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REVIEW

Liquid biopsy in solid tumours: expert opinion paper of the European society of pathology

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Abstract

Liquid biopsy (LBx), particularly plasma circulating tumour DNA (ctDNA) analysis, has become an important use case of molecular pathology in solid tumours. While tissue remains the cornerstone of diagnosis, ctDNA testing provides a minimally invasive alternative using repeatable source of tumour-derived genomic information that complements tissue analysis when samples are unavailable, insufficient, outdated, or unlikely to capture tumour heterogeneity and evolution. In this expert opinion paper, the European Society of Pathology (ESP) defines its vision for LBx implementation across Europe and reaffirms the central role of pathology in blood-based detection of genomic alterations in solid tumours. Two clinical models highlight current implementation of LBx into standard diagnostic work-up. In advanced non-small cell lung cancer (NSCLC), LBx enables rapid detection of actionable somatic genomic alterations, supports treatment decisions when tissue biopsy is not available/sufficient for molecular analysis, and identifies acquired resistance genomic alterations at progression. However, tissue remains essential for histological classification, PD-L1 assessment, recognition of histologic transformation, evaluation of tumour microenvironment, and molecular testing when comprehensive genomic profiling or confirmation of liquid biopsy findings is required. In metastatic breast cancer, LBx has become central to dynamic molecular management of patients with originally hormonally dependent disease. This paper also addresses key technical and interpretative challenges, including assay selection, next-generation sequencing, clonal haematopoiesis, tumour fraction, variant allele fraction, co-mutations, testing logistics, and quality assurance. Given heterogeneity in access to LBx testing across Europe, harmonized standards for validation, reporting, training, and equitable availability are urgently needed. Pathology laboratories have a central role in identifying somatic alterations in cancer tissue, and LBx is an obvious extension of this important task. An integrated morphological-molecular pathology assessment is needed to implement appropriate precision oncology strategies. Incorporation of specific professional personal profiles (molecular biologists, bioinformaticians) to pathology laboratories is therefore essential for such optimal approach.

Keywords Liquid biopsy · ctDNA · Molecular pathology · Precision oncology · Biomarkers · Lung cancer · Breast cancer

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Introduction

Molecular pathology has become an integral component of the clinical management of solid tumours and has unlocked the potential of precision medicine [1]. While tissue remains the cornerstone of cancer diagnosis and biomarker assessment, the rapid evolution of liquid biopsy (LBx) has enabled a complementary and increasingly necessary dimension of pathology [2]. Among the available LBx analytes, plasma circulating tumour (ct) DNA is currently the only clinically validated blood biomarker testing approach in solid tumours with sufficient clinical evidence. It is useful particularly in patients where timely access to molecular information is critical or tissue material is limited, exhausted, unsafe to obtain, or unlikely to reflect tumour heterogeneity [2–4]. In this context, LBx should be viewed as an extension of modern molecular pathology, as it interrogates acquired somatic alterations for diagnostic, predictive, prognostic, and disease-monitoring purposes [5, 6].

The European Society of Pathology (ESP) has a responsibility to provide guidance in this evolving field by defining evidence-based pathways for LBx implementation across Europe, a geographically compact yet highly heterogeneous continent in terms of healthcare systems and access to molecular diagnostics [7–9]. This expert opinion paper therefore aims to articulate the central role of pathology in ctDNA analysis of solid tumours, while promoting clinically responsible adoption. It also recognizes that progress in LBx requires close interdisciplinary collaboration. Strong alignment with other societies is essential to ensure clinical utility and synergy in technical standards [10, 11].

Precision oncology has entered an era in which somatic diagnostics integrate tissue- and blood-based testing. To ground these principles in current clinical practice, this paper includes two disease-focused case studies: non-small cell lung cancer (NSCLC) and breast cancer, where the evidence of the clinical use of LBx is high. In NSCLC patients, ctDNA has reshaped the detection of actionable genomic drivers and resistance mechanisms where rapid therapeutic decisions are required [12]. In breast cancer, ctDNA is increasingly relevant for identifying mechanisms of acquired endocrine resistance and guiding targeted therapies [13]. This paper sets out the vision of the ESP for this evolving landscape.

> Statement 1: Pathology departments are responsible for molecular analysis of somatic alterations in cancer, and LBx is a logical extension of this role. Integrated interpretation of somatic alterations in tissue and blood is highly recommended to support optimal clinical decision-making.

Liquid biopsies for non-small cell lung cancer

The therapeutic innovations in thoracic oncology are significantly dependent on detecting predictive biomarkers, which are used for selecting lung cancer patients who should benefit from personalized schemes, such as targeted therapies, immunotherapies, and more recently, antibody drug conjugates and bi-specific antibodies [14, 15]. Detection of specific genomic alterations (mutations, gene fusions) has been adopted as an integral part of the comprehensive pathological testing of NSCLC. These alterations may be successfully detected not only in tissue samples, but also in ctDNA using LBx [16, 17]. LBx are minimally invasive, and thus easy to obtain and repeatable, they reflect the overall state of the tumour and allow for real-time monitoring, notably in patients receiving targeted therapies, and they represent a source of information needed for an optimal clinical management of the patient [18, 19] **(**Fig. 1**).** Moreover, in certain settings, LBx may be the only way to identify druggable genomic alterations. In NSCLC it is namely in cases, where a tissue biopsy is not available at diagnosis or at tumour progression [20]. For clinical practice, circulating cell-free DNA (cfDNA) is to date the only approved and clinically validated material to assess the genomic profile of druggable alterations [17, 20]. However, it is noteworthy that the analysis of a non-negligible fraction of LBx may give non-informative results and may be associated with biological and technical limitations, depending on the heterogeneous shedding of ctDNA into the blood stream and the tumour stage [21, 22].

Recommendations and guidelines for routine clinical practice

To date, LBx is primarily indicated for locally advanced (Stage IIIB) and metastatic (Stage IV) lung adenocarcinoma and large cell lung carcinoma. No established recommendations currently support its routine clinical use in small cell lung carcinoma (SCLC), squamous cell carcinoma, or for early-stage (Stage I-IIIA) NSCLC [10, 20]. IASCL and ESMO recommend the use of LBx in advanced and metastatic lung adenocarcinoma to identify druggable genomic alterations when tissue biopsy is unavailable, inaccessible, clinically unfeasible, or insufficient for molecular testing because of poor sample quality, low tumour cellularity, or inadequate nucleic acid yield [10, 20]. It is also necessary to consider the time to obtain a tissue biopsy with a bronchoscopy or a transthoracic biopsy, which may not be compatible with an urgent need for treatment decision-making. It is increasingly accepted to obtain LBx on progression of the lung tumour.

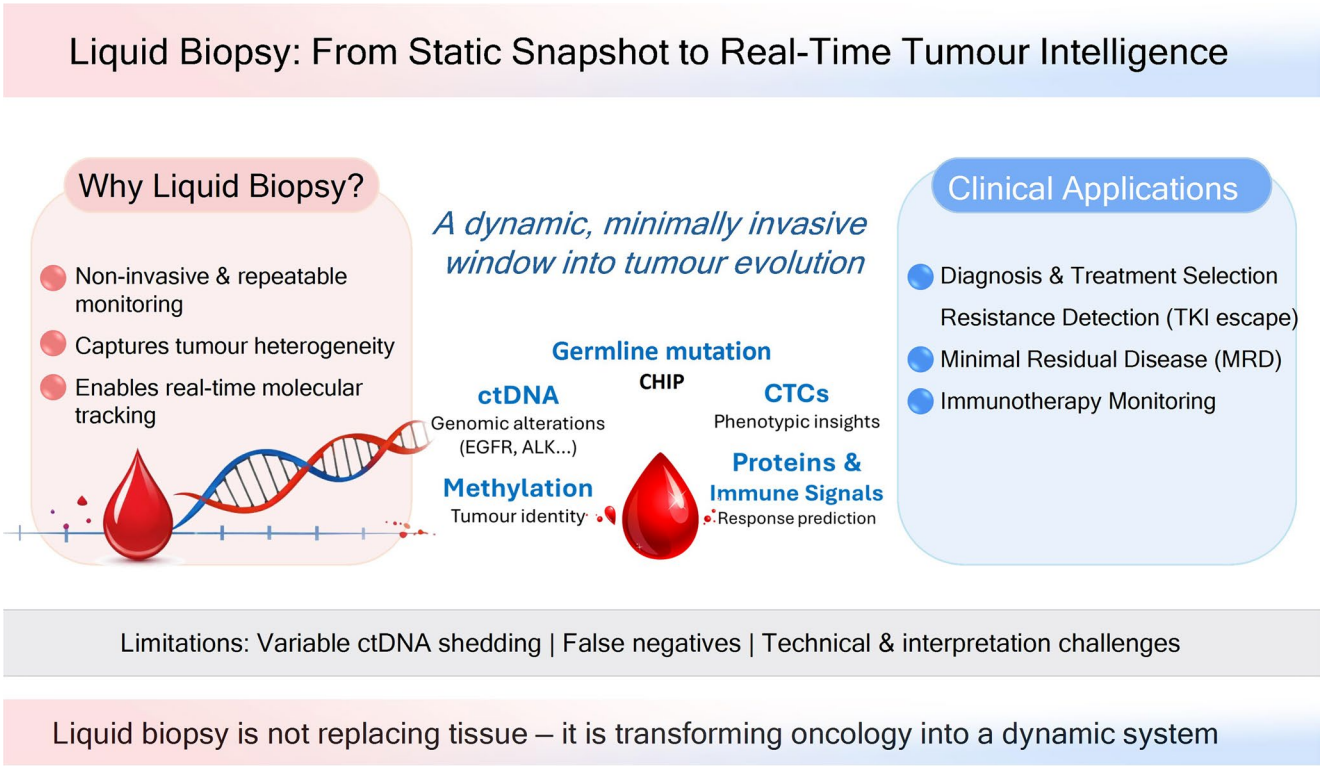

Fig. 1 Current and future opportunities for liquid biopsy (LBx) use in solid tumours for precision oncology. LBx presents several advantages in metastatic tumours at diagnosis (actionable genomic alterations identification), and at disease progression (mechanisms of treatment resistance characterization). LBx can offer better treatment decision making in early-stage solid tumours, monitor cancer progression and detect the minimal residual disease

Therefore, LBx can identify mutations that confer resistance to targeted therapies, particularly in patients treated with tyrosine kinase inhibitors targeting *EGFR* mutations and *ALK* rearrangements [23, 24]. As tissue biopsy and LBx have both their inherent sensitivity and specificity limitations, they should be rather than alternative approaches seen as complementary ones. Therefore, LBx should ideally be performed in parallel with testing of a tissue biopsy [25].

Tissue biopsy remains the gold standard in lung cancer, as it enables histological diagnosis (including the identification of histologic transformation, such as transformation from adenocarcinoma to small cell or squamous carcinoma), PD-L1 assessment, and genomic profiling at baseline and progression [26–29].

Indications and timing for LBx based diagnostic approaches

There are three different strategies, which may be adopted regarding the timing of LBx and tissue biopsy in locally advanced and metastatic NSCLC **(**Fig. 2**).** The first one is the "plasma first" approach, in which LBx is obtained at diagnosis to enable rapid molecular analysis [30]. Identification of a druggable alteration on *EGFR* or *ALK* allows prompt initiation of targeted therapy [12]. As the sensitivity of LBx is still limited, in case of a negative results of the LBx (meaning non detectable druggable genomic alterations according to the assay sensitivity and to the tumour fraction percentage among the cfDNA), molecular testing of tissue biopsy may then be performed as a second step to distinguish true negative from false negative cases [31, 32]. The second strategy is the "tissue first" approach, in which molecular testing of tissue biopsy is performed initially. In case of non-informative results of molecular testing (i.e. insufficient absolute or relative number of neoplastic cells for molecular analysis or failure to detect druggable genomic alterations due to sample quality limitations), it is followed by LBx. Finally, the last strategy is to perform concomitant LBx and tissue biopsies [33–35]. The argument in favour of the latter strategy is to increase the chance of identifying a targetable mutation, knowing that a LBx collects tumour DNA that is shed from multiple tumour sites and thus reflects better the putative heterogenous molecular portrait of a disseminated disease [34, 36]. The downside of the latter approach is the increase of the cost of molecular testing and workload (all patients are tested twice). Of note, no single strategy has been formally endorsed by international societies. The

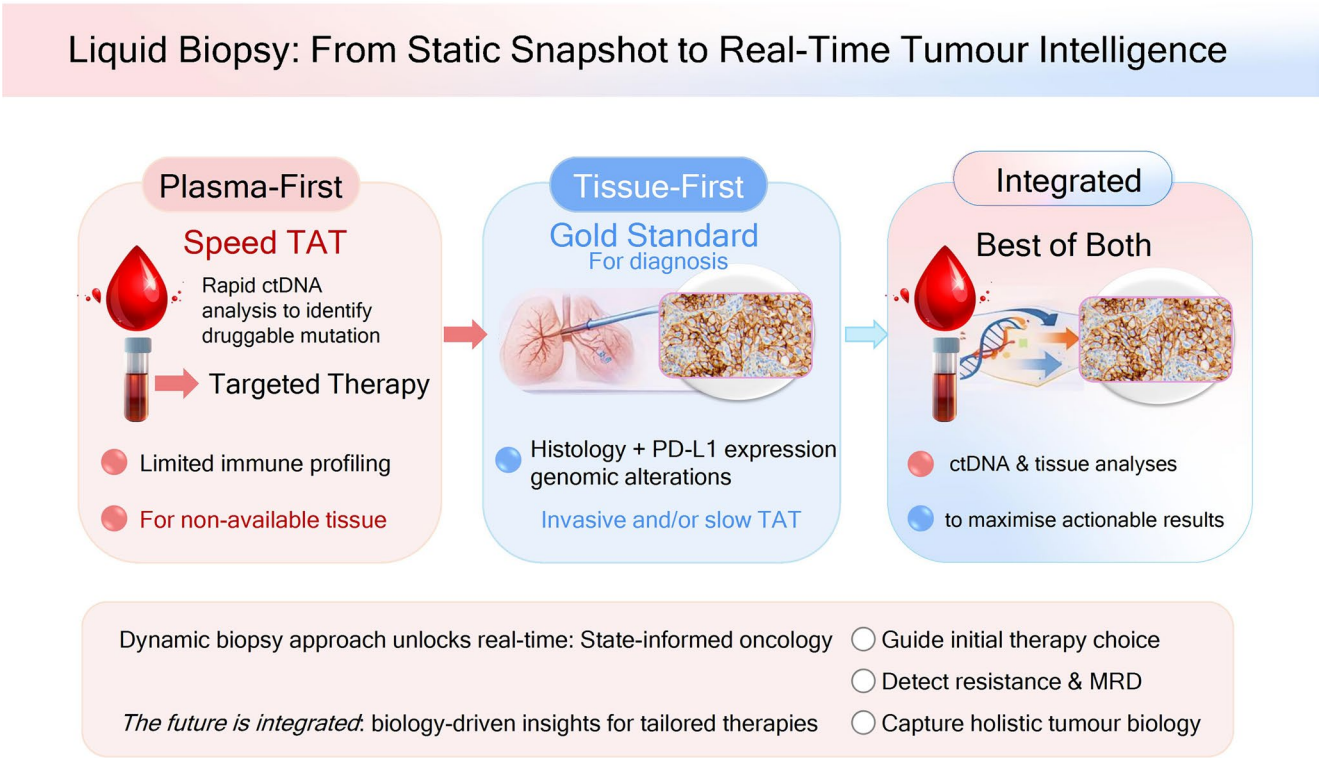

Fig. 2 Different strategies for biomarker testing at cancer diagnosis, according to the sample type, all having advantages and limitations: i) liquid biopsy (LBx) "first" sampling strategy for actionable genomic alteration (AGA) identification; ii) tissue biopsy (TBx) "first" sampling strategy for tumour diagnosis, PDL-1 tumor proportion score assessment and AGA identification; iii) concomitant LBx and TBx sampling strategy allowing to get the entire molecular portrait of the disease

choice should be therefore made within a multidisciplinary thoracic oncology team, according to the clinical scenario, patient performance status, urgency of treatment initiation, tumour accessibility, tissue adequacy, expected biomarker needs, local laboratory turnaround times, and availability of interventional and molecular diagnostic as well as financial resources and reimbursement strategies made at a national level.

Molecular analyses of LBx for NSCLC

Identically to molecular testing of histological or cytological samples, there are two main approaches with LBx, (i) single gene testing (SGT) or (ii) a multiplexed gene analysis using next-generation sequencing (NGS), which identifies alterations of multiple genes at the same time [37]. The SGT approach is based on PCR [RT-PCR and droplet digital (dd)-PCR)] technology [38]. Since ctDNA often represents only a small fraction of total cell-free DNA (cfDNA), sensitive methodologies are required, including ddPCR and NGS. ddPCR offers excellent analytical sensitivity for known hotspot mutations, whereas NGS enables broader coverage. Ideally, assessment of the tumour fraction (TF) in cfDNA should be performed to distinguish true negative from false negative NGS LBx results [39, 40]. However, until now only several commercially available assays offer the possibility to address the TF level in cfDNA. Examples include the Foundation One LBX Dx assay (Roche), the Infinity assay (Guardant Health), or the Tempus xF assay (Tempus). These assays are, however, available only on commercial platforms or in some large academic institutions [41–43]. Therefore, their use would require the outsourcing of LBx testing. Interestingly, new assays, such as Hedera Dx (Hedera) are developed, allowing TF assessment using on-site NGS panels integrating different driver genes [42]. These solutions are, nevertheless, currently intended for research use only and require further large-scale validation. Another solution is the assessment of the of the ctDNA size, as ctDNA fragments are smaller than gDNA. Alternatively, droplet digital PCR can be used to look for methylation of a limited number of genes [21].

SGT provides rapid results (usually within one day or less) and is significantly less costly (both for the reagents and the equipment). It is easy to perform and does not need a high level of expertise for molecular interpretation. However, SGT can lead to false negative results, since this approach does not cover all the variants of a gene (such as for the *EGFR* exon 20 variants) and, by definition, looks

for the status of one gene (or a very few genes) only [37]. Regarding the selection of method used for LBx testing (SGT vs. NGS), several factors, such as availability, cost and reimbursement status and the number of tier I actionable genetic alterations must be considered [10]. NGS identifies in one step multiple druggable genomic alterations associated with drugs available in daily practice, such as ESMO Score of Actionability Targets (ESCAT) I, which are associated with currently available targeted therapeutics [44]. NGS LBx has several limitations which must be taken into consideration. Its sensitivity in detection of copy number variations (CNV) is considered limited when compared to standard tissue testing. LBx has also significant limitations for detecting actionable gene fusions (such as *ALK* and *ROS1* fusions). DNA-based ctDNA assays may have incomplete intronic coverage and technical difficulty capturing complex rearrangements, whereas RNA-based fusion detection is often more sensitive but is challenged by RNA instability in plasma and low circulating RNA abundance. Therefore, a negative LBx does not exclude an actionable fusion (e.g. *ALK*, *ROS1*, *RET*, *NTRK*), and tissue-based testing – preferably including RNA-based NGS – should be pursued whenever feasible. Although *ALK* and other potential gene fusions could be indirectly (by IHC) and directly (by FISH) detected in circulating tumour cells (CTC) harvested from blood, this approach is currently not used in routine clinical practice. CTC testing is facing several methodological challenges, including limited reproducibility due to low CTC numbers, high cost, and technical difficulties hindering the inter-laboratory validation [45, 46]. LBx NGS also needs a high level of expertise both from the technical point of view for the preparation of libraries and sequencing steps and bioinformatic analysis of the sequencing results. NGS is in general more costly than SGT, however, considering that SGT can be repeated if the initial results are negative, this may become comparable or even more expensive than NGS strategy [47]. Finally, sequential SGT can lead to a longer turnaround time to obtain the results compared to NGS, thus becoming a disadvantage for rapid treatment decision-making at tumour diagnosis or progression.

NGS panels for LBx for lung cancer

Current existence of several NGS technologies as well as large number of different NGS panels do not allow adoption of a unique strategy and harmonization of NGS LBx across different practices [21, 48]. Different parameters need to be taken into consideration when selecting the strategy, such as panel size, spectrum of genes covered by the panel, type and capacity of sequencing platform available, number of NGS testing per week, expected turnaround times for obtaining the report according to the clinical setting, as well as expertise of the team members (e.g. for bioinformatic assessment) [49]. Thus, for different clinical needs, different solutions will be most appropriate. Both a medium-sized gene panel (less than 100 genes) and a comprehensive genomic profiling (CGP) testing panel with several hundred genes enabling testing of tumour mutational burden in blood (bTMB), microsatellite instability (MSI) and integration of DNA damage repair genes can be used in LBx [50, 51] depending on clinical needs. The capabilities for sequencing depend on the quantity of ctDNA, varying from a few ng/ml (3–10 ng/ml for amplicon based sequencing approaches) to several dozen ng/ml (20–50 ng/ml for hybrid capture approaches) [52]. Use of the specific protocol for NGS thus depends on the amount of ctDNA, according to the manufacturer's recommendations.

Recommendations for technical considerations of ctDNA analysis in NSCLC

Several caveats should be taken into consideration to optimize LBx use in NSCLC. They have been incorporated into international recommendations [10].

Mutations associated with clonal hematopoiesis of indeterminate potential

Somatic mutations derived from the expansion of clonal populations of blood cells (clonal haematopoiesis of indeterminate potential, or CHIP) may be detected when sequencing cfDNA. The frequency of detection of CHIP-associated mutations in cfDNA increases with age and may become a potential source of false positive error in interpretation of LBx NGS result [53, 54].

Germline variants

Circulating germline DNA (gDNA) originates from the degradation of somatic cells (lymphocytes and neutrophils are the most important source of gDNA) [55]. NGS can detect germline variants in circulating gDNA, which need to be differentiated from somatic mutations in ctDNA, notably when *EGFR* and *TP53* mutations are observed. Some technologies are based on both ctDNA and circulating gDNA sequencing performed in parallel [55].

Variant allelic frequency (VAF) and tumour fraction analyses of ctDNA

The interpretation of different pathogenic variants must consider their variant allelic frequency (VAF) [56]. A high VAF (generally higher than 50%) points to suspected germline variants. In cases with very low VAF, the level

of the circulating tumour fraction must be taken into consideration to eliminate a false positive result [57]. Finally, although a correlation between high tumour fraction in cfDNA and worse prognosis of lung cancer has been demonstrated recently [58], clinical data demonstrating benefit from modification of therapy based on this result are not available so far.

De-centralized versus centralized LBx testing for NSCLC

The equity of access to treatment of NSCLC is linked to the access to molecular testing [59]. In this context, widespread dissemination of molecular testing, avoiding centralization solely to commercial platforms or to large institutions, is a major objective of the current strategy for NSCLC care [59, 60]. De-centralized network providing LBx testing in NSCLC may be a better approach to more accessible molecular testing [59]. However, LBx de-centralization requires an established system of quality control of individual laboratories including participation to external quality assessment (EQA) programs, to guarantee their high-quality performance ensuring reliable results [61]. Several challenges may significantly affect this approach, such as the lack of harmonized technical procedures, pre-analytical challenges and issues in interpreting the molecular data, which can impede significantly the widespread practice of LBx [61–63]. Therefore, the implementation of LBx in routine practice requires qualified personnel - molecular biologists and pathologists who have had extensive training in molecular testing [64–66]. LBx reimbursement is another crucial point [67]. Many patients with stage IV NSCLC can benefit from LBx testing of targetable alterations at diagnosis and/or detection of resistance mechanisms at tumour progression. Reimbursement policies vary greatly from country to country. In some countries (e.g. France or Czechia), until now, LBx testing has only been reimbursed if molecular analyses from tissue biopsies are unavailable. In other countries, such as the USA, reimbursement is available at the time of diagnosis, even if a tissue biopsy testing is performed in parallel. To achieve more widespread LBx reimbursement, multiple stakeholders must get involved in action, including the national authorities and policy makers, diagnostic and pharma companies, health care professionals, the international scientific societies as well as patients' organizations. Added value of LBx must be clearly defined and highlighted. At the same time we sincerely believe that LBx cost will decrease in time, notably the cost of NGS. This should help to speed up and expand the reimbursement of LBx across different countries, eliminating the current inequity in access to this method.

New use cases for LBx for lung cancer

The development of LBx in thoracic oncology opens new opportunities. Therefore, although the results of clinical research are promising, these indications are not ready for clinical adoption yet. However, with rapid development of the emerging technologies, some of these new approaches could be implemented in routine clinical care of lung cancer patients in the near future.

Assessment of the minimal/molecular residual disease (MRD)

Detection of MRD in the peri-operative setting of NSCLC could be used for the optimization of therapeutic strategies and allow modification of the treatment [28, 68–71]. Several challenges regarding both the technology and clinical impact (modification of the treatment on the basis of MRD result) exist, namely in the decision if tumour-informed or tumour-agnostic assay should be used [72]. Tumour-informed LBx assays are tailored for individual patients based on the results of prior molecular analysis of the tissue sample, which must be tested first. These assays are more promising than the less specific generic tumour-agnostic assays. The MRD LBx assays are based on NGS approaches or on PCR testing, particularly ddPCR. The timing of blood sampling varies according to the clinical trials. At this timepoint, evidence demonstrating clear clinical benefit of the modification of the treatment (escalation or de-escalation therapeutic strategy) according to MRD result, is still limited. Several MRD assays are, nevertheless, in development or commercially available. Some of them are now used in routine clinical practice in the USA. However, in Europe MRD assessment is still restricted to research studies and/or clinical trials.

Predictive biomarkers of LBx for lung cancer immunotherapies

Monitoring of ctDNA levels has been investigated in NSCLC patients undergoing neoadjuvant or adjuvant immunotherapy [73], but no definitive data applicable to clinical practice are available so far.

Biomarkers in LBx for small cell lung cancer (SCLC)

Until recently, LBx for SCLC was associated with several translational research projects [29, 74]. Different methylation profiles of ctDNA isolated from SCLC patients have been identified, which may correspond to tumours with different biological behaviour [75]. These promising results could be used in the future for selection of patients for more

individualized treatments. The possibility of characterising and identifying the expression of different proteins on the CTC surface, such as DLL3, could be in the future associated with eligibility for different treatments using antibody drug conjugates (ADCs) [76].

Methylation of ctDNA

Methylation profile of the cfDNA differs not only in individual patients but also shows specific methylation profiles in different solid tumours. Thus, certain specific profiles of ctDNA methylation may point to specific cancer types and can overcome one of the limitations in specificity of ctDNA testing. It may also help to eliminate inability of LBx to differentiate between true negative and false negative result by distinguishing the ctDNA from non-tumorous cfDNA in blood sample [77, 78]. Last but not least, methylation profiling could be also helpful in the diagnosis of different synchronous solid tumours occurring in the same patient.

> Statement 2: LBx is needed in assessing actionable somatic alterations in lung cancer, particularly when tumor tissue is not available or at tumour progression. Technical limitations, especially regarding the detection of actionable gene fusions, should be taken into consideration.

Liquid biopsy in breast cancer

The therapeutic advances in breast cancer are increasingly linked to the necessity to identify predictive biomarkers, mostly related to acquired resistance to endocrine treatment, for novel targeted agents and endocrine therapies [13, 79]. In patients who experience recurrence or progression of carcinoma, which was originally diagnosed as hormonally dependent and has been treated by the blockade of the hormonal axis, tissue re-biopsy remains the method of choice. This is particularly valid for molecular assessment of the *PIK3CA* pathway including *PTEN* loss. However, repeated tissue sampling may be invasive, logistically challenging, or impractical, especially in the metastatic setting where lesions may be inaccessible or involve multiple tumour sites. Accordingly, LBx is increasingly integrated into routine breast oncology practice [80]. Unlike in lung cancer, where LBx is mainly complementary to tissue analysis, in breast cancer represents LBx the guideline-supported strategy for identifying emergent endocrine treatment resistance mechanisms such as acquired *ESR1* mutations. These are typically absent in the primary tumour and develop only during the course of endocrine treatment. Detection of *ESR1* mutations does not necessarily require biopsy of metastatic lesions, as their presence in LBx closely correlates with their identification in the tissue of the recurring tumour [81, 82]. Furthermore, it has become evident that, owing to their accessibility and repeatability, LBx can be used to follow up breast cancer evolution in nearly real time and to inform adaptive therapeutic strategies [83].

Testing for ESR1 mutations as a paradigm for ctDNA-guided precision oncology

ESR1 mutations testing is currently one of the most clinically relevant and paradigm-shifting applications of ctDNA analysis in solid tumours. In hormone receptor (HR)-positive/HER2-negative metastatic breast cancer it links the dynamic resistance mechanism to a therapeutic strategy directly targeting the signalling pathway [84, 85]. *ESR1* encodes estrogen receptor alpha, the central biological driver of luminal breast cancer, and activating mutations within this gene are a recognized cause of acquired resistance to aromatase inhibitors and other endocrine therapies [81, 86]. These alterations predominantly occur within the ligand-binding domain, with recurrent hotspot regions involving codons 380, 536, 537 and 538, and they are typically absent at initial diagnosis but progressively enriched under selective pressure from endocrine treatment during metastatic disease [84, 87]. Accordingly, *ESR1* mutations may be detected in up to 40–50% of endocrine-pretreated patients with metastatic disease, making them a hallmark of treatment-driven tumour evolution rather than a stable truncal event present from disease onset [88]. This biological behaviour has major diagnostic implications: *ESR1* mutations are frequently subclonal, meaning they may be confined only to a fraction of tumour cells, and polyclonal, with multiple independent resistant clones arising in parallel within the same patient [89–91]. Consequently, a single metastatic tissue biopsy does not provide comprehensive information as it may miss relevant resistant populations, while archival primary tumour tissue is poorly informative, as it does not reflect the status of relapsing cancer [92, 93]. In practical terms, *ESR1* is one of the clearest examples in breast pathology, where tissue testing is intrinsically limited and ctDNA offers a more representative, real-time portrait of systemic disease burden and clonal architecture. This paradigm primarily applies to activating *ESR1* point mutations within the ligand-binding domain (LBD). These mutations currently represent the only clinically validated biomarkers used for treatment selection [85, 94]. Activating *ESR1* gene fusions constitute an additional, considerably less common, mechanism of acquired endocrine resistance [95]. These rearrangements typically retain the N-terminal *ESR1* domains while replacing the LBD with sequences from different fusion partners [96].

Currently, *ESR1* fusions should not be considered equivalent to activating *ESR1* point mutations for therapeutic selection: clinical evidence supporting novel oral selective estrogen receptor degrader (SERDs) has been generated in ESR1-mutated tumors [86]. Thus, although uncommon, *ESR1* fusions exemplify the need for multidisciplinary interpretation of the LBx result in the context of the assay design and reference range [40].

This biological rationale has been translated into direct clinical utility. ESMO guidelines now recommend routine *ESR1* testing at recurrence or progression on endocrine therapy to guide subsequent treatment choices [97–99]. The phase III EMERALD trial established the clinical actionability of ctDNA-detected *ESR1* mutations by demonstrating superior progression-free survival for the oral SERD elacestrant compared with standard-of-care endocrine therapy in previously treated patients with *ESR1*-mutated disease [100]. This transformed *ESR1* mutations from a resistance biomarker into a predictive biomarker with immediate direct therapeutic consequences and represented one of the first settings in breast cancer where liquid biopsy was prioritized over tissue analysis for treatment allocation [101].

Equally important, detection of *ESR1* illustrates the value of serial ctDNA monitoring [101, 102]. In the PADA-1 trial have rising *ESR1* mutations during first-line aromatase inhibitor plus palbociclib therapy identified patients benefiting from an early switch to fulvestrant while maintaining CDK4/6 inhibition [103–105]. This approach has been supported in the SERENA-6 trial, designed to assess switching to another SERD – camizestrant-based therapy when emergent *ESR1* mutations are detected in ctDNA prior to clinical progression [106–108]. Together, these studies support a new model of breast cancer clinical management in which LBx actively guides adaptive treatment sequencing based on evolving tumour biology.

From a pathology perspective, *ESR1* testing also exemplifies the technical and interpretative requirements of high-quality ctDNA diagnostics [109]. Since ctDNA often represents less than 1% of total cfDNA, highly sensitive methodologies are required, including ddPCR and NGS. Neither of these methods is perfect - ddPCR offers excellent analytical sensitivity, but covers only known hotspot mutations, while NGS may cover also non-hotspot variants, as well as other actionable alterations, such as *PIK3CA*, *AKT1* or *PTEN* [42, 81, 84, 94, 110], although with higher cost and longer turnaround time. Thus, assay selection must consider reportable range, limit of detection, turnaround time, local expertise, clinical utility and cost-effectiveness [65, 111]. The sensitivity of the test remains one of the main challenges. A negative plasma result does not necessarily exclude mutation presence due to multiple factors (low tumour fraction, limited ctDNA shedding, etc.) [112]. Therefore, *ESR1* wild-type reports should always be interpreted in the context of sample adequacy, analytical sensitivity, and clinical scenario. Standardized reporting should include specimen type, nucleic acid quality metrics, tumour fraction when available, assay characteristics, genomic regions interrogated, variant allele fraction, and clinical interpretation [87, 113].

Overall, detection of *ESR1* mutations constitutes a first of its kind use case of ctDNA implementation in breast cancer treatment strategy in routine clinical practice.

PIK3CA and the PI3K/AKT/PTEN Pathway

Alterations involving the PI3K signalling pathway represent another frequent and therapeutically relevant mechanism of endocrine resistance in HR-positive/HER2-negative metastatic breast cancer [114, 115]. Within this pathway, *PIK3CA* is the dominant factor. Activating mutations are found in approximately 35–40% of HR-positive/HER2-negative tumours [116]. In contrast to *ESR1*, which usually emerges under treatment pressure, *PIK3CA* mutations are generally an early oncogenic event and are often clonally maintained throughout disease evolution [117, 118]. This relative temporal stability explains high concordance between primary tumours and metastatic lesions [119]. However, additional subclonal evolution may still occur during therapy, and contemporary ctDNA testing remains valuable to capture such secondary co-existing resistance mechanisms [120, 121]. The most common *PIK3CA* alterations cluster in hotspot regions involving the helical domain (E542K, E545K) and kinase domain (H1047R/L), although clinically relevant non-canonical activating variants may also occur outside these exons. This fact strongly supports the preference for broader next-generation sequencing rather than hotspot-limited assays [116].

PIK3CA became the first routinely actionable somatic genomic biomarker in metastatic breast cancer in 2019, when the phase III SOLAR-1 trial has demonstrated that the α-selective PI3K inhibitor alpelisib combined with fulvestrant significantly improved progression-free survival compared with fulvestrant alone in patients with *PIK3CA*-mutated endocrine-resistant disease [122]. Thus it established mandatory molecular biomarker testing as part of standard therapeutic sequencing in this disease [123]. LBx emerged as an attractive testing alternative in this context, particularly when a rebiopsy of metastatic tissue was unavailable, limited, or technically unsuitable [79]. As in NSCLC context, positive LBx result became clinically actionable, whereas negative plasma results still require tissue testing because low ctDNA shedding may be causing false-negative results.

Biomarker-driven therapeutic modification of the pathway is no longer restricted to *PIK3CA* as a single gene. The phase III CAPItello-291 trial showed that capivasertib plus fulvestrant improved outcomes in endocrine-resistant advanced breast cancer harbouring various alterations in the AKT pathway, defined as *PIK3CA* mutation, *AKT1* mutation, or *PTEN* alteration [124]. This broadened the clinical concept from single-gene testing toward pathway profiling. The three genes used in CAPItello-291 trial, however, are extremely heterogeneous in terms of biology and molecular testing (Fig. 3). Unlike *PIK3CA*, where multiple hotspot codons and numerous non-canonical variants exist, *AKT1* alterations are narrower in spectrum and often are more clearly clonal. Their detection is therefore particularly suited to multigene NGS panels performed either in tissue or in ctDNA. *PTEN* represents the most complex biomarker within this pathway. *PTEN* inactivation occurs through multiple mechanisms, including truncating mutations, deletions, copy-number loss, epigenetic silencing, and post-transcriptional or post-translational protein loss [125]. This creates a major limitation for the use of liquid biopsy. ctDNA assays can detect *PTEN* mutations or copy-number changes, but cannot measure PTEN protein expression, which may be the biologically decisive event. Consequently, a LBx assay may underestimate true *PTEN* loss [113, 118, 126–128].

From a technical standpoint, ctDNA analysis of the PI3K pathway requires careful interpretation. *PIK3CA* mutations are often detectable at higher VAF than *ESR1* because they may represent earlier clonal events, although subclonal low-frequency variants also occur and should not be missed [129, 130]. No validated VAF threshold excludes benefit from PI3K inhibition or oral SERDs, and even low-level activating mutations are clinically relevant. In this respect, NGS panels are preferable because they allow simultaneous evaluation of *PIK3CA*, *AKT1*, *PTEN*, *ESR1*.

Discussing co-mutations of ESR1 and PIK3CA with the oncologist

Concurrent *ESR1* and *PIK3CA* alterations are increasingly identified in HR-positive/HER2-negative metastatic breast cancer, with prevalence strongly influenced by

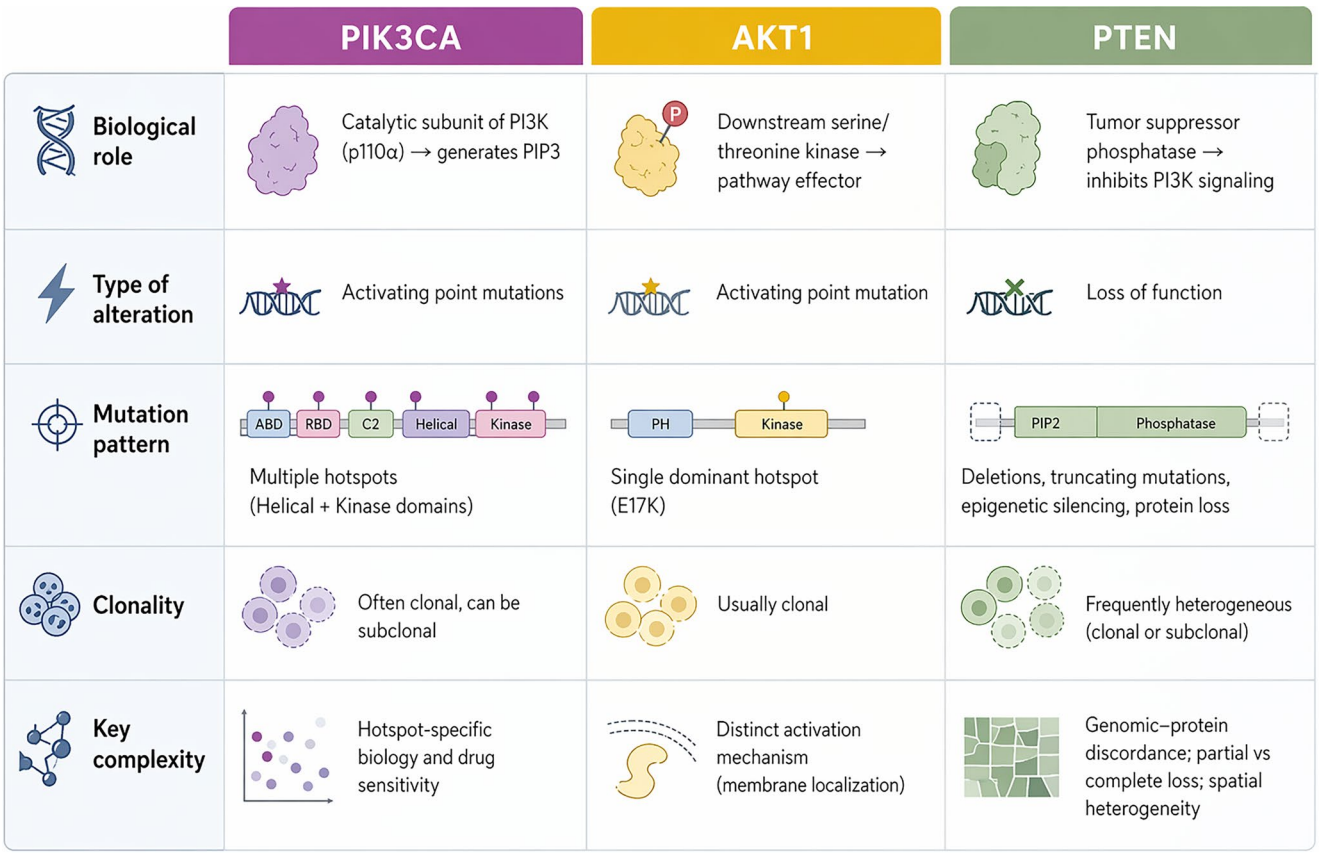

Fig. 3 Comparison of biological and molecular features of PIK3CA, AKT1, and PTEN alterations in the PI3K/AKT pathway. PIK3CA is predominantly altered by activating point mutations. These alterations are often clonal, although subclonal populations may occur. AKT1 is a downstream serine/threonine kinase and pathway effector and is typically clonal. In contrast, PTEN is affected by diverse loss-of-function mechanisms, including genomic deletions, truncating mutations, epigenetic silencing, and loss of protein expression. PTEN alterations frequently show clonal or subclonal heterogeneity and additional complexity arising from genomic–protein discordance, partial versus complete protein loss, and spatial heterogeneity

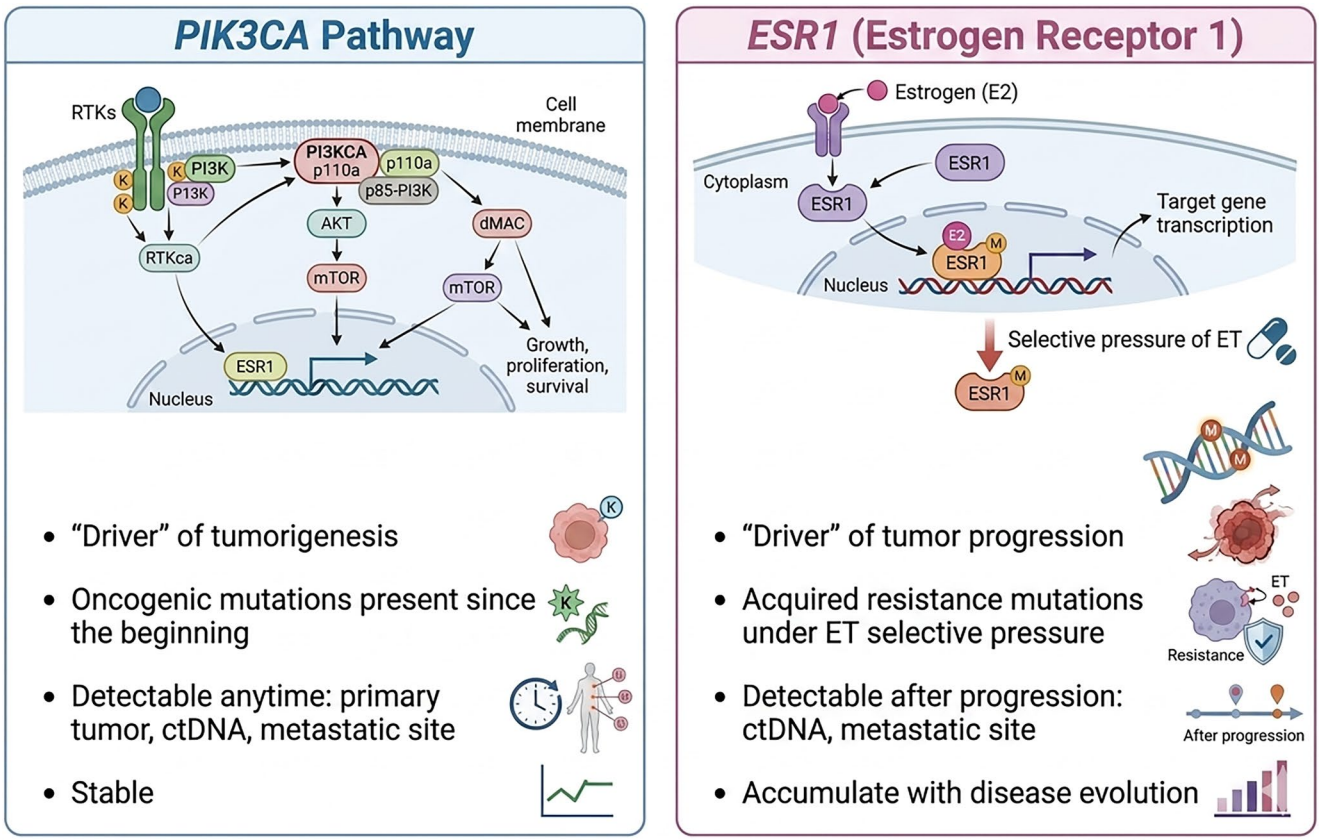

Fig. 4 Distinct temporal and biological patterns of PIK3CA and ESR1 alterations in hormone receptor-positive breast cancer. PIK3CA alterations are typically early oncogenic events that act as drivers of tumorigenesis through constitutive activation of the PI3K/AKT/mTOR signaling pathway. PIK3CA mutations are generally present from the early stages of disease, can be detected in the primary tumor, circulating tumor DNA (ctDNA), or metastatic lesions, and tend to remain relatively stable throughout disease evolution. In contrast, ESR1 mutations primarily represent drivers of tumor progression and acquired resistance to endocrine therapy under the selective pressure of the treatment. ESR1 mutations are more commonly detected after disease progression, particularly in ctDNA and metastatic lesions. These contrasting evolutionary patterns underscore the different implications of PIK3CA and ESR1 testing with respect to timing, specimen selection, and longitudinal molecular monitoring

prior endocrine treatment exposure [94, 131]. In earlier phases of the metastatic process, co-mutations are relatively uncommon, whereas after progression on aromatase inhibitors and CDK4/6 inhibitors they become substantially more frequent, generally observed in approximately 10–15% of cases and potentially exceeding 20% in heavily pretreated patients exposed to multiple sequential endocrine regimens [132].

Coexistence of co-mutations reflects the intersection of two biologically distinct oncogenic processes that arise at different moments of tumour evolution (Fig. 4). While *PIK3CA* mutations are typically early driver events involved in tumorigenesis, they can be detected in primary tumour tissue, metastatic lesions, and ctDNA across different disease stages, and usually show substantial temporal stability [120]. By contrast, *ESR1* mutations are acquired resistance events that emerge later under the selective pressure of endocrine therapy [88]. Accordingly, *ESR1* mutations are often absent in the untreated primary tumour and become detectable mainly after progression [91, 92].

This temporal asymmetry is clinically important. A tumour harbouring both *PIK3CA* and *ESR1* mutations is not simply "double mutant"; it often represents a cancer in which an original oncogenic driver (*PIK3CA*) has persisted while a secondary adaptive mechanism (*ESR1*) has emerged under therapy [86]. LBx is uniquely suited to reveal this layered biology because it captures both stable trunk alterations and newly arising resistant subclones [5, 6]. For cases, where mutations in both genes are present, there is currently no evidence-based rule establishing that one biomarker should automatically supersede the other. As mentioned earlier, VAF should not be used as a stand-alone tool to prioritize treatment between *ESR1*- and *PIK3CA*-directed options [133]. VAF numerical difference may simply reflect tumour evolutionary timing rather than biological dominance or greater therapeutic relevance. Conversely, a rising or detectable low-VAF *ESR1* mutation may still indicate clinically

meaningful endocrine resistance despite representing a smaller clone. Therefore, comparing the percentages of *ESR1* and *PIK3CA* on a ctDNA report can be misleading if interpreted as a therapeutic ranking system.

Overall, interpretation of the molecular findings and their therapeutic impact require multidisciplinary discussion, in which both the pathologist and molecular biologist play a pivotal role alongside the oncologist by contextualizing ctDNA findings within tumour biology, clonality, assay limitations, co-occurring alterations, and prior treatment history [9].

> Statement 3: The integration of validated ctDNA-based LBx into routine molecular diagnostics is required for patients with advanced HR+/HER2- breast cancer, given its established role in guiding ESR1-directed therapy with oral SERDs and its complementary value for PI3K pathway testing.

Perspectives and conclusions

LBx has moved from a promising innovation to a clinically established natural component of molecular pathology. Plasma ctDNA analysis now provides predictive information identifying actionable genomic alterations that can allow personalized treatment decisions. The case studies of NSCLC and breast cancer illustrate two mature yet distinct models of implementation: in lung cancer, LBx supports rapid detection of targetable genomic alterations when tissue is unavailable or insufficient, whereas in breast cancer it has become the preferred strategy for identifying dynamic endocrine resistance biomarkers such as *ESR1* and an important tool for PI3K pathway stratification. Together, these examples confirm that ctDNA testing is fully embedded in clinical decision-making process.

At the same time, ESP reaffirms that testing of the tissue and LBx are not competing but complementary approaches. They represent merely two different methodological approaches in pathology. Histological classification, tumour morphology, immunohistochemistry, PD-L1 evaluation, assessment of protein loss such as PTEN, and many context-dependent molecular questions still require tissue-based diagnostics. The present standard is therefore analysis of both tissue and plasma within an integrated model of somatic diagnostics. In this framework, pathology is uniquely positioned to coordinate the full diagnostic continuum, connecting morphology, immunophenotype, genomics, pre-analytics, assay validation, bioinformatics, and clinically interpretable reporting [134–136]. For this reason, ESP considers that ctDNA testing for solid tumours should be led or co-led by accredited pathology and molecular diagnostics laboratories operating under robust quality systems, with validated workflows, and close multidisciplinary interaction with treating clinicians. As the health care systems in Europe are quite heterogenous, the access to molecular diagnostics, reimbursement pathways, laboratory infrastructure, and turnaround times differ from one country to another. Sometimes there may even be significant differences between different regions within one country [8]. From a global standpoint, the discrepancies are even more pronounced.

> Statement 4: Successful implementation of LBx in routine pathology practice requires adherence to minimum quality standards, participation in external quality assurance programs, standardized reporting, appropriate training of the pathology workforce, and equitable patient access.

For this reason, the ESP supports harmonized European standards for LBx implementation, including minimum quality requirements, EQA programs, reporting standards, training pathways, and equitable access irrespective of geography. Decentralized testing networks with coordinated uniform requirements regarding quality control may represent an effective model in many healthcare systems. The ESP also recognizes that progress in this field depends on sustained collaboration with other scientific societies, regulatory agencies, as well as patients' organizations.

In conclusion, LBx represents one of the most dynamically evolving landscapes in modern pathology. The vision of the ESP is clear: blood-based somatic testing of tumours should be pathology-driven, in close partnership and collaboration with other disciplines to deliver equitable care for patients with cancer.

> Statement 5: Current evidence supports implementation of LBx in lung and breast cancer diagnostics. Emerging clinical evidence supports future extending its use to additional solid tumors and clinical scenarios.

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Declarations

Conflict of interest N.F. has received honoraria for consulting/advisory roles, participation in speaker bureaus, research support, and/or travel support from Abbvie, Alira Health, AstraZeneca, Daiichi San-

kyo, Epredia, Exact Sciences, Gilead, GSK, Leica Biosystems, Lilly, Menarini Group, Merck, MSD, Novartis, Owkin, Pfizer, Pillar Biosciences, Roche, Sakura, Signatur Bio, Sysmex, ThermoFisher, and Veracyte. P.H. reports consulting fees and honoraria for advisory board participation from ThermoFisher Scientist, Illumina, Qiagen, Amgen, Bristol-Myers Squibb, Biocartis, Novartis, Roche, GSK, MSD, Pierre Fabre, Bayer, Biodena, Sophia Genetics, Daichi Sankyo, Pfizer, Eli Lilly, and AstraZeneca. A.R. has received honoraria for consulting/advisory roles, invited lectures and/or travel support from Abbvie, Amgen, AstraZeneca, Bayer, Gilead, GSK, Eli Lilly, Merck, MSD, Pierre Fabre, Roche and Sanofi. R.D. has no conflict of interest to declare. I.E. has no conflict of interest to declare. X.M.G declares Lectures and associated travel expenses: Roche Farma, Qiagen, Ferrer Internacional, Novartis, Menarini, Biocartis, Agilent-Dako, Leyca, Reig Jofre, Sysmex, MSD, Astra-Zeneca, BMS, GSK, Clovis. Eisai, 3D Histech, Abbvie and participation at the Advisory Boards: Astra-Zeneca, Lilly, Amgen, GSK, Janssen, Illumina, MiMark Diagnostics, MSD, Daiichi Sankyo, Inc, Abbvie, CellsIA, Dreampath.

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