Molecular biomarkers in early stage lung cancer

María Rodríguez[1], Daniel Ajona[2,3,4,5], Luis M. Seijo[6,7], Julián Sanz[8], Karmele Valencia[2,4,5], Jesús Corral[9], Miguel Mesa-Guzmán[10], Rubén Pío[2,3,4,5], Alfonso Calvo[2,3,4,11], María D. Lozano[3,4,11,12], Javier J. Zulueta[3,4,13], Luis M. Montuenga[2,3,4,11]

[1]Department of Thoracic Surgery, Clínica Universidad de Navarra, Madrid, Spain; [2]Program in Solid Tumors, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain; [3]Navarra Institute for Health Research (IdISNA), Pamplona, Spain; [4]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain; [5]Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona, Spain; [6]Department of Pulmonology, Clínica Universidad de Navarra, Madrid, Spain; [7]Centro de Investigación Biomédica en Red Enfermedades Respiratorias (CIBERES), Madrid, Spain; [8]Department of Pathology, Clínica Universidad de Navarra, Madrid, Spain; [9]Department of Oncology, Clínica Universidad de Navarra, Madrid, Spain; [10]Department of Thoracic Surgery, Clínica Universidad de Navarra, Pamplona, Spain; [11]Department of Pathology, Anatomy and Physiology, Schools of Medicine and Sciences, University of Navarra, Pamplona, Spain; [12]Department of Pathology, Clínica Universidad de Navarra, Pamplona, Spain; [13]Department of Pulmonology, Clínica Universidad de Navarra, Pamplona, Spain

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Correspondence to: Dr. María Rodríguez. Department of Thoracic Surgery, Clínica Universidad de Navarra, C/Marquesado de Santa Marta 1, 28027 Madrid, Spain. Email: mery.rodriguez.perez@gmail.com; Dr. Luis M. Montuenga. Program in Solid Tumors, Center for Applied Medical Research (CIMA), University of Navarra, Avda Pio XII 55, 31008 Pamplona, Spain. Email: lmontuenga@unav.es.

Abstract: Low dose computed tomography (LDCT) screening, together with the recent advances in targeted and immunotherapies, have shown to improve non-small cell lung cancer (NSCLC) survival. Furthermore, screening has increased the number of early stage-detected tumors, allowing for surgical resection and multimodality treatments when needed. The need for improved sensitivity and specificity of NSCLC screening has led to increased interest in combining clinical and radiological data with molecular data. The development of biomarkers is poised to refine inclusion criteria for LDCT screening programs. Biomarkers may also be useful to better characterize the risk of indeterminate nodules found in the course of screening or to refine prognosis and help in the management of screening detected tumors. The clinical implications of these biomarkers are still being investigated and whether or not biomarkers will be included in further decision-making algorithms in the context of screening and early lung cancer management still needs to be determined. However, it seems clear that there is much room for improvement even in early stage lung cancer disease-free survival (DFS) rates; thus, biomarkers may be the key to refine risk-stratification and treatment of these patients. Clinicians' capacity to register, integrate, and analyze all the available data in both high risk individuals and early stage NSCLC patients will lead to a better understanding of the disease's mechanisms, and will have a direct impact in diagnosis, treatment, and follow up of these patients. In this review, we aim to summarize all the available data regarding the role of biomarkers in LDCT screening and early stage NSCLC from a multidisciplinary perspective. We have highlighted clinical implications, the need to combine risk stratification, clinical data, radiomics, molecular information and artificial intelligence in order to improve clinical decision-making, especially regarding early diagnostics and adjuvant therapy. We also discuss current and future perspectives for biomarker implementation in routine clinical practice.

Keywords: Early lung cancer; lung nodule; screening; biomarkers; radiomics

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Introduction

Multidisciplinarity: a requirement for the current challenges in lung cancer screening and early stage management

Implementation of CT screening for lung cancer, combined with continuous improvements in conventional diagnostics, will undoubtedly increase the number of suspicious pulmonary lesions detected. These improvements highlight the importance of multidisciplinary decision making in the management of these patients (1). Consensus-based recommendations involving thoracic surgeons, radiologists, oncologists, pathologists and pulmonologists are expected to play a central role in the follow up, diagnosis, and treatment of patients with newly detected lung nodules. Screening detected nodules are becoming smaller in size, more peripheral, and frequently less solid when compared to traditionally detected lesions. Furthermore, they can represent a wide variety of histologies, some with potentially indolent growth, for which specific management guidelines are lacking (1). In the present review, we will argue in favor of including molecular biologists, laboratory scientists, and molecular geneticists, in these multidisciplinary teams. We envision that their specific expertise will be increasingly necessary in order to navigate the biological profile of lung tumors in parallel to biomarker discovery and development. In our opinion, biomarkers may help decision making related to risk assessment of asymptomatic individuals, nodule characterization and prognosis, as well as inform surgical management in the treatment of already diagnosed and/or resected tumors. *Figure 1* shows a schematic representation of the different types of biomarkers that are being developed for early detection and management of early stage lung cancer. In the present manuscript we aim to define both the current unmet medical challenges in lung cancer screening and early cancer management, together with the potential contributions that may arise from the field of biomarker discovery and development.

Unmet medical challenges in the screening process

Who should be screened

A growing body of scientific evidence supports lung cancer screening with low-dose computed tomography (LDCT). The pioneer NEJM paper by Henschke and co-workers in 2006 (2) showing that LDCT can detect lung cancer, provided data of the clinical value of screening with LDCT for reducing lung cancer mortality. Since then, multiple randomized trials, including the landmark National Lung Screening Trial (NLST), the Dutch-Belgian NELSON trial, and the Italian MILD trial, have demonstrated a reduction in lung cancer mortality using LDCT as a screening tool (3-5). However, much needs to be done in order to refine recommended screening criteria, largely based on the NLST findings. Screening smokers older than 55 with more than 30 pack years of cumulative exposure to cigarette smoking clearly leaves many individuals at risk out of existing screening protocols (6-8).

The matter of selection criteria is of paramount importance. Screening all smokers may be a daunting task and prove to be quite expensive and increases the risk of performing unnecessary procedures for benign nodules. Therefore, limiting screening to those with the greatest risk may be more cost effective. On the other hand, screening only based on age or smoking exposure, may exclude those who stand to benefit the most, i.e., younger individuals with greater potential for long-term survival, thereby limiting screening benefits on quality-adjusted life years (QALY) and cost effectiveness. A biomarker capable of refining existing inclusion criteria might help in both ways, by excluding subjects theoretically at risk based on age and tobacco exposure, thereby precluding unnecessary screening, while including younger smokers who may be at greater risk than anticipated.

Adequate evaluation of the risk of malignancy, as well as the fitness of a given patient to undergo further intervention or resection, should be a multidisciplinary task centered on improving patient outcomes (1,9,10). Multiple quantitative risk assessment models have been validated in order to evaluate the risk of malignancy, mainly based in epidemiological/clinical criteria. Examples are the Liverpool Lung Project (LLP) risk model (11) or the PLCO2012 risk prediction model (12). Salient clinical features include; age (13,14), smoking status (15), history of prior malignancy (16), presence of chronic obstructive pulmonary disease (COPD), particularly the emphysema phenotype (17), and family history (18). A recent review, mainly directed to primary care physicians addresses this relevant issue (19).

Nodule characterization

LDCT based screening, while very sensitive, has a low specificity. Validated management protocols have been shown to be effective in avoiding excessive unnecessary
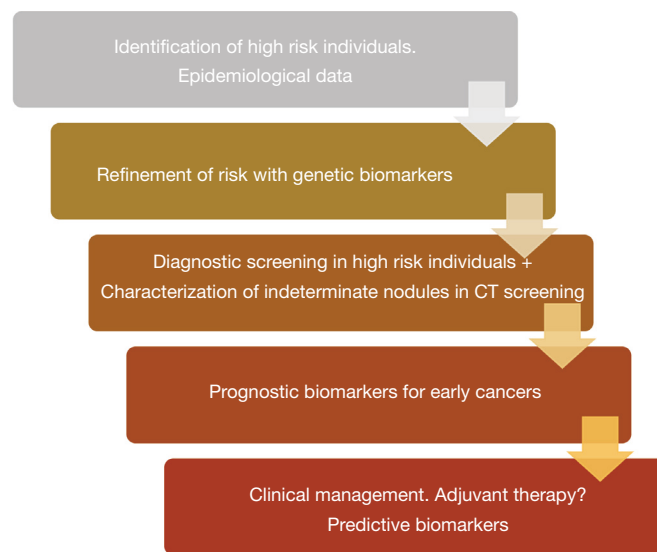
Figure 1 The role of molecular markers in the early lung cancer management cascade.

invasive diagnostic procedures (20). Moreover, a high prevalence of indeterminate nodules detected on LDCT may lead to more testing and anxiety. Therefore, there is an unmet clinical need to develop standardized and robust biomarkers capable of refining screening inclusion criteria and inform decisions based on LDCT screening findings (21). Radiomic and molecular approaches are both currently being explored.

Current management of LDCT identified lung nodules is largely based on serial imaging at specific intervals in order to determine doubling time, which in turn informs decisions regarding follow up or invasive testing. Massion *et al.* have recently demonstrated that the application of a novel deep learning algorithm to chest computed tomography scans is a useful tool to correctly reclassify IPNs into low- or high-risk categories (22). Since most nodules detected by screening are relatively small, a biomarker-based risk assessment can also be helpful in this regard. The development by Vachani and co-workers (23) of a molecular biomarker based on bronchial epithelial gene expression obtained from normal-appearing epithelium during bronchoscopy is an example. This biomarker can stratify the risk of malignancy of suspicious lesions with inconclusive biopsy results, in order to help decision making in the clinical setting. However, this biomarker requires a bronchoscopy, an invasive procedure. Therefore, biomarkers analyzed in samples obtained non-invasively (either in blood, sputum, urine or exhaled breath) would be ideal. We will summarize in the next paragraphs some of

the most relevant candidates.

In addition, biomarkers may prove useful during follow up of patients with screening diagnosed lung cancer, aiding prognosis, defining the need for treatment with adjuvant therapies, and detecting minimal residual disease or second primary malignancies.

Radiological challenges

The potential role of radiomics and radiogenomics

Radiomics are defined as the extraction of quantitative and qualitative data from radiological images to create databases than can help diagnose and predict prognosis and the response to treatment of different malignancies, including non-small cell lung cancer (NSCLC) (24,25). Radiomics have been applied to lung cancer screening in order to increase its sensitivity and specificity (22,26). Several radiomic-based predictive models have been already proposed for CT-based early detection of lung cancer. These models show a great potential as tools for clinical decision-making in lung cancer screening (27,28) although some caveats and limitations such as radiomic workflow lack of repeatability or reproducibility under particular circumstances still need to be considered (29). The field of radiogenomics combines radiomics with genetic biomarker data (30).

A major challenge is to obtain comprehensive information regarding tumor biology based on studying large imaging datasets using artificial intelligence. Unfortunately, due to time, costs and data analysis limitations, large scale-genome

lung cancer characterization is still not available in early stage lung cancers (31). In recent years, different subtypes of lung adenocarcinoma (ADC) harboring actionable mutations or translocations (*EGFR*, *KRAS* and *ALK*) have been identified leading to personalized therapies (32). The search for a correlation between these genetic findings and radiological data is a worthwhile pursuit, albeit thus far yielding somewhat contradictory results. Glynn and cols. (33) were not able to find a relationship between radiological data and *EGFR* or *KRAS*-mutations. On the other hand, Lee and cols. (34) reported an association between CT and PET/CT findings [tumors >4 cm diameter or >5.0 standard uptake value (SUV) or ground glass opacity component <50%] and *EGFR* overexpression. The same group was able to differentiate between the two most common *EGFR* mutations (exon 19 deletions, and exon 21 missense mutations) based on CT-tumor morphology, finding a more prominent ground glass component in patients with exon 21 missense mutations (35). Yano and cols. (36) also highlighted the association between a tumor's ground glass component and the likelihood of an *EGFR* mutation. In the study of Rizzo and colleagues (37) qualitative and quantitative features such as air bronchograms, pleural retractions, smaller size and the absence of fibrosis tended to be associated with *EGFR* mutations while pleural effusion was associated more frequently with *ALK* translocations. Furthermore, a round shape, the presence of other nodules in different lobes and smoking status were associated with *KRAS* mutations. These findings were confirmed by Halpenny and cols. (38), whose study showed that *ALK*-mutated tumors were associated with pleural effusion and mediastinal adenopathy and were larger in size.

RET and *ROS1* mutations, although less frequent, have also been addressed in other models. Plodkowski and cols. (39) found that peripheral tumors were associated more frequently with *ROS1* rearrangements, while solid tumors with spiculated borders tended to harbor *RET*, *ROS1* and *EGFR* mutations. These subgroups of tumors rarely presented cavitation or calcification (39). Nair and colleagues (40), in their attempt to correlate genome expression with different FDG uptakes on PET-CT, found a link between SUV values and nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB), a protein complex related to DNA transcription, cytokine production and cell survival. More recently, in order to try to predict tumor behavior and response to adjuvant treatment, Vaidya and cols. (41) developed and validated a quantitative radiomic risk score and an associated nomogram for early stage NSCLC (stages I and II) capable of predicting disease-free survival (DFS) and the added benefit of adjuvant chemotherapy following surgical resection.

Surgical challenges: the reduction of false positives and the decision on the type of intervention

Limiting false positive findings to a minimum remains an on-going challenge of screening. A high false-positive rate is neither ethically nor economically acceptable. However, a balance must be struck between achieving a high-detection rate with its consequent reduction in mortality, and the lowest possible false-positive rate.

With the advent of more standardized diagnostic algorithms for IPNs, the false positive rates have reduced considerably, with an average of 18% false positives in the context of lung cancer screening programs (42). In our subcohort of I-ELCAP in Pamplona, the rate of interventions for benign disease was 9.2% (43).

Several emerging biomarkers address the issue of reducing the risk of futile surgeries for benign lesions by improving the characterization of indeterminate pulmonary nodules found by LD-CT based screening or routine clinical practice. Section 3 of the present review, devoted to biomarkers in screening, provide detailed examples of these efforts to validate nodule characterization with molecular markers.

As a result of advances in surgical treatment, mortality reported after diagnostic lung surgery is lower than 1% (44,45), or practically nonexistent in the case of video-assisted thoracoscopic surgery (VATS) wedge resection (46,47). Minimally invasive approaches and sublobar resections may become the surgery of choice for diagnostic interventions, always bearing in mind the challenge of identifying and locating these small lesions intraoperatively (48,49). Biomarkers are not standard of care in surgical NSCLC, although well-established and routinely monitored biochemical data are logically considered when evaluating a patient's general status and eligibility for a given surgical approach. The combination of systemic or local (sputum, exhaled breath, etc.) biomarkers with concurrent morbidities such as COPD or emphysema, and other clinical parameters as well as quantitative radiological imaging, will very likely be a useful multidisciplinary approach to help in the surgical decision-making process. In the context of early lung cancer in screening cohorts, the finding of molecular or integrated molecular/clinical/imaging markers that may facilitate the surgical decision

making protocol is still an unmet clinical need.

Challenges in pathology

A histopathological diagnosis, precise staging and, eventually, prognostic information are of the utmost importance in any tumor, and particularly in early cancer stages. For isolated suspicious pulmonary nodules, in the context of screening, it is also important to consider the emerging roles for the pathologist. Rapid on site evaluation (ROSE) of these nodules is an example, highlighting once more the importance of a multidisciplinary approach. The widespread implementation of screening has led to the detection of small nodules, often less than 1 cm, which pose a challenge to frozen section analysis (50). There is an unmet medical need to develop robust, rapidly available and standardized molecular biomarkers to provide unequivocal diagnostic information for ROSE, quick frozen section analysis or fine needle aspiration material. Fast technological development to adapt next-generation sequencing (NGS) and other molecular techniques to cytology samples with very small amount of DNA is currently thriving in NSCLC diagnosis. Examples are the detection of *EGFR* mutations and *ALK* or *ROS1* rearrangements or of the expression of PDL-1 in small cytology specimens (51).

The introduction of screening for lung cancer is increasing notably the proportion of early stage diagnoses, in particular stage I lung tumors. This has posed additional main challenges to the pathologist. The efforts of pathology research in this field are currently focused on the development of more refined algorithms (very likely using artificial intelligence) to: (I) determine definitive diagnostics, including molecular classification of tumor type; (II) refining staging by better predicting outcome and aggressiveness or the potential presence of minimal residual disease in early stage lung cancer patients; (III) advising for the need of adjuvant treatment in a given patient; and (IV) suggesting potential adjuvant treatments based on the biology of the tumor. Very likely, diagnostic, prognostic and predictive algorithms will be based on a combination of image analysis and molecular data. The recent results of the ADAURA trial showing significantly longer DFS in NSCLC patients with stage IB to IIIA *EGFR* mutation positive receiving osimertinib as compared to those who received placebo improves previous results with first and second generation *EGFR*-TKIs (52). The impressive results of this trial strongly advise for routine molecular (at least *EGFR* mutation) testing of NSCLC patients irrespective

of the tumor stage (53). Thus, it is very likely that a call for routine deeper molecular characterization of the early stage lung tumors found in the context of CT-based screening will be around the corner.

Current biomarkers in lung cancer screening

Molecular biomarkers are biological indicators. A good biomarker should be accurately and robustly measured in accessible samples. The rationale to develop molecular biomarkers for lung cancer screening is well established, since altered molecules derived from cancer cells or the tumor microenvironment are released into fluids and can be quantified with highly sensitive technologies. A clinically meaningful biomarker should be quantified with reproducible and cost-effective methods and should provide valuable information for the clinical management of lung cancer (54).

Blood-borne biomarkers would be ideal for screening, because these samples recapitulate tumor heterogeneity and represent both the primary tumor and the different metastatic lesions. They may also reflect the host response to the presence of a neoplastic lesion. Although most of the information included in the present review refers to blood biomarkers, other potential sources of biomarkers include sputum, bronchoalveolar lavage (BAL), or bronchial aspirate samples. The biomarkers found in upper airway secretions may be more closely related to lesions in the central airways, but also more likely to miss peripheral tumors (55). Exhaled breath biomarkers (either measuring volatile compounds or different molecules found in the breath condensate) are also being studied and tested actively (56,57). Saliva (58) and urine (59) have also been explored as potential sources of lung cancer early detection biomarkers, but these studies are still in a very early discovery phase. A recent review has described three biomarker commercially available tests (Early CDT-Lung, Nodify XL2, Percepta) which have undergone extensive validation and are available to the clinician. Clinical applicability and limitations of these tests and future directions of lung cancer screening biomarkers are also discussed (60). Emerging biomarkers and new trends for the future of lung cancer screening biomarker development have been recently summarized elsewhere (61).

Many efforts have been devoted to identify molecular biomarkers, especially in blood samples, but none of the currently available candidates has been fully validated and none has been tested in a trial to show that its use

contributes to a reduction in lung cancer mortality. The clinical use of a given diagnostic biomarker requires a long, tedious and expensive process of validation to demonstrate its clinical usefulness: i.e., its capacity to orient clinical decisions in order to improve patient outcomes. Full clinical validation in the lung cancer screening context has not been reached yet. Nonetheless, massive amounts of biological information are being recorded, including several steps in the clinical front for some of the candidate biomarkers. The tests in intended-to-use (LDCT screening cohorts), the development and completion of properly designed trials, and the increasing interest in developing complementary tools to render LDCT screening more cost-effective, are the engines that will lead promising candidates into routine and reliable clinical use in the upcoming years.

A range of promising biomarkers has been identified in biological samples. *Table 1* and this section summarize those that we consider the most promising to address the two main unmet needs for biomarkers in the context of lung cancer screening, namely risk management and the characterization of indeterminate pulmonary nodules found on LDCT imaging of high-risk individuals. Due to its non-invasive procedure and great potential, liquid biopsy will probably become a standard diagnostic, prognostic and predictive tool in NSCLC (97).

Autoantibodies (AAbs)

AAbs develop in response to tumor antigens and may be found in the plasma of asymptomatic lung cancer patients. An AAbs panel has been validated in different screening cohorts to assess lung cancer risk, and may help differentiate between benign and malignant lesions (64,65,98-100). The high specificity of AAbs indicates that they are suitable to improve the diagnostic performance of mixed panels of biomarkers and complement the results of high sensitivity imaging studies (101). The most advanced test based on AAbs. Early CDT-Lung test has been studied for clinical validation in several cohorts of patients with newly diagnosed lung cancer, showing a specificity of approximately 90% at sensitivities of around 40% (102). Recently, the results of a long time waited trial in Scotland, using the score from the Early CDT-Lung test to randomize more than 12,000 high risk individuals to CT-screening or standard of care follow up when the study begun, have been published. A reduction of the incidence of patients with stage III/IV lung cancer at diagnosis was observed. The intervention arm received the Early CDT-

Lung test and, if test positive, low-dose CT scanning 6-monthly for up to 2 years. Early CDT-Lung test negative and control arm participants received standard clinical care. In the intervention arm, 58.9% lung cancers were diagnosed at stage III/IV compared to 73.2% in the control arm. The hazard ratio (HR) for stage III/IV presentation was 0.64 [95% confidence interval (95% CI): 0.41, 0.99]. Nevertheless, sensitivity in this recent trial was even lower than in previous trials, at 32.1%, with a preservation of specificity at 90.3%. Of note, sensitivity was particularly low for stage III or IV cancers, at 18.2%. There were non-significant differences in lung cancer and all-cause mortality after 2 years (103).

Blood circulating proteins other than AAbs

Several panels of proteins have been proposed for early detection, the most advanced of which is a panel of proteins discovered by mass spectrometry multiple reaction monitoring technique. Initially the authors reported a 13-protein proteomic classifier that gave a 90% negative predictive value (NPV) for benign nodules (104). The same group proposed a 5-marker subset of the original 13 proteins together with 6 normalization markers (105). More recently, the same group discovered that the accuracy of two of the proteins, LG3BP and C163A (independently linked to lung cancer and the inflammatory response to cancer) could be optimized for evaluating lower risk nodules by integrating them with five clinical risk factors (nodule location, size, spiculation and patient's age and smoking history). The classifier was commercialized as XL2^{TM} TEST by Biodesix. An observational clinical trial (PANOPTIC) was carried out with this classifier A subgroup of 178 patients with a clinician-assessed pretest probability of cancer (pCA) ≤50% had a 16% prevalence of lung cancer. The integrated molecular/clinical classifier demonstrated a sensitivity of 97% (CI, 82–100), a specificity of 44% (CI, 36–52), and a NPV of 98% (CI, 92–100) in distinguishing benign from malignant nodules. According to the results in this trial, if the integrated classifier was used to direct care, 40% fewer procedures would be performed on benign nodules, and 3% of malignant nodules would be misclassified (ClinicalTrials.gov; No. NCT01752114) (63).

Complement fragments

The activation of the complement system has been proposed as a potential biomarker for the clinical

Table 1 Candidate biomarkers for lung cancer early detection and for the management of CT-detected undetermined lung nodules [updated from Seijo *et al.* (21)]

Candidates	Biomarker	Target	Phase	References	Trial
Serum/plasma					
Specific proteins/ AAbs	Three proteins (CEA, CA-125, and CYFRA 21-1) and 1 AAb (NY-ESO-1)	RMS	Clinical validation	(62)	
	Two proteins (LG3BP and C163A) and clinical features	DIPN	Clinical validation	(63)	NCT01752114
	Seven AAbs (p53, NY-ESO-1, CAGE, GBU4-5, SOX2, HuD, and MAGE A4)	RMS	Clinical validation	(64,65)	NCT01700257
		DIPN		(66)	
	Four AAbs (FCGR2A, EPB41L3, LINGO1, and S100A7L2)	DIPN	Discovery	(67)	
	Six proteins (CEA, CA-125, SCC, CYFRA 21–1, NSE, and proGRP)	DIPN	Discovery	(68)	
	A signature of complement-derived fragments and tumor-associated proteins	RMS	Discovery	Unpublished	
		DIPN			
	Complement fragment C4d	RMS	Discovery	(69)	
		DIPN		(70)	
miRNA	Ratios among 24 miRNAs	RMS	Clinical validation	(4,71,72)	NCT02247453
		DIPN			
	Signature of 13 microRNA + 6 for normalization	RMS	Clinical validation	(73-76)	COSMOS II trial
		DIPN			
	Signature of 2 microRNA	DIPN	Discovery	(77)	
	Signature of 14 microRNA	RMS	Discovery	(78)	
DNA methylation	SHOX2 and PTGER4 methylation	DIPN	Discovery	(79)	
	Analysis of 11,787 CpG sites across 595 regions in the genome (PanSeer assay)			(80)	
Circulating tumor nucleic acids	ctDNA; NGS technology	RMS	Discovery	(81,82)	NCT02889978
	ctDNA; CAPP-Seq.	DIPN	Clinical validation	(83)	
	ctDNA; Lung-CLiP	RMS	Clinical validarion	(84)	
	ctDNA; Ion Torrent DNA Sequencing technology	DIPN	Discovery	(85)	
	ctDNA; TEC-Seq technology	RMS	Discovery	(86)	
	Signature of 29 genes (RNA)	DIPN	Discovery	(87)	
	ctDNA mutation and proteins (CA-125, CEA, CA19-9, PRL, HGF, OPN, MPO, and TIMP-1)	DIPN	Discovery	(88)	
	Analysis of fragmentation patterns of cell-free DNA			(89)	
mRNA gene expression classifier	Twenty-three gene classifier	DIPN	Clinical validation		NCT01309087, NCT00746759
SNPs	Twenty SNPs for COPD and clinical features	RMS	Clinical validation	(90)	NCT01176383

Table 1 (*continued*)

Table 1 (*continued*)

Candidates	Biomarker	Target	Phase	References	Trial
Bronchoscopy, sputum, breath and urine					
DNA methylation	*SHOX2* and *RASSF1A* methylation	RMS	Discovery	(91)	
MiRNA	Signature of 3 microRNA	DIPN	Discovery	(92)	
Chromosome aberrations	Chromosome regions copy number or fusions (FISH)	DIPN	Discovery	(93)	
Exhaled breath	VOC-nanoparticle biometric tagging (NBT)	DIPN	Discovery		
	VOC-field asymmetric ion mobility spectrometry (FAIMS)		Clinical validation		NCT02612532
Tumor cells	>700 morphological features (by cell CT)	RMS	Discovery		
		DIPN			
	Buccal nanocytology	RMS	Discovery	(94)	
	Porphyrin differential uptake by tumor cells	RMS	Discovery	(95)	
Urine markers	Metabolites	RMS	Discovery	(59)	
Mixed					
Array of biospecimens	Gene expression and proteomic signatures in bronchial airway brushing, proteomic and cytokine signatures in serum	DIPN	Clinical validation	(96)	NCT01785342
		RMS	Clinical validation		NCT02504697

DIPN, diagnosis of indeterminate pulmonary nodules; RMS, risk management in screening context; ctDNA, circulating tumor DNA; NGS, next-generation sequencing; SNP, single-nucleotide polymorphism; COPD, chronic obstructive pulmonary disease.

management and diagnosis of lung cancer. Lung cancer cells activate the complement system through the classical pathway. C4d levels, a downstream molecule derived from this activation, are increased in the biological fluids obtained from lung cancer patients (69,70,106). The value of this biomarker for both lung cancer screening and the management of indeterminate pulmonary nodules has already been reviewed (21). More recently, a novel model based on the quantification in plasma of both complement activation-derived fragments and previously proposed cancer biomarkers has been shown to predict higher risk of lung cancer in asymptomatic individuals enrolled in a CT-screening program. Moreover, the scores derived from this model are associated with higher probabilities of malignancy in patients with indeterminate-risk lung nodules (unpublished data).

miRNAs

Many studies have reported the potential value of plasma miRNA reflecting underlying disease as a biomarker for the clinical management of lung cancer. A plasma signature of 13 miRNA (miR-Test) (73-75) and a signature composed by reciprocal ratios among 24 circulating miRNAs [miRNA signature classifier (MSC)] (4,71,72) have demonstrated high diagnostic accuracy in the screening setting. The prospective validation of these signatures is currently under investigation in three independent screening trials.

Circulating tumor DNA (ctDNA)

The value of ctDNA for the clinical management of lung cancer has been intensely explored in the recent past. Several NGS-based approaches have been proposed for lung cancer diagnosis [reviewed by Abbosh *et al.* (81)]. A prospective, multi-center, observational study enrolling at least 15,000 participants is currently evaluating the value of deep sequencing of circulating cell-free nucleic acids as a biomarker for the detection of different types of cancer at various stages. Recently, a method to improve the sensitivity

of the mutational analysis of ctDNA in blood, based on CAPP-Seq, has been proposed and tested in the context of lung cancer screening. Although levels are very low in early-stage lung cancers, ctDNA is present prior to treatment in most patients and its presence may have prognostic value. Furthermore, most of the somatic mutations found in ctDNA of patients diagnosed with lung cancer and of risk-matched controls reflect clonal hematopoiesis, and are non-recurrent. A tool called 'lung cancer likelihood in plasma' (Lung-CLiP), integrating ctDNA findings with other molecular features, can discriminate early-stage lung cancer patients from risk-matched controls (84). The PanSeer, a noninvasive blood test based on ctDNA methylation, detects lung cancer at early stages (80). Finally, a recent study demonstrate that the fragmentation patterns of circulating cell free DNA may be a useful tool to detect lung cancer (89).

Biomarkers in exosomes

Exosomes are stable, easily collected from body fluids, and are apparently good "concentrators" of otherwise free-circulating biomarkers, thus becoming suitable candidates for liquid biopsies. Furthermore, exosomes are more abundant in cancer patients (107), indicating that tumor or host cells in cancer patients produce more exosomes than healthy individuals (108). Diagnostic studies related to exosomes have mainly focused on miRNA and proteins. miRNAs are the most abundant nucleic acids in exosomes. The following are some examples of discovery efforts of novel diagnostic biomarkers based on molecules stored in exosomes. The level of robustness, reproducibility and maturity of the results of the following examples are heterogeneous and most of them require further validation. miRNA signatures have been considered potential biomarkers in NSCLC since 2009, because miRNA isolated from NSCLC patients' exosomes mirrored miRNA signatures in NSCLC. Several miRNA-panels have been proposed for NSCLC diagnosis in exosomes isolated from plasma/serum (109-117), BAL (118) and pleural fluid (119,120). Expression levels of exosomal MALAT-1, a long non-coding RNA (lncRNA), may have potential value in NSCLC diagnosis (121). Other nucleic acids, such as exosomal dsDNA or mRNA from NSCLC patients have shown potential, but evidence is currently limited to cell lines (122) and animal models (123). Exosome-derived proteins have emerged as a new source of potential biomarkers for NSCLC from early to advanced stages

in blood (124-126), saliva (127), urine (128) and pleural fluid (129). An exosomal lipid profile from peripheral blood can discriminate normal from early and late stage NSCLC patients (130). However, exosomes clearly need further study to reach routine clinical practice, since their current sensitivity and specificity in the diagnosis and treatment of NSCLC are far from ideal. They are also still in a very preliminary phase in the validation arena.

Other possible sources of biomarkers

DNA methylation and polymorphisms, chromosomal abnormalities, RNA, tumor-associated proteins, proteomic signatures, breath volatile compounds, urine metabolites, and cytological analyses have also been proposed as potential biomarkers for the diagnosis of lung cancer (see *Table 1*) in the context of screening. The integration of these models with clinical and radiological (radiomics) features may improve the performance of screening for asymptomatic high-risk individuals and also discriminate between benign and malignant nodules.

Current biomarkers in early lung cancer management: prognostic factors

Correct histopathological evaluation is crucial in NSCLC clinical management. Although either SCC or ADC are considered to have a similar prognosis for a given tumor stage, additional histopathological subtyping has strong prognostic implications. There are three prognostic categories for lung ADCs (131-133), considering the current WHO classification. Adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA), which may soon be considered a single entity (132), followed closely by lepidic predominant tumors, can be classified as low risk. Acinar and papillary predominant tumors may be considered intermediate risk, while micropapillary, solid, mucinous or colloid tumors should be viewed as high risk. Recent studies (134,135) suggest that intermediate risk (acinar and papillary) ADCs can be further subdivided into low and high grade. The cribriform and fused pattern subsets of acinar predominant tumors and the complex papillary patterns are considered high grade.

Squamous cell cancer (SCC) subtypes (keratinizing, non-keratinizing, basaloid pattern, papillary growth, and clear cell feature) have limited prognostic impact. Tumor nest size (tumor budding and single cell invasion), and nuclear grade have been reported as independent prognostic factors

in SCC (136).

Lymphovascular invasion (LVI) (137) is associated with worse recurrence-free survival (RFS) (HR: 3.63, 95% CI: 1.62 to 8.14; 4 studies; 1,147 patients) and overall survival (OS) (HR: 2.38, 95% CI: 1.72 to 3.3). Similarly, a recent meta-analysis suggests that high Ki-67 expression is inversely proportional to OS (HR: 1.59, 95 % CI: 1.35 to 1.88) and DFS (HR: 2.21 95% CI: 1.43 to 3.42) in NSCLC patients (138). The prognostic value of immune cell infiltration of the tumor microenvironment in early-stage lung cancer has been broadly analyzed by several studies. Tumor-infiltrating lymphocytes (TILs) have been evaluated by different methods including the International Immuno-Oncology Biomarkers Working Group consensus proposal. Most studies have shown that high TIL levels are associated with improved DFS and, less frequently, with an increased OS. A meta-analysis of the role of immune cell quantification by immuno-histochemistry performed by Tuminello and cols. (139), revealed that higher levels of CD8+ cytotoxic T cells, CD56/57+ NK cells, and CD20+ B cells are associated with improved OS or DFS. Lung cancers with increased *FoxP3*+ T regulatory cells have worse OS. Additional prognostic and predictive biomarkers for early stage lung cancer have been proposed (140-142). Biomarkers identified by immunohistochemistry (*p53*, *Cyclins*, *Her2*, *EGFR*, etc.), gene expression signatures, methylation or genotyping, although in some cases have been validated (141), have not shown conclusive results and are still experimental. Tumor spread through air spaces (STAS) was proposed in 2015 (143) as an important prognostic factor in lung ADC, especially when sublobar resections have been performed. The surgical procedure-specific outcome can be stratified by STAS (144,145). The expected prevalence of STAS in most studies ranges from 30% to 40% in ADCs. Expertise and careful handling of specimens is required to avoid artefacts that may be misinterpreted as STAS. In our experience, STAS is more frequently found in high grade ADC subtypes (unpublished observation).

Some degree of prognostic value has been shown for more than 200 molecular markers in lung cancer, but their use in routine clinical practice is limited by the lack of both reproducibility and independent validation. Microarray technology has emerged as a powerful tool measuring the expression of hundreds of genes in a high number of samples, and different prognostic mRNA expression signatures have been described in lung cancer. In response to the concern that microarray-based profiles are difficult to

apply in a clinical setting, subsequent efforts have focused on developing quantitative polymerase chain reaction (qPCR)-based molecular signatures. These gene signatures can be analyzed by q-PCR, the gold standard assay for gene expression. Only a few of the initially proposed prognostic qPCR based signatures can withstand the need for quality RNA extraction from fresh frozen specimens. The use of novel RNA extracting and preserving technologies may overcome this limitation, by allowing RNA extraction from formalin fixed, paraffin embedded (FFPE) samples. Xie and cols. developed a 59-gene prognostic signature for NSCLC using genome-wide expression profiling in FFPE samples (146). Kratz and cols. defined a 14-gene prognostic profile for non-squamous NSCLC using qPCR analysis on the same type of samples (147-149). Wistuba and cols. proposed a proliferation-based expression signature of 31 genes for ADC histology subtype (150). Finally, Bianchi and cols. have developed and validated a 10-gene prognostic signature in a large cohort of lung ADC patients using qPCR, also on FFPE samples (151). All of them show promise, but are awaiting further validation.

Recently we have developed and validated two new molecular prognostic signatures for ADC and SCC (152,153) using immunodetection-based techniques to assess the expression of 3 and 5 prognostic proteins (for ADC and SCC respectively). Because of its wide availability (including the type of sample: FFPE) and affordability, immunohistochemistry is still one of the preferred ways to introduce single novel biomarkers into clinical practice (154). Moreover, an IHC-based prognostic signature for SCC is a major advance, since the aforementioned qPCR based signatures have been validated only for non-squamous histologies or ADC. More recently, novel qPCR-based studies have been published for SCC prognostication, but require further validation (155,156). The protein panels developed by our group mentioned above identify a subset of stage I–II patients with a higher risk of recurrence and survival who may need more aggressive therapy or closer follow-up. This classifier improves the prognostic value of TNM staging, and is able to identify a group of early stage ADC or SCC patients as potential candidates for adjuvant chemotherapy. Jin and cols. have also proposed similar protein-based signatures for both ADC and SCC (157). As the lists of proteins in both studies do not overlap, it is possible that a combination of both signatures would have even stronger prognostic value.

Finally, a variety of recently published artificial intelligence-based studies (158,159) also show that complex

comprehensive computational pathological image analysis can give rise to algorithms to predict lung cancer prognosis. The combination of sophisticated deep-learning based image analysis and robustly validated prognostic markers will probably be the way forward to obtain clinically useful prognostic information in the coming years.

Current biomarkers in early lung cancer management: predictive factors for adjuvant therapy

Adjuvant therapy in NSCLC is still a controversial subject, and, although supported by different societies (160-163), its indications, especially in early stage NSCLC, remain unclear. Adequate patient selection in order to maximize the benefit of these therapies, while limiting toxicity, is key (164). Current patient selection is based on the risk of recurrence determined by pathological stage (164), age, comorbidities and performance status.

In the previous section, we have described recent advances to refine personalized prognostic information, which may also be helpful in deciding whether to offer adjuvant therapy. There are ongoing efforts to combine pathological stage with biomarkers to further refine the selection criteria, bust most of them remain investigational and need to be validated in prospective clinical trials (147,152,153).

Modern clinical trials assessing the benefits of adjuvant chemotherapy in early stage lung cancer have yielded controversial results. The Adjuvant Lung Project (ALPI) trial (165), the Big Lung Trial (BLT) (166) and the CALGB 9633 trial (167) found no improvement in 5-year survival and DFS. On the other hand, the International Adjuvant Lung Cancer Trial (IALT) (168), a trial from Canada and the US (169), and the Adjuvant Navelbine International Trialist Association (ANITA) trial reported a benefit in median survival and DFS with adjuvant chemotherapy (170). Side effects of adjuvant treatment have been a constant concern in these trials, as well as the poor compliance with the planned chemotherapy schemes, especially considering the target group are post-resection patients. All of the aforementioned trials included a platinum-based chemotherapy regimen (165-170). Older studies which included patients with non-platinum based regimens, including alkylating agents (mytomicin C and ifosfamide), showed a decreased OS and increased chemotherapy-related complication rates (171). Furthermore, The Lung Adjuvant Cisplatin Evaluation (LACE) meta-analysis (172)

demonstrated that the companion drug administered with cisplatin did not modify the effect of adjuvant therapy.

Adjuvant chemotherapy following complete resection may be associated with a 5.4% increase in 5-year OS for patients with stage II–IIIA NSCLC (HR: 0.83; P=0.004) (172). Although a cisplatin/vinorelbine regimen has been widely used (173), a combination of cisplatin and pemetrexed demonstrated a similar efficacy profile with better tolerability in non-squamous histology (174). Bevacizumab does not appear to improve outcomes in this setting (175), while the role of adjuvant chemotherapy in stage IB NSCLC remains controversial. Only an exploratory analysis from CALGB 9633 (167) demonstrated a significant survival benefit in favor of paclitaxel/carboplatin for patients who had tumors ≥4 cm in diameter (HR: 0.69; P=0.043). Only the non-squamous histology is predictive of efficacy in the context of pemetrexed (176).

Several molecular markers, such as excision repair cross-complement group 1 (*ERCC1*), ribonucleotide reductase regulatory subunit M1 (*RRM1*), breast cancer-specific tumor suppressor protein 1 (*BRCA1*), and receptor-associated protein 80 (*RAP80*), have been assessed, but abandoned for lack of efficacy. The value of thymidylate synthase (TS) has just been studied in the ITACA trial, pending results (177-181). Finally, there has been a great interest in identification of specific gene signatures based on gene expression (147,182), methylation of DNA (183,184), or miRNAs (185,186) that can provide prognostic and predictive guidance: however, none of the studies to date have proven their clinical value in prospective trials.

Since one of the major advances in the management of metastatic NSCLC has been the identification of driver genetic alterations that can be beneficially targeted, *EGFR*-TKIs have also been tested in the adjuvant scenario (187,188). The first reported trial of *EGFR* inhibitors in early stage lung cancer patients was terminated prematurely (189) due to lack of efficacy when compared to placebo. A subsequent trial showed an improved 2-year DFS in stage IIIA patients treated with erlotinib when compared to cisplatin and vinorelbin in patients harboring *EGFR* mutations (190). Osimertinib has very recently proved beneficial for the DFS in the ADAURA trial (4). The difference between the two groups (treated and placebo) was very significant in this trial. At 24 months, 90% of the patients with stage II to IIIa disease in the osimertinib group (95% CI: 84 to 93) and 445 of those of the placebo group (95% CI: 37 to 51) were alive and disease free (overall hazard ratio for disease recurrence or death, 0.17

vs. 0.26; P<0.001). We are now eagerly waiting for the OS results, which also will be relevant in the area of lung cancer screening to address the question on the relevance of targeted adjuvant therapy in early tumors diagnosed in screening cohorts. In addition to TKIs, the role of adjuvant crizotinib in *ALK* positive patients is also being evaluated (191).

In the field of immunotherapy, targeting melanoma-associated antigen-A3 (MAGE A3) (192) was addressed in the MAGRIT study (193). Unfortunately, no benefit in disease-free or OS was found when compared to placebo. A liposome peptide vaccine to mucin 1 (MUC1) glycoprotein known as tecemotide (L-BLP25) has also been studied. A phase III study did not report any advantage in survival of stage III NSCLC patients treated with tecemotide when compared to placebo (194). Pembrolizumab, a PD-1 inhibitor, is approved as first line therapy for metastatic NSCLC in patients with more than 50% of PD-L1 expression (195). Currently, its efficacy as adjuvant therapy in early stage lung cancer is being evaluated in multiple trials (196) with promising results. Ongoing clinical trials, including combined drugs and chimeric antigen receptor (CAR)-T cell treatment (197), will improve our understanding of immune mechanisms related to NSCLC and help develop new therapies to improve early stage NSCLC outcomes.

Future directions of molecular biomarkers in early stage lung cancer: the need for artificial-intelligence based integration

The identification and validation of phenotypes combining clinical, radiological and genetic data have the potential for great clinical impact. The development of these predictive models will not only improve evaluation, risk stratification, diagnosis and treatment of NSCLC patients, but also, make clinicians' work less cumbersome by allowing for an integrated and practical analysis of all patient characteristics (198).

To reach this goal, it is of the utmost importance to establish prospective, independent, and properly audited databases able to capture clinical, radiologic, surgical and genetic information in a standardized, reproducible manner. Furthermore, the processing of the massive amount of data generated will be facilitated by the correct application of these models (198,199). Finally, the optimal design of clinical trials to show evidence based clinical utility of the integrated algorithms combining together imaging

and clinical data need to be discussed and agreed with the regulatory agencies. There are a number of potential designs each with advantages and limitations (55). Despite the wide range of molecular biomarkers available in the laboratory, the fact is that none of them has been integrated in routine clinical practice. Their implementation and validation in early stage lung cancer patients has become the greatest challenge to date. Scant improvement in lung cancer related deaths mandates refinement of current staging systems. Whether or not these refinements will come from prognostic molecular and clinical markers alone is still unclear. The same holds true for our capacity to predict the efficacy and choose the modality of adjuvant treatments. We need a better understanding of the detailed molecular makeup of early stage lung tumors and its correlation with outcomes and response to therapy.

Furthermore, well-implemented and established lung cancer screening programs, carried out by experienced multidisciplinary teams, will set the stage for streamlined cost effective protocols. Integration of the different molecular signatures with clinical and radiological manifestations and the evaluation of their impact in long-term outcomes will be of paramount importance. The design of artificial intelligence models that can help in the integration of all the data and in the decision-making process will definitely be welcome and facilitate specialists' work.

As researchers and clinicians, we need to adapt to the fast pace of current clinical developments. In addition, we need to continue to engage, validate, and facilitate the wide adoption of newly developed biomarkers, prioritizing their predictive ability, cost-efficiency, and generalizability. In summary, what seems clear is the need to integrate and understand the extent of all the data obtained in each patient. Combining clinical features, radiologic images and molecular markers will probably pave the road for further survival improvements in NSCLC patients.

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Footnote

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