Introduction

Most, if not all, lymphoma subtypes display considerable heterogeneity in their clinical and pathological characteristics, intricately linked to the underlying biological heterogeneity. Indeed, the genomic landscape of lymphomas is remarkably diverse, although an increasing number of ‘shared’ genetic lesions have recently emerged, affecting similar mechanisms and processes that in certain instances are
important for ‘generic’ cell homeostasis (e.g. DNA repair) while in others they are ‘lymphocyte-specific’ (e.g. antigen receptor signaling) (Figure 1).

Thanks to next-generation sequencing (NGS), it has become possible to appreciate the panorama of recurrently affected genes that contribute to disease pathogenesis and/or evolution, at least in major lymphoma subtypes. Mounting evidence suggests that certain gene mutations have diagnostic, prognostic and/or predictive impact. However, for most mutations, functional in vitro validation and confirmation in larger patient series are warranted in order to fully elucidate their role in the pathobiology of a particular lymphoma as well as their relevance for routine diagnostics.

In few circumstances, a single recurrent mutation is identified in almost all cases of a given lymphoma and predominates by far in the genomic landscape of that particular tumor, e.g. the MYD88L265P mutation in Waldenström’s Macroglobulinemia (WM)/lymphoplasmacytic lymphoma (LPL)\textsuperscript{2} and the BRAFV600E mutation in hairy-cell leukemia (HCL).\textsuperscript{3} However, for the great majority of lymphomas, including chronic lymphocytic leukemia (CLL),\textsuperscript{4} diffuse large B-cell lymphoma (DLBCL),\textsuperscript{5} follicular lymphoma (FL),\textsuperscript{6} mantle cell lymphoma (MCL),\textsuperscript{7} Burkitt lymphoma (BL),\textsuperscript{8} splenic marginal zone lymphoma (SMZL)\textsuperscript{9} and most peripheral T-cell lymphoma (PTCL) subtypes,\textsuperscript{10-13} NGS studies have revealed a quite diverse and complex mutation pattern, with a limited number of frequently mutated genes accompanied by long tail of genes with low-frequency mutations. In addition, while some genes are biased to certain lymphoma entities, e.g. SF3B1 mutations in CLL,\textsuperscript{14,15} KLF2 mutations in SMZL,\textsuperscript{16-18} ID3 and TCF3 mutations in BL,\textsuperscript{19} STAT3 mutations in large granular lymphocyte (LGL) leukemia,\textsuperscript{11} and RHOA mutations in angioimmunoblastic T-cell lymphoma (AITL),\textsuperscript{20,21} other genes found recurrently mutated, such as genes involved in DNA repair, epigenetic modification and regulation of transcription (Figure 1), can be detected in multiple subtypes (Table 1) and even in other cancer types.

Considering the genetic heterogeneity of lymphomas, also highlighted by diverse results reported in hitherto published NGS studies, it will require large-scale initiatives encompassing thousands of patients to clarify if a specific gene mutation has an impact on the current lymphoma classification/diagnostics and aids clinical decision-making, including therapy selection and response prediction.

In an effort to summarize the current status of this ever-evolving field and provide guidelines regarding the clinical relevance of recent genetic findings, we have established the European Expert Group on NGS-based Diagnostics in Lymphomas (EGNL), with expertise in hematopathology, molecular pathology, clinical genetics, hematology, and oncology. The EGNL Group is supported by the European Research Initiative on CLL (ERIC; Scientific Working Group within the European Hematology Association, EHA) and the European Association for Haematopathology (EAHP).

As our first task, we took advantage of the published literature on lymphomas to identify recurrently mutated genes with a potential clinical relevance that were reported in at least two independent studies; other genomic aberrations, e.g. translocations and copy number aberra-
tions, may also be clinically relevant, though they are not discussed in this review. As the next step, we grouped the identified genes into the following categories based on: (i) immediate impact on treatment decisions, (ii) diagnostic impact, (iii) prognostic impact, (iv) potential clinical impact in the near future, or (v) interest for research purposes only. In the end, only few genes were deemed to have direct therapeutic or diagnostic implications, while a sizeable proportion of genes were judged as prognostic and/or with a potential role for patient management within the next few years (Table 2). Though this latter category is evidently difficult to define, we decided to include genes for which recent evidence strongly suggests a diagnostic and/or predictive role, sometimes limited to retrospective studies, or for which targeted therapy is under development. In the following sections, we outline our arguments for including a particular gene in one of these categories; highlighted genes in each category are summarized in Table 2.

I. Genes with immediate impact on treatment decisions

Currently, there are few genetic lesions with documented impact on therapy selection and patient management in patients with lymphoma. This category is best exemplified by TP53 aberrations in CLL. Such aberrations are due to (i) deletions of chromosome 17p (covering the TP53 gene) seen in 5-10% of patients at diagnosis, which are often associated with TP53 mutations on the remaining allele; and, (ii) in a small fraction of CLL patients (3-6%), mutations within the TP53 gene only. Both types of aberrations (i.e. 17p-deletions and TP53 mutations) are equally adverse in CLL, portending for refractoriness to standard chemotherapies and poor overall survival. Notably, such patients experience major clinical benefit by the recently approved novel agents targeting B-cell receptor signaling, namely, the Bruton tyrosine kinase inhibitor idelalisib and the phosphatidylinositol 3-kinase delta inhibitor idelalisib that are approved for the treatment of patients carrying either of these lesions; however, these patients still constitute a high-risk group with an increased risk of disease recurrence with time.

On these grounds, the assessment of TP53 status is essential for clinical decision-making. Hence, sequencing of the TP53 gene is now recommended for all CLL patients, in addition to FISH analysis, before the start of any line of therapy (except in the palliative situation).

### Table 1A. Mutation frequencies in different B-cell lymphoma entities.

<table>
<thead>
<tr>
<th>Pathway/cellular function</th>
<th>CLL%</th>
<th>MCL%</th>
<th>BL%</th>
<th>FL%</th>
<th>ABC-DLBCL%</th>
<th>GCB-DLBCL%</th>
<th>SMZL%</th>
<th>HCL%</th>
<th>WMP%</th>
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<td><strong>B-cell receptor signaling</strong></td>
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<td>10%</td>
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<tr>
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<tr>
<td>CR2BP</td>
<td>&lt;1%</td>
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<tr>
<td>EP300</td>
<td>&lt;1%</td>
<td>-</td>
<td>-</td>
<td>10-15%</td>
<td>&lt;5%</td>
<td>&lt;10%</td>
<td>4%</td>
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</table>

CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; BL, Burkitt lymphoma; FL, follicular lymphoma; ABC-DLBCL, activated B-cell-like diffuse large B-cell lymphoma; GCB-DLBCL, germinal center B-cell-like diffuse large B-cell lymphoma; SMZL, splenic marginal zone lymphoma; HCL, hairy-cell leukemia; WMP, Waldenström’s Macroglobulinemia.

*Genomic deletions.

**Frequencies are based on the combined cohort included in Puente et al. Nature 2015" and Landau et al. Nature 2015," though the former encompasses more general practice CLL patients and the latter more advanced, clinical trial patients. Based on Bai et al., PNAS 2013; Menzler et al., Blood 2013; Rabah et al., Nat Med 2014; Based on Schmidt et al., Nat Med 2012; Based on Morin et al., Nat Genet 2010; Oboson et al., Nat Genet 2014; Oboson et al., Nat Genet 2016; Based on Companyne et al., Nature 2009; Davis et al., Nature 2010; Nigo et al., Nature 2011; Pasqualucci et al., Nat Genet 2011; de Miranda et al., Blood 2014; Bohera et al., Genes Chrom Cancer 2014; Based on Rossi et al., JEM 2012; Parry et al., Clin Cancer Res 2015; Pica et al., Leukemia 2015; Based on Tracchi et al., NEJM 2011; Based on Bion et al., NEJM 2010; Poulain et al., Clin Cancer Res 2015."
CLL, in order to identify patients with aberrant TP53 for whom the new targeted therapies, described above, represent the current treatment of choice. Furthermore, the fact that the frequency of TP53 mutations gradually increases as the disease becomes more aggressive and chemorefractory supports the need to repeat TP53 mutation analysis prior to any subsequent line of chemotherapy.31-32 Of note, it has also been demonstrated that TP53 microclones at diagnosis, i.e. subclones with a low-allelic burden detected only by NGS but not by Sanger sequencing, were selected by repeated rounds of chemoinmunotherapy and conferred a poor outcome similar to clonal TP53 mutations.33,54 If this finding is confirmed within the context of prospective clinical trials including signaling inhibitors, it is very likely that NGS-based protocols will become the recommended method for TP53 mutation screening in routine clinical practice.

Mutations within TP53 have been described in most other lymphoid malignancies besides CLL, albeit with varying frequency. Although they have been linked to poor clinical outcome in DLBCL,5,6 SMZL6,7 and MCL7,8 this information does not currently have any impact on treatment decisions or follow-up strategies for the individual lymphoma patient.

II. Genes of diagnostic potential

Few lymphoma entities show a predominating, recurrent mutation, such as: the hotspot MYD88L265P mutation in more than 90% of WM/LPL,5,6 the hotspot BRAFV600E mutation in ~90% of HCL,41,42 and the STAT3 mutations detected in up to 40% of LGL leukemia.11,14 None of these mutations are pathognomonic (i.e. exclusive to) a particular entity and can also be found in other lymphomas, though generally at lower frequencies (Table 1). For instance, the MYD88L265P mutation is detected in a significant fraction of DLBCL of the activated B-cell-like subtype (ABC DLBCL),3 as well as in primary cutaneous,43 the central nervous system46 and testicular large B-cell lymphomas44 but also in a minority of patients with CLL.44,45-48 and SMZL16,51 As another example, STAT3 mutations have been reported, albeit rarely, in immune, mainly hypoplastic, bone marrow failure characterizing a subset of patients with severe aplastic anemia or myelodysplastic syndrome.52 Moreover, some gene mutations are mainly found in a specific lymphoma entity at a relatively high frequency, whereas they are rare in other subtypes, e.g. ID3 and TCF3 mutations in BL,53 KLF2 in SMZL16-19, SF3B1 mutations in CLL,17,21 RHOA mutations in AITL and other PTCL with a follicular helper T cell (Tfh) phenotype,20,21,55-59 and, very recently, the novel somatic mutations in RRAGC encoding a Rag GTPase protein (RagC) that were enriched in FL (16%) but were absent in other mature B-cell lymphomas.60

That said, a pattern has started to emerge in recent years where certain lymphoma entities ‘share’ common types of genetic events affecting selected pathways or biological mechanisms (Figure 1). For instance, germinal center derived B-cell malignancies, such as DLBCL of the germinal center B-cell-like type (GCB DLBCL) and FL, appear to have higher frequencies of aberrations in epigenetic-related genes (e.g. EZH2, CREBBP, TET2, IDH2),41,53 while other B-cell lymphomas demonstrate common mutations of members in the NOTCH, B-cell receptor (BcR), and NF-kB signaling pathways (Table 1).60,61 Similarly, different PTCL subtypes, such as mycosis fungoides and Sézary syndrome, AITL and other Tnfr-derived PTCL, and adult T-cell leukemia/lymphoma (ATLL) share frequent alterations affecting both epigenetic regulation and T-cell receptor

Table 1B. Mutation frequencies in different T-cell lymphoma entities.

<table>
<thead>
<tr>
<th>Pathway/cellular function</th>
<th>AITL</th>
<th>MF/SS</th>
<th>PTCL-NOS</th>
<th>LGL</th>
<th>HSTL</th>
<th>P-TLL</th>
<th>ALK-neg ALCL</th>
<th>NKTCL</th>
<th>ATLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-stimulatory/TCR signaling elements</td>
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<tr>
<td>CD28</td>
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<td>-</td>
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<td>-</td>
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<td>60-70%</td>
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*AITL: angioimmunoblastic T-cell lymphoma; MF/SS: mycosis fungoides, SS; Sézary syndrome; PTCL-NOS: peripheral T-cell lymphoma not otherwise specified; LGL: large granular lymphocytic leukemia; HSTL: hepatosplenic T-cell lymphoma; TPLL: T-cell prolymphocytic leukemia; ATLL: adult T-cell leukemia/lymphoma; ALKneg ALCL: ALK-negative anaplastic large cell lymphoma; NKTCL: extranodal NK/T-cell lymphoma, nasal-type. **STAT5B mutations were also reported in 36% of EATL type 2 (Kückelkorn et al Nat Commun 2015).30 Mutations in TET2, RHOA and DNMT3A also reported in the subgroup of PTCL-NOS with Tfh phenotype.38% of systemic ALKneg ALCL patients were reported to carry both JAK1 and STAT5B mutations (Crescenzo et al Cancer Cell 2015).30 Whereas RHOA mutations in AITL almost invariably involve the hotspot G17V, RHOA mutations in ATLL are more widely distributed (Katohoka et al Nat Genet, 2015).30 Nagata et al Blood 2016).30
(TCR) signaling, whereas mutations of members of the JAK-STAT signaling pathway (STAT3, STAT5β) are shared by several cytotoxic T-cell or NK-cell lymphomas, such as T-LGL, nasal NK/T-cell lymphomas, hepatosplenic T-cell lymphoma, enteropathy-associated T-cell lymphoma (type 2) and ALK-negative anaplastic large cell lymphomas, respectively. Nonetheless, caution is required given the retrospective nature of the published studies and the resistance to the anti-CD20 monoclonal antibodies rituximab and alemtuzumab.

Subgroups of patients within a lymphoma entity may also display differential mutation profiles, initially shown for the GCB vs. ABC subtypes of DLBCL (Table 1) and, more recently, also demonstrated in other lymphomas e.g. HCL, where cases expressing the IGHV4-34 gene in the clonotypic B-cell receptor lack the canonical BRAFV600E mutation and instead display enrichment for MAP2K1 mutations with an overall similar profile to the HCL-variant. Similarly, a subgroup of SMZLs with IGHV1-2 expressing B-cell receptor commonly harbors inactivating KLF2 mutations and 7q deletions. Potentially, this might aid future diagnostics aiming to distinguish these subtypes, however the utility and applicability of this approach have to be studied further.

In summary, presently, MYD88, BRAF, ID3, TCF3, STAT3, STAT5β, RHOA, TET2, and IDH2 mutations are the only genes that can be considered as a complement to the current set-up for lymphoma diagnostics. However, the list is rapidly expanding as evidenced by the case of RRA GC mutations, for example.

### III. Genes with prognostic potential

Several gene mutations have been associated with clinical outcome in various lymphoma subtypes. In CLL, besides TP53 and ATM mutations, which are both known to confer poor prognosis, recent high-throughput NGS studies have revealed recurrent mutations within NOTCH1, SF3B1, and BIRC3 for example, that were reported to be associated with poor clinical outcome with higher frequencies in relapsing/treatment-refractory CLL and in Richter’s syndrome. More recent studies have also identified additional gene mutations that may confer a worse outcome in CLL, e.g. NFKBIE, EGR2, and PTPN11, although they have been studied less. In a recent multicenter study conducted within ERIC, sequencing of TP53, NOTCH1, SF3B1, BIRC3 and MYD88 was performed in a large patient series (totaling 3,490 patients), revealing that TP53 and SF3B1 mutations, but not NOTCH1 mutations, remained as independent prognostic markers of shorter time to first treatment in multivariate analysis, even amongst patients expressing unmaturated IGHV genes. A few published clinical trials have also pointed to a prognostic and even predictive role of SF3B1 and NOTCH1 mutations in CLL, where, in particular, the latter confers resistance to the anti-CD20 monoclonal antibodies rituximab and ofatumumab, however, this needs further exploration and validation. Currently, as a new ERIC project, a dedicated NGS-based gene panel (including 11 genes) has been designed with the purpose to test different targeted enrichment techniques and evaluate intercenter variability and reproducibility.

Similar gene panel-based efforts have also been performed for SMZL, revealing a high frequency of TP53, KLF2, NOTCH2, TNP1AIP3, and MYD88 mutations, and demonstrating NOTCH2 and TP53 mutations as independent markers of short treatment-free and overall survival, respectively. Nonetheless, caution is required given the retrospective nature of the published studies and the overall rarity of SMZL raising concerns about potential selection biases.

MCL is characterized by a relatively high number of recurrent secondary genomic aberrations, but few of them have demonstrated additional prognostic value. From recent NGS studies, the prognostic value of NOTCH1/NOTCH2 mutations was recently highlighted in addition to TP53 defects.

In DLBCL, the prognostic impact of MYC translocations critically depends on the second hit, with cases harboring TP53 mutation and MYC translocation showing the worst overall survival, followed by those cases carrying MYC and BCL2 translocation. Indeed, DLBCL with TP53 mutation and MYC translocation is a newly recognized subset of ‘double-hit’ lymphoma, accounting for one-third of MYC translocation positive DLBCL. Hence, it is pivotal to perform TP53 mutation screening, in addition to the detection of BCL2 translocation in MYC translocation positive DLBCL, in order to distinguish the double-hit DLBCL from those with an isolated MYC translocation.

Among PTCL, TET2 mutations inAITL patients were reported to associate with a more aggressive clinical presentation and a shorter progression-free survival, whereas in a recent study of NK-TCL DDX3X mutations conferred a particularly poor prognosis.

Considering the significant differences in mutation frequencies observed between different studies, best exemplified by DLBCL, large-scale efforts are now needed, in particular for rarer entities, to fully understand which genes are the most relevant and will retain independent prognostic impact in relation to other known clinical/molecular markers. This is particularly relevant in light of recent studies pointing to a prognostic relevance of particular combinations of mutations and/or an increasing

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene mutations</th>
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<tbody>
<tr>
<td>1. Immediate impact on patient care</td>
<td>TP53 mutations (exons 4-10) in CLL</td>
</tr>
<tr>
<td>2. Diagnostic impact</td>
<td>MYD88&lt;sup&gt;ww&lt;/sup&gt; mutation in WM/LPL, BRAF&lt;sup&gt;V600E&lt;/sup&gt; mutation in HCL, KLF2 mutations in SMZL, ID3 and TCF3 mutations in BL, STAT3 mutations in LOLL, RHOA, TET2, IDH2 and DNMT3A mutations in ATL and other T&lt;sub&gt;α&lt;/sub&gt; derived PTCL</td>
</tr>
<tr>
<td>3. Prognostic impact</td>
<td>CLL: TP53, ATM, BIRC2, NFKBIE, NOTCH1, SF3B1, MCL: TP53, NOTCH1, NOTCH2 mutations, SMZL: NOTCH2, TP53 mutations, DLBCL: TP53 mutation &amp; MYC translocation, NKTL: DDX3X mutations</td>
</tr>
</tbody>
</table>

*Based on references listed in Table 1. CLL: chronic lymphocytic leukemia; WM: Waldenström’s Macroglobulinemia; LP: lymphoplasmacytic lymphoma; HCL: hairy-cell leukemia; BL: Burkitt lymphoma; AITL: angioimmunoblastic T-cell lymphoma; MCL: mantle cell lymphoma; SMZL: splenic marginal zone lymphoma; DLBCL diffuse large B-cell lymphoma; NKTL: NK T-cell lymphoma.*
number of ‘driver mutations’. In addition, the time point
of mutation screening may also differ depending on the
disease entity and response to therapy.

IV. Genes with potential clinical impact
in the near future

Recent studies in different lymphoma subtypes have
highlighted relevant associations between certain recurrent
gene mutations and response to treatment. The most striking
example is perhaps offered by MYD88 and CXCR4
mutations, which were shown to affect responses to ibrutinib
in WM. Indeed, patients with MYD88<sup>ΔΔ</sup>CXCR4<sup>WT</sup> (with WT indicating wild-type) status had 100% overall
response rate (91% major response rate) as opposed to 86%
(62%) and 71% (29%) for MYD88<sup>ΔΔ</sup>CXCR4<sup>ΔΔ</sup> and
MYD88<sup>ΔΔ</sup>CXCR4<sup>ΔΔ</sup> patients, respectively.<sup>88</sup> Although these results were obtained from a small cohort of patients
with WM, they offer a tantalizing glimpse into the future of lymphoma treatment with novel companion diagnostics tai-
lored to novel therapeutic agents.

From recent studies, we have also learnt that mutations
within the BTK (C481S) and PLCG2 genes may emerge in
CLL patients relapsing after and/or refractory to ibrutinib
treatment, and we foresee that the assessment of these
genes may soon be incorporated in the diagnostic set-up.<sup>89</sup>

Preliminary results also indicate that the divergent responses
of patients with MCL or DLBCL to ibrutinib may be linked to
distinct profiles of genomic aberrations, e.g. BIRC3
mutations in MCL<sup>91</sup> and isolated MYD88 mutation in
DLBCL<sup>92</sup> as well as mutations downstream of BTK, such as
activating mutations of CARD11 in DLBCL<sup>93,94</sup> may make
these tumors resistant to BTK inhibition.

Recent reports of genes linked to particular physiological
processes have revealed new types of mechanisms that
may be suitable for targeted therapy. For mutant BRAF, the
inhibitor vemurafenib is already in clinical trials in
relapsed/refractory HCL, demonstrating high activity even
among heavily pre-treated patients.<sup>95</sup> New types of promising inhibitors have also been developed for EZH2 (a histone methyl transferase),<sup>96</sup> SF3B1 (a splicing factor),<sup>97</sup>
NOTCH1<sup>98</sup> and IDH2,<sup>99</sup> and in some cases have already entered early phase clinical trials. The high frequency of
TET2 and/or DNMT3A mutations in AITL and other T<sub>RI</sub>
derived PTCL may also support the rationale to use
demethylating agents as an alternative way to treat
patients, supported by the results of a recent single report.<sup>100</sup>

More generally, targeting certain epigenetic abnormalities
or alterations in pathways frequently involved in patients
with PTCL (TCR, JAK-STAT, NF-κB; Table 2) represents an
attractive approach, also taking into consideration the over-
all poor outcome of the majority of such patients with con-
ventional chemotherapy-based approaches.

In conclusion, we expect that the list of potential thera-
peutic targets will expand quickly in coming years, once
markers have been functionally validated and new
compounds have been discovered.

V. Genes for research purposes only

Finally, for most recurrently mutated genes identified
thus far, we do not yet understand their functional role
and/or their clinical association, either alone or in the pres-
ence of cooperating events. These genes might still be of
interest, however, before large-scale studies are per-
dorm ed, it will not be possible to discern their potential
contribution to disease pathobiology. Having said that,
and similar to category IV above, this subgroup of genes
will be a ‘moving target’ depending on the evidence level
ascertained for a particular mutation in the coming years,
and genes may be taken out if a certain type of mutation
is judged to have minor impact for a specific lymphoma
entity.

How to move forward in the diagnostic field
using NGS?

As mentioned, targeted NGS allows us to select and test
many genes and samples simultaneously, and this approach
has now been validated for CLL, SMZL and DLBCL,
amongst others. In addition, targeted NGS permits a high
sequence read depth, an important factor for studying
minor subclones and thus clonal heterogeneity, and can also
be adapted to formalin-fixed, paraffin-embedded (FFPE)
tumor material, which in reality is the source of material for
most diagnostic lymphoma specimens.

To gain further insight into NGS-based diagnostics in
lymphoma, the EGNL Group has participated in the design
of a lymphoma gene panel, compatible with FFPE material,
that includes 30 recurrently mutated genes. Our ambition
now is to perform a multi-center validation study using a
defined number of matched FFPE/fresh-frozen lymphoma
specimens to set the technical requirement for NGS-based
diagnostics (including metrics such as sequence coverage,
specificity, sensitivity and reproducibility of the technique).
Once validated, large-scale international collaborative
efforts can be conducted for each lymphoma entity, in par-
ticular within ongoing or planned treatment studies, to fully
understand how to adapt and implement NGS-based diag-
nostics in day-to-day routine diagnostics.

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