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Review

Is It Time to Assess T Cell Clonality by Next-Generation Sequencing in Mature T Cell Lymphoid Neoplasms? A Scoping Review

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Abstract: Background: T cell clonality is commonly assessed in the diagnostic work-up of mature T cell lymphoid neoplasms. Although fragment-length polymerase chain reaction (FL-PCR) assays are most widely used, next-generation sequencing (NGS) of the TRG and TRB genes is increasingly being used to assess T cell clonality. **Objective:** The present work is a scoping review of studies that assessed T cell clonality by NGS for diagnostic purposes, including only studies that provided integrated clinicopathologic diagnoses in comparing FL-PCR and NGS assays to evaluate if it is preferable to use NGS-based assays for T cell clonality evaluation in diagnostic pathology. **Methods:** Papers published from 1992 to 3 August 2024 were searched in PubMed. Twenty-nine cohort studies and five instructive case reports, published from 2013–2024 from the USA, UK, Europe, and Australia that provided integrated clinicopathologic diagnoses and used NGS to evaluate T cell clonality in clinical specimens from patients with mature T cell neoplasms and related non-neoplastic and neoplastic diseases were included, with additional relevant studies. **Results:** Ten (34.4%) of the 29 cohorts included clinical samples from patients having various cutaneous and non-cutaneous T cell malignancies, related neoplasms, and reactive conditions; 2 (6.8%) studies focused on T cell prolymphocytic leukemia, 16 (55%) on cutaneous T cell lymphoma, and one on pediatric pityriasis lichenoides. Eleven (38%) of the 29 cohort studies compared NGS with FL-PCR assays in 908 clinical samples. Eight (72.7%) of the 11 studies compared TRG FL-PCR with TRG NGS (n = 5), TRB NGS (n = 2), and TRG NGS and TRB NGS (n = 1); the remaining three compared EuroClonality/BIOMED-2 FL-PCR (TRG and TRB) with TRG NGS (n = 1), TRB NGS (n = 1), and the EuroClonality-NGS DNA capture assay (n = 1). TRB NGS was used in 16 (55%) of 29, TRG NGS in 6 (20.6%) of 29, and both TRG and TRB NGS in 7 (24%) of 29. Two (6.8%) of the 29 studies compared TRB NGS with flow cytometric immunophenotyping assays for Vβ and T cell receptor β constant region 1. One additional study compared long-read sequencing with NGS for TRG and TRB rearrangements. **Conclusions:** NGS is highly specific and sensitive for assessing T cell clonality. NGS precisely tracks unique rearranged sequences, which FL-PCR cannot. NGS findings for clonality must be interpreted in the context of all clinicopathologic and immunophenotypic findings, like FL-PCR. With such interpretations, NGS is much preferable to FL-PCR for evaluating T cell clonality for diagnostic purposes. It is necessary to reduce costs, increase accessibility, and educate providers about NGS for clonality evaluation. TRB NGS has been primarily assessed in the peripheral blood and skin, whereas TRG NGS has also been evaluated in formalin-fixed and non-cutaneous fresh lymphoid tissues. TRG NGS performed better than TRB NGS in comparative studies.

Keywords: antigen receptors; T cells; gene rearrangement; high-throughput DNA sequencing; polymerase chain reaction; T cell lymphoma; T cell prolymphocytic leukemia; cutaneous T cell lymphoma; anaplastic large-cell lymphoma; Hodgkin's lymphoma; residual neoplasm

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1. Introduction

Lymphoid neoplasms are composed of clonal populations of lymphoid cells. These neoplasms include Hodgkin's lymphomas, non-Hodgkin's lymphomas, and lymphoproliferative disorders. Non-Hodgkin's lymphomas include precursor B or T lymphoblastic leukemias/lymphomas or may arise from mature B or T lymphoid cells, termed mature B cell or mature T cell neoplasms, respectively, as recently reviewed [1–3]. The pathologic evaluation of hematolymphoid clinical specimens often requires an assessment of lymphoid cell clonality to distinguish between benign and neoplastic proliferations in conjunction with other pathologic and clinical features. Unique rearranged antigen receptor genes create enormous diversity in normal mature B and T lymphocytes [4,5], forming the molecular basis for identifying clonal B and T lymphoid cell populations. Gene rearrangements occur sequentially during lymphoid cell development [6,7]. During T cell development, gene rearrangements occur in a highly ordered manner as follows: first, T cell receptor δ (TRD), followed by T cell receptor γ (TRG) rearrangement of its variable (V) and joining (J) region genes, then T cell receptor β (TRB) with the TRB diversity (D) and J region genes rearranged before TRB V-(D)-J rearrangement, and finally, T cell receptor α (TRA) [7,8]. The nomenclature for all human immunoglobulin and T cell receptor genes was approved by the Human Genome Organization (HUGO) Nomenclature Committee (HGNC) [9] in 1999 [10–12]. In contrast with other genes, the immunoglobulin and T cell receptor gene names are not italicized [13]. Both B and T cell clonality have been assessed using Southern blot hybridization since the 1980s and fragment-length polymerase chain reaction (FL-PCR) assays since the 1990s, with guidelines established by the European BIOMED-2 consortium for detecting lymphoid cell clonality by FL-PCR assays since the 1990s [8,14–18]. In the USA, TRG FL-PCR has been preferred for evaluating T cell clonality by FL-PCR, as described earlier [19] (p. 194). TRG is consistently rearranged before TRB and is not deleted at a later T cell developmental stage; TRG also contains a limited germline repertoire of functional V and J gene segments [8], facilitating clonality analysis by FL-PCR assays, as previously described [19].

The diagnosis of mature T cell lymphoid neoplasms can be challenging. In 2001, it was reported that experienced hematopathologists benefited from using molecular clonality assessment to accurately diagnose lymphoid neoplasms in 5–10% of cases [20]; this percentage of cases increased to 10–15% in 2014 [21]. Our understanding of lymphoid neoplasm biology and molecular pathogenesis has advanced exponentially since then. These advances have led to several updates in the classification of hematolymphoid neoplasms since 2001; these updates, including the fifth edition of the World Health Organization (WHO) classification (WHO-HEME5) [22,23] and the International Consensus classification (ICC) [24,25] from 2022 have been described in recent reviews [1–3,26]. More than 100 types of lymphoid neoplasms are described in the WHO-HEME5 [22] and the ICC [24,25]. More than 30 types of mature T cell neoplasms are currently recognized by the WHO (reviewed in [1,3,22,26–28], with terminology differences in WHO-HEME5 and ICC described in [26,28]). Mature T cell neoplasms may present clinically as leukemias involving the blood and bone marrow, cutaneous lymphomas, lymphoproliferative disorders affecting the skin, lymphomas involving lymph nodes (termed nodal lymphomas), and lymphomas or lymphoproliferative disorders involving extracutaneous extranodal sites, including the gastrointestinal tract, liver, spleen, and breast implant-associated tissues. Rarely are the central nervous system and retina involved [29].

Mature (or peripheral) T cell lymphomas are aggressive lymphomas [30]. The most frequent types were peripheral T cell lymphoma (PTCL), not otherwise specified (PTCL-NOS), angioimmunoblastic T cell lymphoma (AITL), and anaplastic large-cell lymphoma (ALCL) {anaplastic lymphoma kinase (ALK)-positive (ALCL, ALK+) and ALCL, ALK-

negative (ALCL, ALK-negative)}, representing 25.9%, 18.5%, and 12.1% (6.6% for ALK+; 5.5% for ALK-negative ALCL), respectively, of all mature T cell neoplasms in a large international cohort [31]. Primary cutaneous lymphomas comprise about 75% of cutaneous T cell lymphomas (CTCL), with mycosis fungoides representing the most frequent type of CTCL (reviewed in [32,33]). Of note, the presence of clonal T cell rearrangements is essential for diagnosing all T cell neoplasms, according to the fifth edition of the WHO classification of hematolymphoid tumors [28] (p. 4) and skin tumors (reviewed in [34]).

Accurate diagnosis and precise subclassification of all lymphoid neoplasms requires integrating histopathologic, immunophenotypic, and genetic information with clinical features, and there is increasing emphasis on genetics in both diagnostic classifications in 2022. In addition to accurate diagnosis, precisely detecting the presence or absence of residual disease and determining whether two different neoplasms in the same patient harbor the exact neoplastic clone or represent a distinct, new tumor is essential for patient management. For example, a cutaneous lymphoma could be a primary tumor arising in the skin, or it may occur secondary to systemic involvement by lymphoma in the body, and the distinction is vital for treatment. Consequently, diagnostic hematopathology increasingly requires tools that allow precise distinction between different diagnoses with objective results that could be standardized between laboratories.

It is critical to note that identifying the presence of a T cell clone in a clinical specimen alone does not indicate the presence of a neoplasm. The presence of a clone in a clinical specimen does not equate with malignancy; conversely, a clone's absence does not rule out a neoplasm. Nevertheless, most T cell neoplasms harbor a T cell clone, and identifying a neoplastic T cell clone by molecular clonality assays can provide a definitive diagnosis of a T cell neoplasm if these findings are present in combination with the appropriate clinical, histopathologic, immunophenotypic, and other molecular genetic features. Lymphoid clonality assessment would also be helpful if molecular somatic mutation analysis is unavailable for evaluating nodal T cell lymphomas and their differential diagnostic entities, which may be the situation even in 2024 for many diagnostic laboratories worldwide. Moreover, somatic gene mutation analysis, even when available, may not even help in diagnosing a mature T cell neoplasm, making T cell clonality evaluation an essential tool for the primary diagnosis of a T cell lymphoma. In the most extensive study thus far, wherein expert hematopathologists used targeted NGS panels to detect somatic mutations to diagnose mature T cell lymphomas, T cell clonality assessment helped to diagnose those cases where the mutational analysis was non-contributory to diagnosis. Further, the authors noted the need for more specificity in the standard means of evaluating T cell clonality in diagnostic evaluation, with T cell clones commonly detected in benign, reactive conditions [35].

It is also important to note that the lineage determination of a lymphoid neoplasm is not recommended based only on the presence of a T cell or a B cell clone since cross-lineage gene rearrangements may occur in lymphoid neoplasms. T cell receptor gene rearrangements have been shown in 90% of precursor B cell ALL [36] and 27% of various types of mature B cell malignancies, ranging from 13% to 50%, depending upon the type of B cell neoplasm [15]. Conversely, 10% of mature T cell malignancies showed immunoglobulin gene rearrangements by BIOMED-2 FL-PCR, most frequently in AITL (32%) and PTCL-NOS (9%); ALK+ ALCL and ALK-negative ALCL cases did not show immunoglobulin gene rearrangements [16].

FL-PCR assays are most commonly used to assess the clonal nature of lymphoid cell populations in clinical laboratories. Pitfalls in detecting T cell clonality by FL-PCR-based assays have been emphasized by BIOMED-2 experts [37]. In the last decade, targeted next-generation sequencing (NGS) or high-throughput screening or immune sequencing of

the rearranged antigen receptor genes in T cells and B cells has emerged as an option for detecting clonal populations in hematolymphoid tissues. Clonal lymphoid populations by FL-PCR assays are determined only by nucleotide size, irrespective of the actual rearranged gene sequences [19]. In contrast, NGS for the rearranged antigen receptor gene specifically identifies the dominant rearranged sequences [19]. Due to quantitative data output, NGS clonality assays can detect small clonal populations even in a polyclonal population of T cells, which is precluded by FL-PCR assays designed with consensus primers to the variable (V) and joining (J) region gene segments [19].

The EuroClonality NGS consortium was formed in Europe to develop, standardize, and validate assays and workflows for clonality assessment, measurable disease detection (MRD) evaluation, and repertoire analysis [38]. Their publications have described their group's development of NGS assays for clonality and MRD evaluation in lymphoid neoplasms [39,40], including quality control targets [41], which are integrated with their bioinformatics platform [42]. They described a multi-center validation of their NGS assays for immunoglobulin gene rearrangements [43], the methods for their NGS assays for immunoglobin heavy chain (IGH) and κ light chain rearrangements [44], and the evaluation of their B cell clonality assays by NGS [45]. Their methods for T cell clonality evaluation by NGS were described in a recent publication wherein they applied their NGS assays for B cell and T cell clonality in a cohort of patients with paired clinical specimens of diagnostic and recurrent classic Hodgkin's lymphoma (CHL) and showed that 29% (n = 10 of 34) of the recurrences were unrelated to the original neoplastic clone [46]; also, T cell lymphomas mimicking CHL were identified at diagnosis and relapse of the earlier diagnosis of CHL [46].

Furthermore, the nature of recurrent B cell lymphomas as clonally related or unrelated was precisely identified in two studies based on NGS-based clonality assays [47,48]. These studies indicate the immense capability afforded to pathologists by integrating findings from NGS assays for clonality with other clinical, pathologic, and mutational analysis findings to render a precise diagnosis, which is critical for appropriate therapies in the era of precision medicine. There are published reports of validation by clinical laboratories that have used commercially available NGS assays to assess T cell clonality in hematolymphoid specimens in the USA [49,50] and Belgium [51] and B cell clonality and MRD assessment in mature B cell and plasma cell neoplasms in the USA [52,53] and in B cell and T cell acute lymphoblastic leukemias in Australia [54].

In the above-described background in the current era of precision medicine, the substantially increased cost of NGS assays is the most cited reason for the continued reliance on FL-PCR assays to evaluate T cell clonality in community medical centers, where most patients are diagnosed and treated. The primary objective of this study was to review the published literature to determine the clinical usefulness of NGS-based T cell clonality assays in evaluating mature lymphoid neoplasms in clinical settings to determine if NGS should be used instead of FL-PCR assays for assessing T cell clonality in the diagnostic evaluation of mature lymphoid proliferations in hematopathology.

2. Materials and Methods

This scoping review was performed based on the preferred reporting items for systematic reviews and meta-analyses (PRISMA) extension for scoping reviews in the cited reference [55]. This review's registration citation in the Open Science Framework public registry website (https://osf.io/dashboard) is provided in the PRISMA checklist (based on [55]), which is submitted as a non-published materials.

A systematic search of the literature was performed on August 3, 2024, using the following search strategy in the PubMed database from 1992 to 2024: ((‘T cell clonality’)

OR ('T-cell clonality') OR ('T-cell clonality by PCR') OR ('T-cell clonality by NGS') OR ('T cell clonality by PCR') OR ('T cell clonality by NGS')) AND (('measurable residual disease') OR ('minimal residual disease') OR ('residual disease') OR ('MRD') OR ('circulating tumor DNA') OR ('ctDNA') OR ('cell-free DNA') OR ('cell free DNA') OR ('cfDNA') OR ('clonality') OR ('lymphoma') OR ('lymphoproliferative disorder')). This search provided 1372 results, which the author reviewed manually to delete publications in non-English language and non-human studies to obtain a list of 1163 publications. The author manually reviewed the titles and abstracts of these 1163 publications to select the following for review: (1) all publications that evaluated mature lymphoid neoplasms and related hematologic disorders and included an assessment of T cell clonality; (2) case reports or cohort studies where the diagnoses were provided for the cases or the patients in whom NGS-based assays were used to assess T cell clonality; (3) case reports with a precise diagnosis in which T cell clonality had been assessed only by FL-PCR assays and the study was instructive for evaluating clonality; and (4) comparative studies between NGS-based T cell clonality assays and flow cytometry assays for evaluating T cell clonality. This step of reviewing 1163 publications was performed in stages and included a full review of many articles that were subsequently excluded or included to narrow the list to 110 publications. Additional manual searches were conducted after reviewing the lists of cited references in reviewed articles to identify additional related publications for review. A PubMed search performed again from 2004 to 23 November 2024, using the same search strategy described above, showed 831 results. These titles and abstracts, and if needed, the articles, were reviewed with a focus on publications from 2010 onwards to ensure that all publications that met the inclusion criteria were included in this review.

Inclusion criteria: The 110 publications described above and additional manually retrieved publications were reviewed to select only those publications that provided a diagnosis based on integrated clinical and pathologic features and analyzed T cell clonality by NGS in those cases for cohort studies and by NGS or FL-PCR for instructive case reports. These final publications were re-reviewed to extract the information presented in the results.

Exclusion criteria: (1) Publications that examined T cell clonality by NGS without a diagnosis provided based on integrated clinical and pathologic criteria for the samples studied were excluded because classifying clinical samples based on whether they are polyclonal or monoclonal based on FL-PCR assays does not provide information to the reader regarding the nature of the disease as neoplastic or non-neoplastic. (2) Publications focused on precursor B or T lymphoblastic leukemia/lymphoma for samples analyzed by NGS for T cell clonality were also excluded. This exclusion was because of two reasons: (i) At diagnosis of acute lymphoblastic leukemia, the neoplastic nature of the presenting disease and the lineage of the neoplastic cells are currently typically determined by flow cytometric immunophenotyping (FCI) and not by molecular assays for T or B cell clonality (reviewed in [2]), and (ii) to review all publications related to MRD evaluation in acute lymphoblastic leukemia would require a separately focused review. (3) Publications evaluating T cell clonality by NGS in non-hematolymphoid cancers were excluded. (4) Publications evaluating T cell clonality in tissue biopsies in non-hematologic diseases were excluded. (5) As stated above, all non-human studies and all studies in languages other than English were excluded.

Figure 1 shows the PRISMA flow diagram for this new systematic review, which included searches of a database and other sources. This flow diagram is based on the cited publication from 2020 [56].

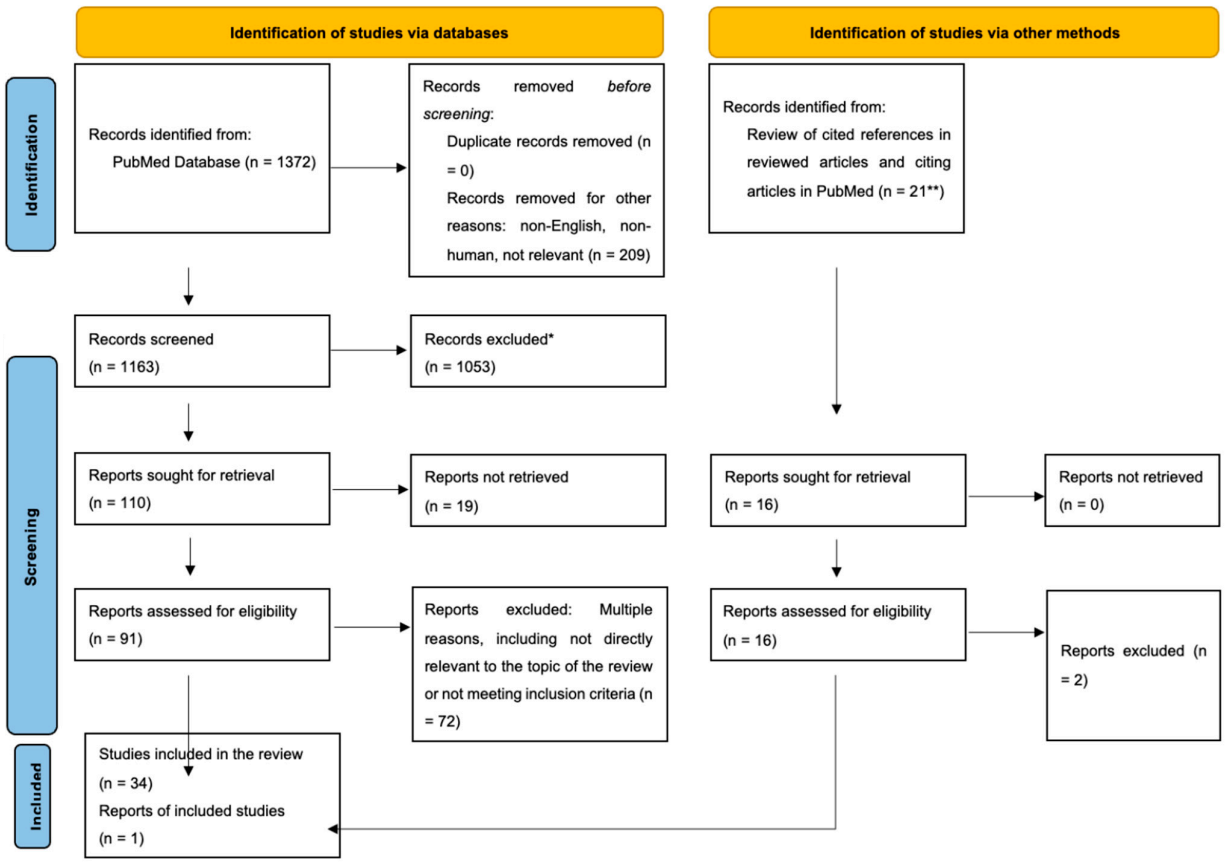

Figure 1. PRISMA 2020 flow diagram for this new systematic review, including the searches of the PubMed database and other sources. Source of the PRISMA flow diagram [56]: Page MJ, et al. *BMJ* 2021;372:n71. doi: 10.1136/bmj.n71. * All records were reviewed by the author manually. No automation tools were used in this review. ** This number is given as a conservative (lowest) estimate.

3. Results

Thirty-four publications met the inclusion criteria described in Section 2, including 29 cohort studies published between 2013 and 2024 and five instructive case reports published between 2016 and 2022. These are described in Section 3.1. Section 3.2 summarizes selected additional publications relevant to assessing T cell clonality by NGS.

3.1. Review of Publications for Evaluating T Cell Clonality in Patients and Clinical Specimens with Mature Lymphoid Neoplasms and Related Diagnoses by Next-Generation Sequencing

3.1.1. T Cell Receptor Clonality Assessment by Next-Generation Sequencing of the Rearranged DNA of the T Cell Receptor Genes in Patients with Mature T Cell Neoplasms and Related Pathologic Diagnoses

In this section, the 29 included cohort studies are summarized in chronological order in Table 1. Table 1 shows the features extracted from those 29 original research publications, including the diagnoses based on integrating clinical and pathologic features, the study purpose, numbers of patients included and their demographics, if available, specimens examined, non-neoplastic specimens if included, targets analyzed by T cell receptor NGS, the specific NGS assays used for T cell clonality, assays compared with T cell receptor NGS, targets for FL-PCR assays and FCI assays for T cell clonality, and relevant results related to T cell clonality evaluated by NGS, including if compared with FL-PCR assays or FCI. Table 2 summarizes the gene targets and target enrichment methods of the commercially available T cell receptor NGS assays used in the studies reviewed. Table 3 focuses on 11 of these 29 cohort studies that compared FL-PCR with NGS assays, including the number and types of samples compared across the 11 studies.

Table 1. Cohort studies (n = 29) that examined T cell clonality by next-generation sequencing of rearranged T cell receptor genes for T cell clonality.

Diagnoses of Studied T Cell Neoplasms	Study Purpose	Patients Studied	Clinical Samples Studied	NGS Assays	FL-PCR and FCI Assays, if Used	Relevant Results	References; Country of Study
Advanced stage mycosis fungoides with Sézary syndrome [a]	To detect MRD by NGS after non-myeloablative allogeneic HSCT in patients with MF and SS	n = 10 (ages >18 <75 y) enrolled in a prospective clinical trial ClinicalTrials.gov #NCT00896493	PB and skin samples collected before and after HSCT as part of the clinical trial; DNA extracted from PB mononuclear cells	TRB V(D)J NGS [57]; Adaptive Biotechnologies, Seattle, USA	FCI for identifying Sézary cells [b]	TRB NGS detected clonal CDR3 sequences in all 10 (100%) pre-HSCT {PB (n = 8) and skin (n = 2)}, [c] including in 6 (60%) patients with no Sézary cells identified by FCI (0.03% to 0.58% of all TRB CDR3 sequences); the percentages of neoplastic clones decreased in all patients immediately post-HSCT	Weng et al., 2013 [58]; USA
Mycosis fungoides	FL-PCR vs. NGS for clonality evaluation	n = 34 14 males, 20 females; ages 37–76 y	Archived DNA samples from skin biopsies with histologic features of MF; no non-neoplastic cases studied	TRG NGS laboratory-developed test	TRG FL-PCR performed previously with 15 clonal and 19 poly-clonal/oligoclonal cases by FL-PCR	29 (85%) clonal by NGS but only 15 (55%) clonal by FL-PCR; 2 (6%) clonal by FL-PCR but nonclonal by NGS (2.4% and 2.5% sequences by NGS); clonal peaks by FL-PCR if 2-fold signal intensity above Gaussian background	Sufficool et al., 2015 [59]; USA
CTCL (as defined by [60]), benign inflammatory skin diseases, and healthy donors	To detect T cell clones in early CTCL and distinguish from non-CTCL diseases	n = 110; 104 patients with CTCL (n = 46), and non-CTCL (n = 58) [d] and 6 healthy donors	DNA from punch biopsies of skin: lesional from CTCL and non-CTCL and from healthy donors	TRB V(D)J NGS and TRG NGS [61]; Adaptive Biotechnologies, Seattle, USA	TRG FL-PCR	NGS showed clones in all 46 (100%) CTCL and had greater sensitivity and specificify than TRG PCR, which was clonal in 27(70)%/39; TRB and TRG NGS were mostly concordant; [e] a single case of γδ T cell CTCL with a clear clone by TRG NGS was nonclonal by TRB NGS; the most frequent clones by TRB and TRG NGS expressed as a fraction of all nucleated cells in skin [f] successfully distinguished CTCL from benign non-CTCL and healthy skin	Kirsch et al., 2015 [62]; USA
T cell prolymphocytic leuekmia (T-PLL)	To detect MRD by NGS as an alternative to detecting by RQ-PCR after allogeneic HSCT	n = 10; 8 males; 2 females; median age at transplant 59 (range 43–72) y	104 samples: PB (n = 91), BM (n = 10) and donor PB (n = 3)	TRB V(D)J NGS; EuroClonality–NGS amplicon-based methods	Clone-specific real-time quantitative (RQ) PCR of clonal TRB, TRG, or both rearrangements [63]	3 patients with the longest follow-up were examined by NGS. In all 3, the diagnostic sample showed 1 or 2 leukemic clonotypes by NGS; MRD evaluation of the leukemic clonotypes by NGS closely followed the kinetics of RQ-PCR-quantified MRD	Sellner et al., 2017 [64]; Germany
CTCL (primarily MF/SS)	To study CTCL with emphasis on early stage CTCL by NGS	N = 309; included 210 (68%) early-stage CTCL, 54 (17%) stage IIB-III MF, 40 (13%) SS and pre-SS	309 lesional skin samples	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	None	The following Vβ families were most frequently used: V20 (13%), V07 (12%), V21 (11%), V06 (9%), V05 (9%), V03 (9%), V10 (5%), V28 (4%), V19 (4%)	de Masson et al., 2018 [65]; USA

Table 1. *Cont.*

Diagnoses of Studied T Cell Neoplasms	Study Purpose	Patients Studied	Clinical Samples Studied	NGS Assays	FL-PCR and FCI Assays, if Used	Relevant Results	References; Country of Study
Benign with no history of any LPD; mature T cell neoplasms (MF/SS, cutaneous CD30+ LPD; T-LGLL, ALK-ALCL, PTCL-NOS); mature B cell neoplasms and immune-dysregulation LPDs (DLBCL, EBV+ DLBCL, PT-LPD, EBV+ MCU); and atypical LPDs	TRG FL-PCR vs. TRG NGS for clonality evaluation	N = 41; 22 females and 19 males; median age 59 y (range 9–87) with integrated clinicopathologic diagnoses; 1 MF with SS, 1 MF, 1 cutaneous CD30+ LPD; 2 T-LGLL, 3 ALK-ALCL, 1 PTCL-NOS); mature B cell neoplasms and immune-dysregulation LPDs (3 DLBCL, 1 EBV+ DLBCL, 2 post-transplant LPD, 1 EBV+ MCU); and 4 atypical LPDs	41 samples (15 PB, 8 BM aspirates, 18 FFPE tissues): 21 benign with no LPD {10 PB, 6 BM, rule out clone: cytopenias (n = 12), other (n = 4), 5 benign lymphoid FFPE tissues}; 9 T cell neoplasms; 7 B cell neoplasms and immune dysregulation-associated LPDs; 4 atypical LPDs (3 EBV+, one suspicious for primary cutaneous CD4+ small/medium T- cell LPD)	TRG NGS; LymphotrackTM, Invivoscribe Inc., San Diego, USA	Two TRG FL-PCR assays (1-tube [66] ᵍ and 2-tube) (Invivoscibe Inc., San Diego, USA)	TRG NGS provided an accurate assessment of all polyclonal, oligoclonal, and clonal T cell populations in 100% of cases diagnosed by integrated clincopathologic features. *False positive FL-PCR*: 3 (30%)/10 benign PB; 1 (16.6%)/6 benign BM aspirate cases; 1 (25%)/4 atypical LPD cases. *False negative FL-PCR*: 3 (30%)/10 benign PB cases; 3 (60%)/5 PB cases in patients with LPDs (1 MF, 1 PT-LPD, 1 EBV+ MCU); 1 (25%) (1 ALK-negative ALCL) in 4 mature T cell lymphomas; 1 PT-LPD in FFPE tissue (~3.5% clone by NGS); and 1 primary cutaneous CD4+ small/medium T cell LPD (25%) of 4 atypical LPDs	Kansal et al., 2018 [19]; USA
Clinical concern for a diagnosis of CTCL; cases in four histologic diagnoses	FL-PCR vs. NGS for clonality evaluation	N = 100 (N = 25 in each of the four histologic categories: 'definitive CTCL', 'atypical lymphoid infiltrate, concerning for CTCL', 'atypical lymphoid infiltrate, favor reactive' and 'reactive lymphoid infiltrate')	100 skin biopsies and concurrent PB samples examined by FL-PCR and NGS; most PB samples also analyzed by FCI	TRB V(D)J NGS; ImmunoSEQ, Adaptive Biotechnologies, Seattle, USA	TRG FL-PCR; FCI for PB samples	In skin, 100% diagnostic specificity of NGS vs. 88% by TRG PCR, with similar diagnostic sensitivity (68% vs. 72% by PCR) and accuracy (84% vs. 80%). In PB samples, TRG FL-PCR and FCI were the least useful for diagnosis. NGS showed non-identical sequences in some identically sized peaks by TRG FL-PCR in concurrent skin and PB samples.	Rea et al., 2018 [67]; USA
T cell prolymphocytic leukemia (T-PLL)	Compare TRB NGS (BIOMED-2 Vβ-Jβ primers) with Vβ FCI	N = 80; 47 (59%) males, 33 (41%) females; median age 64.5 y (range 38–84);	80 diagnostic PB samples	TRB V(D)J NGS using BIOMED-2 Vβ-Jβ primers	Vβ expression by FCI [68,69]	A dominant Vβ domain usage was detected by FC1 in only 41 (51%)/80 samples, but clonality was suspected in all samples by FCI. In 12 (15%) cases, NGS identified the clone missed by FCI. Overall, NGS and FCI results were concordant in 61 (76%) of 80 samples.	Kotrova et al., 2018 [70]; Germany, Czech Repubic

Table 1. *Cont.*

Diagnoses of Studied T Cell Neoplasms	Study Purpose	Patients Studied	Clinical Samples Studied	NGS Assays	FL-PCR and FCI Assays, if Used	Relevant Results	References; Country of Study
CTCL	Quantitate tumor burden in matched skin and PB samples from patients with CTCL and reactive conditions	N = 46; 23 (16 males and 7 females) with histological and clinical diagnosis of CTCL and 23 with reactive conditions	Skin biopsy and concurrent PB samples assessed by TRB NGS in 46 patients	TRB V(D)J NGS; ImmunoSEQ, Adaptive Biotechologies, Seattle, WA	None	7 (30%) CTCL showed identical top frequency skin and PB (1.8–86.8%) clones; 16 (70%) CTCL showed non-identical skin (0–79.4%) and PB clones. In 16 (70%)/23 reactive conditions, NGS detected the top frequency skin clone in PB (0.0012–0.71%); none of these 23 patients had a larger skin clone in PB.	Wang et al., 2019 [71]; USA
Reactive T cell lymphocytosis or lymphoid hyperplasia, T cell NHL-NOS, ALCL, AITL, PTCL-NOS, T cell PLL, T-LGLL, MF, SS, T-ALL/T-LBL, B-NHL and B-ALL, hypereosinophilic syndrome, HL, MDS, non-hematological diseases, and GVHD	FL-PCR vs. NGS for clonality evaluation	N = 121; {Reactive T cell lymphocytosis or lymphoid hyperplasia (*n* = 37), T cell NHL-NOS (*n* = 3), ALCL (*n* = 2), AITL (*n* = 1), PTCL-NOS (*n* = 2), T-PLL (*n* = 1), T-LGLL (*n* = 5), MF (*n* = 1), SS (*n* = 1), T-ALL/T-LBL (*n* = 9), B-NHL (*n* = 6), B-ALL (*n* = 2), hypereosinophilic syndrome (*n* = 7), HL (*n* = 1), MDS (*n* = 2), non-hematological diseases (*n* = 4), GVHD (*n* = 1), unavailable final diagnoses (*n* = 36)}	121 diagnostic samples suspicious for a T cell LPD {85 fresh samples (11 PB, 33 BM, 37 lymph nodes, 4 skin biopsies) with definitive diagnoses established by clinical, morphological, and immunophenotypic data, and 36 referred cases of FFPE tissue sections with unavailable final clinical diagnosis)}	TRG NGS; Lymphotrack^{TM} Dx	TRG FL-PCR	In 94.4% of FFPE, NGS showed reliable results despite unavailable final diagnosis; 55.6% (*n* = 20/36) of FFPE cases non-interpretable by FL-PCR. *False positive FL-PCR cases*: (1) 1 normal case monoclonal by PCR confirmed as polyclonal by NGS; (2) in 14 fresh samples with FL-PCR monoclonal in a polyclonal background, NGS clarified a polyclonal pattern (*n* = 12; normal, B-NHL, and benign), polyclonal with minor clones of uncertain significance (*n* = 1), or monoclonal with only 9.6% clonal sequences (*n* = 1, B-NHL). *False negative FL-PCR*: in 2 of 42 cases polyclonal by FL-PCR (1 benign; one B cell lymphoma), NGS showed clonal sequences (9.2% and 6.3% of total).	Nollet et al., 2019 [51]; Belgium
Advanced MF and SS after allogeneic HSCT	To monitor MRD after HSCT by NGS	N = 35, 13 MF, 22 SS; 21 males, 14 females; median age 60 (20–74) y in a clinical trial (#NCT00896493)	Diagnostic samples available for NGS (in *n* = 30); concurrent PB and skin samples evaluated for MRD	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	None	In PB samples, NGS identified molecular remission (MR), defined as undetectable malignant clone with a sensitivity of 1 in 200,000 nuclear cells.	Weng et al., 2020 [72]; USA

Table 1. *Cont.*

Diagnoses of Studied T Cell Neoplasms	Study Purpose	Patients Studied	Clinical Samples Studied	NGS Assays	FL-PCR and FCI Assays, if Used	Relevant Results	References; Country of Study
T cell leukemia/lymphoma, hematologic malignancy (e.g., AML, MDS), non-malignant inflammatory disorder	FL-PCR vs. NGS for clonality evaluation	N patients unavailable; 72 specimens, majority stated to be from the CTCL clinic	72 samples (20 BM, 21 PB, 14 fresh tissues; 17 FFPE tissues)	TRG NGS; Lymphotrack™	TRG FL-PCR	28 (77.8%) of 36 cases with a T cell neoplasm were clonal by NGS; 3 specimens with *false negative PCR* (having clinicopathologic diagnoses of T cell leukemia/lymphoma) were clonal by NGS. NGS showed a trackable sequence in 11 patients with ≥2 specimens evaluated for TRG clonality.	Lay et al., 2020 [50]; USA
Relapsed or refractory MF/SS treated with mogamulizumab (anti-chemokine receptor type 4 antibody) [73]	Study skin biopsies of mogamulizumab-associated rash examined by NGS or FL-PCR	19 patients who developed mogamulizumab-associated rash; excluded biopsies with dominant TRG or TRB sequences	52 biopsies from 19 patients who developed mogamulizumab-associated rash	TRB V(D)J NGS and TRG NGS; Adaptive Biotechnologies, Seattle, USA [58,62]	TRB and TRG FL-PCR [74] used in only 6 specimens where NGS was not used	20 of 46 biopsies analyzed by NGS showed low levels of neoplastic T cell receptor sequences identified before treatment; in 38 of 43 biopsies, intraepidermal lymphocytes showed an inverted CD4:CD8 ratio ≤1:1 by immunohistochemistry.	Wang et al., 2020 Dec [75]; USA
CTCL (primarily early or partially treated disease) and various non-CTCL conditions [h]	FL-PCR vs. FCI vs. NGS for T cell clonality	N = 55 from the CTCL clinic; 34 males, 21 females; ages 21–85 years; integrated diagnosis of CTCL confirmed in N = 35 after clinical, histologic, immunophenotypic and T cell clonality evaluation	In 102 skin biopsies: 59 FCI tests in *n* = 53 patients, 50 FL-PCR tests in *n* = 50, 26 NGS tests in *n* = 23; In 51 PB samples for FCI (in *n* = 51), 50 FL-PCR (in *n* = 50), 16 NGS tests (in *n* = 16); 15 concurrent FL-PCR and NGS	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	T cell receptor BIOMED-2 FL-PCR using targets Vβ, Dβ, Jβ, Vγ, and Jγ (TRB and TRG)	FL-PCR overall sensitivity 61% in skin and 40% in PB; FL-PCR overall specificity 64% in skin and 61% in PB; TRB NGS overall sensitivity similar (60%) but greater (87%) overall specificity; of note, 28% and 21% of skin samples were insufficient for FL-PCR and FCI, respectively. [i] Specificity in PB: 100% for TRB NGS, 70% for FCI, 62.5% for FL-PCR. [j]	Gibbs et al., 2021 [76]; USA
Various: MF, AITL, PTCL, T-LGLL, other TCL, HL, MZL, CLL, other BCL, reactive, cytopenia, eosinophilia, leukocytosis, other	FL-PCR vs. NGS for clonality evaluation	N patients unavailable {MF/SS (18%), AITL (7%), PTCL (7%), T-LGLL (7%), other TCL (7%), HL (5%), MZL (2%), CLL (1%), other BCL (2%), reactive (5%), cytopenia (21%), eosinophilia (4%), leukocytosis (2%), other (1%), unknown (11%)}	101 DNA samples (48 PB, 46 FFPE tissues, 7 BM aspirates) previously analyzed by TRG FL-PCR as part of the diagnostic workup for suspicion of T cell malignancy or LPD	TRG NGS; Lymphotrack™	TRG FL-PCR (2-tube BIOMED-2 PCR assay lacking the JγP primer [k])	TRG NGS: accuracy 83%; analytical specificity 100%; discordant results between FL-PCR and NGS reported in 25 samples {17 (68%)/25 PB, 1 BM aspirate, 7 FFPE tissues}: 17 samples (11 PB, 6 FFPE) clonal by FL-PCR with discordant results by NGS {*9 false positive PCR* (8 PB, 1 FFPE), *5 false negative NGS* [l] (2 T-LGLL, 1 PTCL, 2 AITL), *2 false oligoclonal NGS* (1 PTCL, 1 AITL), *1 diagnosis unknown*}; 8 samples (6 PB, 1 BM, 1 FFPE) oligoclonal by FL-PCR were polyclonal by NGS. [m]	Ho et al., 2021 [49]; USA

Table 1. *Cont.*

Diagnoses of Studied T Cell Neoplasms	Study Purpose	Patients Studied	Clinical Samples Studied	NGS Assays	FL-PCR and FCI Assays, if Used	Relevant Results	References; Country of Study
T cell and B cell malignancies and reactive lesions	NGS DNA Capture assay vs. standard testing for B cell and T cell malignancy cases submitted by ten EuroClonality–NGS laboratories	N patients unavailable; 204 B- and 76 T cell malignancies {42 T-ALL, 1 T-LBL, and 33 mature T neoplasms (13 ALCL, 9 AITL, 3 MF, 3 SS, 2 EATL, 1 C-ALCL, 1 PTCL-NOS, 1 intestinal TCL-NOS)}; 21 non-neoplastic	DNA from 76 T cell malignancies {42 T-ALL, 1 T-LBL, and 33 mature T neoplasms; 9 HMW DNA, 67 FFPE}; 21 reactive lesions; 14 LPD cell lines; 4 cell line blends	EuroClonality–NGS DNA Capture assay to detect T cell clonality at TRA, TRB V(D)J and TRB DJ, TRD, or TRG loci	The original BIOMED-2 T cell receptor FL-PCR results from the ten participating laboratories for all cases	The NGS assay detected TCR clonality, i.e., ≥1 clonal rearrangements at TRA, TRB, TRD, or TRG loci, in 71 (97%) of 73 T cell malignancies (1 T-ALL and 1 ALCL negative for a clone by FL-PCR and NGS). All 7 mature TCLs clonal by TRG NGS but only 1 clonal by TRG FL-PCR; 6 of 7 clonal by TRB NGS, but TRB FL-PCR clonal in only 3 (43%) of 7.	Stewart et al., 2021 [77]; Europe
CTCL and non-neoplastic	To evaluate the role of TRB NGS in diagnosing CTCL; NGS data from 2013 to 2020 analyzed retrospectively	144 patients: 101 CTCL {52 MF, 35 SS, 8 other TCL, 6 CD30+ LPD (3 ALCL, 3 LyP)} [78] and 43 non-neoplastic	Skin samples from 101 CTCL and 43 non-neoplastic cases; also PB in 9 cases of SS	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	TRG FL-PCR analyzed PB and skin in 9 cases of SS	TRB NGS diagnostic specificity for CTCL: 95% and 100%; sensitivity 89% and 50%, with the top T cell clones comprising 5% and 25%, respectively, of all T cells in skin. PB in 1 (11%) SS case was non-clonal by FL-PCR; in that case, skin and PB were clonal by NGS.	Zimmerman et al., 2021 [79]; France
Refractory or relapsed MF or SS treated with mogamulizumab	To describe the diagnosis and treatment of mogamulizumab-associated skin rash in patients with CTCL	N = 24; including 13 males, 11 females; median age 65.1 (30.4–88.9) years; 17 of 24 developed a rash after treatment with mogamulizumab	Skin biopsies in 14 of those 17 patients who developed a rash after treatment; skin and PB examined by FL-PCR and NGS	Laboratory-developed TRG and TRB V(D)J NGS assays in skin and PB (performed after [19,51] were published)	Laboratory-developed TRG and TRB FL-PCR in skin and PB (completed before [19,51] were published)	All 17 patients showed TRG or TRB clones in skin, PB, or lymph node specimens before treatment by FL-PCR or NGS. After treatment with mogamulizumab, 12 (85.7%)/14 skin and 9 (75%)/12 PB samples examined were absent for a T cell clone.	Trum et al., 2022 [80]; USA
Clinical and histopathologic diagnoses of pityriasis lichenoides	To evaluate NGS in pediatric patients with pityriasis lichenoides	12 pediatric patients (5 females, 7 males; ages 3–16 years)	18 biopsy specimens from 12 patients; five of the 12 patients had two concurrent skin biopsies from different anatomic sites	TRG NGS and TRB V(D)J NGS; LymphoTrack™	None	7 (58%)/12 patients showed T cell clones: TRG in n = 6 (50%); TRB in n = 3 (25%), including n = 2 TRG clonal}; in n = 5 patients with concurrent biopsies, matching TRB clones in n = 2, no TRG or TRB clone in either biopsy in n = 2, and only TRG clonal in 1 of 2 biopsies in n = 1; discordant TRG and TRB results in n = 5 (42%)/12 patients (TRG clonal and TRB nonclonal in n = 4; TRB clonal but TRG nonclonal in n = 1)	Raghavan et al., 2022 [81]; USA
Folliculotropic variant of MF, presenting with the plaque stage	Evaluation of tumor clone frequency by TRB NGS	41 patients, including 24 with progressive disease, including 16 dead of the disease	46 residual FFPE or frozen skin biopsies at diagnosis from 41 patients	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	None	NGS in 41 patients showed a tumor clone frequency of 0.95% to 72.01%, with a median of 11.00%	Van Santen et al., 2022 [82]; The Netherlands

Table 1. *Cont.*

Diagnoses of Studied T Cell Neoplasms	Study Purpose	Patients Studied	Clinical Samples Studied	NGS Assays	FL-PCR and FCI Assays, if Used	Relevant Results	References; Country of Study
Early-stage MF	To study TRG NGS patterns in successive and synchronous skin lesions	7 patients wth early-stage MF	17 skin biopsies in 7 patients (7 sequential in 3 patients, 11 synchronous in 5 and both in 1 patient)	TRG NGS; BIOMED-2- based laboratory-developed assay	Previously assessed by BIOMED-2 TRG and TRB FL-PCR	NGS identified identical dominant TRG rearrangements in all samples spatially and temporally in a given patient, but NGS was minimally more sensitive than FL-PCR.	Bozon et al., 2022 [83]; France
T cell malignancies	Lymphotrack™ MRD assays compared with ClonoSEQ MRD assays for routine MRD monitoring	60 patients {37 CTCL (MF, SS), 8 T-ALL, 6 B-ALL, 2 PTCL, 2 AITL, 1 adult T cell lymphoma, 2 T-LGLL, 1 T cell lymphoma NOS, and 1 T-PLL}	69 follow-up samples (65 PB, 4 BM) analyzed from 60 patients; initial neoplastic clone identification by ClonoSEQ	NGS TRB V(D)J, and TRG MRD; Lymphotrack™ compared with ClonoSEQ TRG and TRB V(D)J MRD (Adaptive Technologies, Seattle, USA)	Concurrent FCI in 32 (46%) samples; no FL-PCR assays	Both manufacturers' TRG MRD assays and FCI performed in 31 samples: 19 (31%) MRD+ by all 3 assays, and 12 (38.7%) MRD+ by both TRG NGS assays but negative by FCI. Both TRB MRD assays and FCI performed in 28 samples: 17 (60.7%) MRD+ by all 3 assays, 11 (39%) MRD+ by both TRB NGS asays but negative by FCI.	Tung et al., 2023 [84]; USA
Mycosis fungoides	To study neoplastic intraclonal CDR3 variants in MF by NGS	N = 3 (2 males and one female) described from a cohort of 21 patients (of 60 reviewed)	23 (22.5%) of 102 MF skin biopsies in 21 patients with ≥2 dominant T cell clones	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	None	6 (26%) of these 23 biopsies from 3 patients showed single base substitutions in the TRB sequences representing intraclonal variations.	Gleason et al., 2023 [85]; USA
MF without PB involvement, defined according to Olsen et al., 2022 [86]	To study T cell clones in skin and PB samples in patients with MF limited to skin	N = 60 with confirmed histopathological diagnosis of MF and no PB involvement; 50 (83%) of 60 early-stage (IA-IIA) MF	PB and lesional skin biopsies in 60 patients	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	None	17 (28%) of 60 patients had a dominant T cell clone in PB by NGS; the clone was identical in skin and PB in 3 (18%) and discordant between skin and PB in 14 (82%, early-stage MF).	Joffe et al., 2023 [87]; USA
Classic Hodgkin's lymphoma at diagnosis and relapse	To study the clonal relationship at diagnosis and relapse	N = 60; median age 26 (4–76) y {ages ≤ 18 y (*n* = 18); >18 y (*n* = 42)}; 37 males, 23 females	130 tissue samples (99 FFPE, 31 fresh frozen); paired tissue samples at diagnosis and relapse in N = 60; T cell clonality in 125 samples in 57 patients	EuroClonality–NGS amplicon-based assays for IGH, IGK, TRB V(D)J, TRB DJ, TRG [39,40,42]	None	14 (11%)/125 samples in 12 (21%)/57 patients showed T cell clones at diagnosis (*n* = 5) and relapse (*n* = 9); 7 (50%)/14 samples in 5 patients were retrospectively diagnosed as TCL based on retrospective pathology review and TCL-associated somatic mutations. [n]	Van Bladel et al. 2023 [46]; Europe
Mycosis fungoides with treatment-related skin rash	Treatment-related skin rash vs. disease progression	N = 3; two females ages 88 y and 90 y, and one male, age 44 y	PB and skin biopsies {mogamulizumab-associated rash (*n* = 2); mechlorethamine gel dermatitis (*n* = 1)	TRB V(D)J NGS; Adaptive Biotechnologies, Seattle, USA	FCI in PB samples (*n* = 2)	A T cell clone similar to the neoplastic clone at diagnosis was absent by NGS in skin and PB of all three patients; no PB involvement by FCI (*n* = 2)	Bhatti et al., 2023 [88]; USA

Table 1. *Cont.*

Diagnoses of Studied T Cell Neoplasms	Study Purpose	Patients Studied	Clinical Samples Studied	NGS Assays	FL-PCR and FCI Assays, if Used	Relevant Results	References; Country of Study
Mature T cell neoplasms, all αβ subtype, CD3+: SS, AITL, ALK-negative ALCL, MF, nodal PTCL-TFH, PTCL-NOS, SCPLTCL, T-LGLL, T-PLL, unclassifiable	FCI for T cell receptor β constant chain 1 (TRBC1) compared with TRB NGS	N = 57 (24 females, 34 males; median age 58 y) 11 SS, 1 AITL, 1 ALK-negative ALCL, 3 MF, 3 nodal PTCL, TFH subtype, 6 PTCL-NOS, 2 SCPLTCL, 1 T-LGLL, 2 T-PLL, 1 unclassifiable	90 samples (48 PB, 37 BM, and 5 lymph nodes), including follow-up samples with variable tumor burden from 57 patients	TRB V(D)J NGS Lymphotrack™ assay in 38 samples with available DNA	TRBC1 by FCI performed in 90 samples	FCI for TRBC1 confirmed T cell clones in 37 (97%)/38 samples involved by a mature T cell neoplasm in 17 patients but also identified T cell clones of uncertain significance in 9 (100%)/9 non-neoplastic samples (5 PB, 4 BM) in 6 (10%) patients. In 17 samples negative for disease, FCI showed polyclonal T cells, and TRB NGS detected T cell clonal disease in 6 patients in 6 (35%)/17 samples. [o]	Nguyen et al., 2024 [89]; Australia
Anaplastic lymphoma kinase (ALK)+ anaplastic large-cell lymphoma	Use real-time quantitative reverse transcriptase PCR to diagnose and monitor MRD in ALK+ ALCL	96 patients {age range 2–80 (median 16) years} with ALK+ ALCL	TRB NGS and TRG NGS used in 23 patients in diagnostic FFPE or frozen tissues	TRB V(D)J and TRG EuroClonality–NGS amplicon-based assays [39] [p]	None	18 (78%)/23 cases showed at least 1 T cell clone; both TRB and TRG were clonally rearranged in 16 (70%); 1 case each was clonal only for TRG and TRB; no clone by TRG or TRB (in *n* = 5). All TRG-clonal cases had 2 major clones; 13 (72%)/17 TRB-clonal cases had 1 major clone; 2 cases showed 2 TRB clones.	Kalinova et al., 2024 [90]; The Czech Republic
Nodal T-follicular helper cell lymphoma, angioimmunoblastic type (or AITL), BM involvement	To study clonal hematopoisis in BM biopsies of patients with AITL	N = 22; {at diagnosis of lymphoma, median age 68 (range 47–83 years); 10 males, 12 females}; 29 BMs in 22 studied	TRB NGS used in 11 BM biopsies from 7 patients with AITL; FFPE tissues from the diagnostic lymph node and clinical staging BM biopsies	Oncomine™ TCR beta-short read (SR) NGS assay for TRB V(D)J (Thermofisher Scientific) [91]	None	Neoplastic lymph nodes in all 7 patients and BMs with definite neoplastic infiltration (*n* = 4) showed high proportions (32–80%) of clonal neoplastic sequences. In 3 patients, 4 BMs negative (*n* = 3) or suspicious for lymphoma (*n* = 1) by morphology showed <1.5% of sequences similar to the neoplastic clone by NGS. [q]	Harland et al., 2024 [92]; Germany

[a] Sézary syndrome was defined as >1000 Sézary cells per microliter (Weng et al., 2013 [58]). [b] By FCI, Sézary cells were defined as dim CD3+, dim CD4+ T cells with diminished or absent expression of CD26, CD7, or both [58]. [c] Experiments using healthy donor blood samples spiked with purified Sézary cells obtained by flow cytometric sorting showed that TRB NGS detected Sézary cells at the level of 1 in 50,000 PBMNCs [58]. [d] Non-neoplastic skin diseases included in Kirsh et al., 2015 [62]: psoriasis (*n* = 23), eczematous dermatitis (*n* = 11), contact dermatitis (*n* = 12), PLEVA (pityriasis lichenoides et varioliformis acuta) (*n* = 12) [62]. [e] In a few cases, the TRB clone showed lower clonal frequencies than with TRG NGS [62]. [f] This denominator includes all nucleated non-T cells in the skin and is different from all T cells in the skin; when total T cells in the skin were used as the denominator, the top clones by NGS could not distinguish CTCL from non-CTCL [62]. [g] The one-tube FL-PCR assay was compared with the BIOMED-2 FL-PCR assay, with similar sensitivity in both assays [19,66]; the Lymphotrack™ TRG NGS assay contains primer master mixes that target regions similar to those targeted in the TRG v2.0 PCR assay [19]. [h] The diagnoses for 20 patients with non-CTCL in the Gibbs et al. report [76] were as follows: pseudolymphoma (*n* = 7), no evidence of, or inactive disease (*n* = 4), atypical lymphoid infiltrate not diagnostic for CTCL (*n* = 1), lymphomatoid papulosis (*n* = 1), superficial perivascular dermatitis (*n* = 2), phototoxic dermatitis (*n* = 1), postinflammatory pigment alteration (*n* = 1), interstitial granulomatous dermatitis (*n* = 1), *Staphylococcus* folliculitis (*n* = 1), and morphea (*n* = 1) [76]. [i] Numbers were extracted from the results text in the publication; separate results for TRG and TRB FL-PCR were unavailable from the publication [76]. [j] Numbers were extracted from the abstract in the publication [76]. [k] The two-tube TRG FL-PCR BIOMED-2 assay used in the study by Ho et al. [49] does not include the JγP primer, which was included in the Lymphotrack™ *TRG* NGS assay used in their comparative study [49]. [l] NGS was considered nonclonal and thus

false negative in these cases by Ho et al. [49] because the frequently identified clonotypes did not have high enough total frequency (>2.5%) and/or sufficient fold-change (5x) over the polyclonal background to meet their criteria for clonal populations by NGS [49]. [m] No clinicopathologic evidence of any hematolymphoid malignancy for these eight samples (Ho et al. [49]). [n] Based on the retrospective review of pathologic features integrated with the somatic mutations showing the presence of T cell lymphoma (TCL)-associated mutations (*RHOA* p.G17V and *TET2* mutations in 3 cases and *STAT3* and *JAK3* mutations in one), the diagnosis at recurrence was retrospectively made as a TCL in 4 cases in Van Bladel et al. [46]. The primary diagnosis in 2 of 5 patients was considered to be a composite lymphoma with CHL and a minor component of TCL [46] and, in one case, as an ALK-negative anaplastic large-cell lymphoma [46]. [o] In Ngyugen et al. [89], among the six patients with polyclonal TRBC1 FCI and monoclonal TRB NGS, previous disease sequences by NGS were present in 2 cases. The TRB NGS sequences in those 2 patients were confirmed to represent MRD that was not detected by TRBC1 FCI [89]. Previous sequences were unavailable for the remaining 4 cases. Still, 1 of these 4 patients had active disease at another site, and another patient had a longstanding rash with indeterminate biopsy results [89], suggesting that clinical correlation was required. [p] The results in the publication by Kalinova et al. show only V(D)J sequences [90]. [q] These findings (Harland et al. 2024 [92]) exemplify the objectivity that NGS achieves compared with subjectivity in morphologic evaluation. *Abbreviations*: AITL, angioimmunoblastic T cell lymphoma; ALCL, anaplastic large-cell lymphoma; BCL, B cell lymphoma; BM, bone marrow; B-NHL, B cell non-Hodgkin's lymphoma; C-ALCL, CD30+ anaplastic large-cell lymphoma; CDR3, complementarity determining region 3; CLL, chronic lymphocytic leukemia; CTCL, cutaneous T cell lymphoma; DLBCL, diffuse large B cell lymphoma; EATL, enteropathy-associated T cell lymphoma; EBC, Epstein-Barr virus; FFPE, formalin-fixed, paraffin-embedded; FCI, flow cytometric immunophenotyping; FL-PCR, fragment length polymerase chain reaction; GVHD, graft versus host disease; HL, Hodgkin's lymphoma; HMW, high molecular weight; HSCT, hematopoietic stem cell transplant; LGLL, Large granular lymphocytic leukemia; LPD, lymphoproliferative disorder; LyP, lymphomatoid papulosis; MCU, mucocutaneous ulcer; MDS, myelodysplastic syndrome; MF, mycosis fungoides; MR, Molecular remission; MRD, Minimal/measurable residual disease; MZL, marginal zone lymphoma; NGS, next-generation sequencing; NHL-NOS, Non-Hodgkin's lymphoma, not otherwise specified; PB, peripheral blood; PCR, polymerase chain reaction; PLL, prolymphocytic leukemia; PTCL-NOS, peripheral T cell lymphoma, not otherwise specified; PT-LPD, post-transplant LPD; RQ-PCR, real-time quantitative PCR; SCPLTCL, subcutaneous panniculitis-like T cell lymphoma; SS, Sézary syndrome; T-ALL, T cell acute lymphoblastic leukemia; TCL, T cell lymphoma; T-LBL, T-lymphoblastic lymphoma; TRBC1, T cell receptor β constant region 1.

All 29 publications included in Table 1 represent studies from the USA (n = 18), several countries in Europe (n = 10), and Australia [89] (n = 1). These studies used T cell receptor NGS to evaluate T cell clonality using only TRG NGS (n = 6) [19,49–51,59,83], only TRB NGS (n = 16) [58,64,65,67,70–72,75,76,79,82,85,87–89,92], or both TRG NGS and TRB NGS (n = 7) [46,62,77,80,81,84,90] gene rearrangements with or without additional antigen receptor gene rearrangement loci. At least six sources of T cell receptor NGS assays were used in the studies in Table 1. The targets examined for TRB NGS were the V-(D)-J regions in all studies except two studies that used the EuroClonality–NGS DNA Capture assay [77] or amplicon-based EuroClonality–NGS [46]. Table 2 shows the gene targets and enrichment methods of various manufacturers' NGS assays for evaluating T cell clonality.

Table 2. Specifications of commercially available NGS assays for T cell receptor clonality analysis.

NGS Assays	Assay Manufacturers	TRB Gene Targets	TRG Gene Targets	Target Enrichment
ClonoSEQ assays [a]	Adaptive Biotechnologies, Seattle, USA	TRB-V(D)J	TRG-VJ	Amplicon-based
Lymphotrack™ assays [a]	Invivoscribe, San Diego, USA	TRB-V(D)J	TRG-VJ	Amplicon-based
EuroClonality NGS-amplicon-based assay	EuroClonality group, Europe	TRB-DJ and TRB-V(D)J	TRG-VJ	Amplicon-based
EuroClonality NGS-capture-based assay (EuroClonality-NDC assay)	EuroClonality group, Europe	TRB-DJ and TRB-V(D)J	TRG-VJ	Capture-based

[a] The differences between the NGS assays developed by these two manufacturers were studied and described by Tung et al. [84] and are summarized in the text in this review.

3.1.2. Comparative Studies for T Cell Receptor Clonality Assessment by NGS and FL-PCR Assays in Patients with Mature T Cell Neoplasms and Related Pathologic Diagnoses

Twelve (41%) of the 29 studies [19,49–51,59,62,67,76,77,79,80,83] used traditional FL-PCR and NGS assays for T cell clonality. One (8%) of these 12 studies used laboratory-developed TRG and TRB FL-PCR and TRG and TRB NGS assays [80]. In that single study, the FL-PCR assays were used before 'NGS was shown to be superior to FL-PCR' in two publications [19,51], and laboratory-developed NGS assays were used after those two publications; further, comparing assay results was not the purpose of that study [80].

Therefore, eleven (38%) of 29 studies compared FL-PCR and NGS assays for evaluating T cell clonality. These 11 studies are shown as distributed in Table 3 according to the compared assays and summarized in the text following the table. Table 3 also shows the numbers and types of clinical samples compared in these studies.

- Five (45%) of these 11 studies compared TRG FL-PCR with TRG NGS [19,49–51,59].
- Two (18%) of 11 studies compared TRG FL-PCR with TRB NGS [67,79].
- One (9%) of 11 studies compared TRG FL-PCR with TRG NGS and TRB NGS [62].
- One (9%) of these 11 studies compared the EuroClonality/BIOMED-2 TRG and TRB FL-PCR assays with TRB NGS [76]. The EuroClonality/BIOMED-2 FL-PCR assays analyze TRB-V(D)J, TRB-DJ and TRG-VJ rearrangements.
- One (9%) of these 11 studies compared the EuroClonality/BIOMED-2 TRG and TRB FL-PCR assays with TRG NGS [83].
- One (9%) of 11 studies compared the EuroClonality–NGS DNA Capture assay for evaluating clonality at the TRD, TRG, TRB, and TRA loci with the participating laboratories' diagnoses based on EuroClonality/BIOMED-2 FL-PCR assays [77].

Table 3. Distribution of the 11 comparative studies with the numbers and types of samples analyzed by TRG and TRB FL-PCR and NGS assays for T cell clonality.

N Studies (% of 11 Comparative Studies), Total N Patients and Samples Compared in the Studies, and the Sample Types Studied for T Cell Receptor Clonality Assessment by TRG and TRB FL-PCR and NGS Assays							
FL-PCR and NGS Assays	**TRG NGS N Cohort Studies and Total N Samples Compared**		**TRB NGS N Cohort Studies and Total N Samples Compared**		**EuroClonality–NGS DNA Capture for TRD, TRG, TRB, TRA**		**Total N Samples Compared**
	N Studies	**Total N Samples**	**N Studies**	**Total N Samples**	**N Cohort Studies**	**Total N Samples**	
TRG FL-PCR	N = 5 (45%) Sufficool et al. [59] Kansal et al. [19] Lay et al. [50] Ho et al. [49] Nollet et al. [51]	Total 369 samples in all 5 studies: 95 PB, 68 BM, 117 FFPE, 89 fresh tissues; patients N = 206 in 3 studies [19,51,59], N unavailable [49,50]	N = 2 (18%) Rea et al. [67] Zimmerman et al. [79]	Total 244 skin biopsies and 109 PB, including 100 concurrent PB [67] and 9 PB in SS [79]	TRG and TRB FL-PCR compared with BIOMED-2 FL-PCR assays in 33 mature T cell neoplasms; results given in 7 (21%) of 33 discordant cases [77]	All 33 samples compared with the EuroClonality/BIOMED-2 FL-PCR assays	Total 832 (369 + 110 + 244 + 109) samples studied
	N = 1 (9%), Kirsch et al. [62]	110 samples from 110 patients [a] [62]	N = 1 (9%), Kirsch et al. [62]	110 samples from 110 patients [a] [62]			
TRB FL-PCR	None	Zero	None	Zero			Zero
EuroClonality/ BIOMED-2 TRG and TRB FL-PCR	N = 1 (9%) Bozon et al. [83]	17 samples (skin) in 7 patients [83]	N = 1 (9%) Gibbs et al. [76]	102 skin biopsies: 50 FL-PCR tests in 50 patients; 26 NGS tests in 23 patients [76]	N = 1 (9%) Stewart et al. [77]	33 samples for mature T cell neoplasms; N patients unavailable [77]	76 (17 + 26 + 33) samples compared by NGS and FL-PCR
Total N cohort studies and samples	7 (63.6%)	Total 496 (369 + 110 [a] + 17) samples in all 7 studies: 95 PB, 68 BM, 117 FFPE, 216 (89 + 110 + 17) fresh tissues	4 (36.3%) [b]	Total 489 (244 + 109 +110 [a] + 26) samples (skin and PB) examined by FL-PCR and NGS	1 (9%)	33 samples (24 FFPE, 9 HMW DNA) for mature T cell neoplasms	Total 908 samples studied (22.5% PB, 7.4% BM, 15.5% FFPE, 54.2% fresh tissues) [c]

The row and column headers in grey color indicate the studies that compared TRG FL-PCR or TRG NGS assays. The row and column headers in light blue indicate the studies that compared TRB FL-PCR or TRB NGS assays. The row and column headers in the light green color include the studies that compared EuroClonality/BIOMED-2 FL-PCR assays or the EuroClonality-NGS DNA Capture assay for all T cell receptor targets. The box in the dark color shows the only study that compared the EuroClonality/BIOMED_2 FL-PCR assays with the EuroClonality-NGS DNA Capture assay [77]. The boxes in light yellow color in the bottom row show the total number of studies for each column. The boxes in dark yellow color show the total number of samples studied for each column and row. [a] The same 110 samples in the study by Kirsch et al. [62] were studied by TRG NGS and TRB NGS. [b] All 4 studies used the ClonoSEQ assay. [c] N and % of samples is based on total 908 samples, including PB = 205 (96 + 109); BM = 68; FFPE = 117; fresh tissues = 493 (216 + 244 + 26 + 7). At least 409 (82.9%) of 493 fresh tissues were skin biopsies. Abbreviations: PB, peripheral blood; BM, bone marrow aspirates; FFPE, formalin-fixed, paraffin-embedded tissues; SS, Sézary syndrome.

As shown in Table 3, other than the samples compared by the EuroClonality–NGS DNA capture assay, which targets TRD, TRG, TRB V(D)J, TRB DJ, and TRA, all four studies that compared TRB NGS with FL-PCR assays analyzed TRB V(D)J using the ClonoSEQ NGS assay (Adaptive Biotechnologies, Seattle, USA). Notably, TRB NGS has only been compared with FL-PCR (TRG or TRB or both in EuroClonality/BIOMED-2 FL-PCR) to evaluate T cell clonality in skin and peripheral blood specimens by the ClonoSEQ assay, not in any FFPE, bone marrow, or non-cutaneous fresh tissue specimens.

In contrast, TRG NGS has been compared with TRG FL-PCR assays in seven cohort studies as follows:

(1) Four comparative studies used the Lymphotrack^{TM} assay (Invivoscribe Inc., San Diego, CA, USA) [19,49–51]. These four studies analyzed 335 clinical specimens, including 95 peripheral blood, 68 bone marrow aspirates, 117 FFPE, and 51 non-cutaneous fresh tissues, including 37 lymph nodes and four skin samples [19,49–51]. The Lymphotrack^{TM} TRG NGS assay primer design targets all functional V and J regions similar to that of the single-tube, single-distribution v2.0 TRG PCR assay (Invivoscribe Inc.), as explained previously [19].

(2) Two studies used separate laboratory-developed TRG NGS assays and compared them with FL-PCR in 34 skin biopsies in one [59] and 17 skin samples in the other [83].

(3) The seventh study compared TRG FL-PCR with TRG NGS and TRB NGS using the ClonoSEQ assay in only skin samples [62]. The ClonoSEQ and Lymphotrack™ NGS assays were compared in a US study [84], described in a subsequent section in this review.

3.1.3. All 29 Cohort Studies According to the Integrated Clinicopathologic Diagnoses for the Studied Clinical Samples

This section describes the relevant findings according to the diagnoses of the clinical samples examined in the 29 cohort studies, which were divided into three groups: cutaneous T cell lymphoma (CTCL), T cell prolymphocytic leukemia, and various neoplastic and non-neoplastic diagnoses.

Cutaneous T Cell Lymphoma

Cutaneous T cell lymphoma (CTCL) with mycosis fungoides and Sézary syndrome as the most common diagnoses among CTCL was the primary focus of 16 studies [58,59,62,65,67,71,72,75,76,79,80,82,83,85,87,88]. A majority of specimens were stated to be analyzed from the CTCL clinic in one additional publication [50]. Pityriasis lichenoides in pediatric patients was the focus of one additional study [81].

A scoping review to evaluate T cell receptor sequencing by NGS in atypical cutaneous lymphoid infiltrates and CTCL was published during the preparation of this manuscript [93]. That review included 13 studies, all of which are included in Table 1 [58,59,62,65,67,71,75,76,79–83], and identified 635 patients with mycosis fungoides and Sézary syndrome, 100 patients with clinical concern for mycosis fungoides, 14 patients with non-mycosis fungoides CTCL, 12 patients with pityriasis lichenoides, and 150 patients with inflammatory dermatoses or healthy donors in their 13 included studies [93].

In the studies summarized in Table 1, an additional 180 patients with CTCL were included in the following eight additional studies:

- 119 patients with mycosis fungoides or Sézary syndrome in four additional studies focused on this disease [72,85,87,88];
- 59 patients with mycosis fungoides or Sézary syndrome in four other studies with various included diagnoses [19,77,84,89]); and
- 2 additional patients with cutaneous CD30+ LPD [19,77].

Skin biopsies from patients with mycosis fungoides can show more than one dominant clone and intra-clonal variation by NGS, which can be challenging to interpret, as described [85]. Nevertheless, the clinical usefulness of NGS in the reviewed studies focused on patients with CTCL is summarized below:

1. TRB V(D)J NGS consistently showed clones in lesional skin biopsies and peripheral blood in these patients, including detecting Sézary cells at 1 in 50,000 circulating mononuclear cells [58,62].
2. In 60 patients with mycosis fungoides without peripheral blood involvement, including 50 (83%) with early-stage disease, TRB V(D)J NGS identified dominant clones in peripheral blood in 28% (n = 17) [87]. TRB V(D)J NGS also clarified the presence of discordance in the clone between the skin and blood in 82% (n = 14) of cases [87], which has a better prognosis, including a longer time to systemic treatment than if there are concordant clones present in the skin and blood [71,87].
3. Identifying molecular remission in both skin and peripheral blood by TRB V(D)J NGS in patients with advanced mycosis fungoides and Sézary syndrome after an allogeneic hematopoietic stem cell transplant significantly reduced the incidence of progression/relapse versus if NGS detected MRD in either skin or blood [72].
4. The absence or low levels of neoplastic T cell receptor sequences by NGS in skin rash associated with the treatment of mycosis fungoides with mogamulizumab, an antibody

to CCR4 (chemokine receptor type 4) or after treatment with mechlorethamine gel, helped to distinguish the treatment-associated skin rash from disease progression [75,88].

5. NGS identified clonal sequences in specimens obtained from different sites and times [83].
6. When analyzed by NGS, peripheral blood samples showed the highest diagnostic specificity of 100% for CTCL, compared with FCI and FL-PCR [76]. Another study found the latter two non-NGS methods to be the least useful for diagnosis and showed 100% diagnostic specificity in skin samples [67]. A French study also showed 100% and 95% diagnostic specificities in skin samples at 25% and 5% tumor cell fraction thresholds, respectively, by NGS [79]. Skin biopsies were often insufficient for analysis by FCI and FL-PCR assays [76].
7. Of note, NGS did not help diagnose pediatric pityriasis lichenoides, since a T cell clone may or may not be present in this non-neoplastic disease [81]. This finding further proves that a T cell clone's presence or absence does not equate to a neoplastic or non-neoplastic disease.

The most important point to note is that the diagnosis of CTCL requires integrating clinical and histopathologic findings, per current guidelines [32], including those published by the American Society for Dermatopathology [94]. For clinical diagnostic purposes, the results of clonality analysis must always be interpreted in the context of other clinicopathologic features, irrespective of the type of molecular assay used, as has been emphasized in numerous publications, including by the BIOMED-2 group [17,95]. Of interest, a US survey of practicing dermatopathologists in 2018 showed that the diagnosis of CTCL was based primarily on clinical, histopathologic, and immunophenotypic findings. Molecular analysis for clonality by FL-PCR assays was only used as an adjunct test, and >95% of the surveyed pathologists did not consider molecular testing for clonality to be essential to diagnosing CTCL [96]. Although FL-PCR-based testing was available to most by sending the test request to external laboratories, it was unclear how the test results, if the test was requested, were used in the final diagnosis [96]. In contrast, both TRG and TRB NGS successfully distinguished between CTCL and non-CTCL and benign skin, as shown by Kirsch et al. [62].

T Cell Prolymphocytic Leukemia

Two of the 29 studies in Table 1 evaluated 90 patients with T cell prolymphocytic leukemia (T-PLL) [64,70], including assessing the presence of MRD by NGS [64]. Two additional T-PLL patients were studied, one each in two other reports [51,84]. TRB NGS successfully evaluated MRD in the first study [64]. In the subsequent, more extensive study, TRB NGS was more sensitive than Vβ flow cytometry analysis in identifying the clones in peripheral blood samples of all patients with T-PLL [70]. Vβ surface expression by FCI uses antibodies directed against the Vβ domains of the αβ T cell receptor [68,69], and this FCI antibody panel covers approximately 70% of the whole Vβ spectrum [70]. Kotrova et al. studied the rearranged sequences by NGS and determined whether they were productive, according to IMGT/V-QUEST [97]. The authors concluded that since TRB NGS covers the whole Vβ spectrum and identifies productive and nonproductive rearrangements, TRB NGS can overcome the limitations of FCI-based Vβ clonality analysis [70]. However, the publication by Kotrova et al. [70] does not clarify the distinction between non-productive (no open reading frame) rearrangements or incomplete TRB D-J rearrangements, which are also not productive.

Various Diagnoses of Lymphoid Neoplasms and Non-Neoplastic Diseases

Ten (34%) of the 29 studies in Table 1 studied clinical samples from patients having various diagnoses of T cell malignancies [19,46,49–51,77,84,89,90,92], including CTCL and non-CTCL T cell neoplasms, and B cell malignancies, and classic Hodgkin's lymphoma [46]. One of these 10 studies was mentioned in the CTCL section as having a majority of samples from the CTCL clinic [50]. Five of these ten studies included benign, non-neoplastic, or reactive lymphoid hyperplasia samples and specimens from patients with neoplastic diseases [19,49–51,77]; these five studies are included in the 11 comparative studies in Table 3 above.

One of these five studies from the USA [19] compared TRG FL-PCR assays with TRG NGS in clinical samples (peripheral blood, bone marrows, and FFPE tissues) in patients with no concurrent or previous history of an LPD, definite diagnosis of an LPD, and atypical LPDs, wherein the diagnosis was suspicious for, but not diagnostic of, an LPD [19]. Of note, in that study, there was a high false positive and a high false negative rate for TRG FL-PCR in the peripheral blood samples, both in patients with no concurrent or previous history of an LPD and in patients with an LPD [19].

Tables 4–6 are derived from previously published data in one previous publication [19] that had reported false positive and false negative PCR assay results in Table 1 above. These tables explain the concepts of false positive and false negative FL-PCR results when comparing FL-PCR and NGS results and interpreting the results in conjunction with the integrated clinicopathologic findings. Of ten peripheral blood samples analyzed from ten patients with no previous or concurrent history of a lymphoproliferative disorder after a clinical request to rule out a T cell clone, six (60%) peripheral blood cases showed a discordance between TRG NGS and TRG FL-PCR results [19]. Table 4 shows those 6 (60%) of 10 peripheral blood cases, including 3 (30%) of 10 with false negative FL-PCR results and 3 (30%) of 10 with false positive FL-PCR results [19].

Table 5 shows five peripheral blood and two cases of bone marrow aspirate samples from seven patients with a diagnosis of LPD, also from the same publication as Table 4 [19]. Three (60%) of the five peripheral blood samples in patients with a diagnosis of an LPD showed the presence of clonal T cell populations by TRG NGS, but the TRG FL-PCR findings were nondiagnostic in these three cases, as depicted in Table 5.

Similarly, 13 FFPE cases with a histologic diagnosis of an atypical or diagnostic LPD in the same study included one false positive TRG FL-PCR case among the atypical LPD cases and three false negative TRG FL-PCR cases among the diagnostic LPD cases (one mature T cell lymphoma, one primary cutaneous LPD, and one post-transplant LPD) by TRG PCR [19]. These false positive and false negative cases are shown in Table 6.

Table 4. Six cases of peripheral blood samples in 6 patients with no history of previous or concurrent LPD and discordant TRG FL-PCR and TRG NGS results [19]. [a] The case numbers are shown according to the publication [19]. [b] Percentages of clonal T cells by NGS, as reported in the publication [19]. The table shows the six discordant cases of peripheral blood samples from six patients among ten patients with peripheral blood samples analyzed to rule out a clone, per the clinical request for the test. Three patients in this table showed a clone by NGS but not by at least one of the TRG FL-PCR assays (shown in the yellow color rectangles in the table): cases PB3 (no clone by both FL-PCR assays) and NLPD3 and NLPD6 (both with no clone in the single-tube FL-PCR assay). These 3 cases represented the 30% false negative FL-PCR cases in the group of ten peripheral blood cases. Conversely, the remaining three cases in this table (NLPD1, NLPD4, NLPD7) showed no clone by NGS, but all three were clonal by the two-tube TRG FL-PCR assay (shown by the text in red font). These three cases represented 30% of false positive cases by FL-PCR in the group of peripheral blood cases with no previous or concurrent history of LPD. Therefore, a total of 6 (60%) false negative or false positive cases were present in the group of 10 cases (Table adapted from the data published in [19]).

PB Cases [a]	TRG FL-PCR 2-Tube	v2.0 TRG FL-PCR Single Tube	TRG NGS [b]
PB3	No clone	No clone	Clone, ~2.4%
NLPD1	Clonal	Not diagnostic for clone; few target cells	No clone, few reads
NLPD3	Clonal	Not diagnostic for clone	Clone, ~5.5%
NLPD4	Borderline clone	No clone	No clone
NLPD6	Clonal	Not diagnostic for clone	Clone, ~7%
NLPD7	Clonal	Not diagnostic for clone	No clone

Table 5. Five peripheral blood and two bone marrow aspirate cases from 7 patients with diagnoses of a lymphoproliferative disorder were analyzed by two TRG FL-PCR and TRG NGS assays [19]. [a] Percentages of clonal T cells by NGS, as reported in the publication [19]. Abbreviations: LPD, lymphoproliferative disorder; PB, peripheral blood; BM, bone marrow; MF, mycosis fungoides; SS, Sezary syndrome; EBV+ MCU, Epstein–Barr virus (EBV)+ mucocutaneous ulcer; T-LGLL, T cell large granular lymphocytic leukemia. The table highlights three (60%) of five peripheral blood cases with clonal T cell populations identified by TRG NGS in patients with known diagnoses of lymphoproliferative disorders and expected T cell clones based on the integrated clinicopathologic diagnoses. These three cases showed nondiagnostic results in at least one of two FL-PCR assays (shown in yellow colored rectangles), representing false negative FL-PCR cases. Both bone marrow cases showed T cell clones by FL-PCR and NGS (Table adapted from the data published in [19]).

Type of Sample	LPD Diagnosis	TRG PCR 2-Tube	v2.0 PCR Single Tube	TRG NGS [a]
PB	MF and SS	Clonal	Clonal	Clonal, at least ~12%
PB	EBV+ MCU	Non-diagnostic	Not diagnostic for clone	Clonal, at least 8%
PB	MF	Clonal	Not diagnostic for clone	Clonal, at least 3%
PB	Cutaneous CD30+ LPD	Clonal	Clonal	Clonal, at least 64%
PB	Post-transplant LPD	Oligoclones	Oligoclones	Clonal, at least 28%
BM	T-LGLL	Clonal	Clonal	Clonal, at least 17%
BM	T-LGLL	Clonal	Clonal	Clonal, at least 12%

Table 6. Analysis of 13 FFPE cases in 13 patients with nine diagnostic LPDs and four atypical LPDs diagnosed by integrated clinicopathologic findings (published in [19]). [a] The case numbers are shown according to the publication [19]. [b] Percentages of clonal T cells by NGS, as reported in the publication [19]. Abbreviations: FFPE, formalin-fixed, paraffin-embedded tissues; LPD, lymphoproliferative disorder; ALK, anaplastic lymphoma kinase; ALCL, anaplastic large-cell lymphoma; NOS, not otherwise specified; DLBCL, diffuse large B cell lymphoma; GC, germinal center; EBV, Epstein–Barr virus. The table highlights two cases: one diagnostic mature T cell lymphoma (case LPD8) and one post-transplant LPD (case LPD16), with a false negative two-tube TRG FL-PCR (shown in yellow colored rectangles), one atypical LPD (case ALPD2) with a false negative single-tube FL-PCR, and one atypical LPD case with a false positive FL-PCR (case ALPD1). The NGS findings in all FFPE cases of diagnostic and atypical LPDs were concordant with the integrated clinicopathologic diagnoses. This table also shows two cases of mature T cell lymphomas, one PTCL-NOS and the other an ALK-negative ALCL (LPD9 and LPD10), which showed a monoallelic clone by NGS but a biallelic clonal pattern by FL-PCR; this discordance is indicated by the text in red font in this table and illustrated in [19].

FFPE Cases [a]	Diagnosis	TRG FL-PCR 2-Tube	V2.0 FL-PCR Single Tube	TRG NGS [b]
Mature T cell lymphomas (*n* = 4)				
LPD8	ALK-negative ALCL, recurrence	Non-diagnostic	Clonal, biallelic	Clonal, ~66%, biallelic
LPD9	Peripheral T cell lymphoma, NOS	NA	Clonal, biallelic	Clonal, ~60%, monoallelic
LPD10	ALK-negative ALCL	NA	Clonal, biallelic	Clonal, ~44%, monoallelic
LPD11	ALK-negative ALCL	NA	Clonal, biallelic	Clonal, ~69%, biallelic
Mature B cell neoplasms and immune dysregulation LPDs (*n* = 5)				
LPD12	DLBCL, non-GC B cell phenotype	NA	Clonal	Clone, ~8%
LPD13	EBV+ positive DLBCL, NOS	NA	No clone, few target cells	Polyclonal, no definite clone in few target cells
LPD14	DLBCL, GC B cell phenotype	NA	Not diagnostic for clone	Polyclonal, no clone, few target cells
LPD15	DLBCL, non-GC B cell phenotype	NA	No clone, few target cells	Few target cells, no clone
LPD16	Post transplant LPD, plasmacytic type	No T cell clone	One borderline clonal peak	Clone, ~3.5%, ample target cells
Atypical LPDs (*n* = 4)				
ALPD1	Atypical LPD, EBV+	Clonal	Not diagnostic for clone	Borderline expansion, no definite clone
ALPD2	Atypical LPD suspicious for but not diagnostic of primary cutaneous CD4+ small/medium T cell LPD	Clonal	Not diagnostic for clone	Clonal, at least 12%
ALPD3	Atypical LPD, EBV+	No clone	Not diagnostic for clone	Polyclonal, no clone
ALPD4	Atypical LPD, post-transplant, EBV negative	No clone	Polyclonal	Polyclonal, no clone

As depicted in Tables 4–6 above [19], the clonality results differed between the two FL-PCR assays in several cases [19], most likely due to differences in the assay design, as has also been shown in other studies [66,98]. Nevertheless, in contrast with FL-PCR results, TRG NGS in all cases in this study [19] showed polyclonal, oligoclonal, or clonal T cell patterns consistent with the diagnoses and provided precise quantitation of the dominant T cell sequences, with their percentages ranging from 2.4% to 69% of all T cells in all cases [19]. For instance, two cases of post-transplant LPDs showed T cell clones comprising ~3.5%

and at least 28% of all T cells by TRG NGS in that study [19], consistent with the previous findings of a T cell monoclonal population present in 50% of all B cell post-transplant LPDs [99]. Similarly, the diffuse large B cell lymphoma (designated LPD12) in Table 6 above showed a small T cell clone comprising ~8% of all T cells, while the other three DLBCL cases did not show a T cell clone [19]. In a BIOMED-2 FL-PCR study published in 2007, 21% of 109 DLBCL cases showed rearranged TRB, and 15% showed rearranged TRG genes [15].

In contrast with the low percentage of clonal T cells in a B cell neoplasm, the percentages of clonal T cells by TRG NGS were much higher in all cases of T cell lymphomas, as shown in Table 6 above. In that study, TRG NGS also clarified the nature of neoplastic T cell clonal populations in the T cell lymphoma cases as biallelic or monoallelic (illustrated in [19]). Likewise, TRG NGS showed a T cell clone comprising ~12% of all T cells in the atypical case, suspicious for a primary cutaneous CD4+ small/medium T cell LPD [32], which is known to harbor a T cell clone (reviewed in [34,100]).

Similarly, the Belgian cohort with integrated clinicopathologic diagnoses showed false positive TRG PCR in 14 (16.4%) of 85 fresh (non-fixed) clinical specimens and two false negative TRG PCR cases, all of which were clarified by TRG NGS [51]. Their study also showed reliable findings in all 36 FFPE cases by TRG NGS [51].

In the multi-center European validation study of the EuroClonality–NGS Capture DNA assay [77], NGS successfully showed T cell clones at the TRG locus in all seven mature T cell lymphomas, in contrast with TRG FL-PCR that had identified only one of those seven cases as clonal. Table 7 shows the BIOMED-2 FL-PCR results compared with the EuroClonality–NGS assay results in seven mature T cell lymphoma cases (five ALCL and two AITL) that showed discordant results between FL-PCR and NGS, based on the publication [77]. As shown, combining both TRG and TRB (V-J and D-J) loci in the BIOMED-2 FL-PCR detected T cell clones in only 4 (57%) of 7 lymphoma cases, including 3 (60%) of 5 ALCL and 1 (50%) of 2 AITL [77].

Table 7. Results of BIOMED-2 T cell receptor FL-PCR assays and the EuroClonality–NGS DNA Capture assay in seven cases of mature T cell malignancies in the 2021 publication by Stewart et al. [77]. [a] Only TRG and the TRB targets were evaluated by FL-PCR in all 7 cases; the TRD (V-J, D-J) and TRA loci were not evaluated by FL-PCR by the submitting laboratories for any of these 7 cases [77].

	BIOMED-2 FL-PCR Results [a] [77]		**EuroClonality–NGS DNA Capture Assay Results [77]**				
Case #	**Clonal for TRG or TRB**	**Polyclonal for TRG, TRB, or both**	**TRD**	**TRG**	**TRB**	**TRA**	**Translocations**
Anaplastic large-cell lymphoma (*n* = 5 cases)							
1	None	Both TRG and TRB polyclonal	Clonal	Clonal	Clonal	Nonclonal	None
2	TRB clonal (V-J)	TRG polyclonal	Clonal	Clonal	Clonal	Clonal	ALK
3	None	TRG and TRB (V-J and D-J) polyclonal	Clonal	Clonal	Clonal	Nonclonal	ALK
4	TRB clonal (D-J)	TRG and TRB V-J polyclonal	Clonal	Clonal	Clonal	Nonclonal	ALK
5	TRB clonal (D-J)	TRG and TRB V-J polyclonal	Nonclonal	Clonal	Clonal	Clonal	ALK
Angioimmunoblastic T cell lymphoma (*n* = 2 cases)							
1	TRG clonal	TRB (V-J and D-J) polyclonal	Nonclonal	Clonal	Clonal	Clonal	None
2	None	Both TRG and TRB polyclonal	Nonclonal	Clonal	Nonclonal	Clonal	None

Five additional publications in 2023 and 2024 [46,84,89,90,92] studied various non-CTCL T cell malignancies by NGS. In the study of 60 patients with T cell malignancies undergoing monitoring for MRD that compared TRG and TRB Lymphotrack™ assays with ClonoSEQ MRD assays [84], the results from NGS assays from the two different manufacturers were not readily comparable due to differences in assay design [84], as was also noted by another group [50]. The primary reason for discordances between the Lymphotrack™ and ClonoSEQ TRB NGS assays was found to be the coverage of TRB pseudogenes, which were covered by ClonoSEQ but not in the Lymphotrack™ assay [84]. In the TRG assays by the same manufacturers, the discordances in the unique rearranged sequences involved JP rearrangements, with a three-base pair difference in the JP primer binding sites between the two assays [84]. The JP primer binding site in the ClonoSEQ assay was three base pairs downstream from the site in the Lymphotrack™ assay [84]. After accounting for these differences, the authors found both assays highly precise, sensitive, and specific [84].

3.1.4. Seven Studies That Used TRG NGS and TRB NGS to Evaluate T Cell Clonality

The seven studies in Table 1 that evaluated both TRG and TRB NGS studied clinical samples from patients with CHL at diagnosis and relapse in 2023 [46], CTCL and non-neoplastic skin diseases in 2015 [62], CTCL treated with mogamulizumab in 2022 [80], pediatric pityriasis lichenoides in 2022 [81], various T cell malignancies [77,84], including in the EuroClonality–NGS validation in 2021 [77] and for MRD analysis in 2023 [84], and ALK+ ALCL in 2024 [90]. Of note is that T cell receptor NGS does not help diagnose one of the diseases, pityriasis lichenoides, examined in one of these seven studies [81,93]. Another study used TRG NGS and TRB NGS, but the publication did not describe comparative results [80].

In the remaining five studies, TRG NGS and TRB NGS performed equally in the study by Kirsh et al., except that only TRG NGS had identified a T cell clone in a single case of γδ T cell lymphoma that was not detected, as expected, by TRB NGS [62].

The multicenter EuroClonality–NGS DNA Capture assay validation study, shown in Table 7 above, showed that TRG NGS performed better than TRB NGS [77].

The US study that evaluated MRD in various T cell malignancies showed that 67.8% (n = 19) of 28 samples were positive for a T cell clone by the TRG NGS assays from both manufacturers [84] (p. 336). In comparison, 60.7% (n = 17) of 28 samples were positive for a T cell clone by TRB NGS assays from both manufacturers [84] (p. 336). In an older FL-PCR study from the same institution that had studied 35 mycosis fungoides and 96 inflammatory dermatoses patients with 69 and 133 FFPE specimens, respectively, TRG and TRB FL-PCR assays for T cell clonality showed identical (64%) sensitivity and (84%) specificity for distinguishing mycosis fungoides from inflammatory dermatoses [74].

Table 8 shows the separate results of TRG and TRB NGS for T cell clonality in the 14 samples from 12 patients with relapsed neoplastic disease after an initial diagnosis of CHL [46]; it should be noted that comparing these results for TRG and TRB targets by a EuroClonality NGS amplicon-based assay was not the purpose of the authors' study.

Table 8. Results of TRB and TRG EuroClonality NGS amplicon-based assay in 12 patients with T cell clones identified retrospectively at diagnosis or relapse of classic Hodgkin's lymphoma (Table adapted from the data in the publication [46]). The entries with clonal results are placed in beige-colored rectangles. [a] The case numbers are shown according to the publication [46]. [b] Same clonotype at relapse as at diagnosis. [c] These diagnostic samples showed the clonotypes present in the relapse samples, albeit in smaller percentages than the defined threshold, and were retrospectively examined in the study [46]; to evaluate the assay performance in identifying the clonotypes, these samples are designated as 'clonal' in this table since the neoplastic clonotypes identified later at relapse were identified in these samples.

Case [a]	Sample at Diagnosis or Relapse	TRB by EuroClonality NGS: Clonal or Polyclonal		TRG V-J by EuroClonality NGS: Clonal or Polyclonal	Sample Conclusion for T Cell Clonality
		TRB V-(D)-J	TRB D-J		
1	Diagnosis	Clonal	Polyclonal	Clonal	Clonal
	Relapse	Clonal [a]	Polyclonal	Clonal [b]	Clonal
5	Diagnosis	Polyclonal	Clonal	Clonal [c]	Clonal
	Relapse	Polyclonal	Clonal	Clonal [b]	Clonal
11	Diagnosis	Polyclonal	Clonal [c]	Clonal [c]	Polyclonal
	First Relapse	Polyclonal	Clonal [b]	Clonal [b]	Clonal
	Second Relapse	Polyclonal	Polyclonal	Polyclonal	Polyclonal
17	Diagnosis	Polyclonal	Polyclonal	Polyclonal	Polyclonal
	First Relapse	Polyclonal	Polyclonal	Polyclonal	Polyclonal
	Second Relapse	Clonal	Polyclonal	Polyclonal	Clonal
23	Diagnosis	Not evaluable	Polyclonal	Clonal [c]	Polyclonal
	First Relapse	Clonal	Polyclonal	Clonal	Clonal
	Second Relapse	Polyclonal	Polyclonal	Polyclonal	Polyclonal
26	Diagnosis	Clonal	Polyclonal	Polyclonal	Clonal
	First Relapse	Polyclonal	Polyclonal	Polyclonal	Polyclonal
	Second Relapse	No specific product	Polyclonal	Polyclonal	Polyclonal
27	Diagnosis	Polyclonal	Polyclonal	Polyclonal	Polyclonal
	Relapse	Clonal	Clonal	Clonal	Clonal
34	Diagnosis	Polyclonal	Clonal	Clonal	Clonal
	Relapse	Polyclonal	Polyclonal	Polyclonal	Polyclonal
39	Diagnosis	Polyclonal	Polyclonal	Polyclonal	Polyclonal
	Relapse	Polyclonal	Clonal	Polyclonal	Clonal
41	Diagnosis	Polyclonal	Polyclonal	Polyclonal	Polyclonal
	Relapse	Polyclonal	Clonal	Clonal	Clonal
50	Diagnosis	Not performed			
	First Relapse	Clonal	Polyclonal	Polyclonal	Clonal
	Second Relapse	Polyclonal	Polyclonal	Polyclonal	Polyclonal
60	Diagnosis	Clonal	Polyclonal	Polyclonal	Clonal
	Relapse	Polyclonal	Polyclonal	Polyclonal	Polyclonal

As shown in Table 8, nine (64%) of the 14 samples with a neoplastic T cell clone were identifiable by the EuroClonality–NGS-amplicon-based assay for TRG. The five samples that were polyclonal by TRG NGS were identified as clonal by TRB V-(D)-J NGS (n = 4) and TRB D-J NGS (n = 1); these two targets for TRB identified all clonal cases by the EuroClonality NGS methods [46].

The fifth study evaluated TRG and TRB EuroClonality NGS in 23 cases of ALCL, of which five were negative for T cell clones by TRG and TRB NGS. Both TRB and TRG were clonally rearranged in 16 (70%) of 23 cases; 1 case each was clonal only for TRG and

TRB [90]. Table 9 shows these seven studies to highlight the heterogeneity in the NGS assays used and the samples examined.

Table 9. Summary of findings in seven cohort studies that used both TRB NGS and TRG NGS to evaluate T cell clonality. The colors in the rectangles depict the different sources of the assays. Abbreviations: CTCL, cutaneous T cell lymphoma; FFPE, formalin-fixed, paraffin-embedded; CHL, classic Hodgkin's lymphoma; ALCL, anaplastic large-cell lymphoma; AITL, angioimmunoblastic T cell lymphoma; PB, peripheral blood; SS, Sézary syndrome [a] Comparing assay performance was not this study's purpose [46].

	TRG and TRB NGS Assays	TRB Targets	Types of Samples, N Cases Compared, and Diagnoses Studied	Comparative TRG and TRB Interpretation Summary
Kirsch et al., 2015 [62]	Adaptive Biotechnologies, Seattle, USA	V(D)J	Skin; N = 110, in CTCL and non-CTCL diseases, and healthy donors	Both assays similar except in one case; only TRG NGS detected the γδ T cell neoplasm
Stewart et al., 2021 [77]	EuroClonality–NGS DNA Capture Assay, Europe	V(D)J and DJ	FFPE tissues and high molecular weight DNA, N = 7, T cell lymphomas (5 ALCL, 2 AITL)	TRG superior to TRB in the 7 cases with separate results available for TRB and TRG NGS; see Table 7
Trum et al., 2022 [80]	Laboratory-developed NGS assays, USA	V(D)J	Skin and PB; N = 14, relapsed or refractory CTCL or SS treated with mogamulizumab	Assays were not compared; comparing assays was not the study's purpose
Raghavan et al., 2022 [81]	Lymphotrack™ (Invivoscribe Inc., San Diego, USA)	V(D)J	Skin biopsies, N = 12, pediatric pityriasis lichenoides	NGS is not helpful in this diagnosis (results can be clonal or non-clonal)
Tung et al., 2023 [84]	Adaptive Biotechnologies, Seattle, USA, and Lymphotrack™ (Invivoscribe Inc., San Diego, USA)	V(D)J in both assays	PB and bone marrow aspirate samples; N = 28; various T cell neoplasms examined for minimal/measurable residual disease	TRG NGS superior to TRB NGS; see above text in this section
Van Bladel et al., 2023 [46]	EuroClonality–NGS amplicon-based assay	V(D)J and DJ	FFPE and fresh frozen tissues, N = 60, CHL at diagnosis and relapse only	TRB NGS better than TRG NGS in 14 samples; [a] see Table 8
Kalinova et al., 2024 [90]	EuroClonality–NGS amplicon-based assay	V(D)J	FFPE and fresh frozen tissues; N = 23; all ALCL, anaplastic lymphoma kinase (ALK) positive only	Both assays similar; TRG and TRB NGS identified one case each as clonal that was not identified as clonal by the other assay

As shown in Table 9, these seven studies alone used at least six different types of NGS assays, which alone could lead to differences in results due to differences in assay design. Only skin tissues, peripheral blood, and four bone marrow samples have been evaluated by both TRG and TRB NGS in the USA [62,84]; FFPE tissues and other fresh tissues have not been evaluated in the USA by TRB NGS. In these US studies, TRG NGS was better at detecting T cell clones than TRB NGS [62,84].

In contrast, in Europe, FFPE, fresh frozen tissues, and high molecular weight DNA have been evaluated [46,77,90] in various T cell neoplasms by the DNA Capture-based assay that evaluated all T cell receptor targets [77] and only CHL at diagnosis and relapse [46] and ALCL [90] by the EuroClonality–NGS-amplicon-based assay [46,90].

3.1.5. Two Studies Comparing NGS with Flow Cytometric Immunophenotyping to Evaluate T Cell Clonality

Two studies in Table 1 compared T cell clonality by TRB NGS with two separate FCI methods for evaluating the presence of monotypic αβ T cells [70,89], with the first one for T-PLL discussed above in Section 3.1.3 [64,70]. The constant chain of T cell receptor β 1 (TRBC1) antibody was identified in 2017 [101] after its original description in 1985 [102]. The Australian study published in 2024 [89] added the TRBC1 antibody to their flow cytometric immunophenotypic evaluation of T cell clonality [89]. The authors studied 90 samples,

including follow-up samples, in 31 patients with diverse T cell (CTCL and non-CTCL) malignancies and compared the results with TRB V(D)J NGS using the Lymphotrack assay to assess T cell clonality [89]. TRB NGS identified T cell clones in six (35%) of 17 samples from six patients that showed polytypic results by FCI [89] (pp. 914–917). In two of those six cases, TRB NGS identified rearranged sequences previously identified in the neoplastic specimens, thus confirming residual disease not identified by FCI [85] (p. 914). In the other four cases, previous sequencing results were unavailable, precluding evaluation of the significance of the NGS findings in those four cases. Still, the presence of active disease was noted in at least two of those four patients, suggesting that the T cell clones identified by NGS could represent disease [89] (p. 914). Interestingly, TRB NGS showed polyclonal results in two cases from patients with mycosis fungoides (n = 1) and PTCL-NOS (n = 1), in whom TRBC1 FCI showed a monotypic pattern; these two cases were also negative for somatic gene mutations by NGS [89] (p. 917).

3.1.6. Selected Recent Instructive Case Reports and Cohort Studies That Assessed T Cell Clonality by NGS or FL-PCR

Table 10 summarizes the patient demographics and clinical significance of five case reports published between 2016 and 2022 in the USA (n = 4) and the UK (n = 1).

Table 10. Summary of five instructive case reports that examined T cell clonality by NGS or fragment-length T cell receptor PCR assays.

Authors, Year Published, Country	Patient Age, Sex	Clinical Significance of the Case Reports
Hwang et al., 2021 [103]; USA	55 years, female	The presence of a false positive T cell clone by FL-PCR led to the misdiagnosis of a benign condition as a hepatosplenic γδ T cell lymphoma [103], which is an aggressive lymphoma [104]. NGS for T cell receptor clonality showed the polyclonal nature of the T cells and a hematopoietic stem cell transplant was avoided in the patient [103].
Zhang et al., 2016 [105]; UK	25 years, male	Acute Epstein–Barr virus (EBV) infection can present with histopathologic features and a clonal T cell population by FL-PCR to mimic a peripheral T cell lymphoma.
Rojansky et al., 2020 [106]; USA	38 and 69 years, males	Two cases of cutaneous T cell lymphoma wherein T cell receptor NGS and T cell receptor FL-PCR did not reveal a T cell clone, and only testing for somatic gene mutations revealed a clone and the neoplastic nature of these cases.
Cho et al., 2022 [107]; USA	81 years, female	Cutaneous lymphoid hyperplasia due to a tick bite with a clonal T cell population by FL-PCR and lambda light chain-restricted plasma cells mimicking primary cutaneous CD4+ small/medium T cell lymphoproliferative disorder and *Borrelia*-associated primary cutaneous marginal zone B cell lymphoma, respectively; the correct diagnosis was established by deeper histologic tissue sections showing tick parts.
Reeder and Wood, 2015 [108]; USA	51 years, male	Erythroderma and pseudo- Sézary syndrome due to antihypertensive medications occurred in the patient with histologic, flow cytometric immunophenotypic, and molecular clonality findings by FL-PCR mimicking neoplastic disease in skin and peripheral blood; the atypical findings resolved after stopping the medications, emphasizing the importance of clinical history in accurate diagnosis.

The case reports in Table 10 included two cases where T cell receptor clonality was examined by NGS [103,106]. The first case summarized in Table 3 had been diagnosed as a hepatosplenic γδ T cell lymphoma by two academic institutions [103]. In that case, NGS showed definite polyclonal T cells, and these NGS results were critical in avoiding the grave mistake of moving to a hematopoietic stem cell transplant for the misdiagnosis of a hepatosplenic γδ T cell lymphoma [103]. Two other cases in Table 3 [105,107] are included

as recently reported examples of infection-related non-neoplastic conditions that can mimic clonal lymphoid populations in lymphoid neoplasms. The final case is an example of a pseudolymphoma due to anti-hypertensive medications mimicking neoplastic disease [108]; a prospective study found drug eruptions to be a common cause of erythroderma with an acute onset [109].

With the same consideration, TRG clones in a polyclonal background were reported by TRG FL-PCR in 15 (18%) of 88 cases of another non-neoplastic lymphoproliferative disease called Kikuchi–Fujimoto disease [110]. This unique non-neoplastic condition (reviewed in [111]) can be accurately diagnosed by careful histopathologic and immunohistochemical examination in conjunction with clinical history. However, its differential diagnosis can be challenging, and this disease is well known to mimic and may be misdiagnosed as peripheral T cell lymphoma [112]. Therefore, as shown in the above-cited study [110], it is essential to be aware that a T cell clone by a commonly used TRG FL-PCR assay can be seen in this benign, self-limiting, non-neoplastic condition.

3.2. Additional Publications Relevant to Evaluating T Cell Receptor Clonality by NGS

This section includes NGS studies relevant to evaluating T cell clonality in patients with cytopenias and graft-versus-host disease (GVHD), an immunological complication of hematopoietic stem cell transplantation [113]. Other studies, including whole exome, transcriptome, and genome sequencing studies in mature T cell neoplasms, are also briefly described.

3.2.1. T Cell Receptor Clonality Assessment by NGS in Patients with Cytopenias

Since T cell receptor clonality is often evaluated in patients with cytopenias, as was seen in one of the publications in this review [19], the following reports are included below to keep in mind when evaluating NGS assays for clonality:

- An NGS study of the T cell repertoire in healthy individuals and patients with cancer showed that a continuous decrease in the diversity of the T cell repertoire in normal individuals begins at the age of 40 years [114]. In untreated patients with hematologic and non-hematologic cancers, there is lower diversity and increased clonality of T cell repertoires. Interestingly, after T cell depleting therapy in patients with cancer, the age-specific repertoire is regained even in older patients [114].
- NGS was used to examine the diversity of the T and B cell repertoires in 25 patients with primary and secondary autoimmune cytopenia who had available follow-up clinical samples. Patients with primary autoimmune cytopenia and 23 patients with active autoimmune hepatitis did not have physiological T cell clusters. These findings suggest that a T cell repertoire signature could act as a biomarker in blood for autoimmune conditions [115].

3.2.2. T Cell Receptor Clonality Evaluation by NGS in Patients with Acute Graft-Versus-Host Disease

Nollet et al. evaluated T cell clonality in at least one case of GVHD [51] in their study. GVHD is an immunologic complication of a hematopoietic stem cell transplant and can be acute or chronic (reviewed in [116]). The skin manifestations of acute GVHD may mimic those of drug hypersensitivity reactions, and it is essential to diagnose acute GVHD correctly [116]. In this context, a small study evaluated T cell clonality by TRB V(D)J NGS (Adaptive Technologies) in FFPE skin samples from ten patients with acute GVHD and seven patients with drug hypersensitivity reactions in the post-transplant setting [117]. The authors excluded polyclonal viral and bacterial exanthems in these patients [117]. A significantly higher T cell clonality was noted in patients with acute GVHD than in patients with drug hypersensitivity reactions [117]. In children with non-malignant diseases, acute

GVHD is associated with γδ T cell oligoclonality after an allogeneic umbilical cord stem cell transplant [118].

3.2.3. T Cell Receptor Clonality Studies by Other Methods, Including Whole Exome, Transcriptome, and Whole Genome Sequencing in Mature T Cell Neoplasms

In research settings, whole transcriptome sequencing was shown in 2017 to help determine T cell clonality in 65 (86%) of 76 PTCL cases [119], despite RNA sequencing only detecting productive gene rearrangements. A study from Singapore showed that copy number variant (CNV)-based analysis of whole genome sequencing (WGS) data could detect monoclonal T cell receptor rearrangements in T cell lymphomas [120]. In that study, WGS detected monoclonal TRG rearrangements in 4 (9%) of 44 cases of T cell lymphomas and 6 (13.6%) of 44 cases that were not detected by EuroClonality/BIOMED-2 FL-PCR for TRG and TRB, respectively [120].

In another study, sequencing after FL-PCR-based T cell receptor clonality assays showed evidence of biclonal or oligoclonal T cell clones arising from a common *TET2*-mutant cell, and a bias in the usage of the TRB variable genes, TRBV19*01 and TRBV27, in AITL and PTCL with a T-follicular helper phenotype [121].

In 2019, two groups studied skin and peripheral blood samples on a research basis [122,123], including exome sequencing and whole transcriptome sequencing [123]. Notably, single monoclonal TRG rearrangements were identified as the neoplastic clonotypes. Also, two to seven TRB clonotypes and multiple TRA clonotypes were identified instead of the expected finding of monoclonal TRB rearrangements. These findings indicated that the origin of the neoplastic cells in mycosis fungoides in at least some cases was at the precursor level after TRG was rearranged but not yet TRB or TRA, and not at the mature T cell stage [123], similar to the findings in the other study [122]. Subsequent studies that have studied the origin of neoplastic cells in mycosis fungoides and Sézary syndrome have also indicated that these mature T cell neoplasms likely arise at the precursor cell stage and subsequently transform in the periphery [124]. Hamrouni and colleagues discussed the clinical implications of this clonal heterogeneity in these neoplastic diseases [122], both for the diagnostic interpretation of a T cell clone in mycosis fungoides and Sézary syndrome by FL-PCR or NGS and for therapies to be directed at precursor cells and not locally at the skin level, for curing patients of these neoplasms [122].

Iyer and colleagues further characterized the T cell clonotypes in eight major subtypes of T cell lymphomas [125]. The authors deduced the stages of neoplastic (clonal) transformation from the relative frequencies of the dominant clonotypes for the rearranged TRG, TRB, and TRA genes. They showed that these lymphomas are oligoclonal in terms of the diversity of the T cell repertoire and that they all arise from precursor cells, not mature T cells [125]. The T cell receptor diversity was most significant in ALCL [125], likely explaining the lack of detection of a T cell clone in a few of these cases in many previous studies (with examples in [16,90]). The authors found a biased use of specific TRB, TRG, and TRA gene segments in various T cell lymphomas, with Vβ20-1 (TRBV20-1) being the most commonly used in cases of mycosis fungoides, Sézary syndrome, adult T cell lymphoma/leukemia, ALCL, and natural killer T cell lymphoma [125]. The authors reasoned that TRBV20-1 is most common due to its ubiquity in normal peripheral blood and other conditions (reviewed in [124]) and not due to antigen selection [125]. Other variable gene segments, TRBV20-1, TRBV4-1, TRBV5-1, TRAV14, TRAV35, or TRAV41, which were preferentially rearranged in T cell lymphomas, were also the most frequently used in normal human thymocytes [125].

3.3. Third-Generation Sequencing Compared with Next-Generation Sequencing for T Cell Clonality Assessment

In November 2024, a German group developed a protocol to evaluate T cell receptor clonality by long-read sequencing [126]. The authors compared Nanopore (ONT GridION platform) long-read sequencing with NGS (Illumina MiSeq platform) using amplicon-based EuroClonality NGS in 45 samples (27 FFPE, 9 fresh-frozen, and 9 isolated CD3-positive cell samples) from 45 patients with the following diagnoses: mycosis fungoides (n = 37), Sézary syndrome (n = 2), folliculotropic CTCL (n = 1), and non-CTCL diagnoses as polyclonal controls (n = 5) [126]. The most frequent clones in the neoplastic cases were identical by both platforms. Of note, the authors reported that TRG and TRB were not always dually rearranged; their patients' data shows that rearranged TRB clones were not identified in 4 (21%) of 19 FFPE cases of mycosis fungoides by either sequencing platform. In contrast, rearranged TRG clones were identified by both platforms in 100% (n = 22) of FFPE cases with the following diagnoses: mycosis fungoides (n = 19), epidermotropic CTCL (n = 1), and Sézary syndrome (n = 2) [126].

4. Discussion

This focused review highlights primary studies that have used NGS assays available for clinical testing with FL-PCR and FCI assays to evaluate T cell clonality in clinical samples of mature lymphoid proliferations. The review also describes other relevant studies evaluating T cell clonality in mature T cell neoplasms. The review indicates that targeted next-generation sequencing of the rearranged T cell receptor genes is precise and sensitive in detecting T cell clones in clinical samples submitted for diagnostic evaluation compared to FL-PCR. Targeted NGS has the unique added advantage of providing unique rearranged T cell receptor sequences termed clonotypes, which can be precisely tracked in samples collected from different sites and times when monitoring patients with lymphoproliferative disorders and mature T cell neoplasms. In contrast, the interpretation of FL-PCR assays is based only on the nucleotide size of the rearranged genes, which can include more than one type of sequence having the same size. FL-PCR results are qualitative.

In contrast with FL-PCR assays, NGS provides quantitative data, allowing for objectivity in evaluating the clonal T cells among all T cells present in the analyzed specimen. When assessed by TRG NGS, these findings apply to various types of clinical specimens, including peripheral blood, bone marrow aspirates, fresh tissues, and formalin-fixed, paraffin-embedded tissues, and they apply to specimens involved in cutaneous and non-cutaneous mature T cell lymphomas and lymphoproliferative disorders. In contrast with TRG NGS, it must be noted that TRB NGS for clonality evaluation in the USA has primarily been used in peripheral blood and skin, not in other formalin-fixed or non-fixed fresh tissues.

Considering the results of this scoping review for CTCL and non-neoplastic skin diseases, both TRG and TRB NGS are superior to FL-PCR in specific diagnostic situations: the diagnosis of early-stage CTCL, prognostication in mycosis fungoides, residual disease detection, and comparing disease clones between clinical samples from different anatomic sites or time, similar to the review focused on CTCL [93]. This review also indicates that *TRG* NGS and TRB V(D)J NGS perform similarly in distinguishing non-neoplastic skin samples from neoplastic skin samples. In these cases, TRG NGS is slightly better than TRB NGS because it can potentially detect clonality in γδ T cell neoplasms, as was seen in the study by Kirsch et al. [62] and the case report of a hepatosplenic γδ T cell lymphoma wherein TRG NGS was critical in avoiding an unnecessary hematopoietic stem cell transplant [105]. Developmentally, the TRG gene is rearranged early at the precursor T cell level and is not deleted at a later stage, unlike TRD, allowing TRG rearrangements

to also detect T cell clones in γδ T cell neoplasms, which TRB NGS cannot since TRB rearranges after TRG [7,127]. This review did not identify examples of dual (B cell and T cell) lineage-rearranged cutaneous lymphoid neoplasms [128] studied by NGS.

The neoplastic cells in most cases of CHL are derived from follicular germinal center B cells (reviewed in [1,26]). A recent study showed TRG clonal peaks by FL-PCR in 4 (4%) of 100 cases of CHL [129], and 3 of these 4 cases showed identical peaks in single microdissected neoplastic cells (Hodgkin and Reed–Sternberg cells), confirming a T cell origin in those cases of CHL [129]. Non-Hodgkin's lymphomas, including mature T cell lymphomas, are often considered in the differential diagnosis of CHL. In the paired diagnostic and relapse specimens in patients with CHL evaluated by a EuroClonality–NGS amplicon-based assay [44], the correct diagnosis of a relapsed T cell lymphoma instead of a CHL was made due to the presence of a T cell clone by NGS in conjunction with somatic mutational analysis showing the presence of T cell lymphoma-associated gene mutations followed by a retrospective review of the histopathology [44]. The treatment of these two neoplasms differs according to the current National Comprehensive Cancer Network guidelines [130–132].

NGS also detected low levels of the neoplastic T cell clone in staging bone marrow biopsies when morphologic evaluation could not identify the neoplastic infiltration in patients with nodal T–follicular helper cell lymphoma, immunoblastic type (or AITL) [92]. According to the response criteria after treatment of lymphoma [133] and the NCCN guidelines [130], 'if there is a response in the lymph node but persistent focal changes in the bone marrow, consideration should be given to further evaluation with magnetic resonance imaging or biopsy or an interval scan' [130,133]. Therefore, the detection of a neoplastic T cell clone in the bone marrow by NGS in a patient with a diagnosis of T cell lymphoma could conceivably be considered as needing follow-up and evaluation.

Precise diagnosis is required before precise treatment for any neoplastic disease and for including patients in appropriate clinical trials for novel and increasingly individualized therapies for each patient. Therefore, in nodal and other non-cutaneous lymphoid proliferations, if the result of the T cell clonality assay would make a difference in the diagnosis after considering all other clinical, pathologic, and immunophenotypic features, then NGS should be regarded as the tool to assess T cell clonality instead of FL-PCR assays. It is of utmost importance to note that the results must be interpreted in the context of other clinical, pathologic, immunophenotypic, and, if performed, somatic mutational analysis for diagnosis. Increased education should also be prioritized to ensure that the clinical care provider who integrates all findings, including for clonality evaluation, correctly interprets the results for accurate diagnosis.

In a French study of FFPE tissues from 82 T cell lymphoma samples and 25 non-neoplastic T cell infiltrates [134], targeted NGS for somatic mutations was more specific than T cell clonality analysis by BIOMED-2 FL-PCR assays [134]. Still, T cell clonality assessment helped diagnose those cases where mutational analysis was not contributory to diagnosis [134]. The findings indicate that T cell clonality analysis is essential in the diagnostic evaluation of mature T cell lymphoproliferations and lymphomas.

The constant chain of the T cell receptor β (TRBC) is expressed as two isoforms, TRBC1 and TRBC1, in αβ T cells. The Australian study comparing anti-TRBC1 by FCI with TRB V(D)J NGS described in this review showed T cell clones of uncertain significance in non-neoplastic cases, and TRB V(D)J NGS identified residual disease that was not detected by FCI [89]. Further, antibodies to both TRBC isoforms were recently used in multiparametric FCI to determine the presence of TRBC-restricted αβ T cells in pathologic specimens as a surrogate for T cell clonal populations, like the detection of κ or λ immunoglobulin light chain-restricted B cells in mature B cell neoplasms [135]. TRBC1-restricted, CD8+ T

cell populations of uncertain clinical significance were reported earlier in patients with no evidence of a T cell neoplasm [136]; these T cell populations of uncertain clinical significance were also present with the dual TRBC1 and TRBC2 staining by FCI [135].

In another study that evaluated TRBC1 by FCI [137], adding TRBC1 to a lymphoid screening tube detected monotypic T cell populations in 11.7% (n = 97) of 830 routinely examined peripheral blood (n = 636) and bone marrow (n = 194) samples by FCI. It is important to note that the monotypic TRBC1-restricted T cells included both monoclonal and oligoclonal T cells, including oligoclonal populations arising from benign non-neoplastic conditions [137]. As concluded by the authors, molecular analysis of T cell clonality is still required and cannot be omitted for assessing T cell clonality [137].

This scoping review highlights several limitations. Only TRG NGS has been evaluated in comparative FL-PCR and NGS studies in various clinical samples. TRB NGS has been assessed in only skin and peripheral blood samples in the USA. Among 908 samples compared by TRG FL-PCR or EuroClonality/BIOMED-2 FL-PCR and TRG, TRB, or both TRG and TRB NGS assays, the majority of samples examined were skin biopsies. These biopsies comprised 45% of all samples or 83% of all fresh tissues examined, with relatively few nodal or other organ biopsies, followed by peripheral blood comprising 22.5% of all analyzed samples. Comparative performance of FL-PCR and NGS is also relatively infrequent for FFPE tissues and bone marrow aspirates. This review also showed considerable variation in both FL-PCR and NGS assays used worldwide to evaluate T cell clonality. The results of NGS assays from the two manufacturers in the USA are not readily comparable [50,84], and comparisons with other NGS assays have not been performed, indicating that switching between assays for any given patient may not provide the same results, which is similar to the situation with FL-PCR assays.

The primary considerations for using NGS assays are accessibility, the lack of networks that can provide these assays, even in the USA, and the increased cost compared to that of FL-PCR assays. The costs and reimbursement for tests vary in the USA depending upon the region, the payer, and the treating institution. The cost of NGS for individual samples could be reduced if the volume of samples for testing is high and if only clonality, not MRD, is being evaluated since MRD testing requires a greater depth of sequencing than only clonality evaluation. Nevertheless, the cost still increases with the number of targets analyzed by NGS. Further, the cost of NGS testing for T cell clonality rises significantly as a send-out test; in the USA, this cost was four or five times greater than performing the same assay at the home institution [84]. While the EuroClonality–NGS group recommends the evaluation of all T cell receptor targets even by NGS, the cost of the test, accessibility to the test, and reimbursement for clinical laboratories and patients are a significant concern in many countries, including the USA. The decision for assay choice would ultimately depend on the individual institution or provider's needs, accessibility, resources, costs, and reimbursement situations and could vary between providers.

Nevertheless, the findings in clinical samples for clonality detected at the TRG and TRB loci, including by NGS and long-read sequencing, suggest that TRG should be tested first if only one T cell receptor target is to be analyzed by NGS. The evidence from studies in the research setting [122–125] corroborates the observations from the reviewed clinical studies. Additional studies are necessary for evaluating TRB NGS in non-cutaneous specimens, at least in the USA.

Since detailed clinical information was unavailable for the cases of mature T cell neoplasms examined by whole exome sequencing [125], additional studies to define the T cell repertoire and oligoclonal T cell populations in well-characterized patients with various types of mature T cell neoplasms and with available detailed clinical information should be pursued to possibly enhance the capabilities for precise diagnosis of mature T

cell neoplasms in the clinical setting. With the ongoing efforts to develop therapies targeted at the structure of the T cell receptor [138–141], such studies could also help to understand the differences between these T cell neoplasms to enable the development of appropriate and effective therapies for these patients.

5. Conclusions

The following conclusions can be made from the evidence available from the current literature:

(1) When NGS-based clonality assay findings are integrated with all other pathologic, immunophenotypic, and clinical features, as required for precise diagnosis and classification of the type of lymphoma (if neoplastic), T cell receptor NGS is highly specific and sensitive. This performance of T cell receptor NGS is highly desirable for accuracy in diagnosing mature T cell neoplasms, which are often aggressive diseases requiring precise diagnosis before appropriate treatment and enrollment in clinical trials.

(2) As with FL-PCR, it is critical that NGS clonality assay findings are interpreted in the context of other clinical, histopathologic, immunophenotypic, and molecular genetic findings; clonality assay findings by any molecular assay for clonality must never be interpreted in isolation.

(3) NGS of the rearranged T cell receptor genes has been evaluated in clinical samples from patients with various types of mature T cell neoplasms, including CTCL and non-CTCL lymphoid neoplasms in the USA, UK, Europe, and Australia. Diagnosing mature T cell lymphoid neoplasms is challenging and, in 2024, requires integrating all clinical, histologic, immunohistochemistry, immunophenotypic information, and molecular genetic findings with T cell receptor clonality analysis as an essential tool for this critical purpose. NGS for T cell clonality assessment is less accessible and costs more than FL-PCR. Still, NGS is superior to FL-PCR assays due to its high specificity, sensitivity, and precision in tracking individual unique rearranged sequences (or clonotypes).

(4) TRB NGS and TRG NGS assays for T cell clonality assessment have been compared primarily with TRG PCR since TRG is the most widely used target for evaluating clonality by FL-PCR.

(5) Skin and peripheral blood specimens comprise the primary specimens compared by FL-PCR and NGS, with fewer nodal or other organ biopsies and FFPE tissues.

(6) FFPE tissues and bone marrow aspirates have only been compared by FL-PCR and TRG NGS, not TRB NGS.

(7) From direct comparative studies, TRG NGS performance is superior to TRB NGS in evaluating T cell clonality, including detecting MRD in patients with mature T cell lymphoid neoplasms.

(8) Based on the currently available evidence, if only one T cell receptor NGS assay is to be used for evaluating T cell clonality in the diagnostic evaluation of mature T cell lymphoid proliferations, it should be TRG NGS.

(9) A reduction in costs, increased accessibility, including test reimbursements, and education are required to apply NGS for clonality evaluation in routine clinical diagnosis.

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