

# The analysis of clonal diversity and therapy responses using *STAT3* mutations as a molecular marker in large granular lymphocytic leukemia

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## ABSTRACT

T-cell large granular lymphocytic leukemia and chronic lymphoproliferative disorder of natural killer cells are intriguing entities between benign and malignant lymphoproliferation. The molecular pathogenesis has partly been uncovered by the recent discovery of somatic activating *STAT3* and *STAT5b* mutations. Here we show that 43% (75/174) of patients with T-cell large granular lymphocytic leukemia and 18% (7/39) with chronic lymphoproliferative disorder of natural killer cells harbor *STAT3* mutations when analyzed by quantitative deep amplicon sequencing. Surprisingly, 17% of the *STAT3*-mutated patients carried multiple *STAT3* mutations, which were located in different lymphocyte clones. The size of the mutated clone correlated well with the degree of clonal expansion of the T-cell repertoire analyzed by T-cell receptor beta chain deep sequencing. The analysis of sequential samples suggested that current immunosuppressive therapy is not able to reduce the level of the mutated clone in most cases, thus warranting the search for novel targeted therapies. Our findings imply that the clonal landscape of large granular lymphocytic leukemia is more complex than considered before, and a substantial number of patients have multiple lymphocyte subclones harboring different *STAT3* mutations, thus mimicking the situation in acute leukemia.

## Introduction

Large granular lymphocytic (LGL) leukemia is characterized by an increased number of clonal cytotoxic T or natural killer (NK) cells in blood and bone marrow.<sup>1</sup> Concurrent neutropenia, anemia, and autoimmune disorders, such as rheumatoid arthritis, further support the diagnosis in unclear cases.<sup>2,3</sup> Leukemic T-LGL cells are terminally differentiated effector memory cells (CD3<sup>+</sup>CD45RA<sup>+</sup>CD62L<sup>neg</sup>CD57<sup>+</sup>) with clonal rearrangement of the T-cell receptor (TCR) genes.<sup>4</sup> According to the World Health Organization (WHO) 2008 criteria, chronic lymphoproliferative disease of NK cells (CLPD-NK) is a separate entity and differs from aggressive, chemorefractory NK-LGL leukemia.<sup>5</sup>

The phenotype of leukemic cells indicates that the expansion originally developed in response to an antigen. However, unlike their normal counterparts, leukemic LGL cells are resistant to subsequent Fas-mediated activation-induced cell death.<sup>6</sup> A current hypothesis postulates that following antigen encounter, the dysregulation of several signaling pathways and cytokines such as interleukin-15, platelet-derived growth factor receptor, and signal transducer and activator of transcription 3 (*STAT3*) sustains a constitutively active clonal cell population.<sup>6,7</sup> The importance of these pathways has been emphasized in the light of the recent discovery of somatic mutations in *STAT3* and *STAT5b* genes.<sup>8-13</sup> The mutations

were located in the Src-like homologue 2 (SH2) domain of *STAT3* and *STAT5b*, caused constitutive phosphorylation of the mutated proteins, and increased the transcriptional activity of *STAT3* and *STAT5b* *in vitro*.<sup>8,9,14</sup> Expression of mutated *STAT3* in mouse bone marrow led to the development of myeloproliferative neoplasm, and recently similar *STAT3* and *STAT5b* mutations have also been discovered in other hematologic malignancies.<sup>12,15-19</sup>

In this project we aimed to analyze the frequency of *STAT3* mutations and the clonal architecture of expanded lymphocytes using both *STAT3* and TCR beta (TCRB) chain deep sequencing methods in a unique cohort of 213 patients with LGL leukemia. In addition, the effect of immunosuppressive treatment on the mutated clones was studied in follow-up samples obtained during therapy to assess if quantitative *STAT3* mutation analysis could be used to monitor therapy response.

## Methods

Additional methods of this study are described in the *Online Supplementary Appendix*.

## Study patients

The study was undertaken in compliance with the principles of the

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Helsinki declaration and was approved by the ethics committees of Helsinki University Central Hospital (Finland), the Cleveland Clinic (Ohio, USA) and the Penn State Hershey Cancer Institute (Pennsylvania, USA). The study population consisted of 213 patients with LGL leukemia as defined by the WHO 2008 criteria: 174 patients had CD8<sup>+</sup> T-LGL leukemia and 39 patients had CLPD-NK. Samples were collected in the Penn State Hershey Cancer Institute (n=93), the Cleveland Clinic (n=89), and Finland (n=31). One hundred and twelve patients with T-LGL leukemia and 30 with CLPD-NK included in the current study cohort were also part of two previous study cohorts analyzing *STAT3* mutation frequency by capillary sequencing.<sup>8,9</sup> All patients gave written informed consent to participation in this study.

### Sample collection and DNA extraction

Mononuclear cells were separated from peripheral blood using Ficoll gradient separation (GE Healthcare, Pittsburgh, PA, US, USA) and cryopreserved in fetal bovine serum with 10% dimethylsulfoxide. Peripheral blood mononuclear cells were labeled with antibodies and sorted into CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell, and NK-cell fractions using antibodies for CD3, CD4, CD8, and CD16/56 (Beckton Dickinson, San Jose, CA, USA). In cases of T-LGL leukemia, the CD8<sup>+</sup> T-cell population was further sorted into clonal/non-clonal cells based on the flow cytometry analysis of TCRB variable chain (Vbeta) expression (IOtest<sup>®</sup> Beta Mark Kit, Beckman Coulter, Brea, CA, US). DNA was extracted using a NucleoSpin Tissue or Tissue XS kit (Macherey-Nagel, Dueren, Germany) and the concentration was measured with Qubit (Life Technologies, Carlsbad, CA, USA).

### Deep targeted sequencing and capillary sequencing of *STAT3* exon 21

*STAT3* exon 21 was sequenced using deep amplicon sequencing and the Illumina Miseq platform as described previously.<sup>10</sup> The data were analyzed with a novel in-house bioinformatics pipeline, which is based on calling of variants with certain count/frequency and filtering out false positives using the estimated error rate and quality data of amplicon reads. *STAT3* amplicon sequencing and data analysis are described in detail in the *Online Supplementary Appendix*. *STAT3* exon 21 capillary sequencing was performed as previously described.<sup>9</sup>

### High-throughput T-cell receptor sequencing

TCRB complementarity determining regions (CDR3 $\beta$ ) were

amplified and sequenced by Adaptive Biotechnologies Corp. (Seattle, WA, USA) using ImmunoSEQ assay Survey level analysis, which is capable of detecting one cell in 40,000 T cells.<sup>20</sup> The TCRB sequencing method is described in the *Online Supplementary Appendix*.

### Statistical analysis

Categorical variants between groups were compared using the  $\chi^2$  test. Comparisons of parametric variables between groups were performed with one-way ANOVA and the Bonferroni post hoc test, or unpaired t test, as appropriate. A *P* value <0.05 was considered statistically significant.

## Results

### *STAT3*-amplicon sequencing is a sensitive and reliable method

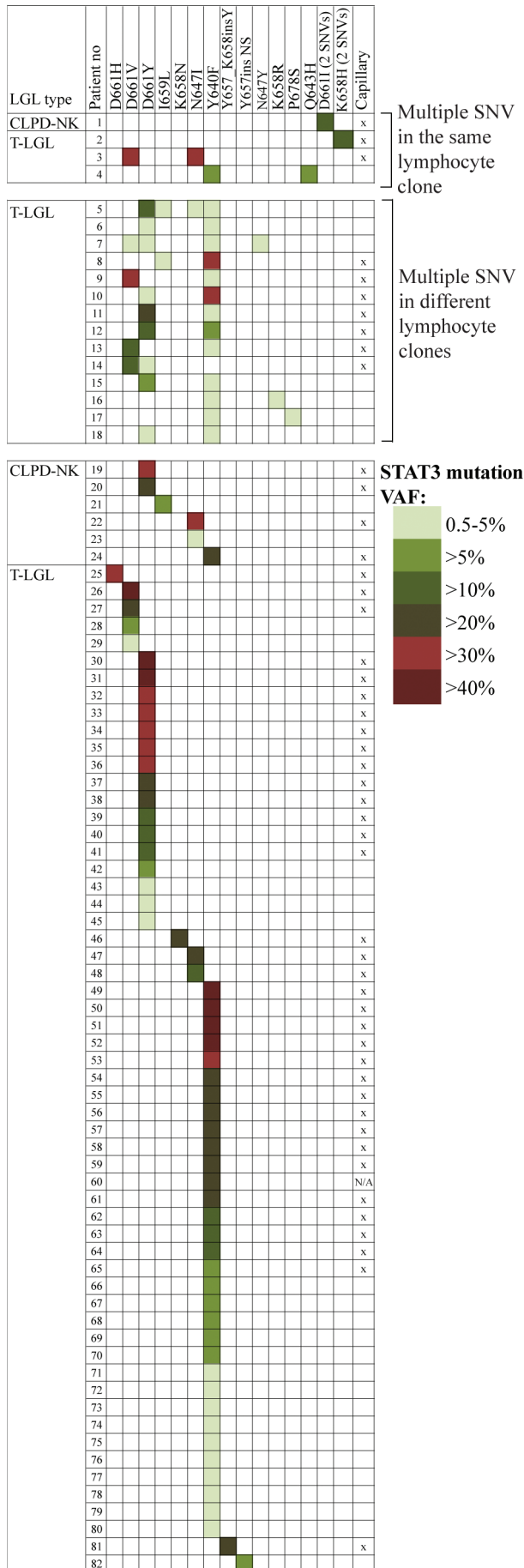
Deep, targeted sequencing is a relatively new method and we, therefore, first compared the sensitivity of amplicon sequencing with the well-established allele-specific oligonucleotide (ASO) real-time quantitative polymerase chain reaction (qPCR) method. The results of ASO-qPCR for *STAT3* mutations Y640F and D661V and amplicon sequencing of *STAT3* exon 21 were congruent in seven of the eight cases analyzed (*Online Supplementary Table S1*). The only discordant case (patient 14, Figure 1) was faintly positive for Y640F by ASO-qPCR, but even the analysis of sorted lymphocyte fractions did not show the Y640F mutation by amplicon sequencing.

As another quality control, we sequenced a dilution series from a CD8<sup>+</sup> peripheral blood mononuclear cell sample (patient 9, Figure 1) with known *STAT3* D661V [variant allele frequency (VAF) 37%] and Y640F (VAF 1.4%) mutations in the CD8<sup>+</sup> fraction: Y640F mutation with low VAF was not seen in the original sequencing of the peripheral blood mononuclear cell sample (*Online Supplementary Table S2*, Figure 1). The D661V mutation was detected at the 2% dilution level with a VAF of 0.633% (frequency ratio 0.896), whereas with the next dilution of 1% and a VAF of 0.433% the frequency ratio dropped under 0.8, being still on the borderline 0.75-0.80. However, the noise exceeded the number of variant alleles

**Table 1.** Clinical characteristics of the patients.

Variable	T-LGL leukemia (n=174)			P	CLPD-NK <sup>1</sup> (n=39)		P
	$\geq 2$ <i>STAT3</i> -mutated clones (n=14)	1 <i>STAT3</i> -mutated clone (n=61)	No <i>STAT3</i> mutations (n=99)		All <i>STAT3</i> -mutated (n=7)	No <i>STAT3</i> mutations (n=32)	
Median age (range)	67 (36-81)	65 (31-84)	66 (17-89)	0.72	68 (58-75)	57 (27-77)	0.077
Males (%)	13 (93%)	33 (54%)	57 (58%)	<b>0.026</b>	6 (86%)	19 (58%)	0.19
Median hemoglobin, g/L (range)	126 (87-154)	114 (74-165)	120 (59-172)	0.44	137 (58-159)	128 (69-168)	0.49
Median leukocytes, 10 <sup>9</sup> /L (range)	4.0 (0.8-27.2)	5.7 (1.0-24.8)	5.9 (1.2-28.5)	0.46	8.8 (4.1-15.0)	9.0 (1.8-21.4)	0.93
Median lymphocytes, 10 <sup>9</sup> /L (range)	3.0 (0.7-23.7)	3.4 (0.4-23.2)	3.2 (0.4-25)	0.15	7.0 (1.9-11.3)	6.0 (1.0-14.6)	0.78
Median neutrophils, 10 <sup>9</sup> /L (range)	0.6 (0.0-2.3)	1.2 (0.0-18.3)	1.6 (0.0-10.2)	0.24	1.3 (0.6-2.7)	1.3 (0.1-10.8)	0.37
Median platelets, 10 <sup>9</sup> /L (range)	217 (115-405)	181 (29-596)	215 (9-627)	0.31	245 (25-478)	208 (22-550)	0.69
Treatment (%) <sup>2</sup>	11 (79%)	43 (70%)	61 (62%)	0.16	3 (38%)	16 (48%)	1.00
Rheumatoid arthritis (%) <sup>3</sup>	6 (43%)	14 (23%)	6 (6%)	<b>&lt;0.0001</b>	NA	NA	

Laboratory values presented in the table were measured at diagnosis. Statistics are compared between three groups of patients ( $\geq 2$  *STAT3*-mutated clones vs. 1 *STAT3*-mutated clone vs. unmutated *STAT3*) in the case of TLGL leukemia, and between two groups in CLPD-NK cases. <sup>1</sup>Chronic lymphoproliferative disease of NK cells. <sup>2</sup>Treatment at any timepoint during follow-up. <sup>3</sup>Rheumatoid arthritis diagnosed at any timepoint during follow-up. Statistically significant differences are shown in bold.



**Figure 1.** STAT3 mutations and variant allele frequencies detected by amplicon sequencing. The patients are grouped based on the number and allelic location of STAT3 mutations, and the LGL leukemia type. STAT3 mutations detected also by capillary sequencing are marked with “x” in the last column. NS: nonsense; VAF: variant allele frequency. SNV: single nucleotide variants.

and the mutation could not be reliably verified (*Online Supplementary Table S2*). The 0.5% cut-off for VAF seemed reasonable and was used in the following analysis.

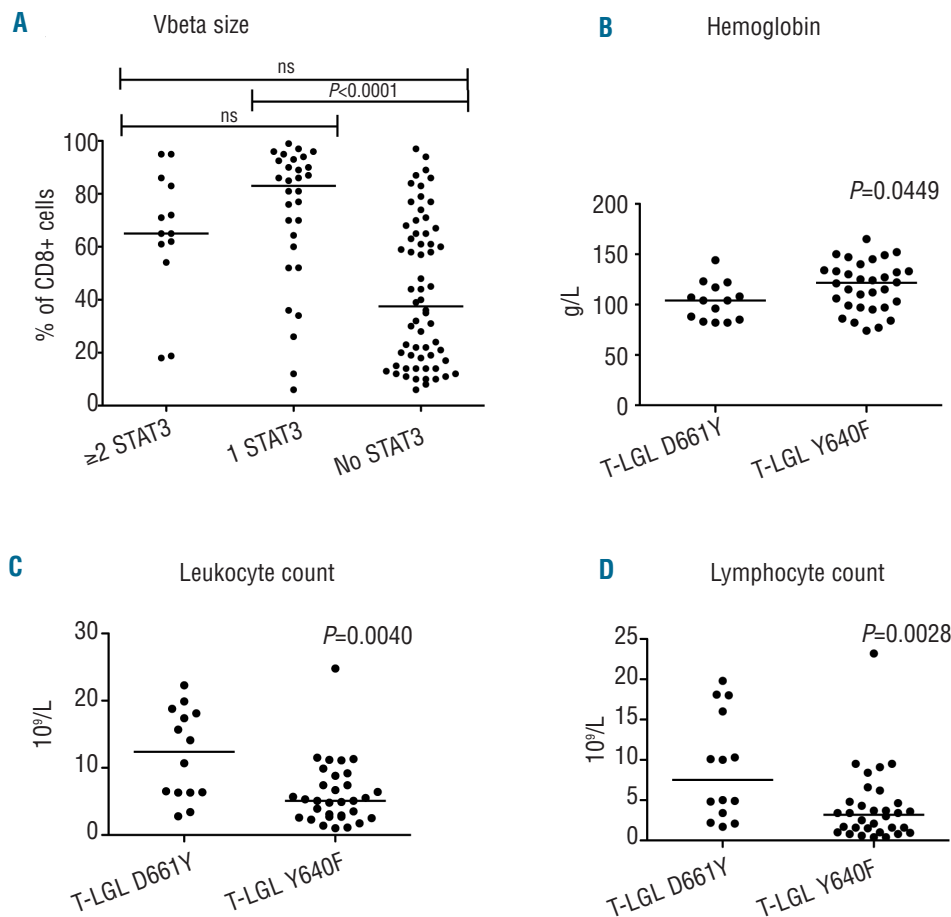
As a negative control, we sequenced samples from eight patients with chronic myeloid leukemia with clonal LGL lymphocytosis during dasatinib treatment (a proportion of dasatinib-treated patients with chronic myeloid leukemia have drug-induced clonal LGL lymphocytosis).<sup>21,22</sup> Sorted monoclonal CD8<sup>+</sup> expansions from four patients (CD8<sup>+</sup> Vbeta clone size 10-69%) and total CD8<sup>+</sup> fractions from four additional cases (CD8<sup>+</sup> Vbeta clone size 10-27%) were analyzed by amplicon sequencing. All tested samples were STAT3 mutation-negative.

**The frequency and pattern of STAT3 mutations in patients with large granular lymphocytosis leukemia as determined by deep quantitative sequencing**

The exon 21 of STAT3 gene was analyzed by both capillary and amplicon sequencing from a cohort of 213 LGL leukemia patients. DNA samples were extracted either from whole blood, peripheral blood mononuclear cells, or CD8<sup>+</sup> T cells. The frequency of STAT3 mutations was 23% (50/213) by capillary sequencing and 38% (82/213) by amplicon sequencing, showing the superiority of amplicon sequencing as a screening assay. The mutations were significantly more common in T-LGL leukemia than in CLPD-NK (75/174, 43% versus 7/39, 18%; *P*=0.0034).<sup>8,11</sup> Capillary sequencing did not detect mutations with VAF smaller than 9% (Figure 1, *Online Supplementary Table S3*). The cohort of patients also included 72 of the 77 T-LGL leukemia patients with a large immunodominant clone (mean clone size 78%; range, 32-99%) described in the original publication of STAT3 mutations in LGL leukemia,<sup>9</sup> and the frequency of STAT3 mutations was as high as 63% (45/72) by amplicon sequencing, compared to 43% by capillary sequencing. The mean clone size in the rest of the patients was 39% of CD8<sup>+</sup> cells (range, 6-97%).

**A substantial proportion of patients with large granular lymphocytic leukemia carry multiple STAT3 mutations**

In the amplicon sequencing analysis 22% (18 of 82) of STAT3-mutated patients harbored multiple mutations in the STAT3 gene (Figure 1). In 17/18 of the cases either Y640 or D661 or both were mutated (Figure 1). As an extreme example, two patients harbored four different STAT3 mutations (Figure 1). Patients with multiple STAT3 mutations could be divided into two different groups based on the allelic status derived from the amplicon data: (i) patients harboring two single nucleotide variants in the same STAT3 allele, resulting in one or two amino acid changes (n=4, Figure 1, patients 1-4), and (ii) patients displaying multiple STAT3 mutations in different alleles and lymphocyte clones (n=14, Figure 1, patients 5-18). Thus,



**Figure 2.** Comparison of different clinical parameters between patients with LGL leukemia patients divided into groups based on *STAT3* mutation status. Horizontal lines define medians of measured variables in all figures. (A) The sizes of the largest clones in CD8<sup>+</sup> lymphocytes detected in T-LGL leukemia patients by flow cytometry (T-cell receptor beta chain variable region analysis). T-LGL patients were divided into three groups: those with  $\geq 2$  *STAT3* mutation (data available from 13 patients), 1 mutation (n=30) or no mutations (n=62). Hemoglobin (B), absolute leukocyte count (C) and lymphocyte count (D) of T-LGL leukemia patients with either D661Y or Y640F mutation were compared.

17% of *STAT3*-mutated cases (14 of 82) had multiple mutated clones in the original screening.

### Correlation of *STAT3* mutations with clinical parameters

Intrigued by the finding of multiple *STAT3* mutations, we studied the correlation between the phenotype of the patients and *STAT3* mutation status to assess whether multiple mutations would affect the clinical characteristics of patients (Table 1). Rheumatoid arthritis, which is the most common autoimmune manifestation in LGL leukemia patients,<sup>23</sup> was significantly more common in T-LGL patients with one or multiple *STAT3* mutations: 43% (6/14) of patients with multiple mutations and 23% (14/61) with one mutation suffered from rheumatoid arthritis compared to 6% (6/99) of patients without mutations ( $P<0.0001$ ) (Table 1). No statistical difference was seen in the prevalence of rheumatoid arthritis between patients with one or multiple *STAT3* mutations ( $P=0.18$ ). Patients who had multiple *STAT3*-mutated clones were almost exclusively males (13/14, 93%) (Table 1). The size of the major expansion in TCR Vbeta analysis was greater among patients with one *STAT3* mutation than among those without mutations ( $P<0.0001$ ) (Figure 2A). Neutropenia is often associated with LGL leukemia<sup>23</sup>, and T-LGL leukemia patients with *STAT3* mutations had a tendency toward lower neutrophil counts at diagnosis, but the difference was not statistically significant (Table 1). No

significant clinical correlations were detected in the CLPD-NK cohort, possibly due to the lower number of patients (Table 1).

The clinical picture was compared in more detail between patients carrying the two most common mutations, Y640F and D661Y (Figure 1). D661Y-mutated patients had lower hemoglobin values at diagnosis (median 104 versus 122 g/L,  $P=0.0449$ ), whereas they had higher leukocyte and lymphocyte counts (12.4 versus 5.1  $\times 10^6$ /L,  $P=0.0440$  and 7.5 versus 3.2  $\times 10^9$ /L,  $P=0.0028$ , respectively) (Figure 2B-D).

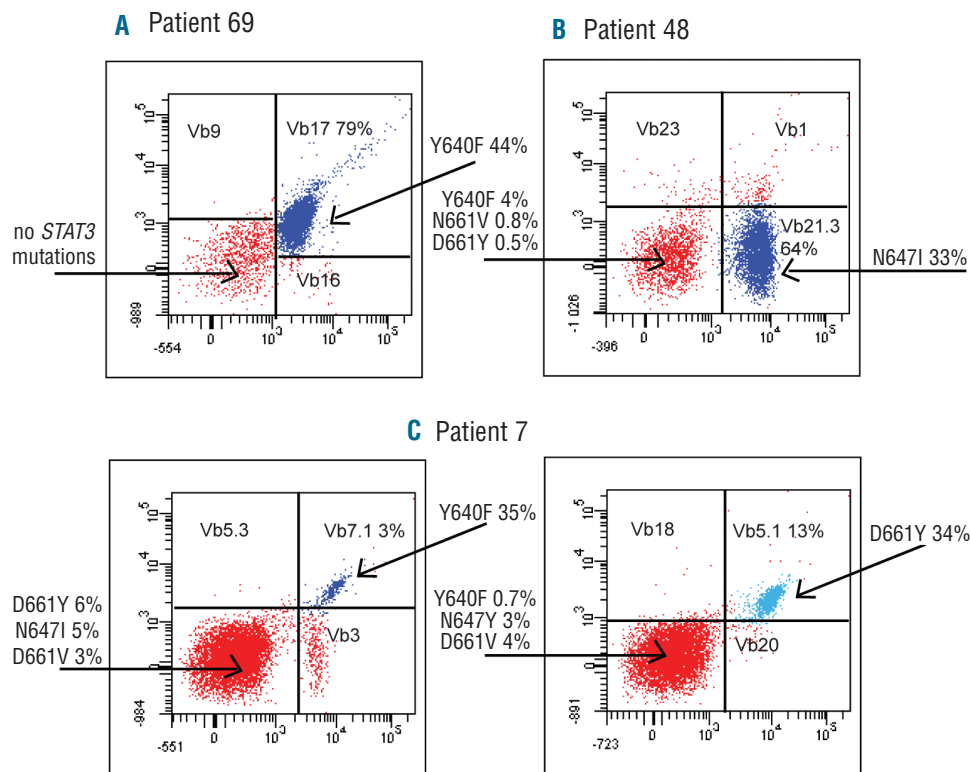
### *STAT3* sequencing of sorted lymphocyte fractions

Screening for *STAT3* mutations was performed on unsorted samples, and the exact location of the mutations and the clonal hierarchy were further analyzed in sorted lymphocyte fractions.

#### Patients with one *STAT3* mutation have a monoclonal pattern in Vbeta analysis

First we sorted and analyzed samples from five T-LGL leukemia patients with a monoclonal pattern in TCR Vbeta analysis (clone size 27-81%) carrying a single *STAT3* mutation in the original screening. In four of the five cases the mutation was located in the major CD8<sup>+</sup> Vbeta expansion, and no other *STAT3* mutations were detected in sorted lymphocyte fractions including CD4<sup>+</sup> T cells, Vbeta expansion-negative CD8<sup>+</sup> cells, and CD3<sup>neg</sup> NK cells and B





**Figure 3.** The flow cytometry Vbeta analysis and STAT3-sequencing results of flow cytometry-sorted lymphocyte fractions from three representative T-LGL leukemia patients. The monoclonal Vbeta antibodies were conjugated with either FITC (x-axis), PE (y-axis), or both PE and FITC (double-positive population), and the Vbeta populations analyzed are marked in each dotplot with the percentage of the clone in CD8<sup>+</sup> cells. STAT3 mutations and their VAF are indicated with arrows. (A) The results of patient 69 after 1.5 years after cyclosporine treatment. (B) STAT3 results of patient 48 with four different STAT3 mutations. (C) Patient 7 (Figure 1) had two CD8<sup>+</sup> expansions at baseline, Vb7.1<sup>+</sup> 10% and Vb5.1 11% (data not shown), and 4 years later during methotrexate treatment the expansions were 3% and 13%, respectively, both harboring STAT3 mutations. This patient also had two minor STAT3 mutations in Vbeta-negative CD8<sup>+</sup> cells. FITC: fluorescein isothiocyanate; PE: phycoerythrin; VAF: variant allele frequency.

cells (Figure 3A, *Online Supplementary Figure S1*). In one case (patient 48, Figure 1), in addition to the N647I-mutated major Vb21.3<sup>+</sup> expansion, three low VAF mutations were detected in the sorted Vb21.3<sup>neg</sup> CD8<sup>+</sup> fraction, which were not discovered in the original screening (Figure 3B).

#### Multiple STAT3 mutations in sorted lymphocytes

A similar analysis was done on cells from two T-LGL leukemia patients who harbored a set of multiple STAT3 mutations already in the original screening (Figure 3C, *Online Supplementary Figure S1*). The patients carried STAT3 mutations in both detected Vbeta expansions and, in addition, an apparently non-clonal CD8<sup>+</sup> population from patient 7 contained two additional mutations, N647Y and D661V (Figure 3C). CD4<sup>+</sup> and CD3<sup>neg</sup> lymphocyte fractions did not harbor STAT3 mutations.

#### Comparison of clonality analysis by STAT3 and deep T-cell receptor beta chain sequencing

Detailed analysis of TCRB CDR3 region rearrangement and clonality was performed in paired samples from three T-LGL leukemia patients who had multiple STAT3 mutations. The patients were not HLA-matched. We assumed that in the case of a heterozygous STAT3 mutation, the size of the mutated clone determined by deep TCRB sequencing would be approximately twice the STAT3 mutation VAF. The number of TCRB CDR3 reads for each sample is shown in *Online Supplementary Table S4*.

#### Deep T-cell receptor beta chain sequencing results are in concordance with the STAT3 mutation analysis

The results of deep TCRB sequencing were in concor-

dance with STAT3 mutation analysis, and they also explained the observed differences between clone size analysis (flow cytometry) and STAT3 amplicon sequencing. Patient 8, who had Y640F-mutated Vb3<sup>+</sup> expansion in the Vbeta flow analysis, correspondingly had one major expansion in the sorted Vb3<sup>+</sup> fraction (73% of TCRB sequences) (Figure 4A, *Online Supplementary Table S5*). The Vb3<sup>neg</sup> fraction was also abnormally skewed and consisted of a single clonal TCRB sequence (74% of reads), which explains the high VAF (25-35% at different time-points) of STAT3 I659L in the Vb3<sup>neg</sup> fraction (Figure 4A). The TCRB rearrangement observed in the Vb3<sup>neg</sup> fraction also appeared in the sorted Vb3<sup>+</sup> sample, due to impurity of the sorting (86% of sorted cells were Vb3<sup>+</sup>) (Figure 4A).

Patient 48 had a large Vb21.3<sup>+</sup> clone in Vbeta flow analysis, but the VAF of N647I was only 34% in the sorted Vb21.3<sup>+</sup> fraction. The sorted Vb21.3<sup>+</sup> population was dominated by a clonal TCRB rearrangement that covered 63% of TCRB sequences, corresponding to the estimated percentage of STAT3-mutated cells from amplicon sequencing (2xVAF of 33%), but in addition, 26% of the sequences came from another rearrangement with the TCRBV20-01 gene (Figure 4B). Interestingly, based on the flow cytometry purity analysis the sorted Vb21.3<sup>+</sup> fraction seemed monoclonal and contained only Vb21.3<sup>+</sup> cells, so both clones with different amino acid sequences were recognized by the same Vbeta antibody (Table 2). The Vb21.3<sup>neg</sup> population was less skewed: the largest productive TCRB rearrangements were 4%, 2% and 2% of sequences, and probably contained the STAT3 mutations detected in the Vb21.3<sup>neg</sup> fraction (Figure 4B, *Online Supplementary Table S5*).

Patient 14 had two CD8<sup>+</sup> expansions (*Online Supplementary Figure S1*) and the TCRB analysis of the larger one (Vb17<sup>+</sup>) showed that 83% of TCRB rearrangement sequences were of a single clone, again corresponding well to the VAF (41%) of the D661V mutation (*Online Supplementary Figure S1, Online Supplementary Table S5*). A smaller D661Y-mutated Vb13.6<sup>+</sup> clone accounted for 11% of CD8<sup>+</sup> cells in Vbeta analysis, but TCRB sequencing could not be done due to the low amount of Vb13.6<sup>+</sup> DNA. A corresponding TCRB sequence was seen in the analysis of the CD3<sup>+</sup> fraction (*Online Supplementary Figure S1, Online Supplementary Table S5*).

**STAT3-mutated clones did not share homologous T-cell receptor beta chain sequences**

In the comparison of the 20 most frequent TCRB CDR3 amino acid sequences of each patient, all sequences were unique and not present in either of the two other cases (*data not shown*). A similar analysis of the clones shown in *Online Supplementary Table S5* was done between patients and 586 healthy individuals (peripheral blood mononuclear cells sequenced, provided by the Adaptive Biotechnologies), and in each LGL leukemia case at least

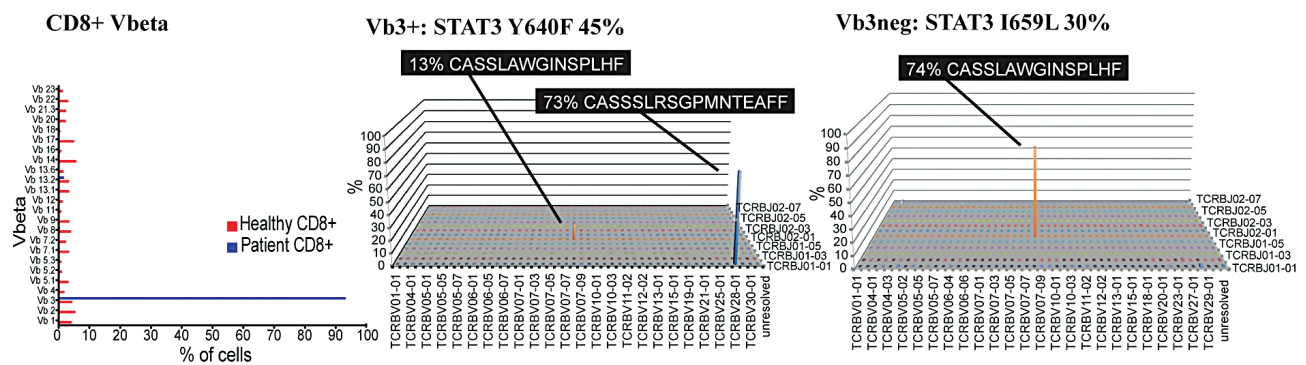
one of the leukemic clones was seen in the healthy controls (the prevalence of the amino acid sequence varied between 0-20% in the healthy control dataset, *Online Supplementary Table S5*). However, the frequencies of these amino acid sequences in individual healthy control samples were low (0.0001-0.005%), corresponding to a single cell per sample.

In the case of multiple STAT3-mutated clones within each patient, the sequences were not apparently homologous, although interestingly the flow cytometry Vbeta antibody recognized two rearrangements in the Vb21.3<sup>+</sup> fraction of patient 48, suggesting that although the amino acid sequences in the CDR3 region are dissimilar, they may share some homology in the three-dimensional structure of the receptors (*Figure 4B, Online Supplementary Table S5*).

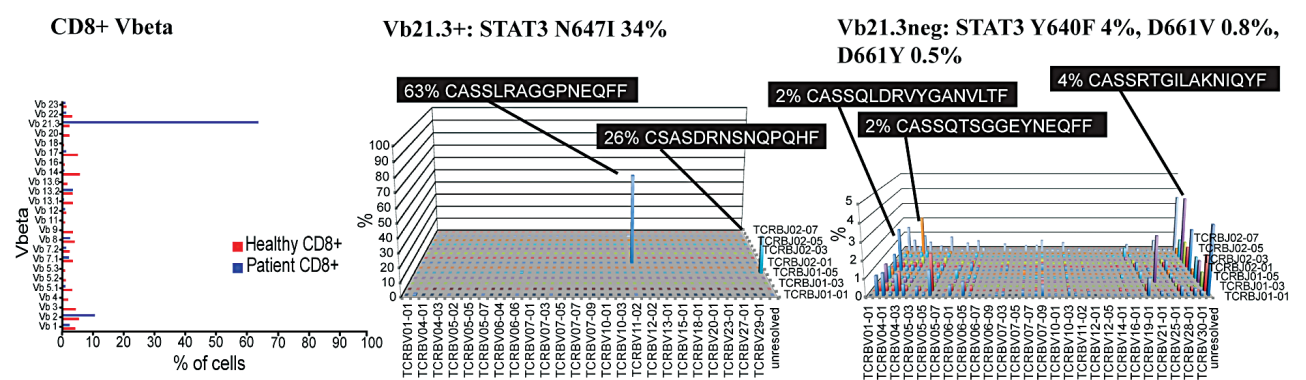
**STAT3 mutation analysis can be used in the follow-up of response to treatment**

Sequential samples were available for STAT3 mutation analysis from six patients including patients with one (n=3) or multiple (n=3) STAT3 mutations. Four patients were diagnosed with T-LGL leukemia, one with CLPD-

**A Patient 8**



**B Patient 48**



**Figure 4.** Flow cytometry Vbeta analysis results and TCRB CDR3 repertoire landscape of two T-LGL leukemia patients. STAT3 mutation VAF are shown in the titles of the analyzed fractions. (A) Patient 8 had one major Vb3<sup>+</sup> expansion (93%) in Vbeta analysis, corresponding to the major TCRB rearrangement in the sorted Vb3<sup>+</sup> fraction. Vb3<sup>neg</sup> fraction appeared polyclonal in the Vbeta analysis, but TCRB sequencing revealed a single major TCRB clone. The TCRB rearrangement observed in the Vb3<sup>neg</sup> fraction was also present in the sorted Vb3<sup>+</sup> sample, due to impurity of the sorting (86% of sorted cells were Vb3<sup>+</sup>). (B) Patient 48 presented with a monoclonal pattern in Vbeta analysis, but TCRB analysis of flow cytometry-sorted Vb21<sup>+</sup> cells revealed two TCRB rearrangements with different amino acid sequences, recognized by the same monoclonal antibody, whereas the Vb21<sup>neg</sup> fraction of the same patient showed only minor TCRB rearrangements. CDR3: complementarity determining region 3; TCRB: T-cell receptor beta chain; VAF: variant allele frequency.

NK, and one patient had both T- and NK cell expansions. Patients were treated with either methotrexate (n=2) or cyclophosphamide (n=3) and one patient was untreated. The median follow-up time was 33 months (range, 24-62).

Patient 4 was untreated, and developed anemia and neutropenia during a follow-up of 28 months: the CD8 percentage increased slightly concomitantly with the development of cytopenias, but the mutated leukemic clone was unchanged and no new *STAT3* mutations evolved (Figure 5A).

The mutated clone(s) persisted during the course of therapy in two T-LGL leukemia patients treated with methotrexate, although improvement of hemoglobin values was observed in both cases during immunosuppression (Figure 5B, *Online Supplementary Figure S2*). However, the partial response achieved was lost after the cessation of methotrexate in the case of patient 8 (Figure 5B).

The disappearance of *STAT3*-mutated clones was associated with complete remission in two patients treated with cyclophosphamide (Figure 5C, *Online Supplementary Figure S2*). In both cases *STAT3* mutation status was the only reliable marker of clonality, as both patients had an aberrant leukemic CD3<sup>neg</sup>CD19<sup>neg</sup>CD16/56<sup>neg</sup> NK-LGL population carrying a *STAT3* mutation (Figure 5C, *Online Supplementary Figure S2*). In the case of patient 44, D661Y-mutated NK-LGL cells were suppressed and normal NK cells were restored at complete remission (Figure 5C). The third patient treated with cyclophosphamide developed marked cytopenias during treatment, and no change was observed in the immunodominant *STAT3*-mutated clone (*Online Supplementary Figure S2*).

## Discussion

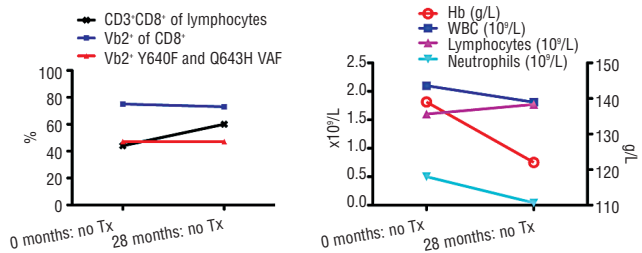
Next generation sequencing methods have enabled the discovery of the molecular background and clonal hierarchy of many hematologic malignancies. Although LGL leukemia differs from acute leukemia, our results show that clonal diversity also plays a role in the former. To our surprise, 17% of all LGL leukemia patients with *STAT3* mutations had multiple mutations in the *STAT3* gene residing solely in cytotoxic CD8<sup>+</sup> or NK cells. The initial mutation-causing event remains undiscovered, but the combined results from TCRB deep sequencing and *STAT3* mutation analysis suggest that a polyclonal immune response may lay the foundation for mutagenesis.

Ultra-deep amplicon sequencing is a novel method for mutation screening, quantitative analysis of the mutated clones, and monitoring of disease burden. Based on our results, the data obtained from amplicon analysis is in good accordance with ASO-qPCR data, and the assay can reliably detect clones down to a VAF threshold of 0.5%, even though higher sensitivities may be reached with ASO-qPCR assays. Compared to ASO-qPCR, the amplicon method can cover a larger part of the gene (one exon, for example, in the case of *STAT3*) and identify previously unknown mutations in a single run, whereas ASO-qPCR is based on mutation-specific primers. However, ASO-qPCR may provide data on common mutations quickly without the need for next-generation sequencing facilities. With the amplicon method, the *STAT3* mutation frequency was 38% in our whole LGL leukemia cohort and significantly higher (over 60%) when only patients with large immunodominant clones were analyzed. Based on these results it

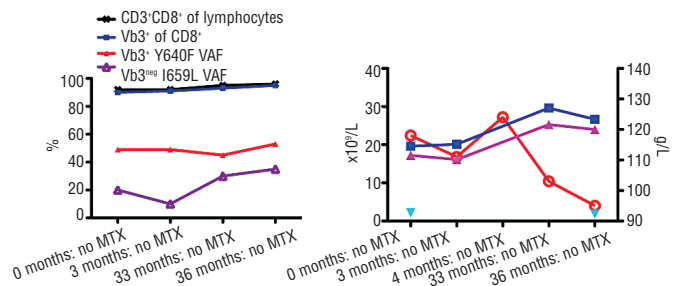
is likely that the diagnosis of LGL leukemia made according to WHO criteria may include different disease subtypes and in some cases also reactive LGL proliferations. Similarly, previous studies have shown that the prevalence of *STAT3* mutations can be as high as 40-70% in T-LGL leukemia, when patients with monoclonal expansions are studied.<sup>9,11</sup> *STAT3* mutations are only rarely found in other hematologic malignancies.<sup>12,15,17,24,25</sup>

The reason for the high incidence of *STAT3* mutations in

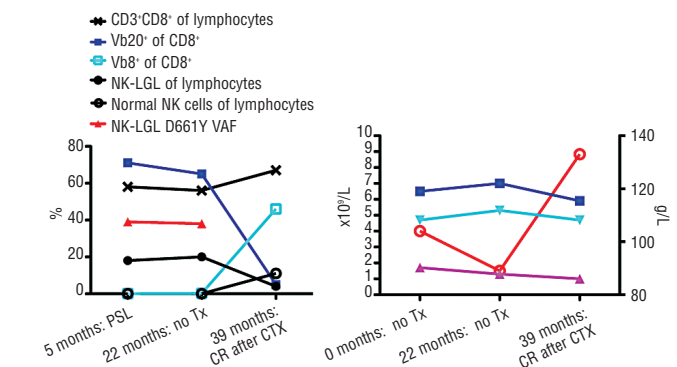
### A Patient 4, no therapy



### B Patient 8, methotrexate



### C Patient 44, cyclophosphamide



**Figure 5.** *STAT3* sequencing results and clinical data of three representative LGL leukemia patients. In each case the first figure shows the size of the LGL clone and VAF of *STAT3* mutation at different time-points, and the second figure presents the hematologic parameters. (A) The results of untreated patient 4, who developed anemia and neutropenia before the initiation of methotrexate (MTX) treatment. (B) Patient 8 was treated with MTX. Leukemic *STAT3*-mutated clones persisted during MTX therapy, and the patient developed anemia after cessation of the therapy. (C) Patient 44 had an aberrant *STAT3*-mutated CD3<sup>neg</sup>CD16/56<sup>neg</sup> NK-LGL population, which was suppressed during remission achieved by cyclophosphamide (CTX) treatment simultaneously with restoration of normal NK cells. In addition, the patient had two CD8<sup>+</sup> clones with unmutated *STAT3*: the Vb20<sup>+</sup> clone was suppressed during CTX administration, whereas Vb8<sup>+</sup> cells were first seen during complete remission. At the last timepoint the number of NK-LGL cells was too low for DNA extraction and *STAT3* mutation analysis. Tx: therapy; VAF: variant allele frequency.



LGL leukemia is unknown. In the detailed analysis of LGL populations, the occurrence of *STAT3* mutations was restricted to expanded lymphocyte clones. During CD8 memory cell development, the *STAT3* pathway is activated, and it may be exposed to mutagenesis during an initial polyclonal cell proliferation.<sup>26,27</sup> As the *STAT3* mutations in LGL leukemia are gain-of-function mutations and are able to cause leukemic transformation in multiple settings,<sup>8,9,14,15</sup> they may give a survival advantage to affected cells, leading to a narrowing of the TCR repertoire and expansion of certain clones. In most cases, patients with multiple mutations had one immunodominant clone and additional smaller expansions, indicating that the clones may have different proliferation capacity or apoptotic resistance, which could be due to the activation potential or temporal variation in the occurrence of the *STAT3* mutation in question. The presence of several *STAT3* mutations could also be related to the clonal drift phenomenon, where the immunodominant LGL clone changes.<sup>28</sup> In addition, *STAT3*-mutated clones may carry somatic mutations in other genes, which affect the proliferative capacity of affected lymphocytes. These mutations could also drive the lymphoproliferation in patients without *STAT3* mutations.<sup>29,30</sup> However, it is interesting to speculate that other factors such as a dysregulated immune system may be needed to sustain the leukemic LGL expansion. In a proportion of cases, these other abnormalities can even lead to the development of LGL leukemia without a somatic driver mutation. This hypothesis is strengthened by a recent study showing that the activated interleukin-6-*STAT3* loop had a significant role in the pathogenesis of LGL leukemia independently of *STAT3* mutation status.<sup>31</sup>

The association between rheumatoid arthritis and multiple *STAT3* mutations in our cohort of patients points toward the involvement of chronic antigen stimulation in the pathogenesis of T-LGL leukemia. Based on our hypothesis polyclonal T cells with autoreactive TCR may represent the starting point for multiple mutated clonal expansions and for rheumatoid arthritis, but other factors are also likely to contribute. In our patients we were not able to address whether in most cases rheumatoid arthritis preceded the diagnosis of LGL leukemia or *vice versa*, but in previous studies, symptomatic rheumatoid arthritis was demonstrated to occur either concurrently or prior to LGL leukemia.<sup>32,33</sup> Further studies with paired follow-up samples and genetically modified mouse models are warranted to understand the detailed pathomechanism and the relation of *STAT3* mutation formation with rheumatoid arthritis.

The deep TCRB sequencing data showed that the results of *STAT3* mutation analysis were in good accordance with the sizes of the clonal CDR3 rearrangements. *STAT3*-mutated clones did not share TCRB CDR3 homology even in the case of multiple *STAT3* mutations in the same patient. However, identical amino acid sequences could be found at a low frequency in healthy controls suggesting that they are common TCR sequences. It was established earlier that the major clones in CD8<sup>+</sup> T-LGL leukemia patients are rarely identical, but the immunodominant clones are sometimes seen at a low frequency in other patients when the whole TCRB repertoire is analyzed.<sup>28,34-36</sup> It should, however, be noted that the specific antigen can be recognized by a large number of different

TCR types depending on the peptide presented, and the variation of HLA genes also affects antigen recognition.<sup>37,38</sup> The presence of a shared antigen driving the LGL proliferation has not, therefore, been ruled out, although our current understanding suggests more private antigens.

During the past years LGL leukemia patients have primarily been treated with immunosuppressive regimens with unsatisfactory results, and over half of the patients relapse on therapy.<sup>3</sup> In clinical studies, the evaluation of response to treatment has included mainly the follow-up of hematologic parameters and TCR PCR testing, both of which are relatively insensitive and unspecific approaches. *STAT3* amplicon sequencing provides a new method to analyze treatment response, and it generates quantitative data on the clone sizes. Although the number of patients we analyzed was relatively small, the sizes of the *STAT3*-mutated clones were in good accordance with clinical results, and the findings may explain why patients relapse. For example, patients treated with methotrexate did not show a marked change in the size of the mutated clone during the observation period. The initial partial responses achieved may be due to the immunosuppressive effect of methotrexate on cytotoxic cell function, but methotrexate therapy is not able to eradicate the mutated clone. Interestingly though, the mutated clones disappeared in two patients who went into complete remission during cyclophosphamide treatment. Concordantly, recent results support the use of cyclophosphamide as a first-line therapy: the overall response rate was 71% (complete response 47%) and relapses were rarely observed.<sup>39</sup> Low relapse rates could be related to the ability of cyclophosphamide to eradicate LGL clones, but further studies with larger patient cohorts are needed to confirm the preliminary findings.

To conclude, ultra-deep *STAT3* amplicon sequencing is a reliable method, which can be used in diagnostics and treatment response evaluation in LGL leukemia. A significant proportion of LGL leukemia patients have multiple *STAT3*-mutated lymphocyte clones mimicking the clonal diversity observed in patients with acute leukemia. Sequential analysis of samples suggested that the current immunosuppressive therapy is not able to eradicate the *STAT3*-mutated clones and novel targeted therapies are, therefore, needed to improve treatment results.

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