Mutational profile dynamics in follicular lymphoma and large cell transformation

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ABSTRACT

Aims Follicular lymphoma (FL) is characterised by significant heterogeneity in both the clinical trajectories and the molecular profiles. This study aimed to investigate clonal dynamics in FL by analysing mutation profiles at various time points during the disease course including at histological transformation (HT), to gain insight into the mutational changes over time.

Methods We retrospectively analysed 76 biopsies from 25 patients, including 13 cases with three or more FL biopsies and 12 cases with subsequent HT. Hybrid capture-based Next-Generation Sequencing (NGS) with the EuroClonality-NGS DNA capture (EuroClonality-NDC) assay was used to examine clonal rearrangements and mutations.

Results A total of 204 (potentially) pathogenic mutations were identified. Only 40% of mutations remained stably present during a median follow-up period of 139 months (range 9–198). *KMT2D* and *CREBBP* were the most frequently mutated genes at diagnosis, exhibiting relative stability in follow-up biopsies. Conversely, *EZH2* displayed a dynamic pattern of mutations gained and lost during the disease course. At HT, pathogenic mutations affecting *B2M*, *MYC* and *TP53* emerged. Changes in mutational burden were observed in both FL-sequential and diagnosis-transformation cohorts, with more pronounced changes in the latter.

Conclusions This real-world study provides insights into the complex molecular pathogenesis of FL and HT. As targeted therapies emerge as treatment modalities, mutational profiles could influence treatment decisions in the future. Therefore, recognising the significant changes occurring in the mutational landscape of FL throughout the disease course is crucial.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Follicular lymphoma (FL) exhibits considerable variability in molecular profiles. While previous studies provided insight into the mutational landscape and molecular subclusters, they predominantly focused on single time points, leaving the dynamic nature of these mutations over the course of the disease less understood.

WHAT THIS STUDY ADDS

⇒ This study highlights the dynamic behaviour of mutational profiles in FL, showing that only 40% of mutations remain stable over time, and highlights the mutational dynamics of *EZH2*. Additionally, it provides evidence of more pronounced changes in the mutational profile during the transformation compared with recurrent FL.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Understanding the evolving mutational landscape in FL will be crucial for future clinical practice as target therapies become more common and ensuring accurate interpretation of genetic data.

INTRODUCTION

Follicular lymphoma (FL) is the most common indolent non-Hodgkin's lymphoma diagnosed in Western countries. Despite a median survival exceeding 15 years, FL's clinical course varies widely, with some patients remaining stable under a 'watch and wait' (WW) approach, while others need multiple lines of immuno-chemotherapy due to relapses.[1 2] Annually, 2%–3% of FLs undergo histological transformation (HT) predominantly to diffuse large B-cell lymphoma (DLBCL), leading to inferior survival.[3]

BCL2 rearrangements and *CREBBP* mutations have been identified as among the earliest events in FL pathogenesis. CREBBP mutations can be detected years before diagnosis, primarily manifesting as missense changes in the lysine acetyltransferase (KAT) domain.[4] Other genetic alterations associated with FL development affect chromatin modifier genes and genes associated with transcription factors, oncogenic pathways and microenvironment pathways.[5–11] Different molecular subclusters of FL have been linked to variations in these pathways.[12] A precursor cell population can exist long before diagnosis and contribute to relapse propagation.[11 13]

At present, FL prognosis relies primarily on clinical findings, molecular risk stratification methods like M7-Follicular Lymphoma International Prognostic Index (FLIPI), Progression of Disease within 24 months Prognostic Index (POD24-PI) and Mutations Associated with Progression (MAP) signature have been proposed but not implemented in clinical practice.[8 14 15] Their predictive value remains unvalidated and may be influenced by treatment and intratumoral subclonal complexity.[11 16–18]

Accurate interpretation of genetic alterations requires a deeper understanding of the genetic changes during the disease course. Generally, genetic complexity increases over time, potentially

Table 1 Patient characteristics

	D-T cohort	FL-seq cohort
Patients	12	13
Biopsies	33	43
Location diagnosis biopsy (%)		
Nodal	11 (92)	13 (100)
Extranodal	1 (8)	0 (0)
Mean age at diagnosis (range)	60 (42–74)	50 (40–61)
Female (%)	5 (42)	3 (23)
Stage (%)		
I–II	1 (8)	0 (0)
III–IV	11 (85)	100 (100)
FlIPI score (% of known)		
Low	1 (8)	1 (8)
Intermediate	8 (67)	9 (69)
High	3 (25)	3 (23)
Ki67		
Mean (range)	22 (10–30)	18 (10–30)
>20 (% of known)	4 (36)	3 (23)
Unknown	1	0
Grade (% of known)		
1–2	12 (100)	11 (85)
3A	0 (0)	2 (15)
BCL2 translocation* (% of known)		
Present	8 (80)	5 (100)
Not present	2 (20)	0 (0)
Unknown	2	8
MYC translocation† (% of known)		
Present at HT	4 (36)	NA
Not present at HT	7 (64)	NA
Unknown	1	NA
First line management (%)		
WW	5 (42)	7 (54)
Systemic treatment	6 (50)	5 (38)
Radiotherapy palliative	0 (0)	1 (8)
Radiotherapy curative	1 (8)	0 (0)
Progressor after diagnosis‡		
Early progressor (%)	4 (33)	5 (38)
Late progressor (%)	8 (67)	8 (62)
Prognostic scores at diagnosis (% early progressor)		
FLIPI high	3 (67)	3 (33)
M7-FLIPI high	1 (100)	2 (0)
POD24-PI high	2 (50)	3 (33)
Treatment lines§		
R-C(H)OP	14	14
Radiotherapy (patients)	5	6
Anti-CD20-bendamustine	5	4
R-chloorambucil	1	2
R-FC	2	0
Stem cell transplantation (auto/allo)	0	7
R-monotherapy	0	2
R-lenalidomide bendamustine	0	1
Idelalisib	0	1
Other	4	1

*BCL2 translocation was considered present if positive in at least one biopsy. Evaluated with fluorescence in situ hybridisation (FISH), PCR or classical cytogenetics.
†*MYC* translocation was evaluated in transformation biopsies by FISH.
‡Patients were classified as an early progressor if a new indication for systemic treatment, histological transformation or death from any cause occurred within 24 months after diagnosis.
§Patients can receive >1 treatment line.
allo, allogeneic; auto, autologous; D-T, diagnosis-transformation; FC, fludarabine, cyclophosphamide; FLIPI, Follicular Lymphoma International Prognostic Index; FL-seq, sequential follicular lymphoma; HT, histological transformation; NA, not applicable; R, rituximab; R-C(H)OP, rituximab, cyclophosphamide, (doxorubicin), vincristine, prednisone; WW, watch and wait.

influenced by factors like time, mutagenic effects and selective pressure from treatment, although this has not been individually assessed in patients with FL.[7 12 15 19–25] Earlier studies found a predominantly branched evolutionary model at HT, while a more varied but predominantly linear model for patients without transformation.[20 23 26] Additionally, (post-treatment) relapses were shown to more commonly originate from a branched evolution of ancestral clones, rather than linear evolution from dominant clones.[6 7 11 20 22 27]

This study analyses mutational profiles in FL at multiple timepoints and at HT, highlighting the mutational dynamics of these mutations, which becomes more relevant as mutational analysis is becoming an increasingly important tool in the diagnosis and treatment of lymphoma.

MATERIAL AND METHODS

Detailed methods are provided in online supplemental file 1.

Participants and outcomes

Adult patients diagnosed between 2000 and 2018 with FL grade 1–3A according to the revised 4th WHO classification were identified from the archives of the Pathology departments of the Radboudumc Nijmegen and Rijnstate Hospital Arnhem, the Netherlands.[28] Patients needed to meet the following criteria: (1) adequate material for DNA isolation at both diagnosis and HT, or from at least three FL biopsies, including the diagnostic biopsy, (2) complete clinical information, (3) no other malignancy at diagnosis and (4) no (immuno-)chemotherapy before diagnosis.

Ultimately, 25 patients were included (online supplement 1, figure 1). They were divided into two cohorts: The diagnosis transformation (D-T) cohort (12 cases, follow-up range 9-180 months) and the FL sequential (FL-seq) cohort (13 cases, follow-up range 15-196 months). Overall survival (OS), time to transformation (TTT), and progression-free survival (PFS) were calculated from diagnosis, with progression defined as systemic treatment, HT or death. Early progressors had PFS shorter than 24 months with an event.

A pathology review was performed for all samples (MvdB, EH). Tumour percentage was assessed on H&E stained sections; excluding samples with <20%.

Molecular- and bioinformatic analysis

Genomic DNA was extracted, samples with DNA quantity <2.5 ng/µL or quality <200 bp were excluded. Library preparation and hybridisation followed the KAPA HyperCap (HyperPlus) Workflow V.3.0 (Roche Sequencing Solutions, Indianapolis, IN, USA) as per the EuroClonality-NDC Assay Quick Guide (Univ8 Genomics, Belfast, UK). The sequencing panel covered the coding sequences or hotspots of 83 genes (online supplement 1, table 1). Pooled enriched libraries were sequenced on the NovaSeq 6000 (Illumina, Cambridge, UK) (performance; online supplemental 1, table 2). Data were analysed using the EuroClonality-NDC analysis app.[29]

Analysis of immunoglobulin rearrangements and sequence variants

Detection of clonal rearrangements and sequence variants was performed as previously described.[29] Samples with matching clonal immunoglobulin sequences (IgH, IgKL, IgL) were deemed clonally related. Samples with no clonal rearrangement were

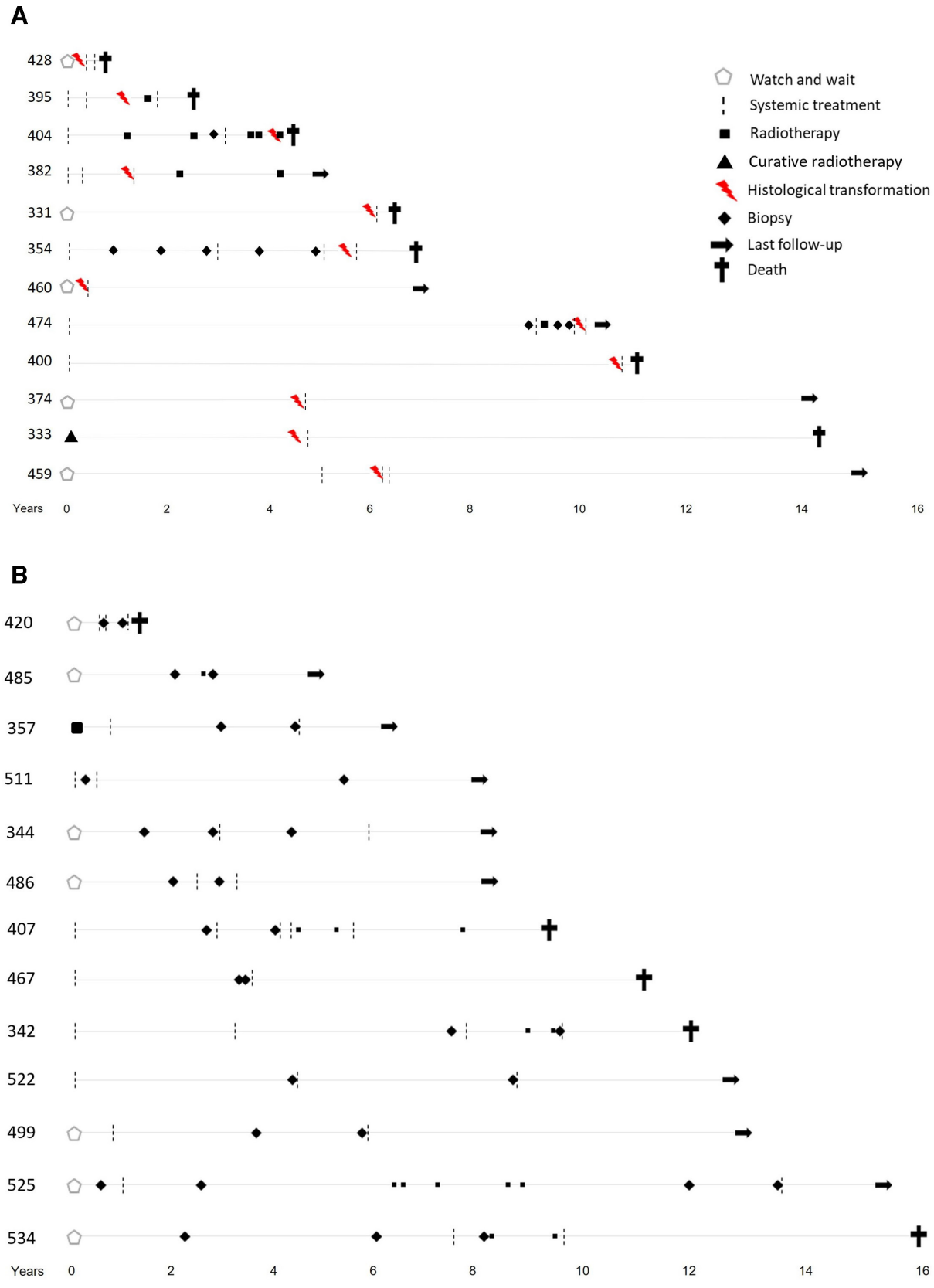

Figure 1 Individual timelines of the diagnosis-transformation (D-T) and sequential follicular lymphoma (FL-seq) cohorts. (A) Individual timelines D-T cohort. (B) Individual timelines FL-seq cohort. Case no. is presented on the Y-axis, and time in years is presented on the X-axis. Legend is presented in the figure.

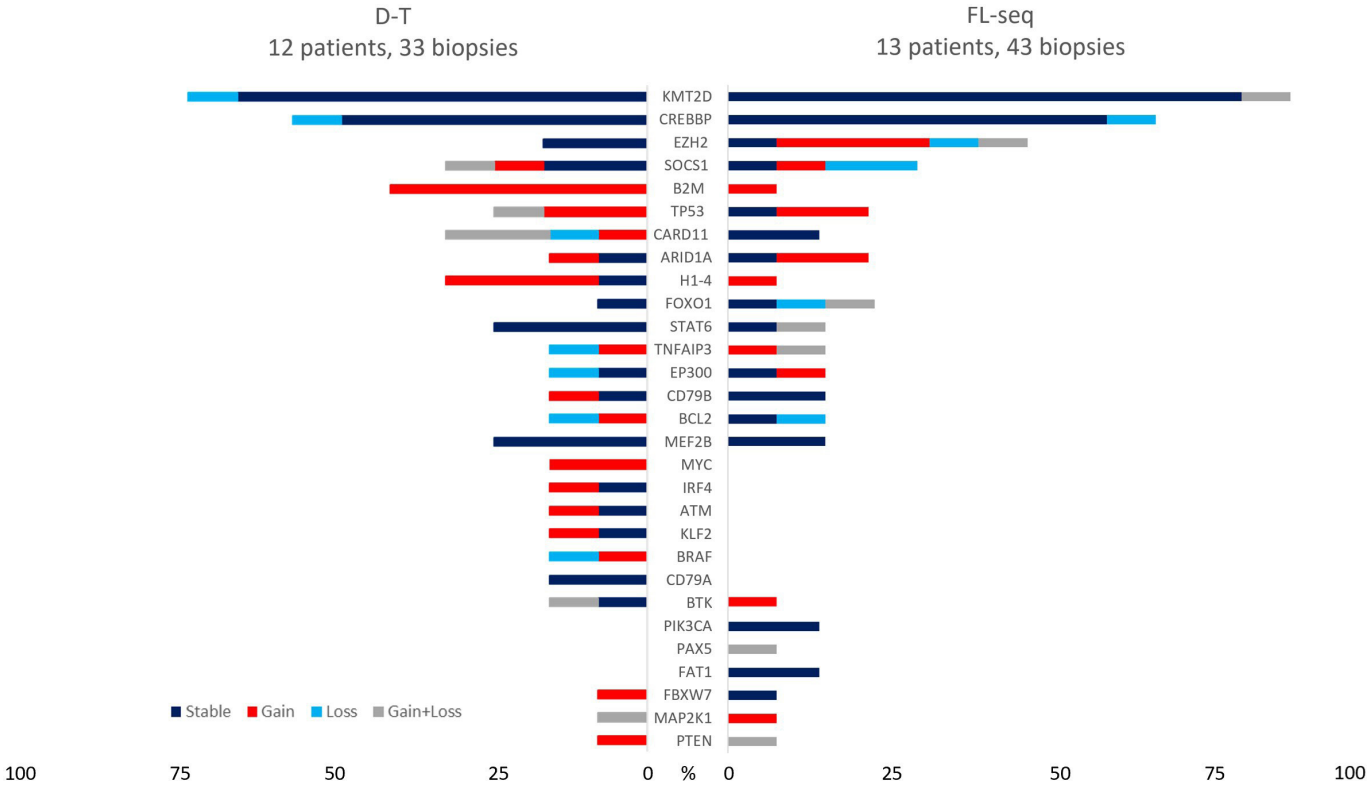

Figure 2 Evolutionary trajectories of affected genes across study cohorts. Presentation of genes affected more than once in percentage of patients. D-T cohort on the left, FL-seq cohort on the right. Gene stability, assessed across the entire follow-up period, indicated by colour. Legend is presented in the figure. D-T, diagnosis-transformation; FL-seq, sequential follicular lymphoma.

excluded. In all cases, the rearranged immunoglobulin confirmed the clonal relationship. Sequence variants were analysed using a ≥4% allele frequency threshold and ≥10 reads.

Assessment of mutations and evolution

Pathogenicity was scored for all mutations, excluding likely benign mutations (online supplement 2, table 3). Evolution was categorised as linear, branched or none based on mutational changes, with delta (δ) calculated as the mean gene changes per patient across biopsy intervals.

Statistical analysis

Mutations showed a non-normal distribution and were analysed using Wilcoxon, Mann-Whitney U and χ^2 tests. Survival outcomes were analysed using Kaplan-Meier and log-rank tests. Statistical significance was set at $p<0.05$, using IBM SPSS Statistics V.29.0.0.0.

RESULTS

Patient characteristics and follow-up

The characteristics of both cohorts are summarised in table 1. The median biopsy interval was 18 months (range 1–132), with 41% of the biopsies being followed by a WW regimen, while 52% received systemic treatment and 7% radiotherapy. First-line immunochemotherapy mainly consisted of rituximab, cyclophosphamide, vincristine and prednisone. Figure 1A,B illustrates individual timelines; online supplement 2, table 4 provides detailed information on treatment modalities.

In the D-T cohort, the median TTT was 54 months (range 1–132), four patients did not receive treatment before HT, and all transformations presented morphologically as DLBCL. The presence of a *MYC* translocation, assessed using fluorescence in situ hybridisation, was known for all cases of transformation except one and was present in 4/11 biopsies. Of these, two biopsies also carried a *BCL2* translocation and can therefore be classified as DLBCL with *MYC* and *BCL2* rearrangements (double hit lymphoma). In the FL-seq cohort, one patient did not need any systemic treatment during follow-up (53 months) and five cases (38%) were classified as early progressors after diagnosis.

Of note, when analysing all cases together, despite the variation in treatment modalities at diagnosis, the FLIPI score, when assessed binary, was predictive of PFS (log-rank p=0.02). In contrast, the m7-FLIPI and POD24-PI scores did not show predictive value for PFS. None of these scores were able to accurately identify early progressors in either of the study cohorts (table 1).

Mutational profile with changes during the disease course

KMT2D (84%) and *CREBBP* (64%) were the most frequently affected genes in both cohorts and mostly remained stable during the disease course (figures 2 and 3A,B). Particularly, missense mutations in the KAT domain of *CREBBP* remained stable in 94% (15 out of 16 mutations). Conversely, *B2M* and *MYC* mutations were solely acquired during follow-up. *TP53* was equally affected in both study cohorts (three cases), with one case in the D-T cohort having a transient mutation in the biopsy before HT, but not in the transformation itself. *EZH2* mutations were more prevalent in the FL-seq cohort (46% vs 25%), with frequent gains or losses of mutations during the disease course; only a single mutation was stably present. Mutations in *MEF2B*, *B2M* and *MYC* were more commonly found in the D-T cohort. These findings suggest a different genetic landscape in both study cohorts,

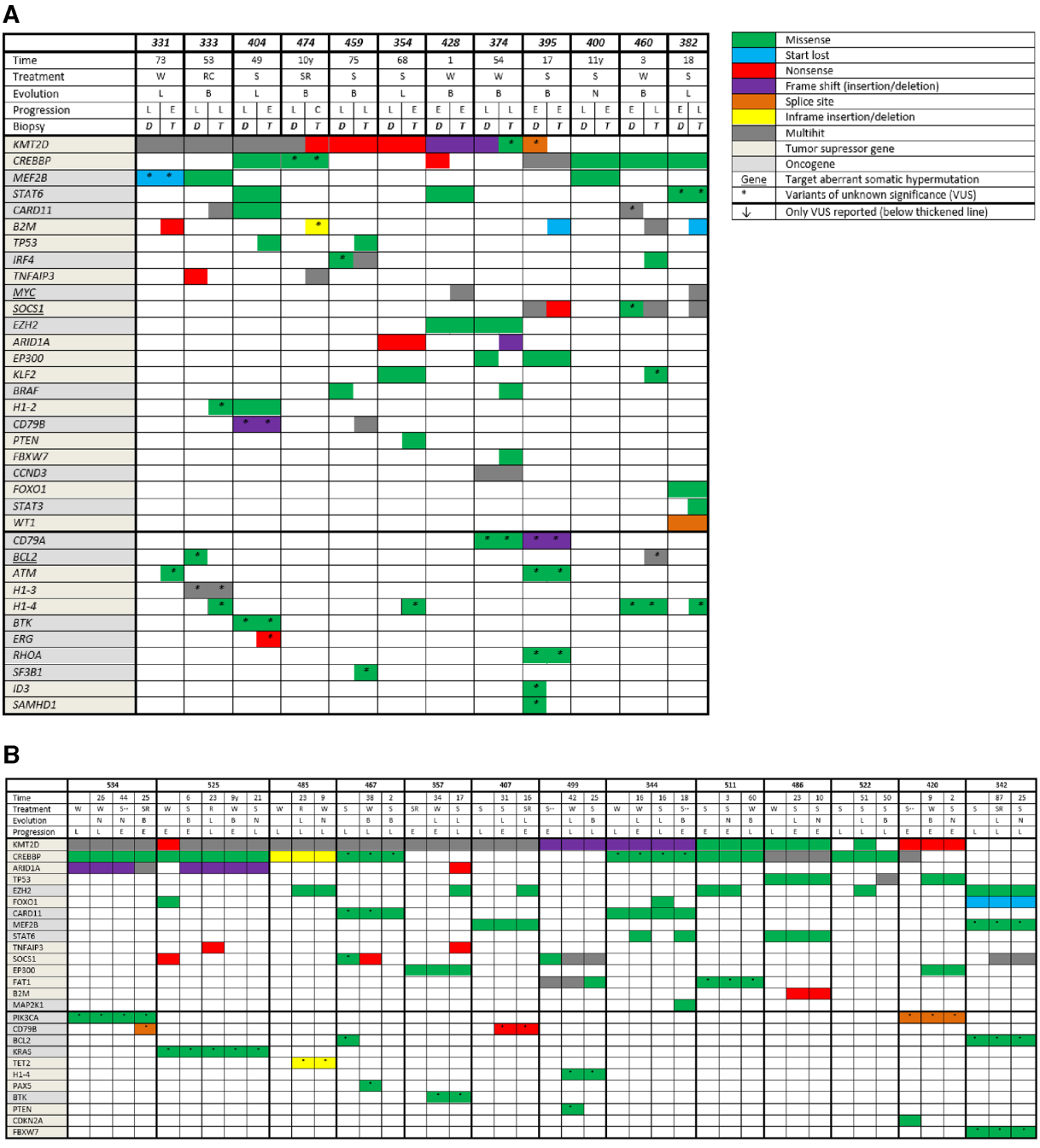

Figure 3 Oncoplots of the diagnosis-transformation (D-T) and sequential FL (FL-seq) cohort. (A) Oncoplot D-T cohort. (B) Oncoplot FL-seq cohort. Clinical parameters: Time: time between biopsies in months, unless exceeding 99 months, in which case it is represented in years denoted by 'y'. Treatment: treatment between biopsies (W=watch and wait, S=systemic treatment, R=radiotherapy, RC=radiotherapy curative, S**=systemic treatment after watch and wait). Evolution: evolution pattern between biopsies (N=no evolution, B=branched evolution, L=linear evolution). Progression: indication for new systemic treatment, histological transformation or death of any cause after biopsy (L=late progressor, no progression within 24 months after biopsy, E=early progressor, progression within 24 months after biopsy, C=censored). D=diagnosis biopsy, T=transformation biopsy. Legend is presented in the figure.

with distinct evolutionary trajectories observed for several genes, highlighting their varied roles in lymphomagenesis.

Clonal evolution in the diagnosis-transformation cohort

The mean number of affected genes was 4.2 (range 2–9) at diagnosis, and 5.7 (range 2–9) at HT, showing a significant difference (p=0.017) (figure 4A). The mean δ of the total cohort amounted to 3.1 (range 0–5). Of note, in two cases, transformation was diagnosed within 3 months of the initial diagnosis. In case ID 428, the acquired *MYC* mutations were already present in the diagnostic biopsy but fell below the detection threshold. In case ID 460, the acquired mutations identified in the transformation biopsy were absent in the diagnostic biopsy. In this case, the transformation biopsy was taken from the bone marrow, so the differences could also reflect spatial heterogeneity.

Branched evolution was most commonly found (n=7), followed by linear (n=4) and no evolution (n=1).

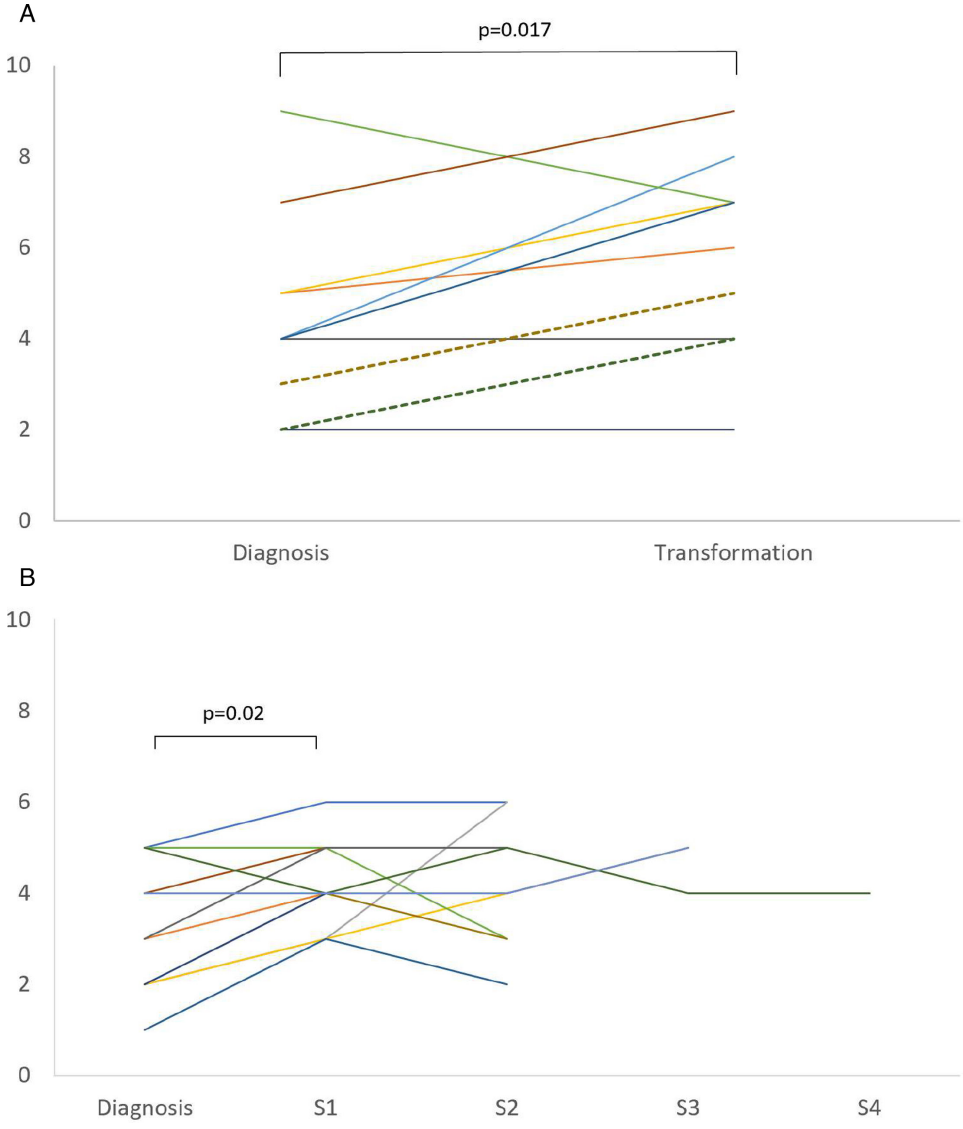

Figure 4 Number of affected genes over time. (A) Number of affected genes over time diagnosis-transformation cohort. (B) Number of affected genes over time sequential follicular lymphoma cohort. The Y-axis depicts the number of affected genes. The X-axis depicts data points corresponding to the time points of diagnosis, sequential biopsy 1 (S1), sequential biopsy 2 (S2), sequential biopsy 3 (S3), sequential biopsy 4 (S4) and at histological transformation. Each line represents an individual patient, and dotted lines represent two patients.

Differences were observed between patients with respect to changes in the mutational profile. Case no. 354 represents an example of a relatively stable mutational profile which was found across seven biopsies spanning nearly 6 years, including three lines of systemic treatment (figure 5A). Notably, a *TP53* mutation is detected in sequential biopsy (S) 4, acquired after second-line systemic treatment. The VAF of approximately 50% of the tumour percentage, comparable to other stable mutations in this biopsy, suggests that the mutation affects a single allele, without evidence of copy number aberrations (CNA) or a subclonal mutation. This mutation was subsequently lost in another biopsy taken from another anatomical site, in the absence of selective pressure due to treatment. In contrast, case no. 474 showed a very dynamic mutational profile (figure 5B).

Taken together, these findings demonstrate that the number of affected genes increases at HT. Moreover, distinct patterns of mutational stability and dynamics, evident in both short and long biopsy intervals, and with and without systemic treatment are apparent.

Clonal evolution in the FL-sequential cohort

The mean number of mutated genes was 3.4 (range 1–5) at diagnosis, 4.2 (range 3–6) at S1 and 4.2 (range 2–6) at S2, showing a significant difference between D and S1 (p=0.02) (figure 4B). The mean δ was 1.5 (range 0–4) from diagnosis to S1 and 1.1 (range 0–3) from S1 to S2. The mean δ per patient throughout the disease course was 1.3 (0.33–2.5). Linear evolution was observed in 12 biopsy intervals, branched evolution in 10 intervals, and no evolution in eight intervals.

Different patterns of clonal evolution are illustrated by case nos. 344 and 525 (figure 5C,D). Case no. 344 displayed stable mutations in *CARD11*, *CREBBP* and *KMT2D*, with additional mutations in *STAT6*, *FOXO1* and *MAP2K1*. Interestingly, the mutation profile in S2 branched from that of S1 while the mutation profile at S3 more closely resembled that of S1, which can be explained by systemic treatment after S2, resulting in the outgrowth of a new subclone. In case no. 525, additional mutations were already found in S1 6 months after diagnosis, but

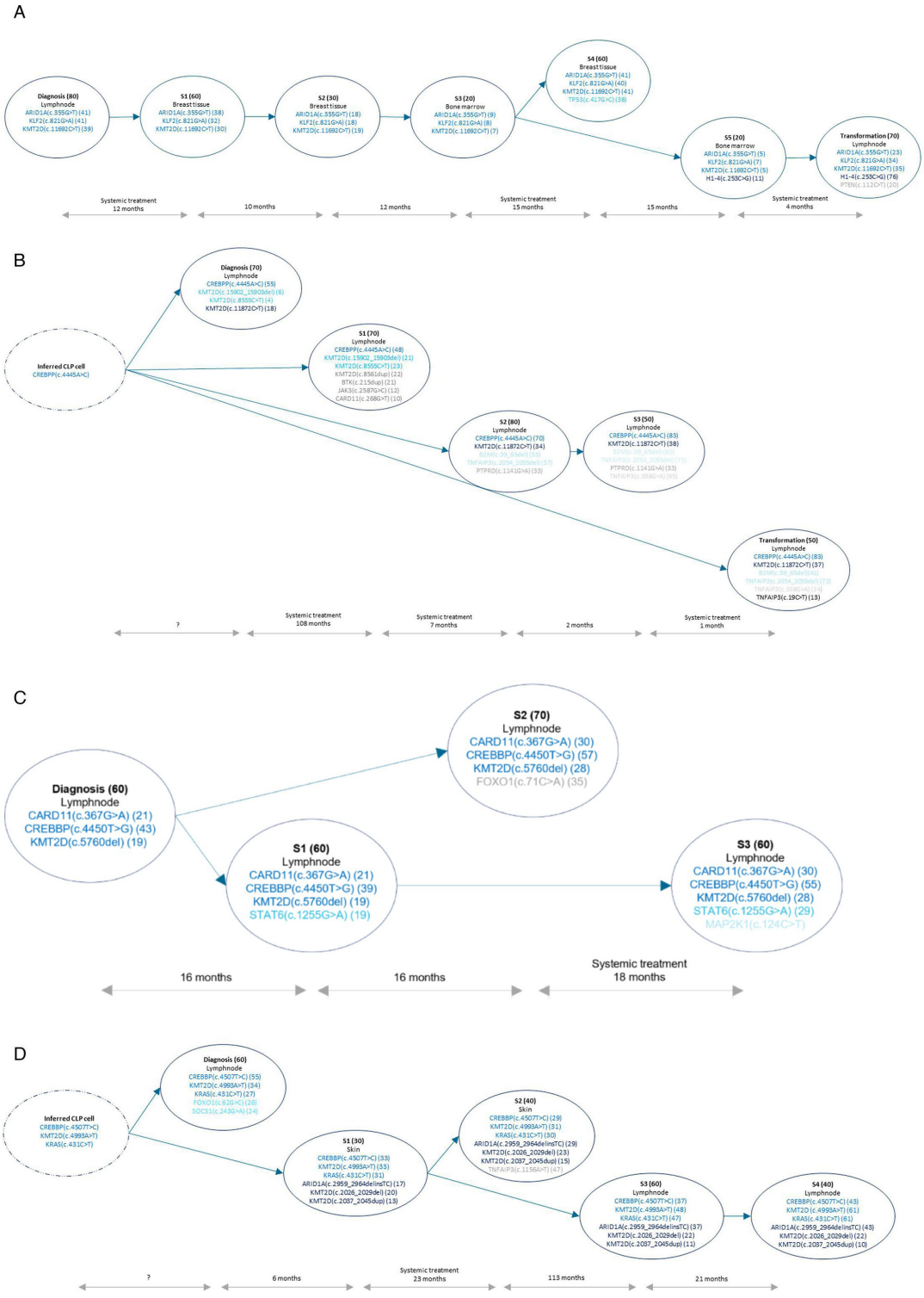

Figure 5 Details of clonal evolution in selected cases. (A) Case no. 354 demonstrates a relatively stable mutation profile across seven biopsies spanning nearly 6 years, including three lines of systemic treatment. Notably, a *TP53* mutation is detected in sequential S4 but subsequently lost in the absence of selective pressure due to treatment. (B) Case no. 474 presents a dynamic mutation profile; S1 shows a branched evolution, 9 years after diagnosis including one line of systemic treatment, possibly originating from an antecedent ancestral clone. S2, S3 and the transformation biopsy show a relatively stable mutation pattern within short biopsy intervals and one line of systemic treatment. (C) Case no. 344 displayed stable mutations in *CARD11*, *CREBBP* and *KMT2D,* with biopsy intervals roughly distributed equally (16, 16 and 18 months). The mutation profile in S2 differed from that in S1. Interestingly, the mutation profile in S3 more closely resembled that of S1, possibly as a result of systemic treatment after S2. (D) In case no. 525, additional mutations were found in S1 6 months after diagnosis, followed by a notably stable mutational profile observed over a 13-year timeframe. Each oval represents a biopsy. Tumour percentages are denoted in bold parentheses after the biopsy title, the anatomical side is specified underneath. Variant allele frequencies are indicated in parentheses after mutations. Corresponding colours are assigned to mutations found in matching biopsies. CLP cell=clonally related lymphoma precursor cell, S1=sequential biopsy 1, S2=sequential biopsy 2, S3=sequential biopsy 3, S4=sequential biopsy 4, S5=sequential biopsy 5.

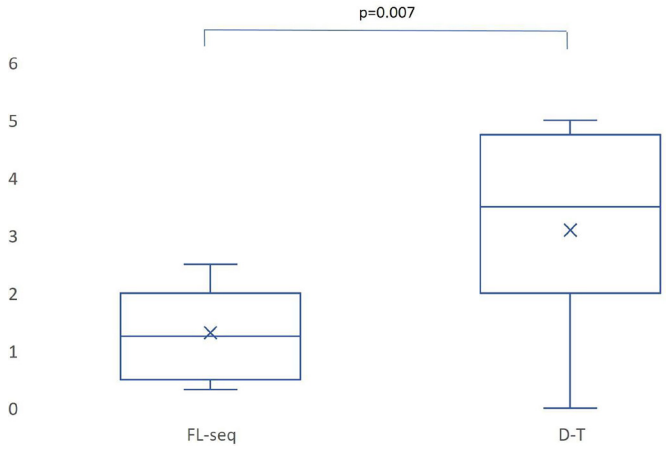

Figure 6 Changes in the mutational profile in the diagnosis-transformation cohort versus the sequential FL cohort. Boxplot illustrating the mean change in mutations (delta, δ) per patient. δ is calculated as the sum of changes in affected genes.

this was followed by a very stable mutational profile observed over a 13-year timeframe. Thus, these exemplify the fluctuating dynamics of mutational profiles within the disease course of FL. Major changes can occur within a short time interval after diagnosis even in the absence of systemic treatment, followed by a stable pattern persisting for years thereafter.

Clonal evolution in transformed FL versus sequential FL

When comparing the D-T and the FL-seq cohorts, the latter was genetically more stable and less heterogeneous (online supplemental 1, table 5). The mean δ of mutated genes per patient was significantly lower in the FL-seq cohort compared with the D-T cohort (p=0.007, mean 1.3 (range 0.33–2.5) vs mean 3.1 (range 0–5)) (figure 6). These data indicate that there is more genetic stability in the FL disease course compared with the evolution towards HT.

DISCUSSION

We explored the clonal evolution by assessing whether genes are affected by potentially pathogenic mutations in sequential FL biopsies and HT over an extensive follow-up period. Despite the limited number of cases, our detailed molecular analysis provides insights into this highly heterogeneous process in both study cohorts. We identified *KMT2D* and *CREBBP* as genes exhibiting relative stability throughout the disease course and at HT, whereas *EZH2*, *MYC*, *TP53* and *B2M* displayed a dynamic pattern. Additionally, we observed distinct evolutionary trajectories that cannot be solely explained by influences of time or treatment-induced selective pressure. When comparing both study cohorts the D-T cohort was genetically more heterogenous and dynamic, with an increase in number of affected genes at HT.

Consistent with prior research, we identified the most prevalent mutations in *KMT2D* and *CREBBP*, genes with roles in histone modification.[12 14 20 22 30 31] *CREBBP* mutations primarily present as missense changes in the KAT domain and can be detected years before FL diagnosis.[4 6 10 31–33] Recently, it has been reported that missense mutations in the KAT domain of *CREBBP* are associated with a reduced rate of HT, although not validated.[15 30] In our analysis, we found no significant difference in the presence of *CREBBP* KAT domain mutations at diagnosis between the D-T cohort and FL-seq cohort, with six cases affected in the D-T cohort and eight cases in the FL-seq cohort (p=0.65).

Similar to previous reports of cooperative mutational pairing between *STAT6* and *CREBBP*, we only detected *STAT6* mutations in the presence of a *CREBBP* mutation.[12 34]

The histone methyltransferase *EZH2* plays a role in upregulation within germinal centre B-cells and is required for the formation of germinal centres. FLs frequently exhibit gain of function mutations, centred around the Y641 hotspot, which are associated with a blockage in terminal differentiation.[35 36] Earlier studies have reported conflicting data regarding the timing and stability of *EZH2* mutations in FL.[6 7 22 31 37 38] For DLBCL, *EZH2* is recognised as a main driver and is present in different genetic subclassifications.[39–41] We indeed exclusively found *EZH2* mutations that affected Y641 which were predominantly present with a high VAF. Interestingly, despite the small number of cases, we observed unstable *EZH2* mutations with frequent changes in the presence or absence of mutations during the disease course, also without systemic treatment. Whether this indicates a different role for *EZH2* in FL compared with DLBCL needs to be elucidated. As *EZH2* inhibitors are becoming available and as *EZH2* mutation status is part of the M7-FLIPI prognostic score, it is important to be aware that *EZH2* mutations can be lost or gained during the disease course.[8 42]

Alterations affecting *B2M* and *TP53* have been associated with early FL progression and are frequently identified at HT.[20 30 43] In our study, *B2M* mutations were reported in 24% (6/25) of the cases, with five cases in the D-T cohort, showing exclusively acquired mutations. The absence of *B2M* mutations at the time of initial diagnosis reduces their potential as a predictor of the disease course. Only one case exhibited a *TP53* mutation at the time of diagnosis but this was followed by an indolent disease course even with a WW regimen. Based on the VAF, this mutation does not appear to represent a minor subclone. Furthermore, another case showed a *TP53* mutation after a second-line systemic treatment but was lost in subsequent biopsies without treatment. In this case, the mutation was not detectable in earlier biopsies, even below the threshold, though an undetectable subclone cannot be excluded. Interestingly, it was subsequently lost in the absence of systemic treatment. Both of these *TP53* mutations were classified as pathogenic.

In chronic lymphocytic leukaemia, *TP53* mutations are well-established as prognostic markers for poor survival.[44] However, transient and indolent disease courses with *TP53* mutations are also described, these mutations typically present with low VAFs, and may expand following systemic treatment.[45–47]

These findings warrant further exploration regarding the potential role of *TP53* as an indicator of disease progression or HT. Mutations in *MEF2B and MYC* were more commonly observed in the D-T cohort, corresponding to previous studies.[5 7 12 15 20 22]

Our analysis of varying evolutionary patterns across both cohorts aligns with previous research findings. In the D-T cohort, we observed predominantly branched evolution in 67% of cases. Conversely, in the FL-seq cohort, evolutionary patterns were more evenly distributed, with linear evolution being most common (40%), followed by branched evolution (33%) and no detectable evolution (27%).

In the FL-seq cohort, we identified a small but statistically significant increase in the number of affected genes from diagnosis to the first subsequent biopsy, with no further significant changes observed in later biopsies. Notably, substantial variability was observed among patients.

Few studies have analysed genetic changes in sequential FL biopsies. Makker *et al* analysed 13 early stage FL patients, including seven cases involved relapsed lymphoma that did not show transformation. While major differences in pathogenic mutations were described, no significant difference was observed in the number of potentially pathogenic mutations detected between diagnostic FL samples and relapsed FL samples.[24] Russler *et al* observed a higher number of mutated genes in relapsed/refractory FL compared with diagnostic biopsies, although they did not assess mutational changes on a per patient basis.[15] Finally, Eide *et al* studied the level of and defined a CNA index as a measure of genomic complexity in 17 FL patients without transformation. They did not find differences in CNA frequencies when comparing the entire groups of initial and late FL biopsies. When looking at individual cases, increased genetic complexity was observed, though this was not significant.[19]

In the D-T cohort, we observed a more pronounced increase in affected genes at HT compared with diagnosis biopsy, consistent with previous studies.[12 15 20] For both cohorts, we observed no correlation between treatment, biopsy interval and mutational changes.

By using a targeted panel for mutation analysis we were able to achieve high sequencing depth despite working with archival material and small biopsies. However, this targeted approach also prevented us from discovering novel genes implicated in disease progression and excluded several genes that have gained relevance and are now part of newly identified molecular clusters and signatures.[15 30 48] Additionally, we did not consider other factors such as morphology, protein expression, tumour microenvironment and epigenetic changes, which likely also affect disease biology.[49] As a retrospective study, variability in biopsy intervals and locations could reflect differences in disease biology but also variances among patients in terms of clinical presentation or treatment. Furthermore, because our samples include biopsies from multiple anatomical sites, location specific factors may have played a role, as mutational profile in FL is known to be different in different locations.[49–51] This heterogeneity complicates interpretation, but it does represent actual clinical practice.

In conclusion, we found a heterogenous and dynamic mutational profile during the disease course of FL and at HT. Understanding these processes is essential for accurate interpretation of genetic data and could enhance predictions of prognosis and treatment response. Our findings underscore the necessity for cautious interpretation of genetic data at a single time-point; when mutational analysis becomes more important for making treatment decisions, our study highlights the need to consider a re-biopsy to obtain up-to-date information on the mutational profile.

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Contributors EH, WBCS and MvdB conceived the project. NMAB and ABvS supervised the project. EH collected materials. EH, WBCS, EvdS and DI collected clinical data. EH and MvdB performed pathology review. JL performed DNA isolations. PJS, DG, LIK and PJTAG performed and interpreted the molecular analysis. EH and MvdB analysed the data. EH wrote the manuscript. All authors discussed the results and contributed to the final manuscript. WBCS is guarantor. In this article, ChatGPT (GPT-4), was minimally used for textual adjustments.

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Competing interests We would like to indicate that one of the coauthors, DG, is founder, director and stockholder of Univ8 Genomics, which provided the Next Generation Sequencing panel used in our study.

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