ELSEVIER

Experimental Hematology

Experimental Hematology 2020;000:1–11

REVIEW ARTICLE

Reading the B-cell receptor immunome in chronic lymphocytic leukemia: revelations and applications

Paul J. Hengeveld[a,b], Mark-David Levin[b], P. Martijn Kolijn[a], and Anton W. Langerak[a]

[a]*Department of Immunology, Erasmus MC, Rotterdam, The Netherlands;* [b]*Department of Internal Medicine, Albert Schweitzer Hospital, Dordrecht, The Netherlands*

(Received 31 July 2020; revised 25 August 2020; accepted 19 September 2020)

B-Cell receptor (BCR) sequencing has been the force driving many recent advances in chronic lymphocytic leukemia (CLL) research. Here, we discuss the general principles, revelations, and applications of reading the BCR immunome in the context of CLL. First, IGHV mutational status, obtained by measuring the mutational imprint on the IGHV gene of the CLL clonotype, is the cornerstone of CLL risk stratification. Furthermore, the discovery of "BCR-stereotyped" groups of unrelated patients that share not only a highly similar BCR on their leukemic clone, but also certain clinical characteristics has provided insights key to understanding disease ontogeny. Additionally, whereas the BCR repertoire of most CLL patients is characterized by a single dominant rearrangement, next-generation sequencing (NGS) has revealed a rich subclonal landscape in a larger than previously expected proportion of CLL patients. We review the mechanisms underlying these "multiple dominant" cases, including V(D)J-recombination errors, failure of allelic exclusion, intraclonal diversification, and "true" bi- or oligoclonality, and their implications, in detail. Finally, BCR repertoire sequencing can be used for sensitive quantification of minimal residual disease to potentially unprecedented depth. To surmount pitfalls inherent to this approach and develop internationally harmonized protocols, the EuroClonality–NGS Working Group has been established.

Over the previous two decades, advances in the field of immunogenetics have propelled the B-cell receptor (BCR) to the center stage of chronic lymphocytic leukemia (CLL) research. Interrogating the nucleotide and amino acid (aa) sequences of the BCR repertoire in CLL has improved our understanding of disease ontogeny, facilitates risk stratification, and guides therapeutic decisions. The more recent advent of next-generation sequencing (NGS) allows for robust characterization of the BCR repertoire in unprecedented resolution, revealing subclonal populations in CLL and opening up new avenues in the ongoing quest for increased measurement depth of minimal/measurable residual disease (MRD). In this review, we discuss the insights and applications that reading the BCR immunome in CLL has provided.

Syntax: synthesis of the BCR

To ensure adaptive immunity against malicious pathogens, evolution has endowed B cells with mechanisms that allow for the synthesis of a virtually limitless repertoire of antigen-recognizing BCRs (Figure 1). In a process called V(D)J recombination, each early-stage B cell attempts formation of a functional immunoglobulin (IG) heavy-chain (IGH) gene through random

JH, MDL, PMK, and AWL wrote the article and approved of the final version of the manuscript.

Offprint requests to: Anton W. Langerak, PhD, Department of Immunology, Erasmus MC, Dr. Molewaterplein 40, 3015 GD, Rotterdam, The Netherlands;; E-mail: a.langerak@erasmusmc.nl

0301-472X/
https://doi.org/10.1016/j.exphem.2020.09.194

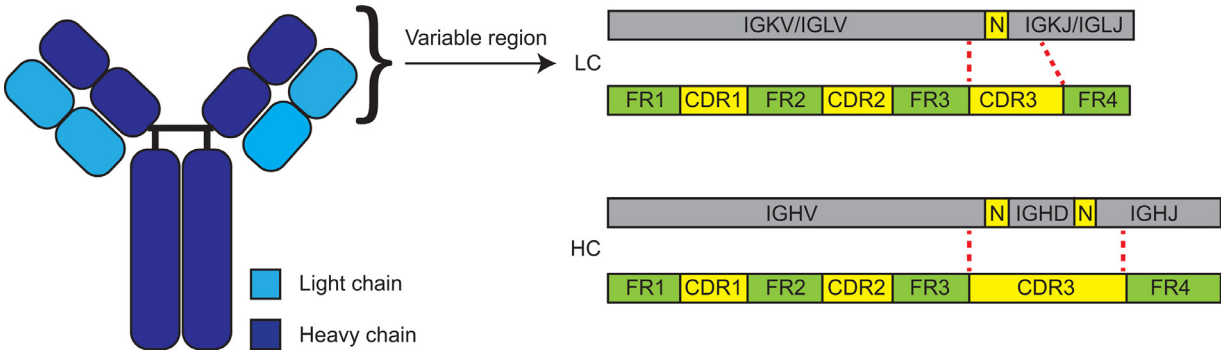


Figure 1. Schematic representation of the BCR. The BCR consists of two identical heavy chains (*dark blue*) and light chains (*light blue*). The variable region constitutes the apical moiety of the BCR, which determines antigen-binding specificity. A HC gene is formed by combining IGHV, IGHD, and IGHJ genes, whereas a LC gene is generated by combining an IGKV or IGLV gene with an IGKJ or IGLJ gene, respectively. Junctional diversity is created through the random insertion and deletion of N-nucleotides. Following successful rearrangement, two identical pairs of HC and LC molecules collectively contribute to the variable regions of the BCR, which determine antigen-binding specificity. The HC constant region, connected to the variable region by mRNA splicing, determines the BCR isotype. By default, antigen-inexperienced B cells co-express IgM and IgD isotypic BCRs. The rearranged IG gene encodes three different CDRs, flanked by four framework regions. As the nucleotide sequence of the CDR3 encompasses all junctional diversity, it is the most variable component of the BCR antigen-binding site, pivotal for determining antigen-binding specificity.

combination of sequentially arranged genes on chromosome 14. This is achieved by rearranging 1 of 27 IGH diversity genes (IGHD) with 1 of 6 IGH joining genes (IGHJ). Subsequently, this DJ combination is recombined with one of approximately 50 IGH variable genes (IGHV), which are subdivided into seven groups (IGHV1–IGHV7) based on relative nucleotide homology. Similarly, functional IG light-chain genes, either of the IG kappa (IGK) or lambda (IGL) variant, are created by combining 1 of approximately 40 IGK variable (IGKV) genes with 1 of approximately 5 IGK joining (IGKJ) genes, or 1 of approximately 30 IGL variable (IGLV) genes with 1 of approximately 4 IGL joining (IGLJ) genes. Additional junctional diversity of the rearranged IG genes is generated by the random insertion and deletion of nontemplated (N) nucleotides at the IGHD–IGHJ, IGHV–IGHD, and IGLV/IGKV–IGLJ/IGKJ junctions. Three moieties in the aa sequence of the variable region of each HC and LC determine antigen-binding specificity. These heavy-chain and light-chain complementarity-determining regions (HCDRs and LCDRs) are flanked by framework regions (FRs), which provide the structural integrity of the molecule. Importantly, whereas the HCDR1 and HCDR2 are encoded fully by the IGHV gene, the HCDR3 crosses the IGHV–IGHD and IGHD–IGHJ junctions and includes the nontemplated nucleotides (N-nucleotides), which introduces significantly more variability. Similarly, the LCDR3 incorporates the variable IGKV/IGLV–IGKJ/IGLJ junction. Consequently, HDCR3 and LCDR3 are the most diverse epitopes of the BCR and play a major role in the determination of antigen-binding specificity [1].

After activation through exposure to an antigen, additional BCR diversity is generated by somatic hypermutation (SHM). During SHM, the enzyme AID introduces mostly single-nucleotide substitutions throughout the IG genes at a rate that exceeds the background mutational rate by approximately a millionfold. These mutations are preferably present in the CDRs, giving rise to mutant BCRs that are selected for enhanced antigen-binding capability in germinal centers, ensuring affinity maturation.

The theoretical diversity of the BCR repertoire, introduced by the aforementioned mechanisms, is enormous, mounting up to 10^{16}–10^{18} unique BCRs [2]. However, in practice, this repertoire is limited by the total number of B cells in the human body to approximately 5×10^9 unique BCRs. Interestingly, recent comprehensive sequencing of the BCR repertoire of healthy individuals has revealed that clonotypes are shared between individuals more frequently than expected, suggestive of a selection mechanism in early B-cell development [2].

The chapters: Composition of the BCR in CLL

CLL cells resemble mature B cells and invariably express a BCR, mostly of the IgM and IgD variety, but occasionally class switched to IgG. According to the degree of SHM imprint on the IGHV gene of the leukemic clone, CLL patients are stratified into two groups: CLL bearing little to no SHM (98%–100% IGHV sequence homology to germline, unmutated or U-CLL) and CLL with significant SHM (<98% IGHV sequence homology, mutated or M-CLL) [3,4]. This division is clinically important: compared with M-CLL,

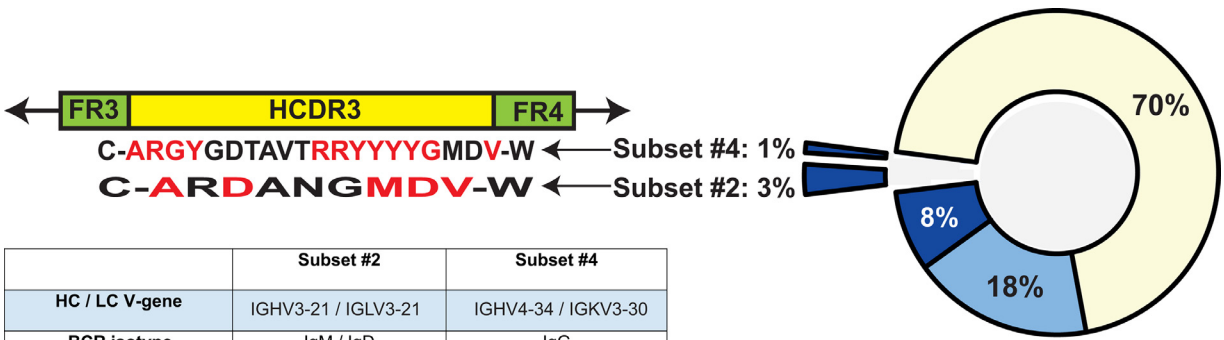
U-CLL is considerably more aggressive and less susceptible to chemoimmunotherapy [5]. Indeed, determination of IGHV mutational status is recommended by the International Workshop on CLL (iwCLL) guidelines for every patient starting therapy and is widely used to guide therapeutic decision making [6,7].

However, the molecular structure of the BCR in CLL has importance beyond its mutational status. IGHV usage in the CLL BCR repertoire is skewed; indeed, some genes are overrepresented in CLL, such as IGHV1-69, IGHV4-34, and IGHV3-21, whereas others, for example, those from the IGHV7 subgroup, are rarely used by the malignant clone [8–10]. Determination of the BCR aa sequences of CLL cells has revealed that subgroups of unrelated patients bear leukemic clones with BCRs that feature very similar HDCR3s, with respect to composition, length, and biochemical properties [11–17]. This has led to the concept of "BCR stereotyped" subsets of CLL patients [18–21]. Such stereotyped rearrangements, overabundant in CLL, are also present in the repertoire of healthy individuals at a low frequency that increases with age, potentially pointing to these cells as possible CLL progenitors [22,23].

In the largest series to date, hundreds of subsets have been defined, to which approximately 30% of CLL cases can be assigned [24,25]. Nineteen of these subsets have been classified as "major," which together represent about 12% of CLL cases [24]. Observing such BCR stereotypy based on chance alone is extremely unlikely. Rather, it supports the idea of (auto)antigenic-driven BCR signaling in CLL ontogeny and, additionally, holds relevance for clinical practice. We will reinforce this point by using subsets 2 and 4, two major CLL subsets, as examples (Figure 2).

Subset 2 is the largest subset in CLL, representing about ~3% of cases. The subset 2 BCR is characterized by the use of IGHV3-21, IGHJ6, and IGLV3-21, with a 9-aa-long HCDR3 [24]. Patients with subset 2 CLL have a high prevalence of del(13q), del(11q), and *SF3B1* mutations and generally suffer a poor prognosis, regardless of IGHV mutational status [26–28]. Contrastingly, CLL cases featuring a subset 4 stereotyped BCR, representing approximately 1% of all patients, have a favorable disease course [24,29]. The subset 4 BCR (IGHV4-34/IGHJ6/IGKV3-30) is frequently IGHV mutated and characterized by a long, positively charged HCDR3, and has always undergone class-switch recombination (CSR) to IgG [14,17,18,24].

One potential mechanism of pathogenicity implicated in CLL is autonomous BCR signaling, driven by HCDR3-mediated binding of an internal epitope of another BCR, effectuating BCR–BCR binding, aggregation, and activation [30]. Examination of the crystal structure of subset 2 and 4 BCRs revealed that both are indeed capable of homotypic interaction [31]. In subset 4, this interaction is dependent on a lysine residue in the constant domain at position 214, only present in IgG, thus explaining the invariable CSR to IgG in this subset [31]. In subset 2, BCR–BCR interaction is dependent on an arginine residue in the light chain, at the splice site between IGLJ3 and the constant region. This position is occupied by a glycine (G) residue in the germline, but a substitution to arginine (R) allows



	Subset #2	Subset #4
HC / LC V-gene	IGHV3-21 / IGLV3-21	IGHV4-34 / IGKV3-30
BCR isotype	IgM / IgD	IgG
Associated cytogenetic and genomic aberrations	del(13q), del(11q), *SF3B1*^{MUT}	del(13q)
Clinical phenotype	Aggressive	Indolent

Figure 2. Subsets in CLL. Approximately 30% of CLL cases can by assigned to stereotyped subsets (major or minor) according to aa sequence similarity of their HCDR3 region. Shown are two examples of HCDR3 aa sequences belonging to subsets 2 and 4, with strongly conserved aa residues highlighted in *red*. The HCDR3 of a subset 2 BCR is always 9 aa long, with a highly conserved acidic residue at position 107 (D107). The HCDR3 of a subset 4 BCR is long and positively charged, with aromatic aa comprising the N1 region and two basic aa in the N2 region, followed invariably by a YYYYG motif encoded by IGHJ6. The BCR of subset 4 CLL patients has always undergone class-switch recombination to IgG. Illustrated in the table are some characteristics of these subsets.

homotypic interaction between the LC and the CDR2 loop of another BCR ([31]. A recently published article reported that the use of IGLV3-21 with this specific substitution (IGLV3-21^{R110} in the authors' nomenclature) facilitates homotypic interaction in allele IGLV3-21*01 only [32]. The prevalence of this specific combination of mutation and allele is not confined to subset 2, but can be detected in up to ~20% of CLL cases, despite being virtually absent in the BCR repertoire of healthy donors. Consequently, the authors define a subset "2L" based on the presence of IGLV3-21^{R110}, which currently constitutes the largest CLL subset, and report the inferior prognosis of patients belonging to this subset in three independent cohorts [32].

Reading between the lines: subclonal IG architecture of CLL

Traditionally, CLL was understood to be a monoclonal disease, stemming from a single parental B cell that underwent malignant transformation. As such, all leukemic cells are expected to express a single, identical BCR. However, how should it be interpreted when not one, but multiple rearrangements dominate the BCR repertoire of a given CLL patient? Indeed, BCR repertoire sequencing has revealed that, while many CLL cases are characterized by a single dominant rearrangement, there is a varying percentage of cases in which the repertoire is characterized by two or more rearrangements that are clearly elevated above the polyclonal background (Table 1). Over the years, multiple explanations for this phenomenon have been proposed (Figure 3), which we discuss in detail.

Productive/nonproductive bi-allelic rearrangements

V(D)J recombination is an error-prone process. The stochastic recombination of genes, including the non-templated insertion and deletion of junctional nucleotides, can lead to nonproductive (NP) rearrangements, which cannot be translated to a functional BCR. Potential causes of nonproductivity are (1) a stop-codon or frameshift in the CDR3 region, (2) large insertions or deletions in the IGHV gene leading to loss of structural integrity, (3) a recombination involving a pseudogene, and (4) replacement or loss of critical "anchor" residues of the HCDR3, such as the cysteine (C) residue at position 104 and the tryptophan residue at position 118 [33]. An exemption to the latter is when the aberrant residue at 118 is immediately followed by an intact glycine–X–glycine motif in FR4. In these cases, productivity is most probably maintained [33].

When V(D)J rearrangement results in an NP sequence, the B cell will attempt rearrangement of its V, D, and J genes on the other allele. If this produces a functional rearrangement, the B cell survives and now bears two rearranged loci: an NP allele and a productive (P) allele. When such a cell suffers leukemic transformation, sequencing the BCR repertoire will reveal both the NP and P rearrangements as co-dominant. Using NGS, such P/NP bi-allelic rearrangements have been detected in 16%–18% of CLL patients [34]. The rate of identification of NP alleles varies depending on the type of genetic material sequenced: NP alleles are identified in greater frequency when analyzing genomic DNA instead of cDNA [33], probably because of nonsense-mediated RNA decay of the NP transcript. The degree of SHM is usually similar in both P and NP alleles, evident from the observation that in 93% of pairs, IGHV mutational status is concordant. However, according to ERIC guidelines, the NP allele should not be used to interpret the IGHV SHM status [33,35]. The presence of an NP rearrangement does not affect prognosis [34].

Failure of allelic exclusion

To ensure monospecificity of B cells, V(D)J recombination is tightly controlled in such a way that only one productive rearrangement is expressed on the cellular surface. This phenomenon is known as *allelic exclusion* [36]. Failure of allelic exclusion (alternatively called *allelic inclusion*), leading to the creation and expression of two productive V(D)J rearrangements in the same cell, has been observed in certain T cells and autoreactive B cells [37–39]. Rassenti and Kipps [40] proposed failure of allelic exclusion as the mechanism explaining the presence of CLL cases characterized by multiple dominant, productive rearrangements (MP cases). Upon studying samples from 108 CLL patients by real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay (RT-PCR–ELISA) and Southern blotting, they identified six MP cases that featured two or more dominant IGH clonotypes from different IGHV subgroups. Sanger sequencing confirmed these rearrangements to be productive and additionally found that each sample expressed only one functional LC rearrangement. Lastly, through the use of flow cytometry with antibodies specific for different IGHV subgroups, both BCRs were confirmed to be expressed on the cell surface. Consequently, the authors concluded that lack of allelic exclusion, leading to dual expression of BCRs, explained the presence of MP rearrangements in these CLL samples, and they postulated that dual BCR expression could confer a selective survival advantage [40].

More recent studies have failed to replicate these findings. Using extensive immunophenotypic and molecular profiling, Plevova et al. [41] examined a cohort of 31 MP CLL cases. In 9 of 31 cases, the presence of two immunophenotypically distinct clones, as well as multiple productive LC rearrangements,

Table 1. BCR sequencing studies in CLL

Study	Study population	Methodology	Notable findings
Rassenti and Kipps 1997 [40]	108 CLL cases	IGHV subgroup analysis by RT-PCR-ELISA and SSeq	6/108 cases (5.6%) reacted with two IGHV subgroup probes on cDNA, productivity confirmed with SSeq All double rearrangements use a single LC gene
Tobin et al. 2003 [11]	265 CLL cases	SSeq	45 (17.0%) of cases have double rearrangements, and 1 case (0.4%) has a triple rearrangement
Tobin et al. 2004 [12]	346 CLL cases	SSeq	52 (15.0%) cases with double and 3 (0.9%) cases with triple rearrangements
Belessi et al. 2006 [72]	760 CLL cases	SSeq	49 (6.5%) cases with double rearrangements, of which 19 (2.5%) have double productive rearrangements
Stamatopoulos et al. 2007 [18]	916 CLL cases	SSeq	11 (1.2%) cases with double productive rearrangements
Murray et al. 2008 [19]	1,939 CLL cases	SSeq	28 (1.4%) cases with double productive rearrangements
Campbell, 2008 [46]	24 CLL cases	Pyrosequencing	6 (25%) cases with phylogenetically related subclonal populations
Bomben et al. 2009 [20]	1,398 CLL cases	SSeq	22 (1.6%) cases with double and 3 (0.2%) cases with triple productive rearrangements
Sutton et al. 2009 [48]	71 CLL cases, biased toward subsets 2 and 4	Subcloning HC genes	28 (39.4%) cases with confirmed intraclonal diversity, especially enriched in subset 4.
Kostareli et al. 2010 [49]	56 CLL cases, biased toward subsets 2 and 4	Subcloning LC genes	22/37 (59.5%) cases with confirmed *IGKV* intraclonal diversity and 6/20 (30.0%) cases with confirmed *IGLV* intraclonal diversity, especially enriched in subset 4
Logan et al. 2011 [47]	6 U-CLL cases	Pyrosequencing	3 (50%) cases show intraclonal diversification
Langerak et al. 2011 [33]	4,154 CLL cases	SSeq	NP rearrangements detected in 377 (9.1%) cases Double productive rearrangements detected in 85 (2.0%) cases
Agathangelidis et al. 2012 [24]	7,424 CLL cases	SSeq	172 (2.3%) cases with double productive rearrangements
Visco et al. 2013 [73]	321 CLL cases	SSeq	Double productive rearrangements in 10 (3.1%) cases
Plevova et al. 2014 [41]	1,147 CLL cases	SSeq	Multiple productive rearrangements detected in 31 (2.7%) cases Definite or very likely biclonality proven in 20/31 cases
Kriangkum et al. 2015 [42]	198 CLL patients	CDR3 profiling, single cell analysis and NGS	Multiple CDR3 sequences in 26 (13.1%) patients 19/26 have an NP rearrangement 7/26 patients feature multiple distinct CLL clones, confirmed by single-cell analysis
Klinger et al. 2016 [51]	30 cases of MBL	NGS	7/29 (24.1%) cases featured double productive unrelated clonotypes 6/29 (20.6%) cases had evidence of intraclonal diversification
Xochelli et al. 2017 [25]	19,907 CLL cases	SSeq	424 (2.1%) cases with double productive rearrangements
Stamatopoulos et al. 2017 [34]	270 treatment-naïve CLL cases 227 CLL cases from FCR trial	NGS	Multiple productive rearrangements in 24.4% of treatment-naïve and 11.5% of trial patients NP in 16% and 18% of the populations, respectively

This table summarizes notable findings regarding subclonal architecture from sequencing studies in CLL.
FCR=fludarabine/cyclophosphamide/rituximab, *SSeq*=Sanger sequencing.

provided definitive proof that distinct clonal populations, rather than failure of allelic exclusion, explained the MP phenotype. In an additional 16 of 31 cases, coexistence of separate clones was deemed highly likely because of the presence of multiple partial or unproductive IG rearrangements, exceeding the allelic capacity of a single clone. Only in the remaining 6 of 31 cases, the authors failed to obtain evidence of the presence of distinct clones, which still is not conclusive proof of allelic inclusion in these cases. Similarly, Kriangkum et al. [42] examined seven MP CLL cases using a single-cell PCR approach, revealing bi- or oligoclonality and maintenance of allelic exclusion in all cases. In summary, more advanced approaches have, in the majority of MP CLL cases, failed to reveal the existence of allelic inclusion. For this reason, violation of allelic exclusion offers an explanation for a small proportion of MP cases at best, and critical reappraisal is required to assess if this phenomenon truly exists in CLL at all.

Intraclonal diversification

Conventionally, the mutational imprint on the IG genes of CLL cells was considered static and shared between

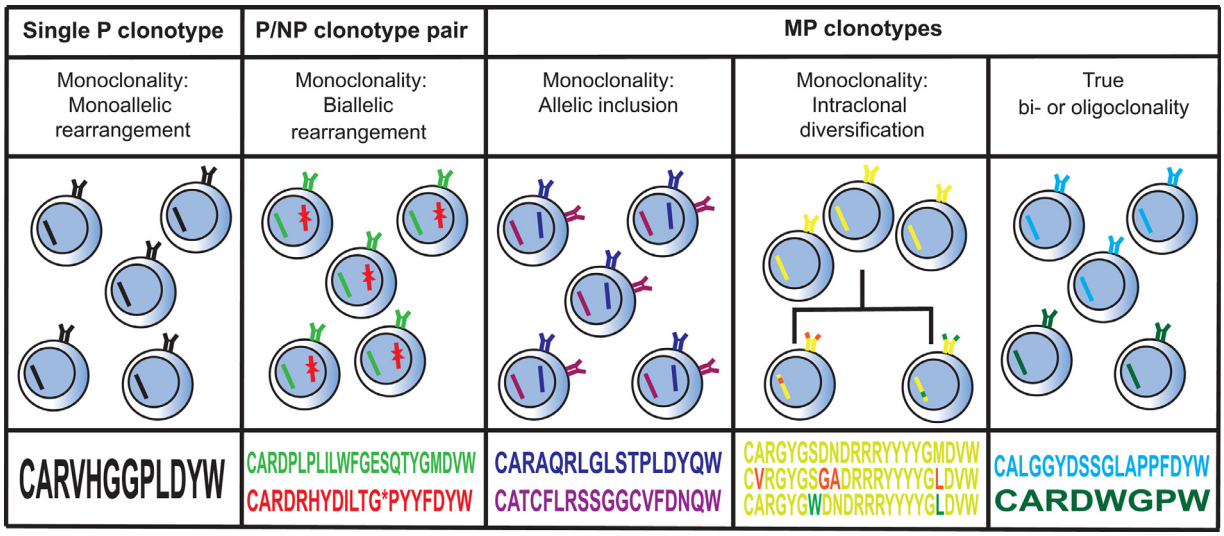


Figure 3. Subclonal architecture of CLL. Panels graphically representing theories of subclonality in CLL. In most CLL cases, IGH repertoire sequencing reveals dominance of a single clonotypic rearrangement (first panel from the left). In CLL cases with bi-allelic rearrangements, repertoire sequencing reveals the dominance of two clonotypes, one of which is nonproductive (second panel). Multiple mechanisms could explain the presence of multiple productive dominant clonotypes in CLL. The theory of allelic inclusion postulates that individual CLL cells can rearrange and express two BCRs, violating the principles of allelic exclusion (third panel). In CLL cases with persistent mutational pressure on the BCR, multiple distinct but phylogenetically related clonotypes can dominate the repertoire, known as intraclonal diversification (fourth panel). Finally, the dominance of multiple productive clonotypes can be explained by the presence of true bi- or oligoclonality, the simultaneous presence of separate, unrelated clonal populations (fifth panel). The bottom panels represent examples of the HCDR3s of the clonotypes dominating the repertoire. The asterisk denotes a stop codon.

all leukemic cells. However, there is clear evidence that in a subgroup of cases, the IG rearrangements are subject to ongoing mutational pressure [43–45]. Using nested PCR amplification and pyrosequencing to characterize the IGH repertoire of 24 CLL patients, Campbell et al. [46] found that the repertoire of 6 patients was characterized by the simultaneous presence of one dominant clonotype and multiple smaller, highly similar, phylogenetically related clonotypes, bearing minimal but outspoken differences in their nucleotide and aa sequences. The presence of these "satellite clonotypes" strongly suggests a common ancestor clone and subsequent intraclonal diversification (ID). Similar ID could be identified in 3 of 6 cases by Logan et al. [47], who used pyrosequencing to characterize the IGH repertoire of CLL patients undergoing allogeneic transplantation

However, robust identification of clonotypes at low frequency by pyrosequencing is somewhat limited because of the errors inherent to this technique, such as the introduction of sequencing artifacts around homopolymer tracts [46,47].

In an extensive subcloning study, Sutton et al. [48] examined the IGH clonotype repertoire in a cohort of 71 CLL cases. Although the majority of cases were characterized by a single dominant clonotype, 28 cases exhibited ID. Interestingly, ID was especially prevalent and extensive in subset 4, which could similarly be identified in the LC genes of these patients [49]. The observed ID in subset 4 exhibited distinctive patterns, clustering in several regions, including the HCDR3, with identical "stereotyped" mutations in multiple unrelated patients [48]. This strongly suggests ongoing SHM, possibly driven by persistent antigen-mediated mutational pressure. Notably, ID can be detected in both M- and U-CLL and even, albeit to a lower extent, in "truly unmutated" cases with 100% IGHV [38,39,41; own, unpublished data]. The discrepant combination of ongoing SHM in the HCDR3 in the presence of a relatively unaffected (unmutated) IGHV gene has as of yet not been explained mechanistically.

Longitudinal follow-up of patients with ID has revealed clonal drift: Whereas some satellite clonotypes persist, others become undetectable at later time points [50]. In addition, ID could already be identified by using NGS on sorted cells in a subgroup of patients with CLL-like monoclonal B-cell lymphocytosis (MBL) [51]. It is currently unknown if the presence of ID in CLL has any impact on prognosis.

True bi- or oligoclonality

A final explanation for MP cases is the existence of two or more phylogenetically unrelated clonal populations ("true bi- or oligoclonality") in one patient. This phenomenon has previously been detected in CLL using flow cytometric immunophenotyping or

conventional BCR Sanger sequencing at a frequency of 1%–3% [33,52]. Contrastingly, deep sequencing the IGH repertoire of purified $CD5^+$ B cells in 270 untreated CLL patients and 227 patients from a clinical trial, Stamatopoulos et al. [34] found this prevalence to be significantly higher, with MP clonotypes present in 24% of the untreated and 11.5% of the trial patients. A comparable study, using NGS on sorted $CD5^+$ cells from MBL patients, identified unrelated MP clonotypes in 24.4% of patients [51]. The implications of the high prevalence of true bi- or oligoclonality for disease ontogeny are currently unclear. Whether these simultaneously existing clonal populations represent pure serendipity, (epi)genetic predilection, recognition of a shared antigen, or accompanying, reactive, benign clonal expansion remains speculative at best.

True bi- or oligoclonality presents a challenge for conventional dichotomization by IGHV mutational status [33]. In an effort to surmount this, Stamatopoulos et al. [34] proposed stratifying CLL patients into five categories, based on the presence or absence of MP clonotypes and their IGHV mutational status: patients with multiple mutated clonotypes (M/M), with a single mutated clonotype (M), with discordantly mutated clonotypes (U/M), with a single unmutated clonotype (U), and with multiple unmutated clonotypes (U/U). Interestingly, median treatment-free survival (TFS) was the longest in M/M patients (20 years) and the shortest in the U/U group (little over 1 year) [34]. Importantly, after treatment with chemoimmunotherapy, U/U patients represented a very poor prognostic subgroup, with a median TFS of only 2 months. Unexpectedly, including NP clonotypes to stratify patients in these subgroups yielded very similar results [34]. Validation of these intriguing findings in external cohorts, including patients treated with novel therapies, is eagerly awaited.

Track and trace: measuring MRD in CLL using BCR immunogenetics

The development of novel, highly effective compounds for the treatment of CLL has sparked interest in sensitively quantitating MRD. Conventionally expressed as the amount of residual CLL cells relative to peripheral blood mononuclear cells (PBMCs) or leukocytes, MRD quantifies the burden of disease and depth of remission when, after successful treatment, leukemic cells are no longer detectable by routine blood counting. Accurately and sensitively measuring MRD serves several purposes. As lower levels of MRD predict longer progression-free survival (PFS) [53], MRD depth can be used to estimate patient prognosis and serve as a shorter-term, surrogate endpoint for clinical trials. Indeed, iwCLL guidelines recommend MRD assessment in every clinical trial that aims for maximizing remission depth [6]. Furthermore, MRD dynamics can be used to adapt therapeutic strategy, for instance, intensifying treatment when MRD persists, de-escalating when MRD levels become undetectable (uMRD), or re-initiating when MRD resurges after a period of undetectability, likely signaling impending relapse.

Reliable and sensitive quantification of MRD requires accurate differentiation between healthy and leukemic cells. As previously mentioned, all leukemic cells carry one or more mostly uniform V(D)J rearrangements. These unique rearrangements can be identified at diagnosis when leukemic burden is high and can subsequently be used as a "DNA fingerprint," through which leukemic cells can be distinguished from their healthy counterparts in an MRD assay. This has led to the development of several immunogenetics-based approaches for MRD quantification, primarily using quantitative PCR (qPCR) or NGS.

MRD assays that employ qPCR generally use allele-specific oligonucleotide (ASO) forward primers and consensus reverse primers to selectively amplify and detect a rearranged target unique to the leukemic cells, on either the IGH or IGK/IGL locus [54,55]. These ASO primers, which can target both productive and unproductive rearrangements, require unique design, tailored to the patient-specific, leukemia-specific V(D)J rearrangement. ASO-qPCR has been used successfully to quantify MRD after both chemoimmunotherapy- and venetoclax-based regimens to a depth of 10^{-4} [56,57], but can potentially reach measurement depths down to 10^{-5}. Although the ASO-qPCR MRD assay has high sensitivity and specificity, the requirement for patient-specific primers implies that the process is often laborious and expensive. Moreover, in a small percentage of CLL cases, no functioning assay can be designed because of intensive SHM in primer-annealing sites [58]. Finally, considering the subclonal variation and ongoing SHM present in some CLL patients, targeting only a single rearrangement could theoretically lead to underestimation of the real burden of disease, a pitfall recognized for many years, for example, in acute lymphoblastic leukemia [59].

An alternative and promising immunogenetics-based approach to quantify MRD uses consensus primers to amplify all IGH rearrangements in a pool of PBMC DNA, followed by NGS to quantify the presence of the leukemia-specific rearrangement. This NGS-MRD approach combines high sensitivity and specificity with extensive applicability, as no primer customization is required. Moreover, deep sequencing allows for characterization of the subclonal landscape, circumventing pitfalls posed by clonal dynamics. Several studies using NGS to quantify MRD in CLL have been performed. Using high-throughput pyrosequencing, Logan et al. [47] longitudinally quantified MRD in 6 patients who

were allografted for aggressive CLL, revealing measurement depths up to 10^{-5} and good correlation with ASO-qPCR [47]. In a follow up study using the LymphoSIGHT platform, a commercially operated Illumina-NGS-based MRD assay by Sequenta, Inc. (South San Francisco, CA) [60], their research group quantified MRD in 40 allografted CLL patients [61]. In these patients, they reported an unprecedented MRD detection depth of one CLL cell per one million PBMCs (MRD 10^{-6}) and reported strong correlation between MRD detectability at 12 months and disease relapse [61]. More recently, the ImmunoSEQ and ClonoSEQ Illumina NGS-based platforms, commercially developed and operated by the Adaptive Biotechnologies Corporation (Seattle, WA), which acquired Sequenta in 2015, have been used to measure MRD in CLL [62–64]. In one study, Thompson et al. [64] used the ClonoSEQ assay for MRD quantification after treatment with chemoimmunotherapy, similarly reporting MRD measurement depths down to 10^{-6}. Interestingly, the ClonoSEQ assay could detect MRD in more than two-thirds of cases that had uMRD as quantified by MFC, and these patients had inferior PFS when compared with patients that had uMRD by NGS. Although these commercially operated platforms provide impressive and thought-provoking results, confirmation by standardized and multicenter validated assays, with robust external quality control, remains pivotal.

Pitfalls of MRD quantification by NGS

NGS-MRD quantification presents several specific challenges. First, the use of consensus primers invariably introduces trade-offs and leads to unequal amplification of rearrangements, determined mainly by primer efficiency. Preferential amplification can lead to considerable errors in MRD quantification, especially after anti-B-cell treatment, when the polyclonal background is severely depleted [58]. Moreover, identifying the correct index clone at diagnosis can be challenging, particularly if amplification of the leukemic rearrangement is suboptimal because of intense SHM in the J-primer binding site and/or if multiple productive rearrangements dominate the repertoire. Lastly, to avoid "false sequencing depth," where the number of reads exceeds the theoretical number of sequenced cells, a minimal threshold of input genomic material needs to be defined.

Translation of NGS-MRD quantification to a clinical setting requires the development of a standardized strategy of library preparation, adoption of a reliable and user-friendly bioinformatics tool, and educational training programs. To tackle these challenges, to ensure stringent external quality control, and to develop standardized protocols, guidelines, and a pipeline for NGS-MRD quantification, the EuroClonality–NGS Working Group has been established [58]. Steps in the development of such a pipeline have recently been published, including validated primer sets [65], the development of spiked in-tube quality control to correct for amplification biases [66], and the ARReST/Interrogate bioinformatics platform for repertoire analysis [67].

Addendum: reading the T-cell receptor immunome in CLL

T lymphocytes likely serve an important auxiliary role in CLL and can induce CLL cell proliferation [68,69]. As there is mounting evidence that, similarly to the BCR repertoire, the T-cell receptor (TCR) repertoire of CLL patients is skewed [70], reading the TCR immunome could be the next chapter in CLL. In fact, by sequencing the TRBV–TRBD–TRBJ repertoire of 32 patients, Vardi et al. [71] found significant oligoclonality in the TCR repertoire in CLL. This skewing was most predominantly present in the $CD8^+$ compartment, where the median cumulative frequency of the 10 largest TRB clonotypes represented 43.6% of the repertoire [71]. Moreover, the authors found that several TRB clonotypes, mostly absent in the healthy population, were shared by multiple CLL patients. Whether this "TCR stereotypy" is driven by the recognition of antigens, either tumor-specific epitopes or, conceivably, the same antigen driving CLL progenitors, will, it is hoped, be elucidated in the future as greater attention is directed to this field.

Conclusions

In this review, we discussed the revelations and applications of reading the BCR immunome in the context of CLL (Figure 4). In CLL, BCR immunome analysis is widely applied, both in research and in clinical practice, to determine IGHV mutational status. Furthermore, the identification of stereotyped subsets of patients with specific HCDR3 configurations has led to the development of an ontological model of CLL in which both antigen-specific and homotypic BCR–BCR interactions are important drivers of disease. More recently, deep sequencing of the CLL BCR repertoire has identified significant subclonal heterogeneity: Whereas the repertoire of most patients is dominated by a single productive rearrangement, intense intraclonal diversity or even true bi- or oligoclonality is observed much more frequently than previously anticipated. The impact of these subclonal variants on disease biology, prognosis, and response to therapy is currently insufficiently characterized and warrants additional research. Finally, the BCR immunome can be exploited to quantify MRD by identifying residual CLL cells based on their unique V(D)J rearrangement. Efforts to develop a standardized, harmonized NGS-MRD pipeline with external quality control, coordinated by the EuroClonality–NGS Working Group, are well underway.

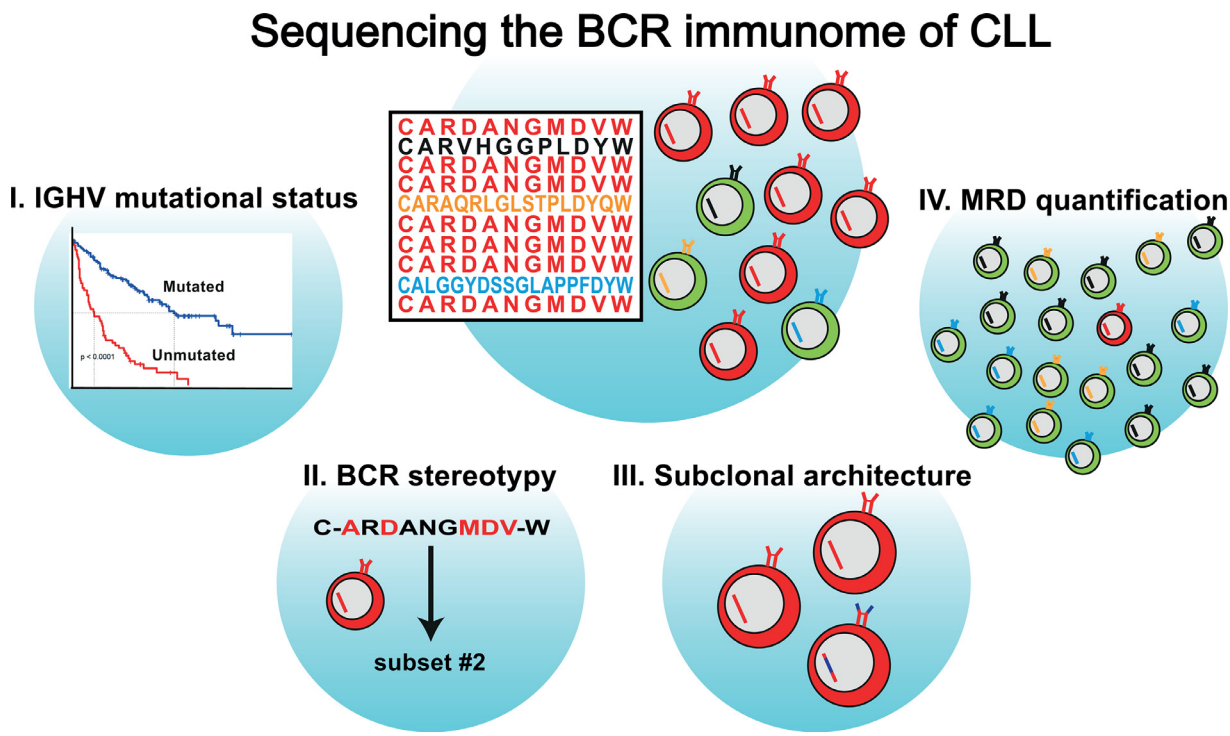


Figure 4. Revelations and applications of BCR immunome sequencing in CLL. Schematic overview of several applications of BCR immunome analysis in CLL. BCR sequencing is widely used to stratify prognosis based on the mutational status of the IGHV gene (I). Additionally, the aa sequence of the HCDR3 region can be used to define BCR stereotyped subsets in CLL, a concept important for disease ontogeny, biology, and clinical practice (II). High-throughput sequencing of the BCR repertoire also allows characterization of the subclonal architecture of CLL, revealing instances of bi- or oligoclonality and intraclonal diversification (III). Lastly, the specific IG rearrangements of the leukemic clone can be used as a "DNA fingerprint" to identify residual CLL cells and quantify MRD.

Conflict of interest disclosure

The authors have no competing interests to declare.

References

1. Xu JL, Davis MM. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. Immunity. 2000;13:37–45.
2. Briney B, Inderbitzin A, Joyce C, Burton D. Commonality despite exceptional diversity in the baseline human antibody repertoire. Nature. 2019;566:393–397.
3. Hamblin T, Davis Z, Gardiner A, Oscier D, Stevenson F. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848–1854.
4. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood. 1999;94:1840–1847.
5. Lin KI, Tam CS, Keating MJ, et al. Relevance of the immunoglobulin VH somatic mutation status in patients with chronic lymphocytic leukemia treated with fludarabine, cyclophosphamide, and rituximab (FCR) or related chemoimmunotherapy regimens. Blood. 2009;113:3168–3171.
6. Hallek M, Cheson BD, Catovsky D, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. Blood. 2018;131:2745–2760.
7. Parikh SA. Chronic lymphocytic leukemia treatment algorithm 2018. Blood Cancer J. 2018;8:93.
8. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 1998;102:1515–1525.
9. Johnson TA, Rassenti LZ, Kipps TJ. Ig VH1 genes expressed in B cell chronic lymphocytic leukemia exhibit distinctive molecular features. J Immunol. 1997;158:235–246.
10. Tobin G, Thunberg U, Johnson A, et al. Somatically mutated Ig VH3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood. 2002;99:2262–2264.
11. Tobin G, Thunberg U, Johnson A, et al. Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted Vλ2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope. Blood. 2003;101:4952–4957.
12. Tobin G, Thunberg U, Karlsson K, et al. Subsets with restricted immunoglobulin gene rearrangement features indicate a role for antigen selection in the development of chronic lymphocytic leukemia. Blood. 2004;104:2879–2885.
13. Messmer BT, Albesiano E, Efremov DG, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 2004;200:519–525.
14. Ghiotto F, Fais F, Valetto A, et al. Remarkably similar antigen receptors among a subset of patients with chronic lymphocytic leukemia. J Clin Invest. 2004;113:1008–1016.
15. Widhopf GF, Rassenti LZ, Toy TL, Gribben JG, Wierda WG, Kipps TJ. Chronic lymphocytic leukemia B cells of more than 1% of patients express virtually identical immunoglobulins. Blood. 2004;104:2499–2504.
16. Thorsélius M, Kröber A, Murray F, et al. Strikingly homologous immunoglobulin gene rearrangements and poor outcome in VH3-21-using chronic lymphocytic leukemia patients

independent of geographic origin and mutational status. Blood. 2006;107:2889–2894.
17. Potter KN, Mockridge CI, Neville L, et al. Structural and functional features of the B-cell receptor in IgG-positive chronic lymphocytic leukemia. Clin Cancer Res. 2006;12:1672–1679.
18. Stamatopoulos K, Belessi C, Moreno C, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: pathogenetic implications and clinical correlations. Blood. 2007;109:259–270.
19. Murray F, Darzentas N, Hadzidimitriou A, et al. Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis. Blood. 2008;111:1524–1533.
20. Bomben R, Dal Bo M, Capello D, et al. Molecular and clinical features of chronic lymphocytic leukaemia with stereotyped B cell receptors: results from an Italian multicentre study. Br J Haematol. 2009;144:492–506.
21. Darzentas N, Hadzidimitriou A, Murray F, et al. A different ontogenesis for chronic lymphocytic leukemia cases carrying stereotyped antigen receptors: molecular and computational evidence. Leukemia. 2010;24:125–132.
22. Muggen AF, De Jong M, Wolvers-Tettero ILM, et al. The presence of CLL-associated stereotypic B cell receptors in the normal BCR repertoire from healthy individuals increases with age. Immun Ageing. 2019;16:1–12.
23. Colombo M, Bagnara D, Reverberi D, et al. Tracing CLL-biased stereotyped immunoglobulin gene rearrangements in normal B cell subsets using a high-throughput immunogenetic approach. Mol Med. 2020;26:25.
24. Agathangelidis A, Darzentas N, Hadzidimitriou A, et al. Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. Blood. 2012;119:4467–4475.
25. Xochelli A, Baliakas P, Kavakiotis I, et al. Chronic lymphocytic leukemia with mutated IGHV4-34 receptors: shared and distinct immunogenetic features and clinical outcomes. Clin Cancer Res. 2017;23:5292–5301.
26. Baliakas P, Agathangelidis A, Hadzidimitriou A, et al. Not all IGHV3-21 chronic lymphocytic leukemias are equal: prognostic considerations. Blood. 2015;125:856–859.
27. Marincevic M, Cahill N, Gunnarsson R, et al. High-density screening reveals a different spectrum of genomic aberrations in chronic lymphocytic leukemia patients with "stereotyped" IGHV3-21 and IGHV4-34 B-cell receptors. Haematologica. 2010;95:1519–1525.
28. Strefford JC, Sutton LA, Baliakas P, et al. Distinct patterns of novel gene mutations in poor-prognostic stereotyped subsets of chronic lymphocytic leukemia: the case of SF3B1 and subset #2. Leukemia. 2013;27:2196–2199.
29. Jaramillo S, Agathangelidis A, Schneider C, et al. Prognostic impact of prevalent chronic lymphocytic leukemia stereotyped subsets: analysis within prospective clinical trials of the German CLL Study Group (GCLLSG). Haematologica. 2019 Dec 26. https://doi.org/10.3324/haematol.2019.231027. [online ahead of print].
30. Minden MD Von, Übelhart R, Schneider D, et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. Nature. 2012;489:309–312.
31. Minici C, Gounari M, Übelhart R, et al. Distinct homotypic B-cell receptor interactions shape the outcome of chronic lymphocytic leukaemia. Nat Commun. 2017;8:1–12.
32. Maity PC, Bilal M, Koning MT, et al. IGLV3-21*01 is an inherited risk factor for CLL through the acquisition of a single-point mutation enabling autonomous BCR signaling. Proc Natl Acad Sci USA. 2020;117:4320–4327.
33. Langerak AW, Davi F, Ghia P, et al. Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. Leukemia. 2011;25:979–984.
34. Stamatopoulos B, Timbs A, Bruce D, et al. Targeted deep sequencing reveals clinically relevant subclonal IgHV rearrangements in chronic lymphocytic leukemia. Leukemia. 2017;31:837–845.
35. Rosenquist R, Ghia P, Hadzidimitriou A, et al. Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: updated ERIC recommendations. Leukemia. 2017;31:1477–1481.
36. Kitamura D, Rajewsky K. Targeted disruption of mu chain membrane exon causes loss of heavy-chain allelic exclusion. Nature. 1992;356:154–156.
37. Li Y, Li H, Weigert M. Autoreactive B cells in the marginal zone that express dual receptors. J Exp Med. 2002;195:181–188.
38. Davodeau F, Peyrat M, Houde I, et al. Surface expression of two distinct functional antigen receptors on human γδ T cells. Science. 1993;260:1800–1802.
39. Balomenos D, Balderas RS, Mulvany KP, Kaye J, Kono DH, Theofilopoulos AN. Incomplete T cell receptor V beta allelic exclusion and dual V beta-expressing cells. J Immunol. 1995;155:3308–3312.
40. Rassenti LZ, Kipps TJ. Lack of allelic exclusion in B cell chronic lymphocytic leukemia. J Exp Med. 1997;185:1435–1445.
41. Plevova K, Francova HS, Burckova K, et al. Multiple productive immunoglobulin heavy chain gene rearrangements in chronic lymphocytic leukemia are mostly derived from independent clones. Haematologica. 2014;99:329–338.
42. Kriangkum J, Motz SN, Mack T, et al. Single-cell analysis and next-generation immuno-sequencing show that multiple clones persist in patients with chronic lymphocytic leukemia. PLoS One. 2015;10:1–15.
43. Gurrieri C, McGuire P, Zan H, et al. Chronic lymphocytic leukemia B cells can undergo somatic hypermutation and intraclonal immunoglobulin VHDJH gene diversification. J Exp Med. 2002;196:629–639.
44. Degan M, Bomben R, Dal Bo M, et al. Analysis of IgVH gene mutations in B cell chronic lymphocytic leukaemia according to antigen-driven selection identifies subgroups with different prognosis and usage of the canonical somatic hypermutation machinery. Br J Haematol. 2004;126:29–42.
45. Volkheimer AD, Weinberg JB, Beasley BE, et al. Progressive immunoglobulin gene mutations in chronic lymphocytic leukemia: evidence for antigen-driven intraclonal diversification. Blood. 2007;109:1559–1567.
46. Campbell PJ, Pleasance ED, Stephens PJ, et al. Subclonal phylogenetic structures in cancer revealed by ultra-deep sequencing. Proc Natl Acad Sci USA. 2008;105:13081–13086.
47. Logan AC, Gao H, Wang C, et al. High-throughput VDJ sequencing for quantification of minimal residual disease in chronic lymphocytic leukemia and immune reconstitution assessment. Proc Natl Acad Sci USA. 2011;108:21194–21199.
48. Sutton LA, Kostareli E, Hadzidimitriou A, et al. Extensive intraclonal diversification in a subgroup of chronic lymphocytic leukemia patients with stereotyped IGHV4-34 receptors: implications for ongoing interactions with antigen. Blood. 2009;114:4460–4468.
49. Kostareli E, Sutton LA, Hadzidimitriou A, et al. Intraclonal diversification of immunoglobulin light chains in a subset of chronic lymphocytic leukemia alludes to antigen-driven clonal evolution. Leukemia. 2010;24:1317–1324.
50. Sutton LA, Kostareli E, Stalika E, et al. Temporal dynamics of clonal evolution in chronic lymphocytic leukemia with

stereotyped IGHV4-34/IGKV2-30 antigen receptors: longitudinal immunogenetic evidence. Mol Med. 2013;19:230–236.
51. Klinger M, Zheng J, Elenitoba-Johnson KSJ, Perkins SL, Faham M, Bahler DW. Next-generation IgVH sequencing CLL-like monoclonal B-cell lymphocytosis reveals frequent oligoclonality and ongoing hypermutation. Leukemia. 2016;30:1055–1061.
52. Sanchez ML, Almeida J, Gonzalez D, et al. Incidence and clinicobiologic characteristics of leukemic B-cell chronic lymphoproliferative disorders with more than one B-cell clone. Blood. 2003;102:2994–3002.
53. Molica S, Giannarelli D, Montserrat E. Minimal residual disease and survival outcomes in patients with chronic lymphocytic leukemia: a systematic review and meta-analysis. Clin Lymphoma Myeloma Leuk. 2019;19:423–430.
54. van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJM. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia. 2003;17:1013–1034.
55. van der Velden VHJ, Cazzaniga G, Schrauder A, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia. 2007;21:604–611.
56. Langerak AW, Ritgen M, Goede V, et al. Prognostic value of MRD in CLL patients with comorbidities receiving chlorambucil plus obinutuzumab or rituximab. Blood. 2019;133:494–497.
57. Fischer K, Al-Sawaf O, Bahlo J, et al. Venetoclax and obinutuzumab in patients with CLL and coexisting conditions. N Engl J Med. 2019;380:2225–2236.
58. Langerak AW, Brüggemann M, Davi F, et al. High-throughput immunogenetics for clinical and research applications in immunohematology: potential and challenges. J Immunol. 2017;198:3765–3774.
59. Germano G, del Giudice L, Palatron S, et al. Clonality profile in relapsed precursor-B-ALL children by GeneScan and sequencing analyses: consequences on minimal residual disease monitoring. Leukemia. 2003;17:1573–1582.
60. Faham M, Zheng J, Moorhead M, et al. Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. Blood. 2012;120:5173–5180.
61. Logan AC, Zhang B, Narasimhan B, et al. Minimal residual disease quantification using consensus primers and high-throughput IGH sequencing predicts post-transplant relapse in chronic lymphocytic leukemia. Leukemia. 2013;27:1659–1665.
62. Porter DL, Hwang WT, Frey NV, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Sci Transl Med. 2015;7:1–13.
63. Rawstron AC, Fazi C, Agathangelidis A, et al. A complementary role of multiparameter flow cytometry and high-throughput sequencing for minimal residual disease detection in chronic lymphocytic leukemia: an European Research Initiative on CLL study. Leukemia. 2016;30:929–936.
64. Thompson PA, Srivastava J, Peterson C, et al. Minimal residual disease undetectable by next-generation sequencing predicts improved outcome in CLL after chemoimmunotherapy. Blood. 2019;134:1951–1959.
65. Brüggemann M, Kotrová M, Knecht H, et al. Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia: a EuroClonality−NGS validation study. Leukemia. 2019;33:2241–2253.
66. Knecht H, Reigl T, Kotrová M, et al. Quality control and quantification in IG/TR next-generation sequencing marker identification: protocols and bioinformatic functionalities by EuroClonality−NGS. Leukemia. 2019;33:2254–2265.
67. Bystry V, Reigl T, Krejci A, et al. ARResT/Interrogate: an interactive immunoprofiler for IG/TR NGS data. Bioinformatics. 2017;33:435–437.
68. Bagnara D, Kaufman MS, Calissano C, et al. A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. Blood. 2011;117:5463–5472.
69. Os A, Bürgler S, Ribes AP, et al. Chronic lymphocytic leukemia cells are activated and proliferate in response to specific T helper cells. Cell Rep. 2013;4:566–577.
70. Vardi A, Agathangelidis A, Stalika E, et al. Antigen selection shapes the T-cell repertoire in chronic lymphocytic leukemia. Clin Cancer Res. 2016;22:167–174.
71. Vardi A, Vlachonikola E, Karypidou M, et al. Restrictions in the T-cell repertoire of chronic lymphocytic leukemia: high-throughput immunoprofiling supports selection by shared antigenic elements. Leukemia. 2017;31:1555–1561.
72. Belessi CJ, Davi FB, Stamatopoulos KE, et al. IGHV gene insertions and deletions in chronic lymphocytic leukemia: "CLL-biased" deletions in a subset of cases with stereotyped receptors. Eur J Immunol. 2006;36:1963–1974.
73. Visco C, Moretta F, Falisi E, et al. Double productive immunoglobulin sequence rearrangements in patients with chronic lymphocytic leukemia. Am J Hematol. 2013;88:277–282.