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Liquid biopsy in detecting early non-small cell lung cancer

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ABSTRACT

Lung cancer screening programs, particularly in the UK, have shown a decrease in lung cancer-related deaths among individuals who underwent low-dose computed tomography (CT) screening. Researchers are now focusing on evaluating cell-free DNA through various methods to determine if pre-diagnostic mutations can be detected years before clinical diagnosis. This could help identify individuals at high risk of developing lung cancer. However, while this approach has successfully identified precursors of follicular lymphoma, the presence of occult lung preneoplasia in non-small-cell lung cancer still requires further investigation.

The TRACERx consortium is conducting extensive research to comprehensively assess the detection and progression of non-small cell lung cancers (NSCLC). Liquid biopsy is being used in advanced stages of the disease to monitor disease progression, predict treatment response, and identify targetable driver oncogenic mutations and fusion genes. Intense research is also underway to identify numerous diagnostic gene signatures with high accuracy for early-stage lung cancer. However, a more focused clinical approach is needed, with a mechanistic focus on the key pathways of cancer development.

Loss of liver kinase B1 (*LKB1*) function and deactivation due to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a metabolite of tobacco-specific carcinogens, could potentially be traced and contribute to the development of new biomarkers. This testing could complement machine-learning approaches. The discovery of epidermal growth factor receptor (*EGFR*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations in healthy lung tissues by TRACERx investigators may also lead to the development of novel diagnostic tools. Tumor protein 53 (*TP53*) loss should also be considered as a marker that could contribute to malignant transformation. Intercepting aggressive non-small-cell lung cancer is a pressing priority.

In this review, we discuss our experience and explore other research on exosomes and plasma circular RNA as potential biomarkers. Circular RNAs, formed through non-sequential back-splicing of pre-mRNA transcripts, play a role in epithelial-mesenchymal transition, with many of them regulated by the RNA-binding protein Quaking. Platelet RNA has shown promise in detecting early and late-stage cancer. The extensive exploration of liquid biopsy aims to provide affordable methods for tracing circulating precursors of non-small-cell lung cancer, highlighting the importance of its mission.

Introduction

Lung cancer is the main cause of all cancer mortality, primarily due to cigarette smoking. The risk of developing lung cancer is associated with various factors, including the cumulative lifetime smoking dose, duration, intensity, and timing of cessation [1]. Mutations in the human lung accumulate with age, with higher levels in the normal proximal bronchial basal cells of smokers. Interestingly, the dose dependency of the mutation

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frequency in smokers, levels-off around 23 pack-years, with mutational burdens in heavy smokers not being significantly different than those in much lighter smokers. These findings posit that intrinsic factors could attenuate lung cancer risk by reducing mutations, either by increasing DNA repair proficiency or by dwindling DNA damage through detoxification of tobacco smoke [2]. Several clinical trials indicate that lung cancer screening with low-dose computed tomography (LDCT) can reduce lung cancer mortality. The Early Lung Cancer Action Project (ELCAP) in North America showed superior capacity of low-dose CT over chest radiography to identify small non-calcified nodules that could be early-stage lung cancers [3]. Randomized controlled trials have been carried out to examine the mortality impact of lung cancer screening with low-dose CT, such as the National Lung Screening trial (NSLT), the Nederlands Leuvens Screening Onderzoek (NELSON), and the German Lung Cancer Screening Intervention (LUSI). In the low-dose CT groups there were 20%, 24% and 26% reductions in mortality for lung cancer compared to the radiography group [4–6]. In the NSLT trial, the number needed to screen to prevent lung cancer-related deaths was 320 [4]. The LDCTscreening study in China involved 1,032,639 individuals of whom 3581 had a lung cancer diagnosis after a median follow-up of 3.6 years. Participants in the LDCT screened group had lower lung cancer mortality compared to those in the non-screened group [7]. The complete list of clinical trials of lung cancer screening with LDCT can be found in Adams et al. [8].

Identification of high-risk individuals is essential to make lung cancer screening more effective. Several selection criteria and risk models have been developed for different populations, including North American, European, and Asian populations [7–9]. A sex-specific scoring system derived from the Harvard Cancer Risk Index has been used to assess the risk of lung cancer for men and women [10]. The score comprises cigarette smoking, self-reported exposure to ambient particulate matter in the past 10 years, level of physical activity, history of chronic respiratory diseases, family history of lung cancer, dietary intake of fresh vegetables in the past 10 years, and history of passive smoking. Men without smoking history were excluded from the high-risk group [7]. Smokers were defined as those who had previously smoked or were currently smoking tobacco more than once per day for at least 6 months. Participants were classified as non-smokers, light smokers (<20 pack-year smoking history), and heavy-smokers (>20 pack-year smoking history), where a smoking pack-year is equal to one packet of 20 cigarettes every day for 1 year. Passive smoking, exclusively measured in women, refers to the involuntary inhalation of other people's tobacco smoke [7]. Occupational exposure to hazardous substances encompasses prolonged exposure to asbestos, rubber, dust, pesticide, radiation, beryllium, uranium, and radon within the occupational setting for a minimum duration of one year. Frequent exercise is defined as engaging in physical activity at least three times per week, with each exercise session lasting over 30 min. The incidence of lung cancer in non-smoking women is most prevalent in South Asia (where 83% of women with lung cancer having never smoked), followed by East Asia (61%). In contrast, only 15% of women with lung cancer in the USA have never smoked [11]; see details in Adams et al. [8].

The analysis of blood-based biomarkers (liquid-biopsy) [12] are an important aid for lung cancer screening, providing guidance in the management of indeterminate lung nodules. The NELSON trial categorized the probability of lung cancer in CT-detected pulmonary nodules based on size criteria. Small nodules (volume <100 mm^3 or diameter < 5 mm) were not predictive for lung cancer, whereas immediate diagnostic evaluation was indispensable for large nodules (>300 mm^3 or >10 mm). Intermediate-sized nodules (volume ranging between 100 and 300 mm^3 or diameter of 5–10 mm) required volume-doubling time assessment [13]. Identifying factors that predict the probability of malignancy in lung nodules detected in the initial LDCT scans remains a clinical dilemma. In the Pan-Canadian Early Detection of Lung Cancer Study (PanCan) and the British Columbia Cancer Agency (BCCA) study, the rates of cancer among individuals with nodules were 5.5% and 3.7%, respectively. Predictors of cancer included older age, female sex, family history of lung cancer, emphysema, larger nodule size, upper lobe location, part-solid nodule type, lower nodule count, and spiculation [14].

Blood-based biomarker studies have demonstrated potential usefulness for early detection of lung cancer through various types of analysis, such as microRNAs, autoantibodies to lung cancer-associated antigens, circulating protein biomarkers, circulating tumor DNA (still under investigation for early detection of lung cancer), DNA methylation, enzyme-linked immunosorbent assay panels, single nucleotide polymorphisms, and others [8]. The characterization of circulating tumor cells (CTCs) and cell-free DNA constitutes two key components of liquid biopsies' applicability [12,15]. Recent reports have highlighted analytes for liquid biopsy in the early detection of lung cancer [16]. The United Kingdom has been at the forefront of addressing logistical issues in lung cancer screening through clinical trials. The English National Health Service (NHS) has invited more than 500,000 participants to date, scanned more than 120,000 participants, and diagnosed more than 1500 lung cancers since 2019. A summary of UK expert opinions has been reported to provide guidance to those organizing and delivering lung cancer screenings in other countries [17].

Tobacco smoking, driven by more than 60 carcinogens in cigarette smoke that directly damage and mutate DNA, is a major cause of lung cancer. The World Health Organization estimates that there are 1.1 billion smokers worldwide and 1.8 million deaths from lung cancer annually [18]. The mutational patterns in healthy lung tissue have been analyzed in non-smokers, current smokers, and ex-smokers through whole-genome sequencing of single cells obtained from lung tissue biopsies.

Non-smoking individuals showed minimal mutations, while current smokers exhibited a high proportion of cells with numerous mutations. The frequency of driver mutations increased with age, affecting 4–14% of cells in middle-aged subjects who had never smoked. In current smokers, at least 25% of cells have driver mutations, but quitting smoking promoted the replenishment of bronchial epithelium with mitotically quiescent cells that had avoided tobacco-induced mutagenesis [19]. This study also postulated that the health benefits of smoking cessation begin immediately, as the bronchial epithelium is replenished with cells resistant to the sustained mutagenic effects of cigarette smoke, thereby restoring the regenerative capacity of the lungs [19]. It is tempting to posit that the immunosuppressive tumor microenvironment plays an important role in lung oncogenesis, where tumor-associated macrophages and myeloid-derived suppressor cells contribute to immunosuppression in the tumor microenvironment. Experimental evidence has shown that agonists of the α2-adrenergic receptors inhibit adenylate cyclase, thereby blocking the immunosuppressive cyclic adenosine monophosphate (*cAMP*)/phosphorylated cAMP response element-binding protein (*pCREB*) signaling pathway [20].

Understanding the biology and exploring new models and microenvironments is considered the holy grail in the early detection of non-small cell lung cancer (NSCLC) [21]. However, the genetic evolution of NSCLC, although inferable, has not been fully elucidated as it has been in the genetic model of colorectal tumorigenesis. In colorectal tumorigenesis, *KRAS* mutations appear to occur in one cell of a pre-existing small adenoma during preneoplasia stages, leading to clonal expansion and the development of larger and more dysplastic tumors [22]. A multiplexed mouse model of oncogenic *KRAS*-driven lung cancer has analyzed the impact of 48 tumor suppressor genes, which are determinants for *KRAS*-driven lung cancer initiation, overall growth, and transition to rapid growth. These genes include *Trp53* (encoding mouse p53), *LKB1, RNA-binding motif protein 10 (RBM10), Kelch-like ECH-associated protein 1 (KEAP1)*, among others, representing different stages of tumor development [23].

One of the major hurdles for the applicability of the liquid biopsy is the lack of predictability in the earliest stages of lung tumorigenesis and the early detection and interception of aggressive, genome-unstable tumors. As some tumors have been described as “born to be bad,” predicting their behavior and progression remains a significant challenge [24]. Large-scale

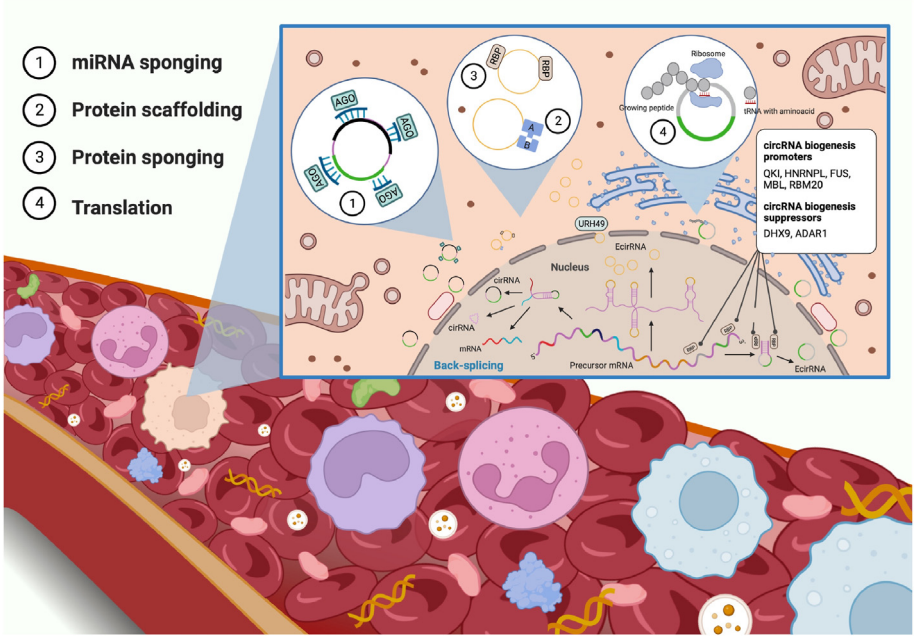

Fig. 1. Synthesis and biological functions of circRNAs. CircRNAs originate in the nucleus by different mechanisms of backsplicing, including the generation of exonic circRNAs (EcircRNA) assisted by RNA binding proteins (RBPs). Once exported to the cytoplasm, they carry on their functions: sponging miRNAs and proteins, serving as protein scaffolding, or translating into small peptides.

pan-cancer studies have pointed out that genomic alterations are present many years before detectable disease [25]. Several studies have demonstrated a near 100% 5-year relapse-free survival rate with sub-lobar resection in early NSCLC cases with tumors measuring 2 cm or less, using radiological criteria to predict pathological non-invasiveness. These criteria include the absence of vascular invasion, lymphatic permeation, and lymph node metastasis. The consolidation-to-tumor ratio (CTR), which considers the longest diameter of the solid part of the tumor divided by the total diameter, including the ground-glass opacity (GGO) on CT scans, is also taken into account [26]. The Japanese Clinical Oncology Group (JCOG) 2001 trial showed that CTR is superior to other variables as a predictor of pathological non-invasive tumors. Segmentectomy with hilar, interlobar, and intrapulmonary lymph node dissection was performed in patients with a tumor diameter up to 3 cm (T1N0), including GGO. The 5-year relapse-free survival was 98.0% [27].

Liver kinase B1 (LKB1) or serine/threonine kinase 11 (STK11)

Liver kinase B1 (*LKB1*) or *serine/threonine kinase 11* (*STK11*) is associated with inactivation mutations in 20% of NSCLC cases, often accompanied by *KRAS* mutations. NSCLC with *KRAS/LKB1* mutations has a response rate of <10% to immune checkpoint inhibitors, compared to >30% in cases with *KRAS/TP53* mutations. *LKB1* deficiency suppresses antigen processing and presentation associated with immunoproteasome activity [28]. Recently, it has been found that the proteasome regulator, *proteasome activator subunit 4 (PSME4*) is upregulated in NSCLC, altering proteasome activity, attenuating antigen diversity and is associated with a lack of response to immune checkpoint inhibitors. In healthy lung tissue there is a homeostatic balance between diverse proteasome complexes that is disrupted in NSCLC altering tumor proteostasis. Cold NSCLC tumors defined by low inflammation and reduced lymphocyte infiltration, have higher expression of *PSME4*. As a consequence of upregulation of *PSME4*, there is an altered protein degradation with reduced presentation of peptides on major histocompatibility complex class I (MHCI) [29].

Loss of *LKB1* is implicated in lung cancer initiation, development, and progression in mouse models [23]. *LKB1* loss of function, in addition to mutations, has been associated with smoking status. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a major metabolite of a tobacco-specific carcinogen, 4-(methylnitrosamino)-1(3-pyridyl)-1-butanone (NKK), induces *LKB1* phosphorylation and its loss of function [30]. The finding that NNAL-mediates *LKB1* phosphorylation and loss of function in lung cancer patients with wild-type *LKB1* could open new avenues for assessment of *LKB1* as an early detection biomarker coupled with the quantification of NNAL in blood samples. In the Bian et al. [30] study, NNAL was detectable in plasma samples from smokers but not in non-smokers, with concentrations ranging from 10.4 pM to 296.0 pM. In *LKB1* wild-type NSCLC cell lines (H1299, H1975 and HCC827), increased phosphorylation of *LKB1* at Ser 248 was observed after exposure to the (R)-NNAL enantiomer. Therefore, the putative effect of *LKB1* loss function in NSCLC. A 120-day NNAL exposure in lung cancer cells, mimics its chronic exposure among smokers, turn out in strong *LKB1* phosphorylation, cell migration and chemoresistance, even in the absence of NNAL, revealing the long-lasting *LKB1* loss of function albeit such effect vanished following NNAL removal for two months [30].

EGFR and *KRAS* driver mutations in healthy lung

Particulate matter (PM) is a key constituent of air pollution. Fine particles <2.5 μm ($PM_{2.5}$) travelling deep into the lung are associated with lung cancer risk. It has recently been proposed that $PM_{2.5}$ favors lung cancer by acting on cells that harbor pre-existing oncogenic mutations in healthy lung tissue. Functional mouse models disclose that air pollutants cause an influx of macrophages into the lungs and release of interleukin-1β, resulting in a progenitor-like cell state within *EGFR*-mutant lung alveolar type II epithelial cells, which promotes lung oncogenesis. The TRACERx Consortium investigators conducted ultra-deep mutational analysis of histologically normal lung tissue from 295 individuals and discovered oncogenic *EGFR* and *KRAS* driver mutations in 18% and 53% of healthy tissue samples, respectively [31]. To examine whether *EGFR* mutations exist in healthy lung tissue from people who have never developed lung cancer in their lifetime, 59 healthy lung samples collected at autopsy (n = 19 patients) from participants in the PEACE study who died of other cancers were analyzed. An *EGFR*-driver mutation was detected in the healthy lung of 16% (3 out of 19) of patients. Also 38 out of 195 (19%) patients harbored activating *EGFR*

mutations in non-cancerous lung tissue that were not detectable in tumor tissue (TRACER x cohort) using digital droplet PCR and MiSeq-based analysis [31]. Based on the frequency of oncogenic *EGFR*-driver mutations identified in healthy lung tissue across all patients in the PEACE and TRACERx cohorts, a Bayesian method estimates the presence of an *EGFR*-driver mutation in lung cells. The calculation showed that 1 in 554, 500 lung cells would harbor an oncogenic *EGFR* mutation [31]. Anthracosis, determined by the presence of anthracitic pigment, is a readout of exposure to ambient air pollution. There was an association between anthracosis and increased variant allele frequencies (VAFs) of *EGFR*-driver mutations. Noticeable is the fact that cigarette smoking is not a risk factor for anthracosis. Smoking status, sex, anthracosis and age of patients in the TRACERx cohort were entered into a multivariable model for the likelihood of an *EGFR* mutation in healthy tissue. Female sex showed the strongest association.

KRAS mutations were examined using an ultradeep sequencing platform in a separate group of patients with or without cancer (n = 81). The authors analyzed 48 samples of non-cancerous lung tissue from the PEACE study (lung cancer, n = 9; other cancer, n = 39). Additionally, 33 samples of healthy lung tissue were obtained from the Biomarkers and Dysplastic Respiratory Epithelium (BRDE) study [31]. The BRDE cohort consisted of patients with suspicious lung nodules identified through CT scans who underwent navigational bronchoscopy for further evaluation. In each patient, a brushing sample enriched for bronchial epithelial cells (>89%) from the uninvolved contralateral lung was taken and used as the source of healthy tissue. The results revealed that 43 out of 81 (53%) samples of non-cancerous lung tissue harbored *KRAS* driver mutations [31].

These findings provide valuable insights for exploring novel strategies to detect *EGFR* and *KRAS* mutations in cell-free DNA for early detection of NSCLC, in conjunction with *TP53* mutation assessment, as *TP53* inactivation is a common early event in occult preneoplasia [32,33].

RNA circularization in early NSCLC

Circular RNAs (circRNAs) are covalently closed molecules of single-stranded RNAs originated by a non-canonical splicing process (known as back-splicing) of the precursor mRNA (pre-mRNA). During this process, a downstream splice donor is covalently joined to an upstream splice acceptor across one or several intronic/exonic regions. Looping and interacting of the region downstream of the splice donor site and the region upstream of the splice acceptor site is necessary for backsplicing. This process of circRNA biogenesis can be mediated by different RNA-binding proteins (RBP), such as the RNA-binding protein Quaking (QKI) [34] (Fig. 1). Of interest is the fact that during epithelial-mesenchymal transition (EMT), dimerization of QKI could favor production of circRNAs involved in cancer metastasis [36]. Back-splicing can also be facilitated by dimerization of other RBPs, or other different back-splicing mechanisms involving inverted repeat intron sequences, including *Alu* elements, or non-repetitive complementary sequences [35]. Among the different RNA-binding proteins (RBPs), *heterogeneous nuclear ribonucleoprotein L (HNRNPL), fused in sarcoma/-translocated in liposarcoma (FUS), muscleblind-like (MBL),* and *RNA-binding motif protein 20 (RBM20)* promote exonic-circular RNA (EcircRNA) biogenesis via exon skipping [34] (Fig. 1). Conversely, *DEAH-box helicase 9 (DHX9)* and *Adenosine Deaminase Acting on RNA 1 (ADAR1)* suppress *Alu*-mediated circRNA biogenesis by destabilizing RNA structures [37] (Fig. 1). Widespread RNA circularization events lead to the genesis of hundreds of circRNAs in different human tissues. Aberrant expression of some of these circular transcripts promotes cell proliferation through functions that could be different from their parental linear RNA in different types of cancer [38]. Increasing evidence suggests that circRNAs could become determinant biomarkers influencing cancer characteristics [38,39]. Moreover, novel techniques have been gradually developed to enable their detection in plasma, which would otherwise go undetected by RT-QPCR assay [40]. Some circRNAs may bind to micro RNAs (miRNAs) in a process known as miRNA "sponging", promoting downstream mRNA expression (Fig. 1).

Other potential functions of circRNAs include serving as protein scaffolds, canonical splicing competitors, or even translating into small peptides through internal ribosome entry sites (IRES) in a cap-independent manner (Fig. 1) [34,41]. Furthermore, certain circRNAs have been identified to lack IRES but to harbor an infinite open reading frame (ORF) with an ATG start codon, enabling continuous translation. During this process, the stop codon is replaced by a system referred to as "programmed-1 ribosomal frameshifting" (-1PRF)-induced out-of-frame stop codon (OSC) [42]. This mechanism, known as rolling circle amplification, is carried out by circ*EGFR* in glioblastoma forming a protein complex designed as rolling-translated *EGFR* (rt*EGFR*). Rt*EGFR* directly interacts with *EGFR*, maintaining *EGFR* membrane localization, hence, attenuating endocytosis and avoiding *EGFR* degradation [43].

Different approaches are currently being carried out for the correct identification and quantification of circRNA from different biosources, including liquid biopsies. In a study led by Pedraz-Valdunciel, from our group, the nCounter platform was tested for the assessment of circRNA expression profiles in NSCLC cell lines and formalin-fixed paraffin embedded (FFPE) NSCLC specimens. The nCounter technology allows the analysis of up to 800 transcripts with a short turnaround time of less than 48 h. During this study, a 78-circRNA nCounter panel was designed, including both highly and low expressed circRNAs that could be involved in lung cancer. For this purpose, each probe was designed to target a flanking exonic sequence between 35 and 55 nucleotides of the circRNA junction site [44]. The study proves the feasibility of determining circRNAs in FFPE tissues using nCounter, and machine-learning (ML) approaches permitted discriminate nontumor control tissue from NSCLC with an 8-circRNA signature [44]. In addition, a four-circRNA signature (circEPB41L2, circSOX13, circBNC2 and circCORO1C) classified early-stage NSCLC samples from nontumor controls, attaining a maximum area under the receiver operating characteristic curve (AUC ROC) of 0.981. Validation of nCounter results were carried out by RT-qPCR and further Sanger sequencing of the junction sites. CircEPB41L2, circSOX13 and circBNC2 were downregulated in NSCLC in comparison with control tissues thus validating nCounter results [44].

Based on the abovementioned results, our group carried out the analysis of extracellular vesicle circRNAs (EV-circRNAs) for the detection of early-stage NSCLC. To this end, Pedraz-Valdunciel and colleagues developed a protocol for EV enrichment from plasma, as well as RNA purification and circRNA analysis using the nCounter platform [45]. Different volumes of plasma, together with different number of pre-amplification cycles were tested. The highest total count was obtained with an input of 500 μL of plasma, and this volume also enabled the detection of a greater number of circRNAs. Our protocol was applied to analyze plasma samples from a cohort consisting of 66 subjects, including 36 early-stage NSCLC patients and 30 non-cancer donors. On average, 40 EV-circRNAs were detected per sample in the control group, while the NSCLC group exhibited 47 EV-circRNAs.

Among the 78 circRNAs included in the panel, 70 were detected in at least one NSCLC sample and 68 in at least one non-cancer control. Four EV-circRNAs were exclusive to NSCLC patients and two to non-cancer donors. In EV-enriched samples from NSCLC patients compared to controls, we observed significant upregulation of eight circRNAs. These include *circular erythrocyte membrane protein band 4.1 like 2* (*circEPB41L2*), *circular core 1 synthase, glycoprotein-N-acetylglactosamine-3-beta-galactosyltransferase 1 (circC1GALT1), circular zinc finger RNA binding protein (circZFR), circular ubiquitin specific peptidase (circUSP3), circular zinc finger CCHC domain-containing protein 6 (circZCCHC6), circular cyclin B1 (circCCNB1), circular DENN domain containing 1B (circDENN1B),* and *circular homeodomain interacting protein kinase 3 (circHIPK3)*ML analysis identified a 10-circRNA signature that was able to discriminate NSCLC patients from controls with an AUC ROC of 0.86. Such study paves the way for employing the nCounter platform for multiplexed EV-circRNA studies in early detection of NSCLC [45].

Similarly, other groups like Luo et al. detected circRNA in lung cancer cell lines and plasma from NSCLC patients by using next-generation sequencing (NGS) or highly sensitive real-time digital droplet PCR (RT-ddPCR). Through NGS analysis of transcriptomes in lung cancer cell lines, Luo and colleagues identified hsa-circ-0000190 (C190) as significantly overexpressed. Moreover, they confirmed an elevated expression level of C190 in the blood samples obtained from NSCLC patients by RT-ddPCR. This heightened expression of C190 was found to be associated

Table 1
Studies exploring the role of blood-based biomarkers for the detection of lung cancer. ADK, adenocarcinoma; ctDNA/cfDNA, circulating-tumor DNA/cell-free DNA; CT, computed tomography; CTCs, Circulating tumor cells; HR, hazard ratio; IHC, Immunohistochemistry; IF, immunofluorescence; LC, lung cancer; LDCT, low-dose computed tomography; NSCLC, non-small cell lung cancer; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase-polymerase chain reaction; SCC, squamous cells carcinoma.

Biomarker	Study	Method	N. of participants	Primary Objective(s)	Results
miRNAs	MILD trial (correlative study) (Sozzi G et al., 2014)	qRT-PCR (microRNA signature classifier-MSC)	939	Diagnostic value of MSC for lung cancer early detection and of combined MSC and LDCT	- Sensitivity 87% - Specificity 81% *Negative predictive value:* - detection 99% - death for disease 99.86% *Combined:* - False-positive rate 3.7% (versus 19.4% of LDCT)
	COSMOS (Montani F et al., 2015)	qRT-PCR	1035	Diagnostic value and identification of "high-risk" population	- Accuracy 74.9% - Sensivity 77.8% - Specificity 74.8%
	BioMILD (Pastorino U et al., 2022)	qRT-PCR	4119	Diagnostic and prognostic value of MSC at baseline in addition to LDCT	*LC incidence at 4 years:* - 0.8% in CT neg/MSC neg - 1.1% in CT neg/MSC pos - 10.8% in CT pos/MSC neg - 20.1% in CT pos/MSC pos *LC mortality rates at 5 years:* - 0.5 in CT neg/MSC neg - 1.5 in CT neg/MSC pos - 4.2 in CT pos/MSC neg - 10.1 in CT pos/MSC pos
Autoantibodies tumor-associated antigens	Early Diagnosis of Lung Cancer Scotland (ECLS) Trial (Sullivan FM et al., 2021)	EarlyCDT-Lung test by ELISA (p53, NY-ESO-1, CAGE, GBU4-5, HuD, MAGE A4 and SOX2)	12208	Predictive value of lung cancer risk of EarlyCDT-Lung plus CT screening versus standard clinical care (symptomatic presentation)	*Lung cancer detection rate:* 1.0% *Incidence of stage III/IV LC:* - Positive-test arm: 58.9% - Negative-test arm: 73.2%
	German Lung Cancer Screening Intervention Trial (González Maldonado S et al., 2021)	EarlyCDT-Lung test	4052	Diagnostic value of EarlyCDT-Lung test	- Sensitivity 13% - Specificity: 1. Baseline control group 88.9% 2. Controls presenting CT-detected nodules 91.1%
ctDNA/cfDNA	LungClip score (Chabon JJ et al., 2020)	Cancer Personalized Profiling by deep Sequencing (CAPP-Seq)	94	Diagnostic value of 'lung cancer likelihood in plasma' (Lung-CLiP) for lung cancer screening compared to risk-matched controls	Stage I/II/III - Sensitivity: 63.0%, 69.0%, 75.0% - Specificity 80.0%
Extracellular vesicles (exosomes)	Jin X et al., 2017	NGS (exosomal miRNAs)	148	Diagnostic value and discrimination between ADK and SCC	- ADK specific: miR-181-5p, miR-30a3p, miR-30e-3p, and miR-361-5p - SCC specific: miR-10b-5p, miR-15b-5p, and miR-320b *Diagnostic accuracy of three combination miRNA panels*: AUC value of 0.899, 0.936, and 0.911 for detecting NSCLC, ADK, and SCC, respectively
CTCs	Zhao Q et al., 2021	IHC, IF, PCR	3997 (data from 21 studies)	Diagnostic value for lung cancer detection	*Pooled* - Sensitivity 0.72 - Specificity 0.96 *Pooled likelihood ratio:* - positive: 16.86 - negative: 0.29
Metabolites/ Proteins	Sun Q et al., 2019	Gas chromatography-mass spectrometry (GC-MS) for metabolomics analyses	60	Identification of altered metabolic pathways in LC compared to healthy subjects	Biomarkers for LC: Indole-3-lactate, erythritol, adenosine-5-phosphate, paracetamol and threitol
DNA Methylation	Kneip C et al., 2011	RT-PCR for SHOX2 methylation	411	Diagnostic value	- Sensitivity 60% - Specificity 90% Higher sensitivity of cancer detection in patients with stages II (72%), III (55%), and IV (83%), compared to stage I

with larger tumor size, a predominant histological pattern of adenocarcinoma with micropapillary and solid growth, as well as advanced stage of the disease. ROC curve analysis shows that C190 discriminates between healthy individuals and stage I-IV NSCLC patients, with an AUC ROC of 0.95. Using 2722 copies/mL as a cut-off value for C190 circRNA expression threshold, the sensitivity and specificity of C190 circRNA for the diagnosis of lung cancer were 0.9 and 0.902, respectively [40]. The circRNA transcriptomes in A549 (*KRAS* mutated) and HCC827 (*EGFR* mutated) NSCLC cell lines demonstrate that among 7494 detected circRNAs (encoded by the genes distributed on all chromosomes) there were 75 upregulated and 131 downregulated circRNAs between HCC827/A549 and BEAS-2B (a normal bronchial epithelial cell line). In addition, C190 was shown to be secreted to the conditioned media by a panel of NSCLC cell lines and detected also by RT-ddPCR. This study further confirms that circRNAs are stable RNA species which could serve as biomarkers for the diagnosis of lung cancer. Although the C190 circRNA may exhibit lower sensitivity in early-stage cases compared to advanced stages, it remains a significant finding that warrants consideration for improving patient selection in lung cancer screening programs. Additionally, it has the potential to aid in estimating the likelihood of malignancy in lung nodules detected through low-dose CT scans [40]. Therefore, the detection of circRNAs secreted into blood plasma is feasible and could represent a very promising liquid biopsy approach.

Recently, the same authors have shown that C190 is upregulated by *EGFR* activation via the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway in A549 and H1299 NSCLC cell lines. Rapid induction of C190 expression occurred in response to *EGFR* stimulation. Furthermore, the study revealed that stable overexpression of C190 significantly enhanced the cell migration and proliferation capacity of lung cancer cells. In addition, C190 exhibited robust overexpression in NSCLC tumors when compared to normal tissue [46]. Bioinformatic prediction identified miR-142-5p-bindings sites present on C190 and predicted that it could target CDK4 and CDK6 [46]. In our opinion, C190 could be an important biomarker of lung oncogenesis and could serve as a surrogate biomarker of *EGFR* up-regulation as a mechanism of resistance to *KRAS* G12C inhibitors [47].

In the context of gastric cancer, a specific circRNA called *circPDIA4* has been identified [48]. It has been found that *circPDIA4* interacts with phosphorylated *ERK1/2* (pERK1/2) in the cytoplasm, thereby inhibiting its dephosphorylation by dual specificity phosphatase 6 (DUSP6). This inhibition results in the activation of the MAPK signaling pathway. Additionally, *circPDIA4* has the ability to bind to *DHX9* in the nucleus. By doing so, it represses *DHX9*, which is a negative regulator of circRNA biogenesis. Consequently, the repression of *DHX9* leads to elevated expression of multiple oncogenic circRNAs, ultimately promoting the progression of gastric cancer [48]. These results highlight that *circPDIA4* competitively binds to the RNA-binding motifs of *DHX9* as a decoy, repressing interactions between *DHX9* and its target RNAs, boosting biogenesis of *DHX9*-controlled circRNAs [46]. Mechanistically, *circPDIA4* binds to *ERK1/2* and serves to sustain the activation of *ERK1/2* by preventing DUSP6-mediated dephosphorylation of phosphorylated ERK (pERK) in the cytoplasm [46]. Finally, the fact that *circPDIA4* interacts with *DHX9* in the nucleus, repressing its functions and inducing elevated expression of multiple oncogenic circRNAs, could serve as guidance for further potential analysis in NSCLC and be tested in liquid biopsies.

Blood platelet-based cancer screening

Nilsson and Würdinger carried out pioneering studies in blood platelets with landmark findings that tumor cells could transfer mutant RNA into the blood platelets in vitro and in vivo. Since platelets do not contain a nucleus, their RNA transcripts, required for functional sustainability, are derived from megakaryocytes in bone marrow during platelet formation. Nilsson et al. [49] discovered that blood platelets take up tumor-derived secreted vesicles that could harbor tumor-associated RNA. Mutant *EGFR*vIII RNA was detected in resected glioma tissues and in platelets from the same patient. A total of 21% of the glioma tissue samples contained the EGFRvIII transcript, which was also found amplified from platelets in 80% of the EGFRvIII-positive patients and absent in platelets from healthy donors. Additionally, the presence of RNA coding for the prostate cancer marker *prostate cancer gene 3 (PCA3)* was observed in platelets from prostate cancer patients, whereas it was absent in platelets from healthy control subjects [49].

The interaction between platelets and cancer cells, leading to the transfer of tumor-associated products, has given rise to the term "tumor-educated platelets" (TEPs). The analysis of TEP-derived profiles using RNA-seq technology has shown promise in differentiating early and late-stage cancer patients from healthy individuals across various tumor types [50,51]. This transfer of tumor-derived RNA into platelets holds potential for detecting, localizing, and molecularly profiling different primary malignancies [52,53]. One enticing aspect is the significant production of platelets within the lungs. This suggests that platelets could serve as a relevant biosource for exploring early detection of occult lung preneoplasia and early-stage lung detection [54]. Intriguingly, platelets are enriched in circRNAs [55]. A platelet-derived circular RNA profile could be relevant in NSCLC [52]. TEP RNA-based blood tests (thromboSeq) correctly detected the presence of cancer in two-thirds of 1096 blood samples from stage I-IV cancer patients [51], although its implementation in the clinic is hampered by its time-consuming and complex library preparation protocol, the difficulty in standardizing methods, cost and laborious data analysis. Conversely, nCounter RNA analysis does not warrant reverse transcription, amplification, nor cDNA library construction. NanoString nCounterhas been utilized for assessment of biomarkers in diverse liquid biopsy biosources, such as circulating free DNA, cell-free RNA, extracellular vesicles and circulating tumor cells [56–58]. Recently, D'Ambrosini et al. [59] used the Nanostring nCounter platform and bioinformatic machine-learning analysis in the platelet transcriptome from lung cancer and non-cancer persons. The combinatorial analysis including mRNA and *circRNA* produces an 8-target signature (6 mRNAs and 2 circRNA) that discriminates lung cancer patients from controls (AUC of 0.92). Furthermore, the combinatorial *mRNA-circRNA* assessment indicates a signature of five biomarkers in early-stage NSCLC, including two *circRNAs* (*circ SLC8A1* and *circCDH9*) and three mRNAs (*PSMN9*, *RUNX1* and *LILRB1*) [59]. In previous research we demonstrated that *EML4-ALK* rearrangements analyzed by RT-PCR in platelets and plasma of 29 patients with *EML4-ALK* NSCLCs treated with crizotinib correlated with progression-free survival and overall survival. Plasma RNA showed lower sensitivity (21%) compared to platelets (65%) for the detection of *EML4-ALK* rearrangements using RT-PCR [60]. This finding is particularly intriguing because it suggests that fusion gene rearrangements, such as *EML4-ALK*, may occur in early life or adolescence in non-smokers, and these individuals may later develop NSCLC driven by fusion genes.

Cell-free circulating DNA in NSCLC detection

Cell-free DNA (cfDNA) derives from hematopoietic cells, but tumor cells release their DNA, named circulating tumor DNA (ctDNA), in the bloodstream. cfDNA and ctDNA have gained attention in the past decade [61–65] as a potential tool for detecting non-small-cell lung cancer (NSCLC). Numerous studies have explored the utility of cfDNA in blood-based screening programs in conjunction with low-dose CT scans (Table 1 summarizes studies carried out, also with other blood biosources).

Early studies have highlighted the potential value of microsatellite alterations, such as the presence of new alleles (shifts) or loss of heterozygosity, in serum or plasma DNA. These alterations could provide valuable insights into tumor staging, management, and potentially cancer detection [66–68]. Premature studies were also able to demonstrate aberrant DNA methylation in the serum of NSCLC patients. Hypermethylation was observed in at least one of the following four genes: p16, death-associated

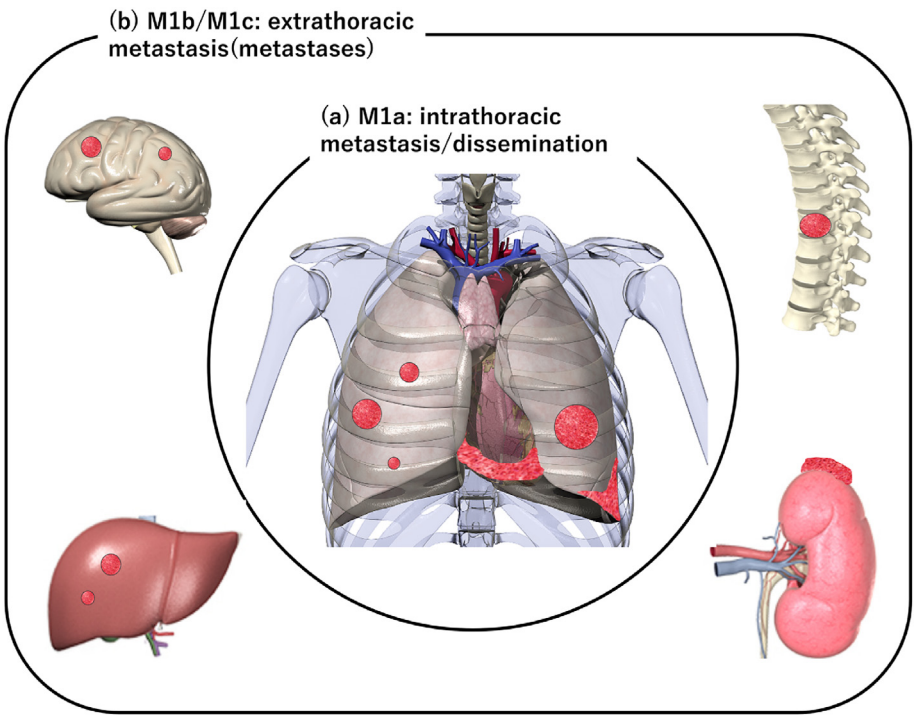

Fig. 2. Illustration implying stage IV condition. Stage IV is defined as status with distant metastasis and subdivided into stage IVA (M1a-1b) and stage IVB (M1c). M descriptor consists of M1a, M1b, and M1c according to the spread of metastasis. (a) Intrathoracic metastasis or dissemination defending M1a status: separate tumor nodule(s) in a contralateral lobe, with pleural or pericardial nodule(s), or malignant pleural or pericardial effusion; (b) Examples of extrathoracic metastasis (in brain, liver, bone, and/or adrenal) suggesting M1b/M1c
M1b: single extrathoracic metastasis; M1c: multiple extrathoracic metastases in one or more organs.

protein kinase, glutathione-S-transferase P1, and the DNA repair gene O6-methylguanine-DNA-methyltransferase. This hypermethylation pattern was detected in 15 out of 22 (68%) NSCLC tumors, while no hypermethylation was found in the corresponding normal lung tissue samples. Furthermore, in 11 out of 15 (73%) NSCLC tumors that exhibited methylation, methylation was also detected in the corresponding serum samples. This study provided the initial evidence supporting the potential validity of serum methylation assays for cancer diagnosis [69]. Additional studies reassure us that plasma DNA could be used for early lung cancer detection using quantitative methylation-specific PCR. In plasma, 73% of patients with cancerous tumors exhibit methylation of at least one of the four gene promoters (*DCC, Kif1a, NISCH* and *Rarb*). Only 22% of patients with ground glass opacity presented methylation of at least one gene. Ground glass opacity is a finding on CT that is defined as hazy increased attenuation of the lung with preservation of bronchial and vascular margins with unclear evolution to cancer. Therefore, this study further reinforces the utility of plasma DNA methylation assays for helping in the distinction between cancerous and non-cancerous radiological CT abnormalities [70]. The Circulating Cell-free Genome Atlas (CCGA) study (NCT02889978) reported the results of a multi-cancer early detection assays by using a whole-genome methylation-based approach, developing machine learning classifiers and deep next-generation sequencing tests [71].

Jameshidi et al. introduced a metric called the clinical limit of detection (LOD), which is defined by the circulating tumor allele fraction (cTAF). The cTAF represents the anticipated fraction of tumor-derived somatic mutations in an acFDNA sample and is determined using a statistical model. The study found that whole-genome methylation was the most effective classifier, and the performance of the classifier improved with higher levels of tumor DNA in the blood, as indicated by the cTAF. Such study [71] represents a paramount endeavor and could obviously contribute to expand the development of blood-based whole-genome methylation tests. The ctDNA fragment derived from cancer cells is reported to be shorter than in non-cancer cells. The length of cfDNA fragments in plasma from healthy volunteers is approximately 167 bp, whereas the cfDNA obtained from cancer patients is more fragmented in comparison with healthy donors [72]. Genome-wide analysis of cfDNA fragmentation indicates a unique fragmentation profile of cfDNA among several types of primary cancers, with a high sensitivity (>90%) and specificity (>98%) in detecting breast, colorectal, gastric, lung, ovarian and pancreatic cancers [73]. Likewise, the size of cfDNA fragment has been suggested to be a predictive factor in several tumors with shortened fragments, lower than 167 bp, correlating with poor clinical outcomes in cancer patients. Distinct methods, such as microfluidics automated electrophoresis-based platforms, can be employed to evaluate the sizes of cfDNA fragments. In short, the average cfDNA level in healthy persons is estimated to be less than 10 ng/mL of plasma. Of interest is the fact that the assessment of size-selected cfDNA facilitates the identification of driver mutations [74]. The enrichment of short cfDNA fragments within the range of 90–150 bp enhances the detection of ctDNA, particularly for low-variant allele frequencies associated with genetic alterations such as *EGFR* exon 20, T790 M, BRAF, and *KRAS* in various cancer types [75].

Recently, the TRACERx consortium, which focuses on tracking cancer evolution through therapy (Rx), has reported five studies examining the profiles of lung cancers captured over time [76–80]. In the study conducted by Abbosh and colleagues [78], the focus was on the development of new technology for the detection of ctDNA, with the aim of utilizing ctDNA monitoring for early detection purposes. The investigators created a computational tool called ECLIPSE that allows to track subclones at low levels of ctDNA. One of the goals for early detection of NSCLC could be the identification of pre-diagnostic mutations that can be detected years before the clinical diagnosis is made. This has been the case in finding follicular lymphoma precursors. The most frequently observed precursor lesions were *CREBBP* lysine acetyltransferase domain mutations, which could be detected a median of 5.8 years before the diagnosis of follicular lymphoma [81]. Early lesions have been identified in situ follicular lymphoma, characterized by alterations in genes that are frequently mutated in follicular lymphoma, including *BCL2, RUNX1, EBF1*, and *CREBBP* [81]. This observation of genetic alterations in circulating precursor cells years before the development of overt follicular lymphoma, particularly with predominant *CREBB* mutations within the lysine acetyltransferase domain, could serve to further encourage the advance of diagnostic strategies in the field of lung cancer. As a reminder, most NSCLCs are diagnosed at an advanced or metastatic stage, as depicted in

Fig. 2 illustrating the clinical staging of NSCLC. This highlights the lack of an early signal in detection, similar to the widespread metastasis observed in leukemias during their early progression.

It is crucial to continue pursuing liquid biopsy endeavors with a specific focus on identifying pre-diagnostic mutations or other alterations that can serve as indicators of the onset of occult advanced or metastatic NSCLC, where an early-stage phase is not present. The analysis of circulating tumor cells [82], circulating miRNAs, and other circulating markers has been extensively reviewed in studies by Serrano et al. [83] and Armakolas et al. [84]. Furthermore, comprehensive reviews on exosomal (extracellular vesicle) circRNA have been reported by Lin and colleagues [85].

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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