Liquid biopsy in lung cancer management

Maria-Anca Irofei Zamfir¹⁻², Laura Buburuzan², Ariana Hudit¹, Bianca Gălațeanu¹, Octav Gînghine³, Daniel Ion³, Natalia Motăș²⁻³, Carmen Maria Ardeleanu², Marieta Costache¹

¹Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Bucharest, Romania
²Department of Molecular Biology, OncoTeam Diagnostic S.A., Bucharest, Romania
³Faculty of Medicine, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania

Abstract
Liquid biopsy is a promising tool for a better cancer management and currently opens perspectives for several clinical applications, such as detection of mutations when the analysis from tissue is not available, monitoring tumor mutational burden and prediction of targeted therapy response. These characteristics validate liquid biopsy analysis as a strong cancer biomarkers source with high potential for improving cancer patient’s evolution. Compared to classical biopsy, liquid biopsy is a minimal invasive procedure, and it allows the real-time monitoring of treatment response. Considering that lung cancer is the most common cause of cancer-associated death worldwide and that only 15–19% of the lung cancer patients survive five years after diagnosis, there is an important interest in improving its management. Like in other types of solid cancers, lung cancer could benefit from liquid biopsy through a simple peripheral blood sample as tumor-related biomarkers, such as circulating tumor cells (CTCs), cell-free nucleic acids (cfNA) [cell-free ribonucleic acid (cfRNA) and cell-free deoxyribonucleic acid (cfDNA)], exosomes and tumor-educated platelets (TEPs) may shed into circulation because of necrosis or in an active manner. More, the detection and analysis of these biomarkers could lead to a better understanding of oncological diseases like lung cancer. The better the tumor profile is established; the better management is possible. However, this approach has currently some limitations, such as low cfNA concentration or low count of CTCs that might be overcome by improving the actual methods and technologies.

Keywords: liquid biopsy, lung cancer, circulating tumor cells, cfDNA, cfRNA.

Introduction
Lung cancer comprises in 19.4% of total deaths caused by cancer worldwide [1] and it has one of the highest mortality rates [2]. General treatments, such as chemotherapy and radiotherapy, contributed to the improvement of patient’s quality of life and their survival rates. However, there still is an acute need to find new ways for an even better understanding of this disease, including the metastasis processes, tumor heterogeneity, earlier recurrence, and real-time treatment response monitoring [3] to increase the overall survival of the patients. In this view, downstream molecular studies using liquid biopsy might open new and useful perspectives.

Liquid biopsy is a non-invasive way to develop new diagnostic tools, which can add value to the classical biopsy outcomes. Biomarkers detection found in body effluents (blood plasma, urine, cerebrospinal fluid, saliva, pleural fluid) has a great potential to extend the understanding of oncological diseases like lung cancer [4] and it has increased the applicability of targeted therapy. These biomarkers are tumor-related markers and are found into the bloodstream due to several releasing processes like necrosis, active shedding, apoptosis, etc. [5]. There has been made an important progress regarding liquid biopsy analysis with respect to circulating tumor cells (CTCs), cell-free nucleic acids (cfNA), with cell-free ribonucleic acid (cfRNA) and cell-free deoxyribonucleic acid (cfDNA) that implies the circulating tumor DNA (ctDNA) detection, exosomes, and tumor-educated platelets (TEPs) detection. CTCs are cells detached from the tumor (primary or metastatic lesions) into the bloodstream, holding the same profile as those in the actual lesion, which makes them valuable targets for liquid biopsy assays [6]. cfDNA consist in a mix of cRNA and cfDNA. cfDNA represents the total amount of DNA molecules present into the bloodstream, while ctDNA represents the cfDNA fraction of interest because it originates from the tumor. Exosomes are extracellular vesicles (EVs) with endocytic origin contributing to the molecular communication between cells. In particular, exosomal RNA analysis can detect molecular alterations associated with tumors [7]. TEPs are another component of liquid biopsy that retain the RNA from the tumor. Besides RNA of tumor origin, TEPs can also retain solubilized tumor-associated proteins, which are considered potential new biomarkers.

Consequently, the liquid biopsy analysis has a great potential in the detection of cancer-associated biomarkers, including lung cancer pathology. However, there still are aspects that might benefit from improvement through further studies.

Lung cancer
Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are the two major histological types of
lung cancer [8]. These two types are different regarding many aspects, such as treatment, prognostic [9]. Twenty percent of all primary lung cancers are classified as SCLC. This type is aggressive and difficult to treat by surgery [10]. NSCLC on the other hand includes adenocarcinoma (ADK), squamous cell carcinoma (SCC) and large cell carcinoma. These types include surgically treatment and adjuvant therapy [11].

Lung cancer affects more men than women in USA and Europe with high-income countries. Most of the patients have smoking history (depending on the duration and number of cigarettes per day) and other causal factors may include air pollution, radon exposure, coal burning emissions, asbestos. The incidence regarding non-smoker patients is lower than smoker patients’ incidence [1].

Due to the complexity of this cancer type, further studies regarding genetic alterations that may be associated are needed. Early diagnosis is very important; this cancer is very aggressive and that is why the studies are directed towards different methods that may help in diagnosis.

**ADK**

ADK is one of the most common types of lung carcinoma and it represent 40% of lung cancer cases and 60% of NSCLC cases [12]. Genes like transforming growth factor beta receptor 2 (TGFBR2), programmed cell death 6 (PDCD6), telomerase reverse transcriptase (TERT), epidermal growth factor receptor (EGFR), tumor protein p53 (TP53), Kirsten rat sarcoma virus (KRAS), neurofibromatosis 1 (NF1), anaplastic lymphoma kinase (ALK) may suffer different types of alterations. Compared to other lung cancers, ADK affects non-smoker patients, especially females [1].

**SCC**

SCC represents 20% of lung cancer cases and it is a smoking-related disease [13]. Ninety percent of the patients are former heavy smokers, especially males; the rest of them are non-smokers or light smokers. ALK and EGFR genes may be mutational drivers in the last-mentioned category of patients (non-smokers and light smokers) [12].

**Large cell carcinoma**

Large cell carcinoma is associated with a relative low number of NSCLC cases compared to ADK or SCC [8]. The higher percent of the patients are smokers. The genetic alterations may include KRAS, EGFR, B-Raf proto-oncogene, serine/threonine kinase (BRAF) and ALK genes [1].

**Adenosquamous carcinoma**

Adenosquamous carcinoma is the NSCLC type that comprises in less than 5% of lung cancer cases [14]. Patients may be smokers or non-smokers. Genetic alterations may include genes like ALK, EGFR, ROS proto-oncogene 1, receptor tyrosine kinase (ROST), Ret proto-oncogene (RET), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), fibroblast growth factor receptor 1 (FGFR1), human epidermal growth factor receptor 2 (HER2), serine/threonine kinase 11 (STK11), AKT serine/threonine kinase 1 (AKTI) [12].

**Neuroendocrine tumors**

Neuroendocrine tumors represent 20–25% of lung cancer cases and are a different type of lung cancer due to their morphological and immunohistochemical aspects [8]. Tobacco smoking may be associated with this type of lung cancer. Genetic alterations may include multiple endocrine neoplasia type 1 (MEN1) gene [13].

**Liquid biopsy**

Liquid biopsy refers to the analysis of different biofluids (blood plasma, urine, cerebrospinal fluid, saliva, breast milk) to identify and characterize specific biomarkers. Peripheral blood samples of patients carrying tumors might contain CTCs, cfNA and exosomes [4] (Figure 1). These representative biomarkers offer an immense potential in oncology. The harvest of the samples for the detection and analysis of these biomarkers is minimal invasive and offers an immense potential to reveal the genetic profile of the tumor [15]. Also called “circularizing biomarkers”, they arrive into the bloodstream from the tumors through different processes like apoptosis, necrosis or as a result of an active shedding mechanism [4].

Despite that the classical biopsy is the “gold standard” in diagnostic, this approach has some limitations related to the amount of tissue available and intra-tumor heterogeneity [7]. More, this analysis reflects the tumor profile at the harvest time only. Compared to the classical biopsy, liquid biopsy is less invasive, it can be performed multiple times
(serial monitoring) and it can offer real-time monitoring. With no doubt, liquid biopsy has its own inconveniences such as limitation of histological analysis, low level of tumor-derived biomarkers that can influence a false negative result [15], etc. However, the analysis of the biomarkers detected from the liquid biopsy offer important information for a better management of the disease.

CTCs analysis in lung cancer

CTCs are cancer cells detached from the primary or metastatic lesions into the bloodstream and represent tumor traits as clonal evolution and intra-tumor heterogeneity. Their abundance varies between lesion types (<10 cells/mL of blood) [16]. The dimension of these cells varies between 8–20 μm [15]. These cells express epithelial markers like epithelial cell adhesion molecule (EpCAM) and cytokeratins (CKs). By comparison with the white blood cells, they do not express the specific cluster of differentiation (CD)45 marker [17]. During the process of invasation, some of the CTCs suffer an epithelial–mesenchymal transition (EMT), while during the process of extravasation, CTCs suffer the reverse process called the mesenchymal–epithelial transition (MET). Consequently, CTCs express different markers associated with EMT or MET status while circulating into the bloodstream [18].

CTCs are important biomarkers because they present different components that can be detected using molecular-based methods, such as DNA (mutation detection), RNA (expression monitoring), proteins, and can also offer important information through immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) analysis [19]. Their phenotype (antigens expressed on their surface) can be analyzed using flow cytometry (FC) methods [19, 20]. The techniques used to detect CTCs based on their phenotype have two important parts: the enrichment part that is the isolation of the CTCs and the detection per se, the identification step that can be performed using FC or molecular biology approaches [21].

CTCs detection and analysis methods

CellSearch® system is the only CTCs isolation system approved by the US Food and Drug Administration (FDA) for clinical use and it is based on immunomagnetic enrichment. In this system, the CTCs are characterized by EpCAM-positive expression and lack of CD45 marker, combined with image processing [20]. FC is another technique that can be used to analyze single cells from samples which are prepared for fluorescence measurement. FC has multiple applications, such as cancer biology (analysis of the cell populations or sorting cell for other analysis) [22]. The cells are stained with fluorochrome-conjugated antibodies that recognize the antigens present on the surface of the cells.

Modular sinusoidal microsystems (BioFluidica) methods involve a chip with 320 sinusoidal microchannels with antibodies. CTCs are captured by antibodies and identified using their phenotype [23]. An impedance sensor is used for CTCs enumeration [20].

Enrichment methods used for the detection of CTCs in NSCLC

Some CTCs isolation (enrichment) methods are based on two biophysical properties of the cells – density and size –, while other methods involve the selection of the CTCs based on the expression of specific markers [2], such as EpCAM+, CKs+, CD45-, etc. Independent of the isolation principle, the methods require high sensitivity and high specificity [24].

Enrichment methods based on biophysical properties

Cell separation based on density refers to a density gradient centrifugation that results in the differential migration, hence separation of the different cell types (based on specific density of leukocytes, red blood cells and cancer cells) into layers [25]. The layer on the top is obtained from the lighter particles as mononuclear and tumor cells and the layer on the bottom contains heavier particles, neutrophils and erythrocytes [25]. In particular, for lung cancer CTCs detection, OncoQuick® and Ficoll-Paque® systems are used [21]. Due to the limitations of the centrifugation method, with respect to its capability to eliminate the possible contamination of CTCs with leukocytes, this method is used as an initial enrichment step when performing enrichment followed by other additional strategies [25], such as positive or negative selection.

Cell separation based on size is a type of enrichment method that uses filtration. The leukocytes (8–10 μm) are smaller than the epithelial cancers-derived CTCs hence, they can be removed by filtration using a polycarbonate porous membrane [21]. For example, systems like isolation by size of tumor cells (ISET®) are used in CTCs lung cancer enrichment step [25].

Immunoseparation techniques

(immunomagnetic separation)

CTCs separation based on immunomagnetic technique is based on the expression of specific markers. Therefore, the system contains a magnetic immuno-bead (monoclonal antibodies coupled to magnetic microbeads) and a ferrofluid [16]. The antibodies against EpCAM and/or CK for positive selection or against CD45 for negative selection, bind the antigens (markers) expressed on the surface of the cells [16].

Immunocytochemical approaches

CTCs detection based on immunocytochemical staining rely on the immunocytochemical fluorescence detection of tumor markers with different cocktails of antibodies [26]. It is a very important and difficult part to choose the antibodies. EpCAM and CK are epithelial antigens, and they are used to detect CTCs with epithelial origin [27]. The primary antibodies with tags or the secondary antibodies tagged (with enzymes or fluorescence) and bound to the primary ones and then are scanned (FC, fiber-optic array scanning technology, laser scanning cytometer) [20]. Staining with multiple epithelial markers and negative selection using CD45 (leukocyte marker) has become the standard to a more reliable identification of CTCs [28]. There are organ-specific markers, such as carcinoembryonic antigen (CEA), thyroid transcription factor-1 (TTF-1), CD56, HER2 and prostate-specific antigen (PSA) and they are useful in CTCs detection, but they are not always present in all tumor cells [28].

NA-based CTCs detection

CTCs detection based on NA refers to the detection of
tumor-associated messenger RNA (mRNA) using reverse transcription–polymerase chain reaction (RT-PCR). Total RNA is isolated and then transcribed into complementary DNA (cDNA) followed by a PCR amplification with negative, positive, and internal controls followed by a nested PCR to increase sensitivity [29]. In lung cancer, for example, the tumor-associated markers that can be detected are CK19 mRNA, CEA mRNA, LUNX mRNA [28]. Due to the rapid degradation of the RNA, the mRNA detected from a blood sample is considered to be from viable tumor cells [20]. Biomarkers like TTF-1, CK19, human telomerase reverse transcriptase (hTERT), survivin, CEA, parathyroid hormone-related protein (PTHrP), LUNX mRNA present a higher detection rate in patients with metastatic lung cancer compared to the patients with primary tumor and no distant metastasis [20, 30]. There has been observed a correlation between these biomarkers’ expression and the metastasis localization. For example, LUNX mRNA has been detected at patients with lymph node metastasis, CK19 mRNA at patients with lymph nodes, adrenal glands, bones, brain metastasis [30]. EGFR gene mutations can be detected from CTCs in patients with NSCLC; patients with NSCLC EGFR positive follow a different scheme of treatment [31]. ALK gene rearrangements are very important markers in lung cancer management. Patients with ALK rearrangements are treated with Crizotinib. One of the methods to detect these rearrangements include filter-adapted FISH [32]. Methods that include ISET® followed by FISH and IHC represent a reliable solution concerning the detection of ALK gene rearrangements in lung cancer patients from CTCs, with a high concordance with the tissue biopsy analysis. Echinoderm microtubule-associated protein like 4 (EML4)–ALK is one of the most frequent rearrangements involving ALK gene in NSCLC patients and it has been detected in CTCs from NSCLC patients’ liquid biopsy sample [33].

cfNA

CFNA contain DNA, mRNA, and microRNA (miRNA) found into the bloodstream. Increased levels of cfNA reflect pathological processes, such as sepsis, inflammatory diseases, benign or malignant lesions, stroke, trauma [4]. The NA shed into the bloodstream from apoptotic and/or necrotic cells. Particularly, the DNA derived from tumors that circulates in the bloodstream is called ctDNA and it contains the genetic and epigenetic tumor traits that are relevant to disease progression, development, and resistance to therapy [34]. Both, wild-type and normal cfNA can be released due to the genetic and morphological heterogeneity of the tumor [35]. The amount of cfNA depends on the state and size of the tumor [34].

Circulating cfRNA

Tumor cells can release RNA into the bloodstream, but its stability may be one of the major cfRNA analysis limitations [34]. RNA may be released during exosome-mediated signaling process (packaging into exosomes). cfRNA may have different tumor cells origin increasing the chance to detect multiple biomarkers [36]. miRNA represents one of the cfRNA fraction that can be detected and analyzed. However, with respect, there are some concerns, such as poor specificity, low interpretability, lack of reproducibility due to pre-analytical processing conditions. mRNA, one the other hand, may be detected and identified using real-time quantitative RT-PCR and next-generation sequencing (NGS). cfRNA analysis is useful to detect fusion transcripts by RT-PCR. For example, one of the most prevalent fusion transcripts of the ALK gene in NSCLC patients (the patients with ALK mutations are eligible to ALK inhibitors treatment) is EML–ALK [37]. This association leads to the occurrence of multiple fusions variants. cfRNA represents a reliable source of analytes for solid tumors (lung, bladder, pancreas) biomarkers detection. The comparison between real-time droplet digital PCR (ddPCR) and NGS-based cfRNA for cfRNA detection in solid tumors led to the observation that real-time ddPCR is an important tool for NSCLC early diagnosis, regardless the mutational status. The transcriptomic changes appear earlier than genomic changes in the process of malignancy.

cfDNA

Cancer patients, including lung cancer patients, present a higher quantity of DNA into the bloodstream [38]. The DNA is released into the bloodstream during different mechanisms, such as apoptosis, active shedding, and necrosis [39]. There are multiple biological factors that may affect the ctDNA concentration in the bloodstream, such as tumor localization and dimension, the stage of the disease, treatment (chemotherapy or radiation therapy), tumor vascularization level, metastasis process or the CTCs lysis process [40]. However, the releasing processes into the bloodstream need further studies and this will help regarding the applicability of the ctDNA analysis [41].

ctDNA represents the DNA from the bloodstream and it comprises the so-called “circulating cell-free tumor DNA”. ctDNA represents less than 0.1–10% from cfDNA [0.01% minor allele frequency (MAF)] [42]. The majority of the ctDNA fragments have approximately 180 nucleotides, uniform fragments compared to ctDNA size, which depends on the releasing process into the bloodstream (short ctDNA fragments mostly carry tumor-specific aberrations). Therefore, ctDNA analysis has great potential regarding the detection of specific genetic alterations that could represent targets for targeted therapy (personalized medicine) [43].

cfDNA analysis may have multiple applications in oncology, such as diagnosis, prognosis, tumor localization, therapy, therapy response and minimal residual disease monitoring depending on the method [41]. The techniques used in ctDNA analysis need optimization and standardization of parameters and pre-analytical steps (blood harvest vials, the centrifugation speed and time, etc.) [44].

cfDNA analysis may offer important information regarding the early diagnosis of lung cancer, treatment response (the occurrence of the resistance mutations can be detected in real-time) [40]. In our days, the liquid biopsy molecular analysis is complementary to the molecular analysis of the classical biopsy. However, considering its major advantages, such as minimal invasive harvest, ability to offer real-time output data and its capacity to picture the whole tumor heterogeneity [45], it gains more and more interest.

cfDNA preparation process is a very important step in ctDNA analysis. The vials used for plasma harvest may
differ. Most frequently, ethylenediaminetetraacetic acid (EDTA) tubes that prevent blood clotting and inhibit DNase activity are used [46]. There are also available tubes developed especially for cfDNA preservation as PAXgene® Blood DNA tubes from Qiagen and Cell-Free DNA BCT® (STRECK tubes) [47]. The first step of the preparation protocol is the centrifugation of the blood: generally, two centrifugation steps with a speed between 800×g and 2000×g for 10–15 minutes to separate plasma. Sometimes, a third centrifugation step may be needed (1000×g for 5 minutes) [48]. The input of plasma and the method used to isolate ctDNA are crucial for the ctDNA analysis outcome.

Different ctDNA analysis methods have been developed and summarized in Table 1: real-time PCR, beads, emulsion, amplification, and magnets (BEAMing), digital PCR and NGS.

| Table 1 – ctDNA detection technologies [49] |
|--------------------------------------------|
| NGS            | Digital PCR | Real-time PCR  |
|----------------|-------------|----------------|
| Deep sequencing| ddPCR       | AS-PCR         |
| Bias-corrected targeted NGS                | BEAMing     | AS-NEPB-PCR    |
| Multiplex PCR NGS                           | PNA-LNA PCR clamp |
| Fast-SeqS                                      | COLD PCR    |
| TAM-Seq                                         |
| Safe-SeqS                                       |
| CAPP-Seq                                         |

AS: Allele-specific; BEAMing: Beads, emulsion, amplification and magnets; CAPP-Seq: Cancer personalized profiling by deep sequencing; COLD: Co-amplification at lower denaturation temperature; ctDNA: Circulating tumor deoxyribonucleic acid; ddPCR: Droplet digital polymerase chain reaction; LNA: Locked nucleic acid; NEPB: Non-extendable primer blocker; NGS: Next-generation sequencing; PNA: Peptide nucleic acid; SeqS: Sequencing system; TAM-Seq: Tagged-amplicon deep sequencing.

Digital PCR platforms include ddPCR and BEAMing, and they are used for point mutations cfDNA detection [49]. ddPCR “splits” the DNA templates into thousands of droplets that will host parallel PCR reactions using water-in-oil (w/o) emulsion droplet technology [50]. In this technology, one droplet contains a single strand of DNA, and this confers high sensitivity [51]. The signal may indicate the absence or the presence of the sequence and that is way it may detect only known mutations [49]. The droplet distribution techniques may differ from capillary action to microfluidic well chips or microfluidic chambers [39]. FC detection based on TaqMan probes may also be used [49].

BEAMing relies on the principle of water droplets in an oil emulsion and the DNA templates are bound to magnetic beads with detection via FC [52]. Even though this technique is specific and highly sensitive, it has a complicated workflow and high costs that make it difficult to implement in clinical work routine [39].

Real-time PCR, on the other hand, is cheaper than the other methods used to detect cfDNA, but it is not as specific and sensitive [53]. That is why there have been developed several variations of real-time PCR mentioned (Table 1).

NGS (Figure 2) is based on parallel sequencing of millions of short DNA sequences and their comparison with a reference genome. This technique is used to detect mutations, rearrangements, and copy number variations [54]. The detection of ctDNA from cfDNA requires high sensitivity and specificity. From this perspective, several NGS variations have been developed (Table 1) using modified NGS techniques, such as cancer personalized profiling by deep sequencing (CAPP-Seq), a quantification method of ctDNA [55]. NGS represent the most reliable technique at the time of diagnosis, but due to its high cost and time-consuming disadvantages, ddPCR method may be considered for monitoring [39].

![Figure 2 – Schematic representation of the NGS protocol using the Ion Torrent platform. DNA: Deoxyribonucleic acid; NGS: Next-generation sequencing.](image)

All the above-mentioned methods are used in ctDNA analysis to detect oncogenic driver mutations. In NSCLC, MET gene amplification is associated with EGFR gene mutations. This is crucial for therapy election because patients may develop resistance to Afitinib and Erlotinib, EGFR tyrosine kinase inhibitor (TKI) treatments. Consequently, the detection of MET amplification for molecular diagnosis is a first important step to detect a possible resistance mechanism [56]. More, in NSCLC, ADK is one of the most common types of lung carcinoma and it is associated with oncogenic drivers in different genes, such as EGFR, K Ras, ALK, MET exon 14, PIK3CA, HER2, BRAF, RET, ROS1 [57]. SCC is associated with different pathogenic variations in genes, such as phosphatase and tensin homolog.
(PTEN), PI3CA and FGFR1 [39]. RAS–RAF–mitogen-activated protein kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway genetic aberrations are responsible for the oncogenic mechanisms associated with diseases like lung cancer and they represent the target for the therapy [58].

**TEPs**

TEPs are blood elements containing tumor-related RNA that is released into the bloodstream [39]. They have an altered function, and they participate in processes like metastasis, tumor cell growth and survival, migration, invasion [59]. They also are associated with inflammatory diseases of the lungs [39]. Their characteristics, size, count and protein markers (e.g., P-selectin) are detected and analyzed and are useful in cancer diagnosis [60]. TEPS can retain EVs released from tumor cells with tumor-related RNA [61]. For example, EML4–ALK translocation may be detected from the RNA localized in TEPS or EVs in lung cancer patients [59].

**Exosomes**

Exosomes are membrane-bound EVs detected in the liquid biopsy with a size between (30–200 nm). They are released into the bloodstream by cells, including tumor cells, and they are involved in angiogenesis, metastasis, cell proliferation [62, 63]. Exosomes may be also derived from normal cells and they play an important role in the homeostasis of the cells.

Biomarkers detected in exosomes are non-invasive biomarkers whom analysis may offer useful information in cancer diagnosis and prognosis along with the detection of ctDNA. NGS methods are used to detect aberrations like ALK translocations from exosomal RNA [64].

**Conclusions**

The great potential of liquid biopsy analysis in oncology has begun to be explored. In the next years, it is estimated that not only lung cancer, but other cancers specificities, will be discovered and established due to the large potential of biomarkers detection of the liquid biopsy. Being such a minimal invasive method and offering so many opportunities as a real-time monitoring method and a tool for determining the whole tumor heterogeneity and early diagnosis, liquid biopsy offers important prognosis factors. Due to its current limitations with respect to low cfNA concentration, low sensitivity, high cost, and low specificity, liquid biopsy is a challenge and it takes further studies to overcome these inconveniences.

**Conflict of interests**

The authors declare that they have no conflict of interests.

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Corresponding author
Natalia Motaš, MD, PhD, Department of Molecular Biology, OncoTeam Diagnostic S.A., 371 Griviță Avenue, Sector 1, 010719 Bucharest, Romania; Phone +4031–226 20 20, e-mail: natalia.motas@gmail.com

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