to amplify targets with high nitrogen base content may exceed the relatively small additional error reduction offered by very high-fidelity polymerase. In conclusion, NGS technique for ctDNA study, however sensitive and specific, is still very expensive and requires a complex work flow by highly specialized personnel. The use in the clinical setting of multigene panels on this biological matrix also requires the use of sophisticated software and, sometimes, the aid of bioinformaticians. It is therefore clear that the choice between the different technologies must take into account their sustainability and clinical use required in the context in which they operate [94-98].

Report

Report is an integral part of the diagnostic procedure and should contain the following information:

1. patient identification;
2. the identification of the physician and of the structure that requested the analysis;
3. the used material for the analysis (type, volume);
4. sampling time (diagnosis, intra- or post-chemotherapy, or biological therapy period);
5. collection date of the used material for the analysis;
6. how to keep the withdrawal;
7. arrival date of the sample in the laboratory that performs the analysis;
8. the used method for performing the analysis with the indication of sensitivity and limits of the test;
9. investigated mutations;
10. test results with specification of the type of mutation eventually detected; and
11. data interpretation and overall evaluation of the analysis with any problems related to the case.

Report must be completed on a pre-established template, dated, and signed by the service manager. In consideration of the impact on therapeutic strategy, the time for reporting must not exceed five working days from request for determination [99]. The test provides information related to the absence or presence in the analyzed sample of hotspot mutations:

1. "positive" result: presence of one or more mutations; it indicates that the test has detected, in the DNA extracted from the blood sample, one or more somatic mutations at the level of one (or more) genes. Mutations detectable through the test can fall into the following prognostic categories:
    a. with known pathological meaning;
    b. with benign meaning, since they are found in normal individuals and are devoid of pathological meaning; and
    c. with uncertain meaning, as not yet known or characterized by medical-scientific community. In this case, further investigations may be needed to clarify the variant meaning. Identification of these mutations may have different implications, in relation to the detected variants. During genetic counseling, the geneticist will explain in detail the meaning of test result, directing the patient to a subsequent consultation with the oncologist specialist. The test is a screening test and has no diagnostic purpose for cancer. In the case of tests with a positive result, specific insights, including diagnostic imaging (CT, MRI, etc.), are recommended as follow-up for the patient;
2. "negative" result: absence of mutations, indicates that the test did not detect any of the somatic mutations sought in the DNA extracted from the blood sample. This result does not mean that there is no cancer present or that there is no risk in the future that cancer may arise; and
3. occasionally, the test could produce a non-optimal or inconclusive result, because the sample does not meet the minimum quality

requirements necessary to be able to consider the obtained result optimal and, therefore, to be able to proceed with the relative issue of report. In this case, a new blood sample will be required to repeat the exam.

The total number of mutant copies of ctDNA present will be indicated on the report. The amounts of cfDNA and ctDNA present in a sample are variable. Each variant is associated with a minimum percentage of ctDNA, compared to total DNA, which represents its LOD. Result interpretation is customized based on the patient's medical history and, optionally, an indication of the possibility of inclusion of the patient in a clinical trial based on test results can be provided. Current DNA sequencing techniques produce results with greater than 99% accuracy. Although this test is very accurate, we must always consider the limits of the exam, which are:

1. the test performs a screening and not a cancer diagnosis;
2. the test does not detect all cancers and cannot be a substitute for tests that are currently the gold standard for diagnosing cancer;
3. the test analyzes only the most frequent mutations of investigated genes. In the case of cancers that, at the time of the test, have not developed the sought specific mutations, the latter will not be detected. It is therefore possible that mutations in untested genes may be causing the patient's disease;
4. the exam is not able to highlight:
    a. mutations localized in gene regions not specifically investigated; and
    b. deletions, reversals, or duplications greater than 25 bp;
5. a "negative" result, the absence of mutations for the investigated genes, does not exclude the possibility that mutations localized in regions of the genome not investigated by the examination are present;
6. a "positive" result should be interpreted in the context of the patient's medical history and related to disease stage, imaging results, therapeutic details, and other laboratory data;
7. in some cases, the result of a genomic analysis may reveal a DNA variant or mutation with a clinical significance that is uncertain or not determinable on the basis of current medical-scientific knowledge;
8. the interpretation of genetic variants is based on the most recent knowledge available at the time of the analysis. Such an interpretation could change in the future with the acquisition of new scientific and medical information on the structure of genome and affect the evaluation of the variants themselves;
9. some of these variants may not yet have been identified or validated by the scientific community and therefore may not be reported as pathogenetic at the time of the analysis;
10. an intrinsic limitation of the used NGS methodology is the lack of uniformity of coverage for each analyzed gene region. This limitation translates into the possibility, inherent in NGS methods, that specific mutations of the selected genes may not have been detected by the test;
11. in the case of cancers that have not yet released tumor DNA into the bloodstream at the time of the test, the sought mutations will not be detected;
12. the test is not aimed at identifying hereditary predisposition to the development of cancer, but only detects somatic mutations in ctDNA;
13. the test was not designed as a diagnostic tool for cancer, but its use must always be accompanied by a careful evaluation of the patient also through traditional methods such as tissue biopsy and imaging techniques; and
14. the test cannot replace the physician's clinical evaluation, imaging studies, or traditional tissue biopsy still considered the gold standard for diagnosing cancer.

The target coverage is the average number of reads obtained from sequencing for each base nucleotide constituting gene. In general, the deeper the coverage of a region, the more sensitive and reliable the analysis is. For the analyzed variants, a coverage of 25000x is necessary for the detection of frequency mutations up to 0.1%. Internal quality control requirements for the test impose a coverage greater than 25000x on more than 99% of the foreseen target bases for the analysis. Mutated allele frequency (MAF) is the frequency of the different mutations (replacements, insertions, and

Table 4: Test Performance Specifications

Mutant Allele Frequency (MAF)/Tumor Fraction	Sensitivity	Positive predictive value (PPV)
≥0.1%	99% (97.2-100%)*	99% (99.4-100%)*

*95% confidence interval (CI).

deletions) identified in the reported sample (Table **4**) [100].

APPLICATIONS OF LIQUID BIOPSY IN GYNECOLOGICAL ONCOLOGY

Breast Cancer

The main applications of LB in gynecological oncology have been developed in the management of breast, ovarian, and endometrial cancer. In the global context, the epidemic of breast cancer is evident for the early 21st century. Evidence shows that national mammography screening programs have sufficiently reduced breast cancer-related mortality. Therefore, the great utility of mammography-based screening is not an issue. However, both false positive and false negative breast cancer diagnosis, excessive biopsies, and irradiation linked to mammography application, as well as sub-optimal imaging, such as in the case of high-dense breast tissue in young females, altogether increase awareness among the experts regarding the limitations of mammography-based screening. Severe concerns regarding mammography as the "gold standard" approach demanding complementary tools to cover the evident deficits led to search innovative strategies, which would sufficiently improve the quality of breast cancer management and services to the patient. Contextually, current clinical data demonstrate the great potential of non-invasive diagnostic tools utilizing circulating miRNA profiles as an adjunct to conventional mammography for population screening and the personalization of breast cancer management [101]. In recent years, breast cancer treatment has become increasingly individualized. The development of cancer treatments has sharply accelerated thanks to the design of *ad-hoc* drugs, directed against specific targets present only on cancer cells. Cancer cells, however, can change over time and acquire characteristics that make them resistant to drugs. Human epidermal growth factor receptor 2 (HER-2)-positive breast cancer is an emblematic case. For this neoplasm, the election treatment is represented by targeted monoclonal antibodies capable of binding to HER-2 receptor, blocking its activation and inhibiting cancer proliferation. The development of "precision" drugs has significantly improved prognosis for this pathology, but problems related to the onset of resistance and recurrence remain. The functioning of new generation chemotherapy drugs can be explained with the "key-lock model". The drug is a key designed specifically to interact with its specific target, namely the lock. If cancer cell changes the target lock, the key drug can no longer recognize and bind its target and treatment becomes ineffective. Being able to monitor the status and changes of cancer cells during therapy is therefore essential to identify the mechanisms underlying resistance development. Patients with HER2+ breast cancer treated with trastuzumab antibody can be followed and monitored both through classic tissue biopsy and through LB to identify molecular markers and specific mutations associated with the development of resistance to treatment. Tissue biopsies are not representative of the heterogeneity and complexity of breast cancer and taking large samples would be too invasive. The use of LB allows to analyze the molecular characteristics of cancer and follow disease evolution over time. Knowing HER-2 breast cancer mutations related to the onset of resistance will allow to anticipate patients' response to treatment and plan more effective therapeutic strategies. Furthermore, clarifying the processes underlying this phenomenon will pave the way for the development of new, more effective drugs and treatments [102].

The majority of breast cancers are hormone receptor positive due to the expression of the estrogen and/or progesterone receptors. Endocrine therapy is a major treatment option for all disease stages of hormone receptor positive breast cancer and improves overall survival. However, endocrine therapy is limited by *de novo* and acquired resistance. Several factors have been proposed for endocrine therapy failures, which include molecular alterations in the estrogen receptor pathway, altered expression of cell-cycle regulators, autophagy, and EMT as a consequence of cancer progression and selection pressure. It is essential to reveal and monitor intra- and intertumoral alterations in breast cancer to allow optimal therapy outcome. Endocrine therapy navigation by the molecular profiling of tissue biopsies is the current gold standard but limited for many reasons. LB such as CTC

and ctDNA offer hope to fill that gap in allowing non-invasive serial assessment of biomarkers predicting success of endocrine therapy regimen and acting as navigators to personalize treatment methods and prevent endocrine treatment resistance in breast cancer [103-105]. A 2015 study [106] pointed out that early stage I to III breast cancer patients positive for PIK3CA mutations (n = 313) showed significantly lower recurrence-free survival (RFS) data than patients negative for the same mutations. Patients with higher amounts of mutated PIK3CA (>29 alleles) showed significantly lower RFS. Beaver *et al*. (2014) [107] found mutations on PIK3CA in 12 of 29 breast cancer patients (stage I-III). Patients with persistent ctDNA even after treatment were more likely to develop clinically evident metastases after 23 months. Dawson *et al*. (2013) [108] detected the presence of ctDNA in almost 97% of patients with metastatic breast cancer, and showed greater sensitivity and specificity of ctDNA, compared to CTC and cancer antigen 15-3 (CA 15-3) levels, in cancer detection [109].

Ovarian Cancer

Ovarian cancer is among the most common types of cancer and is the leading cause of death from gynecological malignancies in western countries. In fact, it accounts for about 30% of all female malignancies and occupies the tenth place among all cancers in women. According to the data of Italian cancer registries, it is estimated that there are about 40,000 women living with this disease in Italy. Unfortunately, ovarian cancer is a very insidious tumor primarily because it is characterized by non-specific symptoms that lead to the diagnosis of ovarian cancer when the disease has already advanced. That's why ovarian cancer is one of those neoplasms that unfortunately is still difficult to treat. The majority of patients are diagnosed in an advanced stage and exhibit resistance to standard chemotherapy. A first risk factor is represented by age, as the peak incidence of the disease is between 50 and 60 years, therefore in women of peri- or post-menopausal age. However, some types of ovarian cancer can occur in younger women. About 15-25% of ovarian cancers have familiarity as their main risk factor. Women with a mother (or sister or daughter) affected by cancer of the ovary, breast, or uterus are more likely to contract the disease. Today, something is starting to move: thanks to DNA analysis, it is now possible to identify the women most at risk – through the search for mutations in breast-related cancer antigens (BRCA) genes – and to customize currently available treatments. BRCA1 and BRCA2 gene alterations of hereditary origin can lead to a more or less important predisposition to the development of ovarian cancer. Not only that, an Italian research recently published has identified in some miRNA the possible indicator of tumor presence. An additional weapon to arrive to an earlier diagnosis [110]. Although significant progress has been made in the use of CTC and cfDNA for ovarian cancer diagnosis, their potential for the early detection or monitoring progression remains elusive. Upon initial diagnosis, the majority of patients present with widespread metastatic growth within the peritoneal cavity. This metastatic growth occurs in stages, with the formation of a pre-metastatic niche occurring prior to macroscopic tumor cell invasion. Exosomes released by the primary ovarian tumor are small extracellular vesicles which prepare the distant tumor microenvironment for accelerated metastatic invasion. They regulate the intercellular communication between tumor cells and normal stroma, cancer-associated fibroblasts, and local immune cells within the tumor microenvironment. Extracellular vesicles are a heterogeneous group of lipid membranous particles released from almost all cell types. Extracellular vesicles contain proteins, mRNA, DNA fragments, ncRNA, and lipids and play a critical role in intercellular communication. Emerging evidence suggests that extracellular vesicles have crucial roles in cancer development and metastasis, as coordinators of the pre-metastatic niche formation, thus holding promise for the LB-based biomarker discovery for ovarian cancer diagnosis and targets of chemotherapy [111,112].

Cancer biomarkers have a potential for improving management of ovarian cancer patients at every point from screening and detection, diagnosis, prognosis, follow-up, response to therapy, and outcome. Literature search has indicated a number of candidate biomarkers have recently emerged that could facilitate the molecular definition of ovarian cancer, providing information about prognosis and predicting response to therapy. These potentially promising biomarkers include immune cells and their products, tumor-derived exosomes, nucleic acids, and epigenetic biomarkers. Although most biomarkers available today require prospective validation, the development of non-invasive LB-based monitoring promises to improve their utility for the evaluations of prognosis, response to therapy, and outcome in ovarian cancer [113]. We believe systemic immune parameters might be a good alternative to tumor biopsy to gain insight in the immunological background of ovarian cancer [114].

Current biomarkers available in the clinical setting are not enough for early diagnosis or for monitoring disease progression of ovarian cancer. LB is a minimally invasive test and has the advantage of earlier diagnosis and real-time monitoring of treatment response. CTC and ctDNA represent the main LB approaches that offer a minimally invasive sample collection. Both have shown diagnostic, prognostic, and predictive value in many types of solid malignancies and recent studies attempted to shed light on their role in ovarian cancer [115]. Molecular medicine offers new possibilities, if not for healing, certainly to obtain an extension of life while maintaining a good quality of the same, even in the presence of advanced disease. For example, results of genetic tests that reveal the possible presence of mutated genes in female DNA (BRCA1 and BRCA2) now allow us to offer targeted and effective drugs to those who carry them. Research in this field goes fast: today we know that up to 25% of patients with high-grade serous ovarian carcinoma have a BRCA mutation, while 5 years ago we thought they were 5%. Knowing this fact allows us not only to offer them more targeted treatments, but also to identify healthy family members to whom to offer preventive measures [116]. But that's not all, because through the analysis of cancer genetic characteristics the goal is to identify patients who will most benefit from the surgical act compared to those who will have to be sent to primary chemotherapy, with the advantage of improving cure and lessen side effects. Little positive signs also come by the front of early diagnosis: a study managed to identify a molecular signature of miRNA in the blood of patients affected from ovarian malignant epithelial cancer, that is the indication of cancer presence obtainable through LB [117]. Results of the study, carried out largely through funding from the AIRC, were recently published. The research was carried out by a large group of Italian researchers (biologists, bioinformatics, and statisticians) belonging to different clinical centers (Department of Biology of the University of Padua, University of Ferrara, Civil Hospital of Brescia, University of the Sacred Heart in Rome, etc.) and was coordinated by the IRCCS Institute for Pharmacological Research "Mario Negri" in Milan. miRNA are small RNA molecules that have important regulatory functions. They are very stable molecules and for this reason it has recently been discovered that they are used by the tumor and the tissues of our organism as important intracellular messengers. In short, they work both inside the cell and after being released into the circulation as messengers of a tumor or inflammatory process. This is a research field still largely unexplored, so the data must be taken with caution and validated by further studies. The comparative analysis of the serum miRNA profiles of 168 patients with high-grade serous cancer and 65 women of similar age, but not affected by the same disease, however, highlighted important and reproducible differences. In particular, there were differences in the expression of three miRNA named miR1246, miR595, and miR2278. The study lays the foundation for subsequent research aimed at assessing whether measurement of these miRNA can be used for the earlier diagnosis of ovarian cancer. Furthermore, subsequent studies will establish whether biomarkers are potentially useful for measuring the effectiveness of therapy in a more sensitive and earlier way compared to traditional radiological assessments. The ability to trace molecules that are released from cancer into the blood of a patient represents a new, valid tool, even less invasive, to improve diagnostic and therapeutic routes [118,119].

Uterine Cancer

Uterine cancer includes endometrial carcinoma and uterine sarcoma. Endometrial cancer is the fourth most common cancer in women in developed countries. It is the most frequent variant and has early symptoms and a solid diagnostic work up, resulting in a rather fair prognosis. However, in case of advanced stage of the disease and relapse, treatment options are limited and prognosis is impaired. So, the identification of sensitive and specific biomarkers to improve the early detection of endometrial cancer is crucial for an appropriate management of this disease, in which 30% of patients are diagnosed only at advanced stages, which is associated with high levels of morbidity and mortality. Despite major efforts and investments made to identify endometrial cancer biomarkers, no protein has yet reached the stage of clinical application. New perspectives in endometrial cancer biomarker research include comprehensive knowledge of previously suggested candidate biomarkers in conjunction with novel mass spectrometry-based proteomic technologies with enhanced sensitivity and specificity not yet applied to endometrial cancer studies and a directed clinical perspective in the study design. These ingredients could be the recipe to accelerate the application of protein biomarkers in the clinical setting [120]. Uterine sarcomas are rare, often lacking symptoms, and no diagnostic tool for a correct pre-operative diagnosis are available. Prognosis is poor. Circulating biomarkers as LB could be beneficial as a diagnostic tool in uterine sarcomas. For both

carcinomas and sarcomas, circulating biomarkers such as proteins, CTC, ctDNA, miRNA, and immune cells could be of use in predicting early disease recurrence [121].

CONCLUSIONS

One of the greatest challenges of modern medicine would be to develop a universal test, simple to perform, so that it could perhaps be done by all healthy people once a year. Technological progress today has put the scientific community in a position to have detailed information in the molecular field, which was not reachable before. The fact of knowing some aspects of cancer's genetic "identity card", however, does not mean knowing how to correctly use this information yet. The term "LB" refers to the use of biological fluids as a substitute for neoplastic tissue to obtain useful information for diagnostic and prognostic purposes, or to predict the response to therapy with specific anticancer drugs. Today, the analysis of ctDNA contained in cfDNA, which can be isolated from peripheral blood, represents the main LB approach used in clinical practice. However, it is possible that in the future other blood derivatives, such as CTC, circulating RNA and miRNA, platelets, and exosomes, as well as other biological fluids such as urine and cerebrospinal fluid, may be used in clinical practice to obtain more information than that obtainable through ctDNA analysis. cf/ctDNA ratio can vary both from the point of view of sample collection and from the patient's clinical condition. In current clinical practice, LB analysis is generally referred to the identification of driver mutations present in ctDNA deriving from both cancer and CTC. ctDNA is however a fraction, sometimes extremely small, of total cfDNA that can be isolated from the plasma of neoplastic patients and which also contains DNA deriving from untransformed cells. Some commercial systems (with CE-IVD mark) available on the European and Italian market report ctDNA as target rather than others cfDNA, given that when carrying out analysis there is no certainty of the presence of DNA of cancer origin until the possible detection of a mutation. For this reason, in the report document reference will be made to cfDNA more generally, and to ctDNA in case of positivity for the mutations object of the analysis.

LB consists of collecting blood, urine, but also saliva, and looking for various cancer components. The search for cancer DNA mutations released into the circulation could anticipate the discovery of a neoplasm when it is not yet visible with diagnostic methods such as CT or MRI. It is necessary to distinguish well between reality and hope, between what we can do today, that is to use LB to "choose the right drug" in certain cancers, and what instead is being tested, that is, the use as a means of early diagnosis or as an alternative to tissue biopsy. LB has some obvious advantages over traditional tissue biopsy, namely:

1. the procedure is not invasive, since it is a simple blood sampling which is practically uncomplicated;

2. it can be repeated over time to monitor the molecular evolution of the disease, although there is no evidence to date to change the therapeutic choice in the absence of clinical disease progression; and

3. it is able to represent in a more comprehensive way than tissue biopsy the molecular heterogeneity of the disease containing, at least potentially, tumor DNA deriving from different areas of the same cancer and the possible sites of disease.

However, LB has also some limitations:

1. the amount of ctDNA in the context of cfDNA is often extremely limited, depending on both volume and localization of the disease, and this can result in false negatives; and

2. in the presence of tumor heterogeneity, LB provides little information on the representativeness, in the context of cancer, of the identified biomarker.

With LB, for now, it is not possible to make a diagnosis and tissue analysis remains fundamental, especially in the early stages, for a correct diagnostic classification. LB is a complementary tool, useful for obtaining cancer molecular characterization, for finding biomarkers that allow us to choose, in some cases, a drug instead of another. Understanding which is the most valid drug in the individual patient is in fact increasingly crucial, not only for the patient who benefits from it, but also for the health system, because in this way new expensive drugs can be prescribed only in the appropriate cases [122]. Both tissue biopsy and LB can provide complementary information about the risk of disease progression. In the future, LB will be used not only as a substitute or alternative to tissue biopsy but rather as a valid supplement to better decide which therapy to administer. Of course, to remain in the

wake of prescriptive appropriateness, it will be always responsibility of the oncologist to recommend the execution of this test. An international initiative for quality control on the execution of LB test which will allow to certify a list of centers capable of performing the test with high levels of quality has been launched. It is a difficult and peculiar analysis because small quantities of ctDNA are extracted from blood and this makes the execution very complex, which must be carried out in specialized centers where a strong technical experience and high availability of technologies are concentrated.

The last half-decade has been marked by a rapid expansion of research efforts in the field of LB, thereby investigating the potential of blood-derived ctDNA markers for their application in clinical oncological management. The analysis of cfDNA appears to be particularly attractive for treatment monitoring purposes, while in terms of early cancer diagnosis and screening the potentials are just starting to be explored. Challenges, both of biological and technical nature, need to be addressed. One such challenge is to overcome the low levels of ctDNA in the circulation, intrinsic to many early-stage cancers. Although many studies report encouraging results, further technical development and larger studies are warranted before the application of ctDNA analysis may find its place in the clinical setting [123]. Progress in cancer treatment made by the beginning of the 21st century has shifted the paradigm from one-size-fits-all to tailor-made treatment. The popular vision, to study solid tumors through the relatively non-invasive sampling of blood, is one of the most thrilling and rapidly advancing fields in global cancer diagnostics. From this perspective, immune-cell analysis in cancer identifying the presence of microsatellites high instability, a parameter that allows to predict the patient's response to immunotherapy, could play a pivotal role in oncology practice. This approach is driven both by rapid technological developments, including the analysis of circulating myeloid-derived suppressor cells (cMDSC), and by the increasing application of immunotherapies, the success or failure of which may depend on the effective and timely measurements of relevant biomarkers. Although the implementation of these powerful non-invasive diagnostic capabilities in guiding precision cancer treatment is poised to change the ways in which we select and monitor cancer therapy, challenges remain [124-133].

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