Review

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Biomarkers in the management of lung cancer: changing the practice of thoracic oncology

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Abstract: Lung cancer currently represents a leading cause of cancer death. Substantial progress achieved in the medical therapy of lung cancer during the last decade has been associated with the advent of targeted therapy, including immunotherapy. The targeted therapy has gradually shifted from drugs suppressing general mechanisms of tumor growth and progression to agents aiming at transforming mechanisms like driver mutations in a particular tumor. Knowledge of the molecular characteristics of a tumor has become an essential component of the more targeted therapeutic approach. There are specific challenges for biomarker determination in lung cancer, in particular a commonly limited size of tumor sample. Liquid biopsy is therefore of particular importance in the management of lung cancer. Laboratory medicine is an indispensable part of multidisciplinary management of lung cancer. Clinical Chemistry and Laboratory Medicine (CCLM) has played and will continue playing a major role in updating and spreading the knowledge in the field.

Keywords: biomarkers; immunotherapy; lung cancer; targeted therapy.

Introduction

Both laboratory medicine and medical oncology have undergone an unprecedented transformation that was certainly not imagined (and even not imaginable) 60 years ago, changing their standing among other medical and surgical specialties from marginal to central due to the technological progress as well as advances in the understanding of molecular pathogenesis of human diseases, including cancer [1]. In contrast to medical and surgical specialties devoted to the study of organ systems, e.g. gastroenterology, dermatology or urology, laboratory medicine and medical oncology tackle problems across virtually all organ systems and in daily practice require multidisciplinary interaction with other branches of medical and surgical science. The systemic and multidisciplinary nature represents a fundamental feature of both laboratory medicine and medical oncology. Among other bilateral and multilateral multidisciplinary interactions, the collaboration between laboratory medicine and medical oncology is of particular significance from the point of view of both specialties. The role of laboratory medicine in the practice of medical oncology has obviously evolved, in particular during the last two decades. With the advent of targeted therapy medical oncologist rely on the identification of druggable targets associated with tumor growth and progression. The best current example of the new role of laboratory medicine in guiding the medical therapy of cancer is probably the management of lung cancer.

Lung cancer currently represents a leading cause of cancer death [2, 3]. The incidence of lung cancer increased markedly during the 20th century, mostly due to an increase in the smoking of tobacco [4]. More recently, a decrease in the incidence of lung cancer has been documented in some populations, probably due to a change of life-style habits [5].

The unprecedented progress in the management of solid tumors during the past two decades is due to improved diagnosis that widened the possibility of curative treatment options as well as advances in surgical, radiation and medical therapy, including supportive care. Substantial progress has also been achieved in the treatment of lung cancer. In fact, a decrease of mortality exceeding the decrease in incidence has been documented for non-small cell lung cancer (NSCLC) in temporal association with the advent of targeted therapy [5].

In the present review, current status of the management of patients with lung cancer is briefly reviewed leading to the discussion of the role of biomarker determination in shaping the therapeutic strategy in the targeted therapy era.
Epidemiology, biology and clinical presentation of lung cancer

Based on histology, lung cancer is divided into two principal types, small cell lung cancer (SCLC) [6] and NSCLC [7]. Although both SCLC and NSCLC can be further divided into a number of subtypes, defined mostly by molecular characteristics, the distinction between SCLC and NSCLC has been historically of fundamental importance for the management strategy.

Tobacco smoking is by far the most predominant etiologic factor in both NSCLC and SCLC [3, 4]. An important aspect of lung cancer is the presence of significant medical comorbidities [8]. Besides chronic obstructive lung disease, tobacco smoking, the principal etiologic factor of lung cancer, also markedly increases the risk of cardiovascular diseases, including ischemic heart disease, stroke or ischemic disease of lower extremity, as well as other disorders like rheumatoid arthritis [4]. Tobacco smoking also increases risk of many primary tumor outside the lung, and second primary tumors are common in survivors of lung cancer, and, vice versa, lung cancer is common in survivors of tobacco-associated extrapulmonary tumors. This adds a complexity to the management of patients with lung cancer, including the use of biomarkers.

Much progress has been achieved in understanding the molecular pathogenesis of lung cancer. In particular, driver genetic changes have been identified that are responsible for malignant transformation in individual cases of NSCLC [9]. The knowledge of molecular pathogenesis has led to remarkable achievements in the targeted therapy outlined in the following paragraphs [10].

As summarized below, the diagnosis of lung cancer may be difficult and is still mostly being made in patients who present with symptoms rather than within screening programs in asymptomatic individuals. The improvement in survival of patients with NSCLC is mostly due to improved therapies [5], and the principal role of biomarkers in the management lies in guiding the systemic therapy [10].

Diagnosis of lung cancer

Although screening programs using low-dose computed tomography (CT) are being slowly introduced [3, 11], the majority of cases of lung cancer are still being diagnosed in symptomatic individuals, and, consequently, most patients with lung cancer still present at an advanced stage. Patients can present with hemoptysis, dyspnea, or chest pain as well as systemic manifestation like weight loss or recurrent infections or manifestations of the distant metastases in the central nervous system, skeleton or liver, or paraneoplastic disorders including hypercalcemia or hyponatremia [6]. All the symptoms and disorder listed above should arise suspicion of lung cancer, in particular in a person with history of tobacco smoking, but are also non-specific and accompany other non-neoplastic disorders.

The diagnostic approach in patients with lung mass typically involves an imaging study and bronchoscopy. Tumor biopsy is essential not only to establish the diagnosis, but also to guide therapy as will be outlined below [10]. Circulating tumor markers are being widely used to complement the diagnosis and to follow the disease course, but have limited role in establishing the diagnosis [3, 12]. Alterations in a number of protein or carbohydrate antigens [13–17] have been described in patients with lung cancer, and some of these biomarkers may be used in the clinical practice, e.g. pro-gastrin-releasing peptide be used to support the diagnosis of SCLC [18]. The diagnostic utilization of circulating tumor markers may be complicated by the fact that many of these biomarkers are increased in different primary tumors, e.g. human epididymis protein 4 is increased not only in lung cancer [17], but is mostly used in the management of ovarian cancer [16]. Cytokines like stem cell factor and granulocyte-macrophage colony stimulating factor that have also been proposed as lung cancer biomarkers [19] also lacked specificity. Other non-specific biomarkers include 8-hydroxydeoxyguanosine, a biomarker of DNA turnover [20]. Recently, artificial intelligence methods have been introduced as an aid in the diagnosis of lung cancer, but this approach remains experimental [21].

The screening strategy based on low-dose CT has limits to its use because of the logistics, cost and also concern about stochastic effects of radiation [10, 11]. Considerable research has been devoted to potential circulating biomarkers for early lung cancer detection, but so far with little success [22]. Circulating protein or carbohydrate protein markers are not useful in lung cancer screening. More promising screening approaches across a spectrum of solid tumors include, among others, detecting mutations of circulating DNA [23], messenger RNAs [24], non-coding RNAs [25, 26], epigenetic changes like methylation pattern of specific genes [22, 27], or lipidomic profiling [28]. Despite these efforts there is currently no biomarker that could be used routinely in population screening. As outlined below the molecular genetic approaches associated with liquid biopsy are used to detect minimal residual disease or for the follow up in patients in whom lung cancer has already been diagnosed.
Of particular interest are the studies of potential biomarkers in the exhaled air [29, 30]. There are even studies on canine olfactory detection, i.e. using dogs to detect cancer [31, 32]. These approaches are obviously attractive because of the non-invasive nature, but at present remain only investigational.

Current treatment strategies in patients with lung cancer

As is the case for tumors of other primary locations, the management strategy in patients with lung cancer is based on a multidisciplinary approach [28]. This tenet is also true for the treatment that encompasses surgical resection, radiation therapy and pharmacotherapy. The patients are evaluated initially before the start of any treatment to assess the extent of disease and general physical fitness. The extent of disease is reflected in disease stage, and imaging studies are used to define stage in individual patients. Positron-emission tomography (PET-CT) has been shown to be the superior imaging method in preoperative evaluation [33]. In patients in whom surgery is planned a general medical examination should include the assessment of lung function [34]. Although the cornerstones of initial evaluation are imaging by PET-CT and functional spirometry studies, routine laboratory studies are an integral part of this assessment [12, 35].

Surgery

Surgery remains the mainstay of curative therapy of lung cancer. Surgery is the principal curative treatment modality in early NSCLC [34]. Surgery should be considered by the multidisciplinary team in every NSCLC patient in whom there is no evidence of metastatic disease. However, even many patients in whom no distant metastases are detected do not ultimately undergo surgery. There are two principal categories of circumstances that preclude radical surgery, technical inoperability and medical inoperability [36, 37]. Surgical approaches have undergone a substantial evolution in the past decades towards minimally invasive procedures [34]. The determination of biomarkers has currently limited role guiding surgical therapy of NSCLC. In contrast to NSCLC, SCLC is considered a systemic disease, and surgical therapy has limited role in the management of SCLC [6].

Radiation therapy

Radiation therapy may be used either alone or in combination with other therapeutic approaches as part of multimodality treatment. In medically inoperable NSCLC patients, radiation treatment, in most cases stereotactic radiotherapy, is an alternative to surgical resection [36]. Patients with technically inoperable tumors have more advanced disease that usually cannot be treated by stereotactic techniques, but other techniques of conformal radiotherapy may be used [38]. Target volume include the primary tumor as well as involved mediastinal lymph nodes. When possible, external beam radiation is combined with concurrently administered platinum-based chemotherapy [37]. More recently, the combination of external beam radiation and chemotherapy has been complemented with sequential administration of immunotherapy (durvalumab) [39]. Radiation therapy is also an important component in the management of SCLC [38]. In patients with metastatic tumors, external beam radiation is used to control brain metastases or symptomatic skeletal metastases [40], and may even be used with curative intent in the setting of oligometastatic disease [41]. Similarly to the surgical therapy, biomarkers still play limited role in planning radiation therapy or follow up of patients during this treatment [37].

Medical therapy

Medical therapy of both NSCLC and SCLC has evolved substantially over the last decade [10]. In the management of lung cancer hormonal therapy has been limited to the use of corticosteroids as part of symptomatic treatment, and until recently, medical therapy of NSCLC and SCLC has been dominated by cytotoxic chemotherapy. The management strategy has been transformed fundamentally with the advent of targeted therapy, including immunotherapy, and medical management of lung cancer, in particular NSCLC, currently represent an example of successful implementation of the paradigm of precision medicine [42].

Chemotherapy

Cytotoxic agents have been shown to be effective in both early and advanced lung cancer [43, 44]. In both cases, systemic disease in the form of microscopic (adjuvant therapy in early disease) or macroscopic (therapy in advanced disease setting) rather than the primary tumor is being treated. The backbone of chemotherapy regimes in both SCLC and NSCLC, and in NSCLC in the adjuvant, neo-adjuvant and advanced disease settings remains platinum (cisplatin). Cisplatin-based doublet chemotherapy regimens have been shown to improve overall survival after radical surgery in patients with NSCLC [45, 46].

In untreated patients with advanced or metastatic disease, platinum-based combination doublets remain the
standard of cytotoxic chemotherapy of untreated lung cancer, both NSCLC and SCLC [47]. In general, cisplatin is preferred over carboplatin as it is considered to be slightly superior in efficacy, but because comorbidities are common in patients with lung cancer, carboplatin is used in many cases [48].

In SCLC platinum-etoposide remains the standard chemotherapy backbone [44, 49]. Patients with early (formerly called limited-stage) SCLC are treated with the combination of systemic therapy and radiation [50]. In patients with SCLC who progress with some delay after prior platinum chemotherapy, re-challenge with platinum-based regimen may be considered. In other patients, single agent topotecan or paclitaxel may be used, but the efficacy is limited [51, 52].

In NSCLC a number of drugs have demonstrated comparable activity in combination with cisplatin, including taxanes (paclitaxel or docetaxel), gemcitabine, vinorelbine or pemetrexed [47, 53–55]. In patients with non-squamous histology the combination of pemetrexed with cisplatin was associated with slightly superior outcomes [56]. Triplet combinations did not show an advantage over doublet combinations featuring platinum and one of the agents mentioned above [57–59]. In the chemotherapy era, the median progression-free survival was less than six months with median overall survival being less than a year [47]. Docetaxel was the most commonly used second-line cytotoxic agent, but the activity was limited [60].

Biomarkers play a limited role in planning chemotherapy or follow up of patients during the treatment. If circulating tumor markers are elevated, serial measurement may be used to assess response, but imaging studies remain the standard method of response evaluation. In patients with early tumors treated with primary surgery, examination of gene expression using microarrays or real time reverse-transcriptase (RT)-polymerase chain reaction (PCR) has been introduced to aid with decision on adjuvant treatment across the range of solid tumors. While in some tumors, e.g. breast cancer these results are of relevance for treatment planning [61], these gene-signature profiles have found limited use in daily clinical practice [62]. In patients with NSCLC, the expression of excision repair cross-complementation group 1 (ERCC1) protein has been proposed as a biomarker of resistance to platinum-based chemotherapy in NSCLC [63], but clinical application has been hampered by problems associated with antibody specificity [64]. Other experimental approaches to identify included serum protein profiles detected with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry [65], but this approach also did not find utilization in the clinical practice. Thus, there is currently no biomarker that would reliably identify patients with tumors sensitive or resistant to cytotoxic agents.

**Targeted therapy**

As mentioned above, the advent of targeted therapy translated into fundamental advances in the management lung cancer. Conceptually, the targeted drugs could be divided into two broad categories. The agents of the first category target molecular pathways or mechanism common to most (if not all) malignant tumors. These drugs have a broad spectrum of activity across different tumors types, but the magnitude of the therapeutic effect is usually limited, and these agents are most active in combination with cytotoxic or hormonal drugs, or other targeted agents. Moreover, it has been difficult to identify predictive biomarkers for this category of agents. The second category is represented by agents that aim at targets that are essential for the growth and progression of a given tumor, usually a driver mutation. These agents are active in a much smaller proportion of patients, but the magnitude of treatment effect is typically much greater, these agents are active as monotherapy and, most importantly, have molecularly defined biomarkers that predict the efficacy. Table 1 lists principal agents of both categories currently used in the treatment of NSCLC. Currently, more than 20 different targeted agents are available for the treatment of NSCLC and the list is rapidly expanding. Currently available agents include bevacizumab [66], ramucirumab [67], nintedanib [68], gefitinib [69], erlotinib [70], afatinib [71], dacomitinib [72], osimertinib [73], crizotinib [74], ceritinib [75], alectinib [76], brigatinib [77], ensartinib [78], lorlatinib [79], dabrafenib [80], trametinib [80], vemurafenib [81], selpercatinib [82], pralsetinib [83], capmatinib [84], tepotinib [85], entrectinib [86], larotrectinib [87], sotorasib [88], adagrasib [89], amivantamab [90], or trastuzumab deruxtecan [91], administered alone or in combinations. The table does not include other targeted agents used in NSCLC based on tumor-agnostic paradigm in the presence of BRCA or phosphatidylinositol 3-kinase mutations. Some agents, e.g. epidermal growth factor receptor (EGFR) kinase may be viewed as falling into both of the categories mentioned above. In fact, EGFR inhibitors have been first introduced in NSCLC patients non-discriminately as agents targeting a common cell signaling pathway resulting in limited efficacy [70], but when NSCLC patients were selected for the treatment based on the presence of EGFR driver mutations a remarkably higher activity was observed in comparison with chemotherapy [92], hitherto the standard of care. On the other hand, some agents may target more than one driver genetic changes, e.g. crizotinib or entrectinib. Most
of the agents are small molecules inhibiting the tyrosine kinase activity of target proteins, but some monoclonal antibodies or monoclonal antibody conjugates have also been recently introduced [91]. Amivantamab, a bispecific monoclonal antibody with specificity against EGFR and mesenchymal-epithelial transition factor (MET) that may also engage the immune response represents a new concept in the targeted therapy [90].

A fundamental feature of targeted therapy is that it is essentially tumor agnostic [93], meaning that the same treatment regimen may be effective in tumors with different primary origin because of targeting general mechanisms of tumor growth and progression or tumors bearing the same genetic changes driving the malignant transformation. The tumor-agnostic nature of targeted therapy is of particular importance in the management of patients with rare driving mutations. As outlined below, same drug combination regimen is used in relatively rare patient with BRAF-mutated NSCLC as in much more common BRAF-mutated skin melanoma [94]. On the other hand, the situation may be more complex in NSCLC with some molecular targets, e.g. human epidermal growth factor receptor 2 (HER-2) [95], in contrast to other tumors like breast carcinoma. Three mechanisms of HER-2 activation have been described in NSCLC, including mutation, gene amplification and protein over-expression. High response rate to the treatment with trastuzumab deruxtecan has been demonstrated in patients with HER-2 mutated NSCLC [91].

In SCLC the identification of druggable targets is lagging behind NSCLC. Despite promising activity of some agents, e.g. alisertib, an inhibitor of aurora kinase A [96], none of these therapies have been established in the clinical practice. Thus, identification of targeted therapies for SCLC remain an unmet medical need.

**Immunotherapy**

Cancer immunotherapy may be regarded as a form of targeted therapy, and, similarly to other targeted agents the introduction of immune checkpoint inhibitors has resulted in substantial improvement of outcomes in NSCLC and SCLC. Immunotherapy with immune checkpoint inhibitors is also, by the essence, tumor agnostic. Activity of immune checkpoint inhibitors administered as monotherapy or in combination regimens has been proven for most common solid tumors, including both NSCLC and SCLC [97–99]. When administered in properly selected patient population, immune checkpoint inhibitors not only improved the efficacy outcomes, but were also associated with significantly decreased toxicity [99, 100]. Similar to the other targeted agents, the lists of drugs active in patients with lung cancer is rapidly expanding (Table 2). Immune checkpoint inhibitors have shown activity in both NSCLC [99–103] and SCLC [104]. In NSCLC, immunotherapy, as monotherapy or in combination regimens, is used in advanced or metastatic disease [99, 100, 103, 105] as well as in adjuvant or neoadjuvant [106, 107] settings. All agents currently available are monoclonal antibodies, including ipilimumab [103], nivolumab [99, 100], pembrolizumab [97], atezolizumab [101, 102, 104], durvalumab [39], and cemiplimab [108].

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### Table 1: Targeted agents currently used in the treatment of NSCLC.

<table>
<thead>
<tr>
<th>Target</th>
<th>Molecular genetic basis</th>
<th>Drug</th>
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<tr>
<td>Common mechanisms</td>
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<tr>
<td>Angiogenesis (VEGF)</td>
<td>Complex</td>
<td>Bevacizumab</td>
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<tr>
<td></td>
<td>Inhibition of growth signals (multiple receptor tyrosine kinases)</td>
<td>Ramucirumab</td>
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<tr>
<td>Growth signals</td>
<td></td>
<td>Gefitinib</td>
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<td></td>
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<td>Erlotinib</td>
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<td></td>
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<td>Nintedanib</td>
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<tr>
<td>Individual driver genetic changes</td>
<td>Mutation</td>
<td>Gefitinib</td>
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<td></td>
<td>First generation</td>
<td>Erlotinib</td>
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<td></td>
<td>Second generation</td>
<td>Gefitinib</td>
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<td></td>
<td>Third generation</td>
<td>Gefitinib</td>
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<tr>
<td>EGFR</td>
<td>Rearrangement (fusion)</td>
<td>Crizotinib</td>
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<tr>
<td></td>
<td>First generation</td>
<td>Crizotinib</td>
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<tr>
<td></td>
<td>Second generation</td>
<td>Crizotinib</td>
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<tr>
<td></td>
<td>Third generation</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>ALK</td>
<td>Translocation</td>
<td>Crizotinib</td>
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<tr>
<td>ROS1</td>
<td>Mutation</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>BRAF</td>
<td>Rearrangement (fusion)</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>RET</td>
<td>Rearrangement (fusion)</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>MET</td>
<td>Mutation or amplification</td>
<td>Crizotinib</td>
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<tr>
<td>NTRK</td>
<td>Rearrangement (fusion)</td>
<td>Crizotinib</td>
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<tr>
<td>KRAS</td>
<td>Mutation</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>HER-2</td>
<td>Mutation</td>
<td>Crizotinib</td>
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</table>

ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; HER-2, human epidermal growth factor receptor 2; KRAS, Kirsten rat sarcoma; MET, mesenchymal-epithelial transition factor; NTRK, neurotropic tyrosine kinase; VEGF, vascular endothelial growth factor.
Biomarkers in lung cancer

Historically, the utilization of biomarkers in the management of lung cancer patients has been rather limited. Determination of circulating tumor markers has been used for decades to support the diagnosis and to monitor therapeutic efficacy [12], but laboratory investigations currently provide, at best, only auxiliary information, and therapeutic decisions rely almost exclusively on imaging studies. Concentrations of biomarkers of immune and inflammatory response such as C-reactive protein or neopterin might have been used for the monitoring of complications of therapy or to assess prognosis [109]. The measurement of different analytes has been used in the clinical routine during the diagnostic process and to assess and monitor the complications of the disease course and treatment [35].

With the advent of targeted therapy, the determination of biomarkers became an essential and indispensable part of the management strategy and treatment decisions [42]. Although the determination of biomarkers is gradually gaining some role in the surgical therapy and radiotherapy, by far the most important is the role of molecular biomarkers in shaping the strategy of systemic therapy [110].

Sample matrix: the concept of liquid biopsy

Sample matrix is an issue of fundamental importance in the assessment of biomarkers in lung cancer. In contrast to other solid tumors, there are several points that have to be considered in patients with lung cancer [111]. First and most importantly, the amount of tumor tissue available is limited as relatively few patients are treated with surgery, and specimens of resected tumor are commonly not available. In the majority of the patients, tumor tissue specimens are obtained either with endoscopic biopsy or transthoracic biopsy, and the nature of these procedures has obviously implications for the size of the tissue sample that can be obtained. An associated issue is the quality of the sample, and it is not infrequent that the biopsies contain high amount of necrotic tissue that is difficult to analyze. Thus, limited sample size represents the principal difficulty in biomarker studies in lung cancer that is further compounded by heterogeneity within a tumor as well as differences between the primary tumor and metastases and, as mentioned above, with the presence of necrotic areas. On the other hand, advanced lung cancer is commonly associated with pleural effusion, and pleural fluid represents another potential sample matrix for biomarker studies [23]. Lung cancer also commonly spreads to central nervous system, and cerebrospinal fluid is another sample matrix of interest in patients with advanced disease [112]. Thus, the issue of sample type is of fundamental importance in the management of lung cancer [111].

Consequently, sample matrices investigated in patient with lung cancer include, besides tumor tissue, peripheral blood (usually serum or plasma), pleural fluid [23] or cerebrospinal fluid [112] also exhaled air [30, 113], bronchoalveolar lavage [114], induced sputum [115] and urine [20]. Among these sample matrices, samples derived from the peripheral blood are the most widely used because these can be easily obtained virtually under all circumstances. Moreover, medical therapy of lung cancer targets primarily the cancer as a disease associated with systemic tumor spread that is accompanied with the presence of circulating tumor cell or release of tumor cell components into the bloodstream. For the identification of tumor cells or tumor cell component from the samples of peripheral blood, the term “liquid biopsy” has been coined [116].

Historically, the evaluation of tumor cell properties, including molecular characteristic, has been a domain of pathology. Immunohistochemistry and later molecular biology methods have been used to determine the presence of molecular determinants of response, e.g. hormone receptors. This approach paradigm has been shaped by the experience with the management of breast cancer. In breast cancer, the availability of a tumor tissue sample sufficient for molecular analyses rarely represents a problem. As outlined above, the paradigm of precision medicine based on histological evaluation of tumor specimen by the pathologist is more difficult to apply in patients with lung cancer, and laboratory investigations using alternative tissue matrices complement the traditional histology-based diagnostic approach in the multidisciplinary management of lung cancer, resulting in wider involvement of laboratory medicine.

Liquid biopsy is emerging as a potential tool to follow the disease course in lung cancer patients. The term liquid biopsy covers widely different methodological approaches including circulating tumor cells [117, 118], circulating

![Table 2: Immunotherapy agents used in the therapy of NSCLC.](image-url)
tumor DNA, messenger RNAs [24], non-coding RNAs [25], or even proteomic and lipidomic analyses [28]. Liquid biopsy obviously relies on the ability to detect in an unequivocal manner the presence of tumor-derived structural elements in biological fluids. Circulating tumor cells represent a tiny proportion of circulating cells, and different approaches have been introduced for circulating tumor cell isolation combining tumor cell enrichment with the depletion of other cell types [117–119]. Because of the technical difficulties associated with the isolation of circulating tumor cells, most current liquid biopsy approaches use isolation of cell free circulating tumor DNA and subsequent search for gene mutations, gene rearrangements or epigenetic changes like altered methylation patterns [22, 27, 120].

Considerable attention has been focused on evaluating microRNAs as biomarkers of lung cancer [25, 121]. microRNAs are small non-coding RNAs involved in the post-translational regulation of gene expression. For microRNA analysis, unprocessed serum or plasma, or isolated exosomes have been used [26]. Different microRNA species can be upregulated as well as downregulated in cancer patients, and differences were observed in microRNA profile between metastatic and non-metastatic NSCLC [26]. Besides the presence of driver mutations, DNA of tumor origin can also be distinguished by epigenetic changes like altered methylation patterns. The potential use of distinct methylation patterns, that may involve hyper- as well as hypomethylation, in the diagnosis and evaluation of lung cancer has been extensively investigated in tumor samples, induced sputum or biological fluids [27, 115, 122].

At present, the results of liquid biopsy could be used, at best, only to support the diagnosis of lung cancer obtained by other methods. There are currently two principal uses of liquid biopsy approach. First, liquid biopsy may be used to detect molecular biomarkers (e.g. the presence of mutation) determining the response to targeted agents [23]. Second, liquid biopsy may be used to follow the course of the disease, including the evaluation of efficacy of therapy. The use of liquid biopsy in this setting is linked with another important concept, i.e. minimal residual disease [123]. The term minimal residual disease was coined to denote cancer cells detected morphologically in distant organs (e.g. in bone marrow biopsy), but this concept is currently used more in association with liquid biopsy. Minimal residual disease is thus defined as evidence of presence of cancer cells by classical morphological or molecular biology methods after potentially curative cancer treatment, i.e. surgery or radiotherapy. The detection of minimal residual disease has obvious implications for decisions throughout the course of treatment. In colorectal carcinoma detection of minimal residual disease has been used for the selection of patients for adjuvant therapy (or, rather, the absence of minimal residual disease to select patients in whom adjuvant chemotherapy could be safely omitted) [124]. However, the molecular methods used for liquid biopsy assessments are relatively novel, and their role in the management of cancer patients is still under investigation.

The use of biomarkers in the selection of targeted agents

The very concept of targeted therapy is based on the presence of a molecular target that distinguishes cancer cell from normal cells and plays a role in tumor growth and progressions. These targets are linked with the concept of hallmarks of cancer, i.e. fundamental mechanisms responsible for tumor growth and progression [125]. Most of the targets in NSCLC are driver mutations or other genetic changes responsible for malignant transformation. These driver mutations or other driver genetic changes differ in individual NSCLC cases. On the other hand, the same driver mutations are encountered in tumors of different primary location. For example, BRAF mutations that are found in about half of malignant melanoma of the skin are also detected in 2–3% of patients with NSCLC, and the same medication may be used. According to the principles of tumor-agnostic cancer medicine mentioned above same treatment regimen may be used and is active in the treatment of both advanced skin melanoma or NSCLC, in this case the combination of BRAF inhibitor (e.g. dabrafenib) and MEK inhibitor (e.g. trametinib) [80, 94].

The first driver mutations that could be targeted with drug therapy were discovered in the EGFR gene, and the story of EGFR development during the last two decades illustrates the successful application of the paradigm of targeted therapy. As mentioned above, the first-generation drugs targeting EGFR, gefitinib and erlotinib, have been originally introduced in non-selected patients with advanced or metastatic NSCLC, resulting in limited efficacy. Based on seminal observation of the association between the response to gefitinib and EGFR mutations [126], trials have been directed to this subgroup of patients, and much higher efficacy has been observed in selected cohorts of patients with EGFR driver mutations [127]. The subsequent advent of second and third generation EGFR inhibitors has resulted not only in increased efficacy in treatment-naïve patients, but also provided effective second line treatment options. Finally, efficacy of EGFR inhibitor osimertinib has been demonstrated in the adjuvant setting in patients after lung resection [73]. The initial observation of an association between EGFR mutations and efficacy of cognate tyrosine kinase inhibitors introduced the necessity for screening NSCLC patients for the presence of EGFR mutations [128].
Exon 19 deletions and exon 21 L858R mutation that constitute the overwhelming majority of EGFR mutations are sensitive to EGFR inhibitors [129]. The identification of mutations associated with primary resistance like exon 20 insertion has gained significance with the emergence of effective therapy, i.e. amivantamab [90, 130]. As outlined below the identification of acquired resistance associated with T790M mutation is important because of efficacy of osimertinib in this setting [129].

Consequently, considerable attention has been devoted during the past two decades to the methodology of detecting EGFR mutations in paraffin embedded tissues, cytological specimens, plasma, cerebrospinal fluid, bronchoalveolar lavage or malignant effusions using different methods [114, 131, 132]. Liquid biopsy is being frequently used to determine the EGFR mutation status. In a study comparing paired plasma samples with other body fluids, including pleural or pericardial effusions, cerebrospinal fluid and ascites from the same patient it was demonstrated that the concentration of circulating free DNA and the detection rate of EGFR mutations was markedly higher in other body fluids [133]. In pleural effusions, both supernatants and cell pellets can be used as sample matrices, and real-time PCR has been shown to represent an effective method of detecting EGFR mutations in pleural effusion [134]. Other detection methods involved fluorophore-labeled peptide nucleic acid probes [135]. Along with EGFR mutations, PCR-based methods were shown to be successful in demonstrating the presence of mutations of KRAS and BRAF in cerebrospinal fluid [112]. KRAS mutations are the most common driver genetic changes in NSCLC. Determination of serum p21ras protein was found not to be feasible in lung cancer patients [136]. On the other hand, methods have been developed to detect specific KRAS mutations in the serum and tumor tissues [137], long before the advent of drugs targeting KRAS G12C mutations. The exact identification of KRAS mutation is currently of importance as mutations other than G12C remain undruggable.

Rearrangements (fusions) of anaplastic lymphoma kinase (ALK) gene are found in a small proportion of NSCLC patients. Similar to EGFR-mutated NSCLC, the advent of targeted therapy has changed the natural history of these tumors. Determination of ALK rearrangements is now part of routine assessment of NSCLC patients, and considerable attention has been devoted to the development of laboratory methods [138].

Methods using fluorescence in situ hybridization or PCR-based methods can be used to study alterations of a single or few genes. With the increasing number of targetable driver genetic changes, a broader strategy to identify patients who might benefit from a specific medication became a necessity. Classical Sanger sequencing is not practical for routine clinical use. With the advent of next generation sequencing (NGS) a genome-wide search for specific alterations became available in routine clinical practice [139]. In NSCLC NGS may be used in a liquid biopsy approach to search for the presence of driver mutations in circulating free DNA [140]. The introduction of NGS into the routine clinical practice of lung cancer management has opened the ways for the use of precision medicine, allowing for targeting the common as well as less common driver genetic changes.

The determination of biomarkers plays a crucial role in the management of patients with acquired resistance to targeted agents. The clinical experience with EGFR inhibitors has again highlighted the progress in the area of resistance to targeted agents. One of the principal mechanisms of acquired resistance to EGFR inhibition is the emergence of the T790M mutation. Liquid biopsy plays an even more important role in the detection of acquired resistance mutation, and different methods have been introduced to detect the T790M mutation in patients treated with EGFR inhibitors in daily clinical practice [141].

In patients treated with agents targeting the programmed cell death receptor ligand (PD-L)-1 the expression of PD-L-1 identifies a population of patients more likely to benefit from the administration of pembrolizumab compared to platinum-based chemotherapy [98], in particular among patients with non-squamous NSCLC [142]. However, prediction of response to immune checkpoint inhibitors remains an extremely complex issue. There is an association with tumor mutational burden, and some studies extended the identification further up to the identification of individual neoantigens [143, 144]. Although this approach would certainly result in further refinement of the selection of therapeutic agents, it remains at this moment impractical for clinical routine. However, tumor mutational burden testing is now routinely available in the clinical practice. In a prospective trial, a substantial proportion of patients had high tumor mutational burden, and among patients with high tumor mutational burden the combination of nivolumab with ipilimumab was shown to be markedly superior compared to platinum-based chemotherapy, with less associated toxicity [145]. The current practice of using PD-L1 for decision on the use of immune checkpoint inhibitors is complicating by several outstanding problems. First, different methodologies, including differences in tissue processing, different antibodies and scoring systems that differ in every aspect, including the definition which cells should be counted.
The cutoff levels used in clinical decisions are arbitrary and without any biological rationale. Importantly, PD-L1 expression changes in time, and is extremely heterogeneous even within a single tumor sample. These analyses are performed on histological slides, and limitations of sample size outlined above are of obvious consideration here, and in many patients this analysis cannot be performed. A test based on the liquid biopsy concept would be of advantage here, but remains elusive. On the other hand, increased correlations of biomarkers of systemic inflammatory response have been associated with inferior efficacy of immunotherapy [146], but the utilization of this information in the decision on the management of an individual patient may be rather difficult.

**Biomarkers of immune and inflammatory response in patients with lung cancer**

It has been known for decades that increased concentration of biomarkers of the activation of immune and inflammatory response, e.g. C-reactive protein (CRP) or neopterin is associated with worse prognosis in cancer patients across the range of primary tumors, including lung cancer [109]. In particular, increased activity of indoleamine 2,3-dioxygenase that is reflected in increased circulating kynurenine and decreased tryptophan concentrations has been associated with resistance to the immune checkpoint inhibitors [147].

Lymphopenia is another parameter associated with poor outcomes in cancer patients. In patients with advanced cancer, lymphocyte counts inversely correlate with biomarkers of immune activation like neopterin [148]. In the last decade lymphocytes have been extensively determined in cancer patients as part of peripheral blood leukocyte count determination. Neutrophil-to-lymphocyte, platelet-to-lymphocyte and lymphocyte-to-monocyte ratios are basically relative lymphocyte counts [149]. Because peripheral blood cell count is determined routinely during the patient visits, the peripheral blood cell count-derived ratio have been studied extensively, mostly retrospectively, in patients almost all primary tumors [149], including lung cancer [150]. As mentioned above lower relative lymphocyte counts were associated with inferior response to immunotherapy [146]. Decreased ratio of neutrophils to lymphocytes was reported to be associated with favorable response to pembrolizumab and higher tumor lymphocyte infiltration [151].

**Future perspectives**

Given the fast pace of advances in the medical therapy of lung cancer, future directions of research and clinical practice may be difficult or even impossible to predict. Despite this limitation, some the principal areas of unmet medical need should be defined and possible direction of future research may be outlined.

With novel driver mutations or other driver genetic changes targeted with new classes of drugs new molecular predictors will inevitably emerge that would need to be determined in the daily clinical practice. With the prevailing tumor-agnostic paradigm liquid biopsy will probably assume an important role in the biomarker studies.

Primary (neoadjuvant) systemic therapy is being increasingly used across the spectrum of solid tumors. The paradigm of neoadjuvant therapy has been developed in the management of breast cancer, but is being introduced into the therapeutic strategies of other solid tumors, including NSCLC [106]. The neoadjuvant model is of advantage in the development of novel therapies because an outcome parameter, pathological response, that can be evaluated almost immediately after the completion of the therapy is associated with long-term efficacy outcomes like relapse-free survival and overall survival. The same advantage of the neoadjuvant approach also pertains to biomarker research.

Despite the data demonstrating the role of PD-L1 determination in predicting the response to immune checkpoint inhibitors targeting the PD-1/PD-L1 interaction, more predictive biomarkers of immunotherapy outcome are urgently needed, in particular in patients with squamous NSCLC.

Among the outcomes evaluated, almost all effort in biomarker research have been concentrated on association with parameters of efficacy like objective response rate, progression-free survival and overall survival. However, targeted therapy, in particular immunotherapy is associated with relatively rare, but sometimes serious or even life-threatening toxicity. Evaluation of toxicity with laboratory methods is simple for hematologic toxicity or endocrine side effects. For example, for patients treated with immune checkpoint inhibitors, hypothyroidism is so common that many medical oncologists perform screening for this condition by determining circulating thyroid stimulating hormone levels at every visit, similarly to the use of peripheral blood cell count. However, data supporting the use of biomarkers that would help in detecting or even predicting other serious side effects of treatment like gastrointestinal toxicity or pneumonitis are scanty in medical oncology in general and for targeted treatments in particular. Measurements of intestinal permeability or determination of circulating citrulline concentrations has been investigated in patients treated with chemotherapy or combination of chemotherapy and radiation [152, 153], but the data on the use of these laboratory approaches
in patients treated with targeted agents are more or less scanty [154]. A decrease of circulating gamma-tocopherol concentrations has been documented in a small cohort of patients treated with EGFR inhibitors that included patients with NSCLC [154], but it is not clear whether there is an association of these changes with the dermatitis that accompanies the treatment with EGFR inhibitors. Reliable biomarkers should also be investigated for other serious and potentially fatal toxicities of targeted agents like pneumonitis. One commonly determined biomarker associated with lung function is gamma-glutamyl transferase [155], but more biomarkers are needed for this relatively common and unpredictable complication of immunotherapy and other targeted therapies.

With improved survival of lung cancer patients, the significance of competitive causes of morbidity and mortality will inevitably increase. Similarly to the situation in some other tumors with high cure rate like testicular cancer [156], or breast cancer [157], an increase in the prevalence of risk factors, manifestations and serious complications of atherosclerosis might be expected in long-term survivors of lung cancer. The association with important risk factors of atherosclerosis is stronger in long cancer compared to many other tumors [4]. Moreover, disorders of lipid metabolism associated with atherosclerosis risk like increased concentrations of lipoprotein (a) [158] or hyperhomocysteinemia [159] have been documented in lung cancer patients. Future studies should systematically investigate the prevalence of risk factors of atherosclerosis in lung cancer survivors, and large population studies should follow cardiovascular events in these patients.

Conclusions

Cancer management is multidisciplinary and involves clinicians specialized in the diagnosis, surgical, radiation and medical therapy. Current management of lung cancer is not imaginable without the involvement of laboratory medicine. With the advent of targeted therapy, the identification of molecules at which aim the precision drugs became a foundation of success or failure of given treatment. CCLM has followed the developments in the wide field of lung cancer biomarkers throughout the past six decades by publishing many important papers reviewed above, from early studies on circulating tumor markers to the identification of biomarkers of targeted therapies, and will undoubtedly continue to do so in the years to come.

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