The value of liquid biopsy in diagnosis and monitoring of diffuse large b-cell lymphoma: recent developments and future potential

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Review
The value of liquid biopsy in diagnosis and monitoring of diffuse large b-cell lymphoma: recent developments and future potential

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Abstract

Introduction: Diffuse large B-cell lymphomas (DLBCL) represent a heterogeneous subset of non-Hodgkin lymphomas (NHL) that demonstrate many molecular alterations and somatic mutations, all of which are targets for the recent development of biomarkers that use various molecular biological techniques. These non-invasive emerging biomarkers will be used in the next few years to better monitor the response to immunochemotherapeutic treatments with the aim of completely eradicating the disease in order to cure it.

Areas covered: In this review, the authors conducted a literature search to identify and summarize the major advances in liquid biopsy techniques for DLBCL that are useful for diagnosis and monitoring minimal residual disease (MRD). The authors report on the major technological leaps represented by the main MRD tools (sequencing of clone-specific rearrangements of immunoglobulin genes and sequencing of somatic mutations in circulating tumor plasma DNA) and present the expected future developments and the impact of these new tools on clinical practice.

Expert commentary: The monitoring of somatic mutations in tumor plasma cell-free DNA represents a promising tool for liquid biopsy, which will in the future allow non-invasive monitoring that will be used at any time to follow the response to the treatment.

Keywords: DLBCL, biomarkers, liquid biopsy, cfDNA, MRD
1. **Introduction:**

1.1. Histopathology

Diffuse large B-cell lymphoma (DLBCL) is the most common form of aggressive non-Hodgkin lymphoma (NHL) worldwide and accounts for 30-58% of newly diagnosed lymphoma cases. The annual incidence of DLBCL in the European Union is 3–4/100,000 people and increases with age from 0.3/100,000 (35–39 years) to 26.6/100,000/year (80–84 years) (1). Molecular techniques such as gene expression profiling (GEP), comparative genomic hybridization (CGH) and next generation sequencing (NGS) have identified distinct molecular signatures and pathways that define three major molecular subtype categories of DLBCL: the activated B-cell-like (ABC) subtype, the germinal center B-cell-like (GCB) subtype, and the primary mediastinal BCL (PMBL) (2–4). These three major subtypes of DLBCL emerge from B cells at different stages of maturation and differentiation. All subtypes express the antigen receptor of B lymphocytes (BCR), which is composed of paired heavy and light immunoglobulin chains (IgH and IgL, respectively). However, the molecular phenotypes of the subtypes are very different. The GCB subtype is characterized by the more frequent presence of the translocation t(14;18) (q32;q21) BCL2-IGH, alterations in EZH2 and the loss of PTEN. The ABC subtype is characterized by a higher prevalence of translocations t(3;14)(q27; q21), mutations in MYD88 and CD79A/B, and deletions of INK4A-ARF or amplification of BCL2. In contrast, PMBL is a separate entity of aggressive B-cell lymphoma that is clinically and biologically distinct from the other molecular subtypes of DLBCL, the PMBL subtype has a genetic profile that is closer to that of classical Hodgkin's lymphomas (cHL) including XPO1 mutations (5), amplifications of the JAK2 loci and deletion of SOCS1. PMBL-specific gene expression profiles have been reported, but in clinical practice, the diagnosis of PMBL remains essentially based on clinical, pathological, and immunophenotypic characteristics that lack specificity (3,6).
In addition to their molecular heterogeneity, these subtypes of DLBCL have distinct outcomes, with the ABC subtype being clearly associated with an unfavorable prognosis whereas the GCB DLBCL has a more favorable prognosis (7,8). The limited ability to collect appropriate fresh tumoral DNA from DLBCL tissue biopsies from the patients has hindered the active adoption of somatic gene mutation results in prognostic or theranostic strategies for routine daily use. At the dawn of the era of personalized medicine, also termed "precision medicine," the main difficulty lies in integrating the results of high-throughput sequencing of tumor DNA into clinical practice and management to determine the best treatment for patients based on the genetic profile of their tumors at diagnosis or relapse. DLBCL cell of origin (COO) subtypes can be accurately classified using molecular alterations identified in the DNA from tumor biopsies, including formalin-fixed paraffin-embedded tissue (FFPE) (8). However, these techniques are still not routinely used due to their high cost and the time to perform the analyses. Immunohistochemical (IHC) algorithms developed in the last decade are not very reproducible and have no current impact on therapeutic decisions (9,10). Of note, the Visco-Young' algorithm (11) is used in many countries and is more precise than Hans' in identifying COO.

1.2. Treatment and staging

Six to eight cycles of cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) combined with anti-CD20 rituximab (R) every 21 days is the current standard treatment (12,13) for patients with DLBCL (14). This front-line R-CHOP regimen has greatly improved long-term disease control, with complete response (CR) rates ranging from 75% to 80% (15), and more than half of both elderly and younger patients are still in CR 5 years after initial treatment (12,15,16). However, despite the improved accuracy of the
molecular/histopathological classifications and risk-modified chemo-immunotherapies, there are important clinical heterogeneities among DLBCL patients: 20% of the patients are refractory to the primary R-CHOP treatment, and 30% experience disease relapse after achieving complete remission (16–18). These refractory/relapse patients have poor prognoses and are not usually cured by the current salvage treatment, which involves a combination of high-dose chemotherapy (HDC) for eligible patients followed by autologous stem cell transplantation (ASCT) (20). To date, there is no standard procedure for the prompt and accurate detection of relapses. Positron emission tomography (PET) associated with computed tomography (PET/CT) represents the current gold standard for the initial staging of all patients as well as the evaluation of the response at the end of treatment. It has not yet been possible to clearly demonstrate the utility of a positive interim PET (iPET) as an intermediate biomarker that would indicate the necessity to intensify the treatment (21,22). However, an early PET assessment has a prognostic value at mid-treatment and is useful for patients younger than 60 years with high-risk DLBCL, based on the results of the GELA LNH 07-3B (23) and GOELAMS 075 trials (24). These trials showed that early responders to standard immunochemotherapy do not benefit from therapeutic intensification.

It has long been known that the results of routine blood tests such as lactate dehydrogenase (LDH) performed during scheduled clinical visits do not detect recurrence of the disease before clinical symptoms appear (25), but these tests are still performed in routine practice. In addition, PET/CT has not yet demonstrated its utility in the systematic follow-up of patients in remission after the first-line treatment because the majority of DLBCL relapses are detected outside of the planned follow-up period and the outcomes are not affected (26). Furthermore, PET/CT raises concerns regarding the cost to society and the repeated irradiation of patients (27,28). Finally, a large retrospective study of DLBCL demonstrated
that the utilization of routine surveillance CT scans after the end of the treatment did not result in any improvement in the survival compared with standard clinical evaluations (29).

All of these results mandate the development of biological techniques and new methods that will be complementary to PET/CT to enhance its sensitivity, to better stratify patients with respect to risk, to detect early relapse and to improve patient survival.

Moreover, new targeted therapies and anti-tumor immunotherapy strategies are currently being developed to overcome the mechanisms of therapeutic resistance of the tumors. However, these strategies currently benefit only certain types of patients (30), and the current routine clinical tools are inadequate to predict which patients might benefit from such targeted therapy for relapse treatment.

1.3. Liquid biopsy and molecular profiling

The current gold standard method for molecular profiling of tumors is based on lymph node or tissue biopsies, but it has several disadvantages, notably in terms of the risks associated with the procedures (hemorrhage, infections, anesthetic risks). However, there are also risks of bias due to the spatial heterogeneity of DLBCL tumors (31). Furthermore, in certain tumor types, biopsies are particularly difficult to obtain, especially in the case of PMBL, primary central nervous system lymphoma (PCNSL) or bone lymphoma. In this context, the available histological material is often of poor quality, with artifacts related to the sampling. In addition, the small amount of tumor material increases the difficulties of accurate pathological diagnosis.

Given these limitations, the use of "liquid biopsy" in which molecular tumor characterization is performed using a simple peripheral blood sample is currently increasing. This technique represents a major technological leap in the non-invasive management of cancers (see Table 1). Such an approach could have an impact on the cure rate for aggressive B lymphomas through increased monitoring of clinical development using tools for the early detection of
treatment failures and relapses of the disease. The main obstacle to date has been the difficulty in identifying targets that are applicable to the wide diversity of NHL subtypes. For example, recurrent IGH-BCL2 translocations are only detectable in the blood of patients with follicular lymphomas (FL), transformed FL (t-FL) and a rare subset of de novo DLBCL, but allele-specific PCR fails to detect all rearrangements in the blood due to variability of the breakpoint regions. Furthermore, the majority of DLBCL patients do not harbor any recurrent translocations susceptible to molecular monitoring.

The concept of liquid biopsy in patients has been well known for several years mainly with solid tumors, for which it has been possible to demonstrate the presence of circulating tumor cells (CTC), plasma cell-free DNA (cfDNA), and circulating micro-RNAs (31–34). It is thought that the plasma cfDNA is mainly released by apoptotic tumor cells but may also be released by necrotic tumor cells or actively secreted by tumor cells, as illustrated by the large range of DNA fragment sizes that are detected (from 0.150 to several kilobases) and the presence of tumor-specific gene alterations.

Furthermore, the value of liquid biopsies was recently highlighted in a study of a series of DLBCL patients in whom high-throughput sequencing of a panel of target genes was performed. This study demonstrated the successful detection of somatic variants in both the tumors and in the plasma (36). These results indicate that cfDNA can be used to detect somatic variants in DLBCL, which validates the use of liquid biopsies for this type of tumor, even though this condition is not normally characterized by the presence of CTC or leukemic phases (37). Moreover, cfDNA is detectable in 96% of DLBCL patients (38), which argues for its major importance as a widely applicable biomarker. The advantages of using cfDNA for the detection of tumor mutations are obvious and include (see Table 2) (i) noninvasive collection, (ii) availability at any time during the course of the disease, (iii) real-time detection and monitoring of biomarker dynamics, and (iv) potentially fewer heterogeneity issues than
tumor tissue testing (39–41). The accuracy of the molecular methods for the evaluation of cfDNA provides lower detection limits than CT and may overcome the current limitations of both CT imaging and tissue biopsies.

In this review, we conducted a literature search using PUBMED to summarize and highlight major recent advances in biomarkers for DLBCL and discuss their potential impact on clinical practice and in routine patient management in the coming years.

2. Major liquid biopsy techniques

2.1. VDJ rearrangements

Normal and tumor B-cells express the BCR throughout their lifespans. The diversity in this characteristic molecule results from the random rearrangement, at the DNA level, of the variable-diversity-joining (VDJ) genes in the B-cell progenitor. This process produces unique clonotypes, and each DLBCL tumor contains a singular clonotypic VDJ immunoglobulin rearrangement that can serve as a "barcode" for the immediate detection of disease relapse in the peripheral blood. Indeed, NGS-based tests are able to identify and measure circulating tumor DNA that encodes the VDJ junctions of the immunoglobulin receptors in the patients’ blood prior to treatment in most of the patients with DLBCL (42). Emerging evidence suggests that these NGS tests (Lymphosight® technology) can be used widely to predict relapse in DLBCL patients several months earlier than conventional imaging (43,44).

Furthermore, previous studies have shown that DLBCL relapse can occur through either of two scenarios: an early divergent scenario in which the relapsing clone independently evolves from a common progenitor and a late divergent scenario in which the relapsing clone is usually derived from the clone identified in the original diagnosis (45). These results have been recently confirmed in a high-throughput immunoglobulin sequencing study that used...
peripheral blood mononuclear cell DNA. The study added new data on this topic that suggested that oncogenic events under the selection pressure of the chemotherapeutic intervention may be the main forces that drive relapse (46). Nevertheless, these high-throughput VDJ sequencing techniques have several limitations. Specifically, VDJ rearrangements are not detectable in all patients (for instance, in the immunoglobulin-negative phenotype of PMBL as well as in some de novo DLBCL with unproductive VDJ rearrangements) (47), and the technology is not useful as a means of tailoring a targeted therapy or detecting the emergence of resistant clones.

2.2. High-throughput sequencing of somatic mutations in cfDNA

Deep-sequencing analysis of the rearrangement of the immunoglobulin heavy chain gene (VDJ-seq) is able to trace the clonal evolution patterns of DLBCL relapse (48). In addition, whole exome sequencing is also capable of characterizing the somatic mutation profiles of DBLCL tumors that are resistant to chemotherapy (49). This study of the exomes of 38 biopsies of refractory DLBCL revealed clonal expansions that affected the regulatory genes of the NF-κB pathway whose mutations had not been previously associated with DLBCL (NFKBIE and NFKBIZ) as well as mutations that could affect the sensitivity to new drugs, such as MYD88 and CD79B mutations in ABC patients and STAT6 (JAK/STAT pathway) in GCB patients.

In addition, previous studies (50–53) have demonstrated that targetable activating mutations are very common in GCB and ABC DLBCL, which indicates that evaluations of a limited number of somatic mutations in plasma cfDNA could help tailor optimal treatment strategies in DLBCL, now that many pharmacological inhibitors are in development. In this context, an NGS "lymphopanel" has been designed to identify somatic mutations in 34 genes that were selected from a review of the literature regarding the genetic diversity of DLBCL (54) and
based on whole exome sequences of patients with relapsed/refractory (R/R) DLBCL (55). This lymphopanel was then tested on 215 patients with de novo CD20+ DLBCL (56) that had previously been characterized using GEP Affymetrix GeneChip Arrays. This lymphopanel was informative for 96% of the DLBCL patients in whom it identified the most frequent somatic alterations, some of which could potentially be affected by targeted therapeutics (EZH2 Y641N, MYD88 L265P, XPO1 E571K) and/or serve as molecular biomarkers of residual disease. Interestingly, the lymphopanel could also be used as a tool to evaluate the liquid biopsies of patients' plasma cfDNA, where it could reveal similar mutational profiles in the blood and tumors of the patients (36). Nevertheless, many technical validation steps for these methods will still have to be completed in the next few years, before the NGS can be integrated into routine clinical practice and therapeutic decision support.

2.3. Digital PCR

A recent study of many solid cancers in a large cohort of patients confirmed the effectiveness of the digital polymerase chain reaction (dPCR) to identify somatic KRAS mutations in cfDNA and to monitor the effect of targeted therapies through the detection of newly developed EGFR mutations (57). Several studies have demonstrated that dPCR is more sensitive than quantitative PCR (qPCR) for the detection of circulating biomarkers (58, 59). An interesting study with droplet dPCR (60) demonstrated the clinical value of cfDNA as a noninvasive monitoring tool for the treatment response of gynecologic cancers. This method appears to be as sensitive and specific as CA-125, a well-known protein biomarker of ovarian cancer. In this study, the somatic mutations that were identified by sequencing each tumor were then measured in the plasma cfDNA with a 93.8% detection rate. These markers were detected in the plasma cfDNA 7 months before evidence could be obtained by CT imaging. Furthermore, undetectable levels of cfDNA were associated with both improved PFS and OS.
Because dPCR is a fast, easy, and cost-effective method that requires little DNA, it a useful tool for plasma cfDNA analysis. A highly sensitive and specific probe-based dPCR assay was recently designed for the detection of three common mutations in DLBCL: (i) the exportin-1 (XPO1) E571K mutation, which was recently described as present in 31.8% of PMBL (5) and which potentially affects the prognosis and sensitivity to Selective Inhibitors of Nuclear Exports (SINEs such as selinexor); (ii) the activating EZH2 Y641 mutations, which are present in approximately 24% of GCB DLBCL (61,62) and include a subset consisting of p.Y641N, which can be targeted by EZH2 inhibitors that are currently being developed (e.g., EPZ-6438) (63); and (iii) the oncogenically active MYD88 L265P mutation, which is found in 29% of ABC DLBCL (53,64) and can potentially modulate the response to ibrutinib (65,66). Another recent study described new multiplex droplet dPCR assays that allowed the simultaneous detection of distinct recurrent somatic mutations in fresh tumors, FFPE and liquid biopsies of DLBCL patients (67). These studies confirmed that the dPCR technology is a powerful complementary technique in the emerging context of noninvasive somatic mutation detection (68). Specifically, the strengths of dPCR include rapid implementation, low cost, short turnaround times, and high sensitivity, making it a valuable tool on its own and in addition to NGS. Nevertheless, several limitations of dPCR should be considered: (i) it is not appropriate for determining MRD using single point mutations in cases of subclonality, in particular for actionable alterations because an effective treatment might eradicate those subclones and mutation-negative clones could persist yet be undetectable by this assay; (ii) the low amount of cfDNA in DLBCL blood samples may limit the ability to detect MRD; (iii) there are concerns regarding false-positives and the detection limits for plasma cfDNA (69,70); and (iv) a multicenter assessment program has not yet been established, and thus, it is difficult to evaluate the reproducibility of these dPCR approaches, their sensitivity and their specificity.
2.4. CAPP-seq

CAncer Personalized Profiling by deep Sequencing (CAPP-seq) is a highly sensitive new method for quantifying circulating tumor DNA that uses high-performance, high-throughput sequencing and permits the rapid detection of very rare circulating somatic variants in various tumors (71). In the proof-of-concept study, the authors detected cfDNA in 100% of patients with stage II–IV NSCLC and in 50% of patients with stage I, with 96% specificity for the mutant allele fractions down to ~0.02%. In another remarkable study, CAPP-seq technology was used to analyze tumor biopsies and cfDNA from 92 lymphoma patients and 24 healthy subjects (72). The authors identified distinct modes of clonal evolution that differentiated indolent follicular lymphomas from those that transformed into DLBCL, which could lead to a promising noninvasive means to assess histological transformation in the future. Perhaps even more impressively, the same team was able to accurately determine the DLBCL COO subtypes using somatic alterations that were detectable in circulating cfDNA using the CAPP-seq technology. In this study (73), the concordance between the tumors and plasma COO classification was excellent (88%). Compared to dPCR, CAPP-seq can not only distinguish point mutations but is also able to identify indels, structural variations including translocations, and copy number variations and can analyze many loci in the same experiment (74).

This is a promising technique that may overcome some of the limitations of the GEP- and IHC-based approaches, including the need for invasive biopsies, lack of tissue, and inadequate assay efficiency. It is interesting to note that the cfDNA data obtained by this CAPP-seq technique are highly dynamically correlated with the tumor volume assessed using TEP-scan imaging during the treatment, and there was an excellent relationship between the plasma cfDNA changes, the therapeutic response after two cycles of chemotherapy and the clinical
outcome in DLBCL patients (75). Another study used both the CAPP-seq method and droplet dPCR in DLBCL patients treated with panobinostat. Of the 14 patients who showed progression of the disease, 10 subjects displayed increasing cfDNA levels, and it appeared that the cfDNA fluctuations were correlated with the response to treatment (38). This was the first clinical trial using cfDNA monitoring to measure the response rate in DLBCL. Very recently, CAPP-seq technology was used in a prospective longitudinal trial that included 50 DLBCL patients who were treated with the standard R-CHOP protocol (76). This convincing report demonstrated a rapid loss of DLBCL mutations from cfDNA among the responsive patients and the persistence of basal DLBCL mutations in plasma cfDNA of the refractory patients. In the latter group, new mutations were observed during the immunochemotherapy treatment, which represented distinct resistant clones that were revealed with a real-time approach that demonstrated a >90% sensitivity and an ~100% specificity.

In summary, this CAPP-seq technology has demonstrated its utility in disease burden measurement, MRD monitoring, non-invasive genotyping, detection of early relapse and the development of mutations that are associated with treatment resistance.

2.5. Liquid biopsy limitations

All innovative liquid biopsy techniques have some limitations, which include technical limitations. It is important to be aware of these limitations before designing a clinical trial that will use these methods. The CAPP-Seq limit of detection (0.02%) is constrained by three major factors: the total input amount of cfDNA, sample cross-contamination, and PCR or sequencing errors.

The quality of the pre-analytical preservation of plasma samples is also a major concern for teams interested in the topic of liquid biopsy and cfDNA. Of note, there might be cases where
CT or PET scan is positive, with negative plasma surveillance. The few discrepancies between the detection of the mutation in the plasma, the tumor, and PET scan results can be explained partly by the poor quality of some biopsies with tumor cell scarcity, potentially rendering the mutation detection in biopsy-extracted DNA impossible, and secondly by the absence of tumor DNA release in plasma by certain tumors and the short half-life (10-15 minutes) of circulating DNA in plasma (77).

These limitations also apply to the dPCR and NGS techniques. Furthermore, we currently do not know whether cfDNA is delivered at equal rates from the various lymph nodes, this may be of concern with respect to the determination of tumor burden and clonal evolution if different tumors or clones release their DNA at different rates. This technology also requires bioinformatics processes and dedicated computational validation methods to avoid false-positive results, and there is currently no specific informatic framework for CAPP-seq data analysis. Another important condition for the monitoring of cfDNA levels to detect tumor burden is that cfDNA can demonstrate only that residual tumor tissue is present, but it cannot inform the physician where the residual mass is located. Of note, there are discrepant results regarding the prognostic value of the presence of residual anatomical disease indicated by CT in PET-negative DLBCL patients (78,79). The use of cfDNA may help to address this concern, and its analysis should be utilized in a manner that is complementary to PET imaging for residual disease monitoring. Nevertheless, the final meaning of “molecular relapse detection” is totally unknown. Indeed, there exist patients that remain positive by NGS in plasma cfDNA and will never relapse. Second, there is no data about pre-emptive therapy based on these techniques, and no clue on which therapy might be used in this onset.

2.6. Additional biomarkers in DLBCL
Additional alternative biomarkers, not based on DNA-sequencing techniques, are currently being developed for DLBCL and are described here. First, flow cytometry-based evaluation of blood lymphocytes may be useful to clarify the prognosis for newly diagnosed DLBCL patients. Specifically, increased numbers of B cells and decreased levels of Tregs and apoptotic cells after treatment might predict a poor clinical outcome in patients treated with RCHOP (80). Second, interest in soluble markers for the risk stratification of DLBCL has recently increased. In particular, the serum concentrations of soluble LR11, which is a tumor-derived biomarker, seem to be related to the tumor mass and had an unfavorable prognostic value in a multicenter prospective analysis (81). Another well-known biomarker in oncology clinical practice, serum carbohydrate antigen 125 (CA-125), seems to be correlated with serosal effusions and PFS in DLBCL patients (82), but this marker has limited sensitivity (73.8%) and specificity, although this could be greater in the case of human ovarian carcinoma.

Third, the interleukin (IL)-10 concentration in cerebrospinal fluid (CSF) has been confirmed to be a useful biomarker for the diagnosis of PCNSL and assessment of the therapeutic response when combined with magnetic resonance imaging in this very-difficult-to-manage disease. Another controversial biomarker that is currently being studied in DLBCL is the lymphocyte-to-monocyte ratio (LMR). Two recent meta-analyses with >5000 patients established that patients with low LMRs displayed shorter PFS and OS (83,84). However, the optimal cutoff for the LMR and the technical applicability of this ratio for the individual stratification of patient risk and the development of therapeutic strategies are still unknown. More substantial and meaningful trials that include the COO classification of the patients are justified to better define the prognostic impact of the LMR in DLBCL. Fourth, various serum or tissue micro RNAs are currently being widely studied in DLBCL, with various prognostic
impacts (85–87). Fifth, new proteomic approaches have also implicated new candidate biomarkers, such as HSP70 and adiponectin. However, these proteomic profiling strategies remain exploratory, and these preliminary data need to be confirmed in large prospective studies to firmly establish their value.

Finally, all of these novel biomarker results are promising but are still too recent to be included in large prospective clinical trials or to have a significant clinical usefulness for risk stratification at the time of diagnosis but also during treatment in daily practice, mostly due to low sensitivity and specificity.

3. Conclusion

The liquid biopsy approach has great potential for the detection and surveillance of disease relapse, and many choices for the quantitation of mutations or VDJ rearrangements in circulating tumor DNA are currently available. Despite the fact that many of these techniques have been developed as valuable noninvasive, appropriate, and real-time monitoring tools, none of these techniques has yet been translated into the clinical practice. Further understanding of the biology of circulating tumor DNA could help to improve its relevance as a biomarker. Molecular monitoring of cfDNA is an extremely promising technique and might become a new indispensable tool for the management of DLBCL. Therefore, it is essential that dedicated well thought-out studies confirm the current results and further assess the roles of cfDNA in the interim monitoring, routine follow-up and treatment response evaluation.

There is no doubt that the progress of emerging monitoring biomarkers based on cfDNA analysis will be valuable to DLBCL patients and improve the clinical outcome in the next several years.

4. Expert commentary
It is an exciting time in the field of noninvasive management of DLBCL, and there is currently increasing evidence that circulating tumor cfDNA can be accurately detected and quantified and that it is able to predict relapse or disease progression several months before standard imaging. The new results for cfDNA follow-up for the VDJ sequences in DLBCL patients are convincing and show a direct link between decreases in the cfDNA, therapeutic response and improved PFS (68, 88). These tests will soon have an important influence on the surveillance procedure after treatment. Although there are currently insufficient data to establish the value of liquid biopsy compared to conventional PET monitoring, based on the limitations of PET and CT imaging with respect to sensitivity, specificity, societal cost and radiation hazard, we can speculate that cfDNA could greatly reduce the reliance on PET imaging for the diagnosis of molecular relapses before there is clinical evidence of disease progression. Additionally, cfDNA could be a valuable tool for evaluation of drug activity that could be used to make prompt decisions regarding treatment with targeted drugs and for the surveillance of MRD during maintenance therapy. We may assert that liquid biopsy monitoring of tumor-specific somatic mutations or VDJ rearrangements will represent in the near future a paradigm switch in the evaluation of the response to treatment and in the surveillance for relapse in DLBCL (See Figure 1). Currently, the main rationale for the immediate incorporation of these non-invasive techniques in large prospective therapeutic trials is the commercial availability of the main technique (from Adaptive Biotechnologies®), which has been proven to be superior to classical follow-up by CT-scan. Moreover, the use of the liquid biopsy technique could help for the diagnosis of certain forms of difficult-to-biopsy DLBCL. Indeed, in the near future, dPCR could potentially be a useful diagnostic tool for the identification of lymphoma subtypes that are usually associated with poor biopsy quality, such as Primary Central Nervous System Lymphoma (PCNSL) or PMBL. Specifically, these
subtypes include (i) PCNSL with *MYD88* L265P mutation detection (89) and (ii) PMBL with *XPO1* E571K mutation detection (5,90). However, these somatic mutations are not specific for distinct lymphoma types and a least a partially preserved morphology will be necessary to make a proper first diagnosis in addition to mutational analysis.

Furthermore, the approach of classifying the COO subtype using plasma DNA sequencing is highly consistent with classification by immunohistochemistry (IHC) and the associated clinical outcomes and may potentially pinpoint high-risk patients including those with double-hit lymphomas (*MYC* and *BCL2* and/or *BCL6* rearrangements) at the time of diagnosis (91). This method may also overcome the limitations of GEP and IHC, reduce the requirement for invasive biopsies and the limitation of tissue availability, and help to tailor future therapy decisions in DLBCL based on the COO (73). Nevertheless, various important technical obstacles and considerations remain to be addressed. These include the lack of standardization of various steps of the methodology: preferred sample type, storage conditions, candidate molecules and appropriate detection limits. In addition, technical errors associated with the PCR procedure may introduce contaminant molecules into the samples, which could lead to misinterpretation and statistical errors. Therefore, standardization of all experimental steps of the techniques should be strongly encouraged. It is also necessary to understand the mechanisms associated with the variations in the cfDNA concentrations (cfDNA base level according to disease stage, circadian variation, half-life, and impact of the inflammatory reactions on the cfDNA level).

5. **Five-year view**

To date, no clinical impact of a strategy that involves monitoring of cfDNA has been directly demonstrated, no recommendation exists for the implementation of a salvage treatment in the event of molecular detection of relapse, and there are no recommendations on the timing of
blood collections to be made after the end of the initial treatment or on the duration of such monitoring. However, the future will certainly not be without PET/CT or without tumor biopsy, but with complementary liquid biopsy techniques, side by side with PET and histological results.

The roles for cfDNA analysis in relation to PET imaging at diagnosis, during treatment and at end-of-treatment, still need to be determined in large dedicated prospective studies, although the current data seem to link tumor metabolic data and molecular profiling of the tumor (92). Interventional clinical trials based on non-invasive cfDNA MRD could soon be considered: the primary endpoint could be the impact on PFS of early detection of a molecular relapse, with an early salvage treatment strategy, when MRD became detectable by NGS but PET remains negative. Another type of clinical trial that could be suggested is a trial of a targeted agent that would be used in maintenance therapy in patients with positive MRD at the end of standard 1st line treatment.

Finally, it is very likely that monitoring of MRD by cfDNA analysis will become important in decision-making within the next 5 years and will be part of "multidisciplinary molecular diagnostics meetings" (MMDM). These meetings are already used in some centers for refractory/relapsed DLBCL patients in which NGS techniques that use the DNA of frozen or paraffin-fixed biopsies are presently used to identify somatic mutations that have prognostic and theranostic impact. High-throughput sequencing of a targeted gene cluster, specific to DLBCL and clinically relevant, from plasma circulating tumor DNA, with rapidly available and inexpensive results will soon find its place within DLBCL daily management at the dawn of the age of personalized cancer medicine.

6. **Key issues**
• Non-invasive emerging biomarkers will be used in the future to monitor the response to immunochemotherapeutic treatments
• Liquid biopsy may replace histological diagnosis and biopsy in the future
• Liquid biopsy might serve as an MRD tool for monitoring of DLBCL in clinical trials
• Circulating DNA allows molecular tumor characterization using a simple peripheral blood sample
• NGS, dPCR and CAPP-seq are the major techniques currently used for non-invasive tumor characterization, somatic mutation analysis and MRD monitoring
• To date, no clinical impact of a strategy that involves monitoring of cfDNA has been directly demonstrated.
• Liquid biopsy will be used in the future at any time to follow the response to the treatment and to identify the emergence of new resistant clones at an early stage before clinical relapse.
• The quality of the pre-analytical preservation of plasma samples is a major concern for teams interested in the topic of liquid biopsy and circulating DNA

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**Declaration of Interest**
H. Tilly discloses work with Roche, Celgene, Janssen and Karyopharm. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
References:

Papers of special note have been highlighted as:

* of interest
** of considerable interest


-> This article reports the current standard Clinical Practice Guidelines for DLBCL management


->This study showed that immunotherapy and PD-1 blockade produced significant antitumor activity in highly pretreated lymphoma patients.


This article demonstrated the feasibility and the clinical interest of noninvasive DLBCL monitoring with a VDJ rearrangements based technique.


This article established the pertinence and prognostic impact of NGS-based molecular circulating cfDNA monitoring in a large retrospective cohort of 126 DLBCL patients.


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-> This landmark study including 50 DLBCL patients treated with the standard R-CHOP demonstrated a rapid clearance of mutations from cfDNA among the responsive patients using CAPP-seq technology and the persistence of basal mutations in plasma cfDNA of the refractory patients.


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**Tables**

**Table 1:** Relevance of circulating tumor cell-free DNA in DLBCL

<table>
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<tr>
<th>At diagnosis</th>
<th>During and at the end of treatment</th>
<th>During follow-up</th>
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<tbody>
<tr>
<td>Eligibility for targeted therapy</td>
<td>Quantitative assessment of tumor burden decrease</td>
<td>Early discovery of disease relapse</td>
</tr>
<tr>
<td>Prognostic biomarker</td>
<td>Early detection of infraclinic disease progression</td>
<td>Minimal residual disease monitoring</td>
</tr>
<tr>
<td>Genetic screening of the disease</td>
<td>Molecular complete remission assessment</td>
<td>Molecular personalized medicine: determination of appropriate salvage treatment</td>
</tr>
<tr>
<td>Less heterogeneity than tissue testing</td>
<td></td>
<td>Clonal evolution follow-up</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Noninvasive liquid biopsy for hard-to-biopsy tumors</td>
</tr>
</tbody>
</table>
Table 2: Example of follow-up monitoring approaches after 1st line therapy in DLBCL

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical exam</strong></td>
<td>Economical</td>
<td>Low sensitivity and specificity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late detection of relapse</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase (LDH)</strong></td>
<td>Low cost</td>
<td>Low sensitivity and specificity</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td><strong>Computed tomography (CT)</strong></td>
<td>Easily accessible</td>
<td>Radiation concerns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No demonstrated survival benefit</td>
</tr>
<tr>
<td><strong>Positron emission tomography (PET)</strong></td>
<td>Upgraded sensitivity over CTQuite available in cancer centers</td>
<td>Radiation exposure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>False-positive findings</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Costly</td>
</tr>
<tr>
<td><strong>Circulating tumor cell-free DNA</strong></td>
<td>Non-invasive Liquid biopsy Minimal residual disease (MRD) tool Tumor-characteristic Real-time dynamic assessment</td>
<td>No standardized technique</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No prospective clinical validation</td>
</tr>
</tbody>
</table>
Figure 1: Schematic representation of circulating tumor cell-free DNA (cfDNA) monitoring in DLBCL.