Liquid biopsies in lung cancer: The new ambrosia of researchers

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Abstract

In the last decades the approach to cancer patient management has been deeply revolutionized. We are moving from a "one-fits-all" strategy to the "personalized medicine" based on the molecular characterization of the tumor. In this new era it is becoming more and more clear that the monitoring of the disease is fundamental for the success of the treatment, thus there is the need of new biomarker discovery. More precisely in the last years the scientific community has started to use the term "liquid biopsy". A liquid biopsy is a liquid biomarker that can be easily isolated from many body fluids (blood, saliva, urine, ascites, pleural effusion, etc.) and, as well as a tissue biopsy, a representative of the tissue from which it is spread. In this review we will focus our attention on circulating tumor cells, circulating tumor DNA, exosomes and secretomes with the aim to underlie their usefulness and potential application in a clinical setting for lung cancer patient management.

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Contents

1. Liquid biopsies: definition, usefulness and potential applications ........................................... 539
2. Clinical perspectives and research opportunities from liquid biopsy in lung cancer ......................... 541
3. CTCs, ctDNA, exosomes and secretomes in NSCLC: what's new? .................................................. 541
  3.1. Circulating tumor cells (CTCs): clinical data in NSCLC and SCLC .................................................. 541
  3.2. Circulating tumor DNA (ctDNA): quantitative and qualitative alterations of ctDNA in lung cancer .... 542
  3.3. Exosome and miRNA in NSCLC .................................................................................................... 543
  3.4. Secretome in lung cancer ............................................................................................................ 545
4. Conclusion .................................................................................................................................. 545
References ........................................................................................................................................ 545

1. Liquid biopsies: definition, usefulness and potential applications

The term liquid biopsy [1] was originally introduced to define circulating tumor cells (CTCs). Currently it is also used for circulating tumor DNA (ctDNA) [2,3] as well as for exosomes [4] (Fig. 1).

Liquid biopsy analysis is currently a rapidly expanding field in translational cancer research as it might be useful at different points of the diagnostic/therapeutic course of cancer patients. They may be used for
(a) early diagnosis, (b) estimation of the risk for metastatic relapse or metastatic progression (prognostic information), (c) stratification and real-time monitoring of therapies, (d) identification of therapeutic targets and resistance mechanisms (predictive information), and (e) understanding metastasis development in cancer patients [1].

CTCs, ctDNA and exosomes are likely to contain a wider presentation of genomic data from multiple metastatic sites, whereas mutations present in a single biopsy or minor sub-clone may be missed [5].

Moreover, they may be obtained from almost all body fluids (blood, serum, plasma, urine, pleural effusion, ascites, etc...).

CTCs are formed by cell detachment from the primary tumor mass, determining the migration of tumor cells to secondary sites via the lymphatic and blood system. The presence of CTCs has been demonstrated in the blood of patients with various solid tumors [6] and it has been associated with poor outcome in metastatic non-small cell lung cancer (NSCLC) patients and small cell lung cancer (SCLC) as well as in other tumors [7,8].

As aforementioned, CTC analysis might be useful for both, understanding the metastatic process and also for monitoring the disease during treatment. In this regard there is a wide interest in the utility of circulating nucleic acids (CNAs) and proteins as biomarkers of cancer. Thus far, the majority of research has focused on circulating miRNAs, mRNA and DNA (defined also as cell-free DNA, cfDNA) due to their stability and easy isolation. Circulating miRNA has been extensively studied as a noninvasive biomarker for human disease. The signature profile of circulating miRNA has been shown to distinguish healthy from diseased individuals [9]. Recently the other non-coding RNA (ncRNA), the long-non-coding RNA (IncRNA) is being investigated as a novel biomarker in cancer patients [10].

ctDNA is released from both healthy and cancer cells but several studies demonstrate that the tumor-released ctDNA (also identified as circulating tumor DNA, ctDNA) is increased in cancer patients when compared to healthy controls and its concentration further increases with advanced disease [11]. The ctDNA can be used for molecular characterization and its exosome-wide analysis could also complement the current invasive biopsy approaches to identify mutations associated with acquired drug resistance in advanced cancers [3] or for treatment outcome monitoring.

Exosomes are small (40 to 100 nm) membrane derived vesicles that are released extracellularly following the fusion of multivesicular bodies or mature endosomes with the cellular membrane [12,13]. Exosomes are released from normal, diseased, and neoplastic cells and are present in the blood and other body fluids [14]. They contain a variety of molecules such as signal proteins and/or peptides, microRNAs, mRNAs, and lipids [15]. Molecules contained within these small vesicles can therefore move through the bloodstream and reach distant sites, enabling the exchange of “information and materials” between different sites. mRNA-containing exosomes have been shown to be functional, as murine exosomes “taken up” by human cells resulted in the synthesis of mouse proteins [15]. Because exosomes provide signals to distant cells, they act as a nanoparticle-based communication system [16] and could be fundamental for metastasis outcome [17]. Furthermore metastases harbor unique genomic characteristics that may not be detectable in the corresponding primary tumor of the same patients. Thus, the
mern molecular analysis of the resected primary tumor alone (current standard practice in oncology) or, even the evaluation of tumor characteristics based on the biopsy of the most accessible metastasis may not reveal sufficient information for treatment decisions [18].

Recently, Thakur et al. have demonstrated that exosomes also contain double-stranded DNA, defined as exoDNA, that could be characterized, as well. In the same paper the authors also assessed that exoDNA is representative for the whole genomic DNA and this finding strengthens the translational potential of exosomes as circulating cancer biomarkers [19].

The “secretome” is referred to as the rich, complex set of molecules secreted from living cells. More loosely the term also includes molecules shed from the surface of living cells. Secretome proteins play a key role in cell signaling, communication and migration [20]. The current protein-based serum biomarkers in lung cancer clinical practice include carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA 21-1), tissue polypeptide antigen (TPA), pro-gastrin releasing peptide (ProGRP), neuron-specific enolase (NSE) and tumor M2 pyruvate kinase [21].

2. Clinical perspectives and research opportunities from liquid biopsy in lung cancer

Cancer is not a “molecularly stable” disease, instead it is characterized by a high heterogeneity. In particular for lung cancer it is known that many patients with advanced EGFR-mutated or ALK-rearranged tumors do benefit from treatment with specific tyrosine kinase inhibitors (TKIs, such as gefitinib, erlotinib, crizotinib and ceritinib) [22–26]. Unfortunately the length of disease control is notoriously short due to the acquisition of resistance mechanisms. EGFR TKI-acquired resistance is mostly (50–60%) due to a point mutation in exon 20 (T790M) and occasionally (5–10%) due to c-MET amplification, among others [27,28]. Thus primary tumor and metastases might be molecularly different. Furthermore, metastasis is the leading cause of death in cancer patients while early-stage disease is starting to be considered curable, or, at least, is characterized by a remarkably high survival rate. A detailed characterization of metastases is therefore needed to improve patient outcome even in advanced-disease stages. The main issue in characterizing metastasis is its intrinsic nature, since it is often tricky and difficult for sampling. Liquid biopsies offer the chance to investigate primary tumor and metastasis through a non-invasive and simple blood tests. Another interesting feature of liquid biopsy, mostly of ctDNA analysis, is the opportunity to study “tumor dormancy” phenomenon. Cell-free DNA might offer the opportunity to monitor patients with no clinically detectable disease after surgery and standard therapy. It has been demonstrated that T790M mutations are detectable in the blood before radiological and symptomatic relapse [3,29,30]. Thus in the near future disease monitoring might also be integrated with ctDNA testing and CTC molecular characterization (Fig. 2).

3. CTCs, ctDNA, exosomes and secretomes in NSCLC: what’s new?

3.1. Circulating tumor cells (CTCs): clinical data in NSCLC and SCLC

Since they were first described by Thomas Ashworth in 1869 [31], the presence of circulating tumor cells has been suggested to be a characteristic of cancer by various early studies [32]. CTCs are cancer cells released from tumors into the bloodstream. Over the past years CTCs’ presence has been associated with worse prognosis in several major cancer types, including breast, prostate, colorectal and lung cancer. Through a proposed process known as the epithelial–mesenchymal transition (EMT), epithelial cells of solid tumors undergo cellular changes that enable them to escape their structural confines via increased mobility and invasiveness, to enter into the bloodstream, and to adhere to endothelial cells and give birth to distant metastases [33,34].

Thus, isolation and characterization of CTCs represent a very attractive challenge, as they may comprise both the phenotypic and genetic compositions of the primary tumors and potentially serve as a liquid biopsy for any metastatic or non-metastatic tumors. The analysis of CTCs’ compositions of the primary tumors and potentially serve as a liquid biopsy for any metastatic or non-metastatic tumors.

Fig. 2. Clinical application of CTC and ctDNA analyses in cancer patients. Modifications of CTC and ctDNA levels in plasma during disease progression. After diagnosis and treatment initiation CTC and ctDNA levels dramatically drop down according also to tumor growth decrease. At relapse both CTC and ctDNA levels increase and are more sensitive (ctDNA > CTCs) in relapse detection than CT/PET scan analysis.
increase in the number of cells predicts tumor progression. Furthermore, in NSCLC patients the total count of CTCs before chemotherapy initiation is associated with staging (higher detectable number of cells in stage IV patients) and the progression-free survival (PFS) and overall survival (OS) seem to be correlated to CTC count. In the study from Krebs G. et al., PFS and OS were higher among NSCLC patients with fewer than five CTCs per 7.5 ml of blood when compared to patients with five or more CTCs [7] and they conclude that CTC number is the strongest predictor of OS. In addition, the presence of circulating cells is associated with a significantly higher risk of lymph node metastasis but not with tumor histology in NSCLC [37]. Recently it has been proved the prognostic value of CTC enumeration in patients with SCLC [8,38]. The reduction of CTC count higher than 89% after one chemotherapy cycle improves prognostic accuracy and is associated with lower risk of death. Furthermore CTC count is strongly associated with the number of metastatic sites [39]. In Table 4 are listed the major clinical investigations of liquid biopsy in lung cancer.

CTCs might be prognostic markers in cancer patients and, in the future, therapy decision should also be made taking into account CTC analysis, especially for the selection of those patients at a higher risk of relapse who might benefit from adjuvant therapies.

Currently, there is only one technology approved for CTC enrichment and enumeration. This technique has been used in various clinical trials (breast, prostate and colon cancers) to establish in which subset of patient CTC count above a known threshold might be used as a prognostic marker and a predictor of patient outcome [40–42]. Based on these clinical trials, the US Food and Drug Administration (FDA) licensed the CellSearch® technology (Veridex, LLC, Raritan, NJ, USA) for CTC analysis in clinical practice. This system uses immunomagnetic purification with antibodies against epithelial cell adhesion molecule (EpCAM) from peripheral blood. The specificity of CellSearch® isolation is only based on EpCAM expression on CTC surface. Consequently, CTCs with low or absent expression of EpCAM are easily missed. Thus, tumor cells that have undergone EMT, and which are probably the most aggressive, are not detectable with CellSearch® technology. There is an increasing number of papers that, by using alternative detection technologies on the basis of EpCAM or other antigens, have demonstrated an apparent greater sensitivity than CellSearch® [43,44] but further studies in the context of larger clinical studies are warranted. In Table 1 are shown the most promising and interesting new techniques for CTC isolation.

While all these new methods of separation provide the advantage of enriching for viable CTCs, which may be collected and stained for cytopathological analysis or subjected to RNA or DNA analyses, the technologies still need to be improved because they are quite elementary and imprecise (especially density gradient and filtration methods). Thus the main issue is to strike a balance between lower number of CTCs but high purity and greater number of CTCs but reduced purity.

<table>
<thead>
<tr>
<th>Enrichment methods</th>
<th>Product</th>
<th>Cell viability</th>
<th>Antibody-based</th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>Downstream application</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biological properties</strong></td>
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<tr>
<td>Immunomagnetic bead</td>
<td>CellSearch System</td>
<td>x</td>
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<td>x</td>
<td>x</td>
<td>CTCs enumeration and analysis</td>
<td>[1]</td>
</tr>
<tr>
<td></td>
<td>Magsweeper</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>Molecular analysis</td>
<td>[2]</td>
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<tr>
<td></td>
<td>CTC–chip</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>CTCs enumeration and analysis</td>
<td>[3]</td>
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<tr>
<td></td>
<td>GILUPI Nanodetector</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>CTCs enumeration and analysis</td>
<td>[4]</td>
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<tr>
<td><strong>Physical properties</strong></td>
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<tr>
<td>Density gradient centrifugation</td>
<td>Immunorosettes</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>CTCs enumeration and molecular analysis</td>
<td>[5]</td>
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<tr>
<td>Filtration</td>
<td>ISET</td>
<td>x</td>
<td>x</td>
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<td>x</td>
<td>CTCs isolation and molecular analysis</td>
<td>[6]</td>
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<tr>
<td></td>
<td>CellSieve</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>CTCs isolation and molecular analysis</td>
<td>[7]</td>
</tr>
<tr>
<td>Dielectrophoresis</td>
<td>APOstream</td>
<td>x</td>
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<td>x</td>
<td>CTCs isolation and molecular analysis</td>
<td>[8]</td>
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<td></td>
<td>DEPArray</td>
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<td>x</td>
<td>x</td>
<td>x</td>
<td>CTCs isolation and molecular analysis</td>
<td>[9]</td>
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### 3.2. Circulating tumor DNA (ctDNA): quantitative and qualitative alterations of ctDNA in lung cancer

Currently the evaluation of specific predictive biomarkers is mandatory for a proper treatment of NSCLC patients according to the molecular characterization of the disease. Indeed, the EGFR mutation status assessment in NSCLC patients is definitely embedded in clinical practice. Nonetheless, molecular profiling can be difficult due to the very small amount of tumor tissue available for the analysis (as most are obtained by fine needle aspiration procedures). Many investigators have tried to solve this problem, leading to the development of more sensitive techniques to detect EGFR mutations [45,46]. ctDNA testing might be an ideal solution.

cDNA originates from both healthy and tumor cells [47,48] and may have broad clinical applications because it is non-invasive, convenient and ctDNA based assays can be performed repeatedly [49]. DNA released from necrotic malignant cells varies in size, whereas DNA released from apoptotic cells is uniformly truncated into 185- to 200-bp fragments. Because the main source of free circulating DNA in healthy individuals is from apoptotic cells, a preponderance of longer DNA fragments could be a marker for malignant tumor detection [50,51]. Cell-free DNA levels have been demonstrated to be higher in cancer patients compared with healthy controls [11,52–54].

cDNA can also be used for mutational analysis and there are already several proof-of-concept papers on the potential of ctDNA testing in the management of NSCLC patients. The whole exome sequencing of ctDNA through Next Generation Sequencing (NGS) has been demonstrated to provide relevant information about the molecular status of the tumor. Recently Murtaza et al. have performed whole exome sequencing of plasma DNA of six patients with advanced cancers (two with breast cancer, three with ovarian cancer and one with NSCLC). Interestingly in NSCLC patients, the analysis of the EGFR gene in ctDNA has shown

Table 1
CTC enrichment and isolation methods: A comparison of the available techniques.

<table>
<thead>
<tr>
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<td>x</td>
<td>x</td>
<td>x</td>
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<td>x</td>
<td>x</td>
<td>x</td>
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<td>x</td>
<td>x</td>
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<td><strong>Physical properties</strong></td>
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<td>x</td>
<td>x</td>
<td>x</td>
<td>CTCs isolation and molecular analysis</td>
<td>[9]</td>
</tr>
</tbody>
</table>

Table 2
ctDNA analysis.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative analysis</td>
<td>High</td>
<td>Low</td>
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<tr>
<td>Qualitative analysis (based on DNA specific mutation)</td>
<td>Sanger sequencing</td>
<td>Very low</td>
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<td></td>
<td>Pyrosequencing</td>
<td>Low</td>
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<tr>
<td></td>
<td>*AMS</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>*BEAMing</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Digital PCR</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>*TAM-Seq</td>
<td>High</td>
</tr>
</tbody>
</table>

* qPCR: quantitative polymerase chain reaction (PCR); NGS: Next Generation Sequencing; AMS: amplification refractory mutation system; BEAMing: beads, emulsion, amplification and magnetic; TAM-Seq: tagged-amplicon deep sequencing.
the occurrence of the resistance mutation T790M at progression, but not at the time of treatment initiation [3]. cfDNA quantification may also be associated to tumor burden estimated by Positron Emission Tomography/Computerized Tomography (PET/CT) [55]. The evaluation of the KRAS mutation in cfDNA from NSCLC individuals has demonstrated that patients with a detectable plasma-KRAS mutation had a significantly shorter OS and PFS compared to patients showing wild type (WT) plasma-KRAS. Thus, the detection of KRAS mutations in plasma may earn a prognostic as well as a predictive value [56].

Despite the promising results obtained in support of the feasibility of ctDNA testing there is still a lack of widely accepted and approved methodologies for ctDNA analysis. To successfully detect the presence of cfDNA in plasma or serum, a suitable methodology needs to be selected. This consists of several essential steps: 1) processing collected blood samples with normal lung tissues [67]. On the basis of this genetic profile, exosomal miRNA as a diagnostic marker for lung cancer. Comparisons between peripheral circulation miRNA-derived exosomes and miRNA-derived tumors indicated that the miRNA signatures were not

3.3. Exosome and miRNA in NSCLC

Exosomes are small membrane vesicles of endocytic origin and were initially isolated from the peripheral circulation of patients with cancer in 1979 [62,63]. Exosomes appear to play a central role in cell-to-cell communication by a direct activation of surface-expressed ligands or by transferring molecules between cells. Exosomes can have a bimodal role in cancer: they can either manipulate the local and systemic environment allowing cancer growth and dissemination or modulate the immune system to elicit or suppress an anti-tumor response [64,65]. Exosomes are rich in RNA fragments, proteins, mRNA and microRNA (miRNA). Interestingly enough, exosomal RNA has been shown to determine horizontal transfer of genetic information between cells [66]. In recent years, studies on exosome's prognostic and predictive value in NSCLC have evolved and new evidences have emphasized their potentiality in clinical practice. Biological molecules (protein, RNA and miRNA) well protected by a lipid bilayer membrane that confers high degree of stability, are contained in exosomes and can be isolated and analyzed quite simply. The genome-wide expression profiling of miRNAs has been shown to be significantly different among primary lung cancers and corresponding noncancerous lung tissues and thus has shown to have a potential role as a diagnostic marker. An overexpression of 12 specific miRNAs (hsa-miR-17-3p, hsa-miR-21, hsa-miR-106a, hsa-miR-146, hsa-miR-155, hsa-miR-191, hsa-miR-192, hsa-miR-203, hsa-miR-205, hsa-miR-210, hsa-miR-212, and hsa-miR-214) has been revealed comparing the miRNA expression profile of lung cancer samples with normal lung tissues [67]. On the basis of this genome-wide expression profile, Rabinowits et al. [68] investigated the role of exosomal miRNA as a diagnostic marker for lung cancer. Comparisons between peripheral circulation miRNA-derived exosomes and miRNA-derived tumors indicated that the miRNA signatures were not

### Table 3

<table>
<thead>
<tr>
<th>Isolation methods</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differential centrifugation</td>
<td>It consists of multiple steps:</td>
<td>- Is considered the gold standard method</td>
<td>- This procedure cannot achieve absolute separation by size because sedimentation also depends on the density and content of vesicles.</td>
<td>[9–11]</td>
</tr>
<tr>
<td></td>
<td>- Depletion of cells, cell debris and large apoptotic bodies by low-speed centrifugation (300 × g 10')</td>
<td>- Is widely used to isolate vesicles from body fluids and conditioned medium</td>
<td>- The pellet could contain proteins, complexes, lipoproteins and other contaminants.</td>
<td></td>
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<tr>
<td></td>
<td>- Larger vesicles are collected after a higher speed spin (10,000 × g)</td>
<td></td>
<td>- Extremely cumbersome process (4-5 h).</td>
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</tr>
<tr>
<td></td>
<td>- Smaller vesicles (exosomes) are then pelleted at high speed (100,000–120,000 × g).</td>
<td></td>
<td></td>
<td>[9]</td>
</tr>
<tr>
<td>Size exclusion</td>
<td>Cell fragments and larger vesicles can be excluded by a filtration step with a 0.8 μm pore size filter. To collect smaller vesicles (exosomes), a 0.2 μm pore size filter can be used.</td>
<td>- Allow the separation of vesicles based on size</td>
<td>- Forcing vesicles through a filter could cause their deformation or breakup.</td>
<td>[12–14]</td>
</tr>
<tr>
<td>Immunofinity isolation</td>
<td>The presence of characteristic surface proteins on different vesicle populations, allows the specific isolation of desired vesicles through the immunofinity isolation, in which antibodies to surface proteins (CD9, CD63, CD81, MHC) are used to select desired vesicles population.</td>
<td>High specificity and characterization of vesicles</td>
<td>- Lower yields because some markers are not recognized on all vesicles.</td>
<td>[15,16]</td>
</tr>
<tr>
<td>Microfluidic devices</td>
<td>Micro- and nano-fluidic methods separate nanoparticles based on physical properties.</td>
<td>- Reduce material costs and samples volume</td>
<td>- The obtained population cannot be used for further functional assays.</td>
<td>[17,18]</td>
</tr>
<tr>
<td>Polymeric precipitation (ExoQuick)</td>
<td>Volume-excluding polymers, such as PEGs, are capable of differentiating precipitating vesicles thereby allowing exosome isolation by low-speed centrifugation.</td>
<td>- Single step capture</td>
<td>- It is not suited for isolation of large amount of vesicles.</td>
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<td></td>
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<td>- Technically easy</td>
<td>- The purity of the isolated material is not certain.</td>
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<td></td>
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<td>- Require small amount of sample</td>
<td>- High contamination from lipoproteins.</td>
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<td>- Short time of isolation</td>
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<tr>
<td></td>
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<td>- High yield of RNA</td>
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</tbody>
</table>
The table summarizes the major clinical investigation of liquid biopsy in NSCLC disease.

<table>
<thead>
<tr>
<th>First author</th>
<th>Liquid biopsy type</th>
<th>Detection method</th>
<th>Correlation with reported outcome</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs G. et al. [20]</td>
<td>CTC</td>
<td>Monitoring of tumor genotypes changing during the course of treatment</td>
<td>X</td>
<td>Monitoring of tumor genotypes changing during the course of treatment</td>
</tr>
<tr>
<td>Maheswaran S. et al. [19]</td>
<td>CTC and ctDNA</td>
<td>Pre and post-treatment</td>
<td>X</td>
<td>Pre and post-treatment</td>
</tr>
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<td>Igawa et al. [23]</td>
<td>Igawa et al. [23]</td>
<td>Pre- and post-treatment</td>
<td>X</td>
<td>Pre- and post-treatment</td>
</tr>
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<td>Nyagaard et al. [25]</td>
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<td>Pre-treatment</td>
<td>X</td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>Rabinovits et al. [27]</td>
<td>Exosomal microRNA</td>
<td>Exosomal microRNA</td>
<td>X</td>
<td>Exosomal microRNA</td>
</tr>
</tbody>
</table>

**Sampling time**

- Multiple-time points
- Before and after administration of one cycle of standard chemotherapy
- Pre and post-treatment
- Pre and post-treatment
- Pre treatment
- Prior to first-line therapy and at relapse

**Correlation with reported outcome**

- Correlation between CTC counts and instrumental response in extensive SCLC
- Correlation between CTC counts and instrumental response in extensive SCLC
- CTC count predictive response to chemotherapy in SCLC
- EGFR is not simple marker for tumor
- Circulating exosomal miRNA can be used as screening test for lung adenocarcinoma
- Prognostic role in extensive SCLC
- Plasma genotype allows non-invasive assessment of response and resistance
- Marker for diagnosis and prognosis
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- Marker for diagnosis and prognosis

**Conclusion**

- Monitoring of tumor genotypes changing during the course of treatment
- Monitoring of tumor genotypes changing during the course of treatment
- CTC count predictive response to chemotherapy in SCLC
- EGFR is not simple marker for tumor
- Circulating exosomal miRNA can be used as screening test for lung adenocarcinoma
- Prognostic role in extensive SCLC
- Plasma genotype allows non-invasive assessment of response and resistance
- Marker for diagnosis and prognosis

**Significance**

The data presented in Table 4 illustrate the clinical utility of liquid biopsy in NSCLC disease. The table indicates that multiple methods have been utilized for the detection of tumor cells and circulating tumor DNA (ctDNA). These methods include cell-based approaches (e.g., CTC) and DNA-based approaches (e.g., ctDNA). The correlation between these methods and clinical outcomes is varied, with some studies reporting a significant correlation and others not. The conclusion is that liquid biopsy can be a valuable tool in the management of NSCLC, but further research is needed to optimize the methods and interpret the results accurately.
functions of exosomes in cancer progression and will aid in the development of novel diagnostic tools for NSCLC [78].

3.4. Secretome in lung cancer

The term “secretome” was introduced by Tjalsma et al. in a study of the secreted proteins in *Bacillus subtilis* [79] and includes proteins released by a cell, tissue or organism through different secretion mechanisms [80]. The cancer secretome was first mentioned in 2006 [81]. Secreted proteins participate in various physiological processes such as immune defense, blood coagulation and cell signaling and also play crucial roles in pathological processes including cancer angiogenesis, differentiation, invasion and metastasis. Proximal biological fluids (e.g. ascites fluid of ovarian cancer or pleural effusion of lung cancer) that are in contact with the tumor may be enriched with proteins secreted or shed by cancer cells. Such proteins could enter the circulation and be detected in body fluids such as blood and urine [20]. The malignant pleural effusion is a clinical complication that occurs relatively often in NSCL. This malignant effusion has been analyzed to identify useful biomarkers in lung cancer and recently a “secretomic-profile” of malignant pleural effusion from lung adenocarcinoma patients has been proposed. Researchers were able to identify 482 non-redundant proteins. Through subsequent in vitro analyses, 4 potential protein biomarkers significantly associated with malignancy were isolated. These findings collectively suggest that the malignant pleural effusion proteome (secretome) from adenocarcinoma NSCLC provides a useful data set for malignancy biomarker research [82]. Several groups have studied the proteomic profile of secreted proteins derived from NSCLC cell lines [83,84] and have tried to translate the obtained results into clinical practice. Despite the fact that the interest in secretome analysis of NSCLC patients is progressively growing, there is still a gap between in vitro secretome testing and clinical applications.

4. Conclusion

Our understanding and knowledge of cancer have changed dramatically over the last 30 years. The discovery of oncogene addiction has paved the way for the development of “intelligent” drugs, able to target only cancer cells. The introduction of molecular biomarkers in clinical practice has changed the natural history of many tumors (c-KIT and PDGFRα in GIST, EGFR in lung cancer, BRAF in melanoma and KRAS in colorectal cancer) and the molecular characterization of cancer through NGS still holds many surprises. Nevertheless, the major limitation of these drugs is the rise of resistance mechanisms that impair and slow down the development of genotype-directed therapy in solid tumors. Tumor cells never reach a steady state. They are continuously changing and they acquire new mutations to survive in an adverse environment. The “dark side” of a targeted agent is due to the selective pressure that favors the selection of most aggressive cells. These cells are likely the ones that escape from the primary tumors and colonize distant districts causing metastasis onset. Since these findings, metastatic cells are thought to be a clone of the primary tumors with a more aggressive behavior. It is now becoming increasingly clear that metastasis and tumors are extremely heterogeneous and therefore, metastatic disease has been proposed as a different condition. From these considerations it is clear how important is the characterization of the new lesion through a re-biopsy. Unfortunately re-biopsy is not always feasible due to many reasons (e.g. comorbidity of the patients, the lack of a safe access to the lesion). In this context, liquid biopsies could be an effective solution. Recently, Oxnard et al. [30] have demonstrated that cell-free plasma DNA is suitable for monitoring treatment response but also for the early detection of acquired resistance mechanisms (e.g. T790M in NSCLC). These and other studies are setting the basis for the development of cfdNA as a new and non-invasive biomarker that can be used routinely as predictive biomarker for targeted therapies.

The value of CTC analysis is, at present, mainly correlated to prognosis but there is an increasing interest in the development of new techniques for their molecular characterization. CTCs are cells that detach from the primary tumors and migrate through the bloodstream. It is not fully understood whether CTCs are the only cause of metastasis but it is well known from clinical studies that their count is proportional to a poorer prognosis in various tumor types. The prognostic value of CTCs is then almost widely accepted while there is still the need of effort for their molecular characterization.

Exosome analysis in cancer is a new field and there are still many questions about their function. Exosomes can be considered as “operating/active secretions” of cancer cells and, as secretome, they might be representative for the functional status of tumor cells. So, these microvesicles are more than simple exophytic budding of cell membrane. Thus, the analysis of molecules contained in exosomes can provide additional information about the tumor biology.

These new insights are paving the way for a deep changing in the approach of patients’ management. Analysis based on tissue biopsies will be complemented with the information obtained from cfdNA and exosome characterization. This information, together with the one obtained from the imaging analysis, will probably guide treatment decision and will improve the outcome for lung cancer patients. Another important aspect of liquid biopsy is that, due to their non-invasive nature, they might be applied also in a diagnostic setting. It is well known that lung cancer is an insidious disease that most frequently occurs in advanced stage. This is mainly due to the lack of a screening test for the disease and liquid biopsy can be a useful tool for diagnostic purpose.

Nevertheless there are still several limitations for the introduction of liquid biopsy in a clinical setting. Except for CTC enumeration with the Veridex system (FDA-approved), there are no validated techniques both for cfdNA and exosome analysis. cfdNA evaluation is not yet standardized and, for instance, the optimal sampling specimen (plasma or serum) is not yet known. Moreover among laboratories that work with cfdNA there are not yet optimized, standardized and widely approved procedures for blood collection and downstream analysis. Following this consideration, the increasing effort in liquid biopsy development is understandable, even if the road is still long and winding, but hopes are high to see them included in clinical practice.

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