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REVIEW

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Assessing prognosis of chronic lymphocytic leukemia using biomarkers and genetics

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ABSTRACT

Introduction: Chronic lymphocytic leukemia (CLL) is a clinically and genetically heterogenous disease. Genomic studies have deciphered the pathogenesis of CLL and has allowed the identification of prognostic and predictive biomarkers. During the last decade, the treatment options for CLL have expanded significantly, posing the need for the identification of molecular predictors for treatment tailoring.

Areas covered: This review focuses on biomarkers revealed by investigations of CLL molecular genetics and immunogenetics, and that may help optimizing therapy for individual patients. In addition, the manuscript discusses minimal residual disease (MRD) assessment and its potential application as a prognostic biomarker and as a new tool to guide treatment duration.

Expert opinion: The availability of a variety of treatment options, including chemoimmunotherapy (CIT) and biological drugs that inhibit the B cell receptor (BCR) and the B cell lymphoma 2 (BCL2) antiapoptotic protein, has significantly improved survival of CLL patients. In this therapeutic landscape, the identification of different CLL risk groups based on the presence of specific molecular lesions and/or immunogenetic features has allowed treatment tailoring in terms of choosing the most appropriate drug. The combination of genetic and immunogenetic biomarkers together with MRD assessment may allow one step forward in the precision medicine approach to CLL.

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia in Western countries with an annual incidence of 5.1/100,000 [1]. CLL is a markedly heterogeneous disease from both a biological and a clinical standpoint [2]. Genomic studies in CLL have allowed to decipher the disease pathogenesis demonstrating that CLL is not characterized by a common genetic lesion but, conversely, is characterized by many different genetic abnormalities responsible for CLL initiation, development and progression [2–7]. The molecular heterogeneity of CLL indicates that not all patients may benefit from the same treatment and strengthens the importance of identifying molecular predictors, i.e. biomarkers that provide information on the likely benefit from a specific treatment and thus may allow treatment tailoring in every single patient [8]. By combining different clinical and biological prognosticators, several prognostic scores have been developed in order to stratify the outcome of CLL patients [9–15].

Till now, two molecular predictors, namely the mutational status of immunoglobulin heavy chain variable (IGHV) genes and abnormalities of the *TP53* tumor suppressor gene (including 17p deletion and *TP53* mutations), are routinely used in the clinical practice for treatment decision making in CLL patients [16]. Accordingly, patients with mutated IGHV genes devoid of *TP53* disruption are the ones who can benefit most from chemoimmunotherapy (CIT), whereas *TP53* disrupted patients need to be treated upfront with biological agents that can, at least in part, circumvent their chemorefractoriness [2,16].

Recent studies have identified novel molecular features with potential applicability in view of precision medicine for CLL, including B cell receptor (BCR) stereotypy and mutations of the *BIRC3, NOTCH1, BTK, PLCγ2* and *BCL2* genes [17–19]. Moreover, minimal residual disease (MRD) assessment is becoming a potential tool to predict patients' outcome and also to identify the best treatment duration with biological drugs that, till now, are in most of cases administered continuously until progression [20,21]. Here we review the use of the main molecular prognosticators and predictors in CLL. The literature search was performed on PubMed and Cochrane Central Register of Controlled Trials using as keywords 'CLL, prognostic markers, predictive biomarkers' (last search date: 25 June 2020).

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Article highlights

- Genomic studies in CLL have allowed to decipher the disease pathogenesis and to identify prognostic and predictive biomarkers
- Two molecular predictors, namely the mutational status of the IGHV genes and abnormalities of the *TP53* tumor suppressor gene, are currently used in clinical practice for treatment decision making in CLL
- The availability of a variety of treatment options, including chemoimmunotherapy and inhibitors of the BCR and of BCL2, has significantly improved survival of CLL patients
- The identification of novel predictors (e.g. *BIRC3* mutations) combined with the availability of novel treatment options may further improve patients' outcome
- The correlation between molecular predictors and patient reported outcomes may aid in making more informed and individualized treatment decisions in the daily practice

This box summarizes key points contained in the article.

2. IGHV mutational status

The rearrangement of IGHV genes represents the hallmark of monoclonality for all B cell malignancies. An important feature of IGHV rearrangements in CLL is the degree of identity of the IGHV gene utilized by the leukemic clone compared to the sequence of the gene in its germline configuration [22]. The degree of IGHV gene identity to germline depends upon the so called somatic hypermutation (SHM) process. SHM occurs in germinal centers (GC), that are dynamic microanatomical compartments of lymph nodes where B cells are challenged by a foreign antigen and represents the primary site for clonal expansion and antibody affinity and maturation [22]. SHM is primarily caused by activation-induced cytidine deaminase (AID), an enzyme that induces random changes in the nucleotide sequence of the IGHV genes and may increase, decrease, or leave unaltered the affinity of the BCR for a foreign antigen [22].

SHM of IGHV genes in CLL has implications for disease pathogenesis as well as implications as a predictive biomarker [2,16]. In approximately 60% of CLL, the sequence of IGHV genes shows a homology to the normal counterpart of less than 98%. These cases are defined as IGHV mutated CLL whose origin is postulated from B cells that have undergone SHM in the GC [22]. Conversely, 40% of CLL patients displays unmutated IGHV genes, defined as a homology to the normal counterpart equal to or higher than 98%. These cases are defined as IGHV unmutated CLL and are postulated to derive from naïve B cells that have undergone maturation independent of the GC reaction [22].

The SHM status of IGHV genes remains one of the strongest independent prognostic markers in CLL (Table 1) [23,24]. Using the cut off suggested by the European Research Initiative on CLL (ERIC), i.e. ≥98% identity for unmuted IGHV genes and <98% identity for mutated IGHV genes, the mutational status of IGHV genes is not only a prognosticator, but also a predictive biomarker [25]. Retrospective studies have demonstrated that patients with mutated IGHV genes and devoid of *TP53* abnormalities are the ones who can benefit the most from CIT [26–28]. Prospective phase 3 clinical trials, comparing CIT *versus* B cell receptor inhibitors (BCRi) and B cell lymphoma 2 inhibitors (BCL2i), have validated these results, both in young and fit CLL treated with fludarabine, cyclophosphamide and rituximab (FCR) as well as in elderly patients or patients with comorbidities treated with obinutuzumab-chlorambucil [29–31]. More precisely, in all such clinical trials, the overall outcome of patients treated with BCRi or BCL2i was significantly improved compared to the outcome of patients treated with CIT. However, this difference was not seen in the subgroup of IGHV mutated patients, suggesting that a fixed duration CIT regimen may still be a valid option for CLL patients with mutated IGHV genes [29–31]. More recently, evidence from a trial comparing acalabrutinib, a novel BCRi with minimal activity against alternative targets, with or

Table 1. Impact of gene mutations on PFS in CLL phase 3 clinical trials for treatment naïve patients.

Trial	Interventions	Unmutatad IGHV	*TP53* mutations	*BIRC3* mutations	NOTCH1 mutations
CLL14 trial [73]	Venetoclax + Obinutuzumab	HR 1.16 (95% CI 0.51–2.62) p = 0.73	HR 3.08 (95% CI 1.31–7.25) p = 0.01	HR 1.10 (95% CI 0.15–8.13) p = 0.92	HR 1.57 (95% CI 0.69–3.58) p = 0.28
	Chlorambucil + Obinutuzumab	HR 3.45 (95% CI 1.95–6.10) p < 0.01	HR 2.74 (95% CI 1.50–5.00) p < 0.01	HR 4.03 (95% CI 1.73–9.37) p < 0.01	HR 1.74 (95% CI 1.06–2.88) p = 0.03
RESONATE [81]	Ibrutinib	HR 0.80 (95% CI 0.43–1.52)	HR 1.42 (95% CI 0.85–2.46)	HR 0.78 (95% CI 0.37–1.65)	HR 1.00 (95% CI 0.59–1.71)
	Ofatumumab	NA	NA	NA	NA
COMPLEMENT1 [78]	Ofatumumab + Clorambucil	HR* 1.46 (95% CI 1.09–1.95) p = 0.01	HR 2.02 (95% CI 1.18–4.35) p < 0.01	HR 1.63 (95% CI 0.69–3.87) p = 0.23	HR 1.39 (95% CI 1.04–1.86) p = 0.03
	Clorambucil				
CLL8 trial [55]	FCR	5 years PFS 33.1%	Median PFS 15.4 months for mutated and 59.0 months for WT patients (p < 0.001)	NA	Median PFS 34.2 months for mutated and 57.3 months for WT patients (p = 0.013)
	FC	5 years PFS 19.4%	Median PFS 12.1 months for mutated and 35.9 months for WT patients (p < 0.001)	NA	Median PFS 33.9 months for mutated and 32.8 months for WT patients (p = 0.743)
CLL11 trial [77]	Obinutuzumab + Clorambucil	HR 3.0 (95% CI 1.80–4.80) p < 0.001	HR 3.36 (p < 0.001)	Not statistally significant	HR 1.08 (p = 0.697)
	Rituximab + Clorambucil		HR 2.28 (p < 0.001)	HR 1.69 (p = 0.023)	HR 1.42 (p = 0.03)
	Clorambucil	NA	NA	Not statistally significant	HR 1.52 (p = 0.103)

CLL, chronic lymphocytic leukemia; HR, hazard ratio; CI, confidence interval; p, p-value; NA, not avalilable; WT, wild type; *Data from multivariate analysis.

without obinutuzumab *versus* CIT points to the superiority of acalabrutinib-obinutuzumab also in IGHV mutated CLL [32]. Notably, it should be kept in mind that all clinical trial data concerning IGHV mutation status and CIT are derived from subgroup analysis and not from the primary endpoints of the trials [29–32].

Interestingly, cases with a percentage of IGHV identity between 97% and 97.99% are defined as borderline cases by the ERIC guidelines and their clinical outcome remains controversial [25]. In fact, the 98% threshold is purely mathematical rather than biological, although, from a clinical perspective, the 98% threshold is useful to define CLL subgroups with statistically distinct outcomes [33]. To date, the analysis of borderline cases has yielded conflicting results on whether these patients have an outcome similar to patients with a < 98% identity to the normal counterpart or *vice versa* [33–36]. Moreover, the IGHV mutation status seems to maintain its clinical importance also when used as a continuous variable [37]. Since this is an area of ongoing research, CLL guidelines for usage in the clinical practice currently use the original cut off value of ≥98% identity for unmuted IGHV genes and of <98% for mutated IGHV genes [16].

Another important feature of IGHV genes in CLL is that the repertoire of IGHV preferentially involves certain IGHV genes (e.g. IGHV1-69, IGHV3-7, IGHV4-34) over others, consistent with the fact that antigen selection is a driver for CLL pathogenesis and development [38,39]. The role of antigen selection is not only suggested by the biased IGHV repertoire, but is also reinforced by the finding of highly homologous antigen binding sites across groups of patients. In fact, approximately 50% of CLL cases that utilize the IGHV3-21 gene display highly similar, if not identical, antigen binding sites and *quasi*-identical heavy chain complementarity-determining region 3 (CDR3) [40]. The restriction in antigen binding site sequences is not unique to the IGHV3-21 gene but may occur also in other IGHV rearrangements. These highly homologous IGHV rearrangements in unrelated CLL patients have been termed stereotyped BCR [41].

Many different stereotyped BCR subsets have been identified; to date, the so called BCR subset #2 is the one with greatest potential clinical implications [42]. Subset #2 is characterized by IGHV3-21 rearrangement and by a CDR3 of 9 amino acids. Subset #2 includes both mutated and unmutated IGHV genes and is one of the largest stereotyped subsets, overall accounting for 2.5–3% of CLL cases and for almost 5.5% of CLL patients requiring therapy [42]. From a translational point of view, subset #2 is associated with an unfavorable prognosis, irrespective of IGHV mutational status [43,44]. The poor prognosis of subset #2 CLL patients has also been validated in the context of prospective clinical trials with CIT run by the German CLL Study Group, suggesting that subset #2 should be recommended as a novel predictor mandating treatment other than CIT in the clinical practice [44]. Results from clinical trials with BCRi and BCL2i are needed to assess whether these drugs can mitigate the negative prognostic impact of subset #2, and whether this immunogenetic feature may become a predictive biomarker especially in patients with mutated IGHV genes.

Recent evidence has shown that also the immunoglobulin light chain genes play a role in the pathogenesis and in the prognosis of CLL patients [45,46]. CLL subset #2 is known to express a light chain of the lambda isotype that utilizes the IGLV3-21 gene, and IGLV3-21 usage has also been associated with poor prognosis in CLL [45,46]. Most patients carrying a IGLV3-21 rearrangement express an IGLV3-21 with a nonsynonymous mutation that affects codon R110 [46]. The R110 residue is indispensable for the homotypic BCR–BCR interaction, and the R110 mutation enhances this interaction that may promote CLL proliferation [46]. From a clinical point of view, by comparing the wild-type IGLV3-21 and R110-mutated IGLV3-21, CLL patients expressing the R110-mutated IGLV3-21 represent a distinct subset with poor prognosis independent of IGHV mutations and of the assignment to BCR subset subset #2 [46].

3. *TP53* abnormalities

TP53 abnormalities, including 17p deletion and *TP53* mutations, are seen in approximately 5–7% of newly diagnosed CLL cases, in 10% of CLL patients requiring treatment, and in up to 35% chemo-refractory patients [22,47–49]. The *TP53* gene codes for a central regulator of the DNA damage response (DDR) pathway and is the target of the genotoxic effect of chemotherapeutic agents. The target genes induced by *TP53* are involved in different biological processes including: *i)* DDR (*DDB2* and *XPC); ii)* cell cycle arrest (*CDKN1A* encoding p21 and *GADD45A); iii)* apoptosis (*PUMA* and *BAX*); and *iv)* metabolism [50]. Chemotherapy acts by inducing DNA damage, thus activating the *TP53* pathway that leads to the apoptosis of CLL cells. Conversely, when *TP53* is disrupted by mutation and/or deletion, chemotherapy fails to induce apoptosis in CLL cells, that, consequently, may proliferate at a sustained pace and become free to accumulate multiple additional genetic lesions promoting progression and clonal evolution [51]. Whereas some *TP53* mutations cause a simple loss of function of the protein encoded by the affected allele, other *TP53* missense mutations may result in a gain-of-function (GOF) phenotype (i.e., R175H and R273H) reflecting a highly oncogenic activity of the altered protein [52]. A pivotal mechanism of the *TP53* GOF mutations seems to be an interference with *TP53* related proteins (i.e., p63 and p73). Alternatively, or in parallel, some *TP53* GOF mutations have been shown to upregulate genes that support cancer progression (i.e. NF-κB) or cause reduced therapy efficacy by upregulating P-glycoproteins involved in the metabolisms of some drugs used in CLL therapy [53].

Most of *TP53* mutations in CLL represent missense substitutions, which mainly occur in the DNA-binding domain of the protein [49]. Different studies have shown that mutations can also occur in exons outside the DNA binding domain, albeit with low prevalence [54]. From a clinical perspective, patients harboring missense mutations in the DNA binding domain are characterized by a shorter survival compared to patients with mutations outside the DNA binding domain [53]. The distinction between mutations affecting the *TP53* DNA binding domain of the protein versus mutations affecting other *TP53* regions is not currently taken into consideration for clinical decisions [54].

CLL patients carrying *TP53* disruption have a very poor outcome when treated with CIT and these results were confirmed both by retrospective studies and by prospective

clinical trials (Table 1) [26–28,55]. Before the introduction of the BCRi and BCL2i, few compounds were effective in the presence of *TP53* disruption. Alemtuzumab, an antibody targeting the lymphocyte specific surface marker CD52, has been demonstrated to be active also in relapsed/refractory patients with *TP53* abnormalities [56]. However, due to concerns regarding infectious complications, utilization of alemtuzumab has decreased over time [57]. The predictive value of *TP53* abnormalities has been confirmed also in prospective phase 3 clinical trials comparing CIT *versus* BCRi and BCL2i [29–31]. Consistently, CLL guidelines mandate treatment with biological drugs in *TP53* disrupted patients [16]. Importantly, since *TP53* abnormalities might be acquired also at the time of relapse, they must be tested at every subsequent line of treatment, especially in patients who had previously scored negative [16,58,59].

According to the ERIC guidelines, the mutational analysis of *TP53* might be performed either with Sanger sequencing or with Next Generation Sequencing (NGS) using the 10% cut off of variant allele frequency (VAF) for variant calling [54]. The ultra-deep-NGS has a significantly lower detection threshold of mutations compared to Sanger Sequencing and is capable of detecting *TP53* mutated subclones with a VAF <10%, that: *i)* occur in a significant fraction of newly diagnosed CLL; *ii)* have the same unfavorable prognostic impact as clonal *TP53* mutations; and *iii)* are associated with a worse outcome in patients treated with CIT [60,61]. The broader use of NGS analysis of *TP53* may be informative for an accurate prediction of outcome of patients with *TP53* subclones but, till now, their analysis is not recommended by guidelines [16].

Over the past few years, several assays have been developed to test the function of different *TP53* mutations analyzing the expression of responsive targets at the microRNA or protein level. These assays include: *i)* the FACSp53-p21 assay, that utilizes flow cytometry to determine basal TP53 protein level and TP53 levels after p21 induction following irradiation [62–64]; *ii)* reversed transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) that is used to measure *TP53* targeted genes at the RNA level [65,66]; *iii)* reverse transcription polymerase chain reaction (RT-PCR) of mir34a, a master regulator of tumor suppression induced by *TP53* [65–68].

4. *BIRC3* mutations

Physiologically, the NF-κB signaling pathway plays a pivotal role in regulating important cellular processes closely linked to cancer, such as inflammation, cell survival, and proliferation [69]. Not surprisingly, aberrant NF-κB signaling is also a key component for CLL pathogenesis and progression [70]. Two NF-κB pathways exist, namely the canonical and the non-canonical pathway [70]. *BIRC3* is a negative regulator of the non-canonical NF-κB pathway, and *BIRC3* disrupting mutations in CLL lead to aberrant and constitutive activation of the non-canonical NF-κB pathway promoting proliferation and survival (Figure 1) [2]. *BIRC3* mutations are present in approximately 3–4% of newly diagnosed CLL and, similar to *TP53* mutations, are enriched at relapse, accounting for 25% of fludarabine refractory patients [71]. *In vitro* studies have demonstrated that *BIRC3* mutated CLL cells isolated *ex vivo* from patients are characterized by fludarabine resistance [72]. Moreover, the chemorefractoriness induced by *BIRC3* mutations has been validated, *in vivo*, in a retrospective multicenter cohort of FCR treated CLL patients (Table 1) [72]. Consistently, *BIRC3* mutated patients have a poor outcome similar to that of patients with *TP53* disruption, that currently represents the strongest predictor of chemorefractoriness in CLL [72]. These pivotal findings in a retrospective series have been recently validated by the molecular analysis of the prospective CLL14 phase 3 clinical trial comparing chlorambucil-obinutuzumab with venetoclax-obinutuzumab in patients with previously untreated CLL (Table 1) [73]. In this study, *BIRC3* mutated patients treated in the CIT arm were associated with a shorter progression free survival (PFS) [73]. Conversely, venetoclax-obinutuzumab was able to overcome the dismal prognosis associated with *BIRC3* disruption [73].

5. *NOTCH1* mutations

NOTCH1 mutations are present in approximately 10% of CLL patients at diagnosis and are increased in relapsed/refractory patients [74]. The *NOTCH1* gene codes for a transmembrane receptor that, upon ligand binding and migration of the NOTCH1 intracellular domain to the nucleus, induces the transcription of pro-survival and antiapoptotic genes [75]. *NOTCH1* mutations disrupt the PEST domain of the protein that is essential for NOTCH1 proteasomal degradation. As a consequence, NOTCH1 is no longer ubiquitinated and can activate transcription of *NOTCH1* target genes [75]. NOTCH1 signaling may also be enhanced by mutations of *FBXW7*, a gene coding for a NOTCH1 ubiquitinase whose disruption impairs the ubiquitination of the NOTCH1 protein [76].

NOTCH1 mutations seem to associate with shorter survival compared to wild type patients when treated with CIT (Table 1) [55,77,78]. In particular, sub-analysis of the CLL8 trial, comparing FCR *versus* fludarabine, cyclophosphamide (FC) as first line therapy in CLL patients, has demonstrated that *NOTCH1* mutated patients may not benefit from the addition of the type 1 anti-CD20 antibody rituximab [55]. This initial finding was corroborated by the molecular analysis of the COMPLEMENT1 trial, a phase 3 trial comparing chlorambucil *versus* ofatumumab-chlorambucil in patients ineligible for fludarabine-based therapy [78]. This study confirmed a lower efficacy of ofatumumab (a second generation type 1 anti-CD20 antibody) in *NOTCH1* mutated CLL patients compared to wild type cases [78]. Conversely, the novel type 2 anti-CD20 antibody obinutuzumab appears to overcome the refractoriness to anti-CD20 therapy in CLL carrying mutations of *NOTCH1* [77]. The biological reason for the lower efficacy of rituximab and ofatumumab in *NOTCH1* mutated CLL is not completely understood. Notably, *NOTCH1* mutated CLL cells are characterized by lower CD20 expression and by a lower cell lysis induced *in vitro* by rituximab compared to *NOTCH1* wild type cells [79].

At present, the clinical impact of *NOTCH1* mutations in patients treated with BCRi or BCL2i has been addressed by two large prospective studies (Table 1) [73,80,81]. Results from the CLL14 and the RESONATE clinical trials indicate that arms containing venetoclax (in the case of CLL14) or ibrutinib (in the case of RESONATE) overcome the negative prognostic

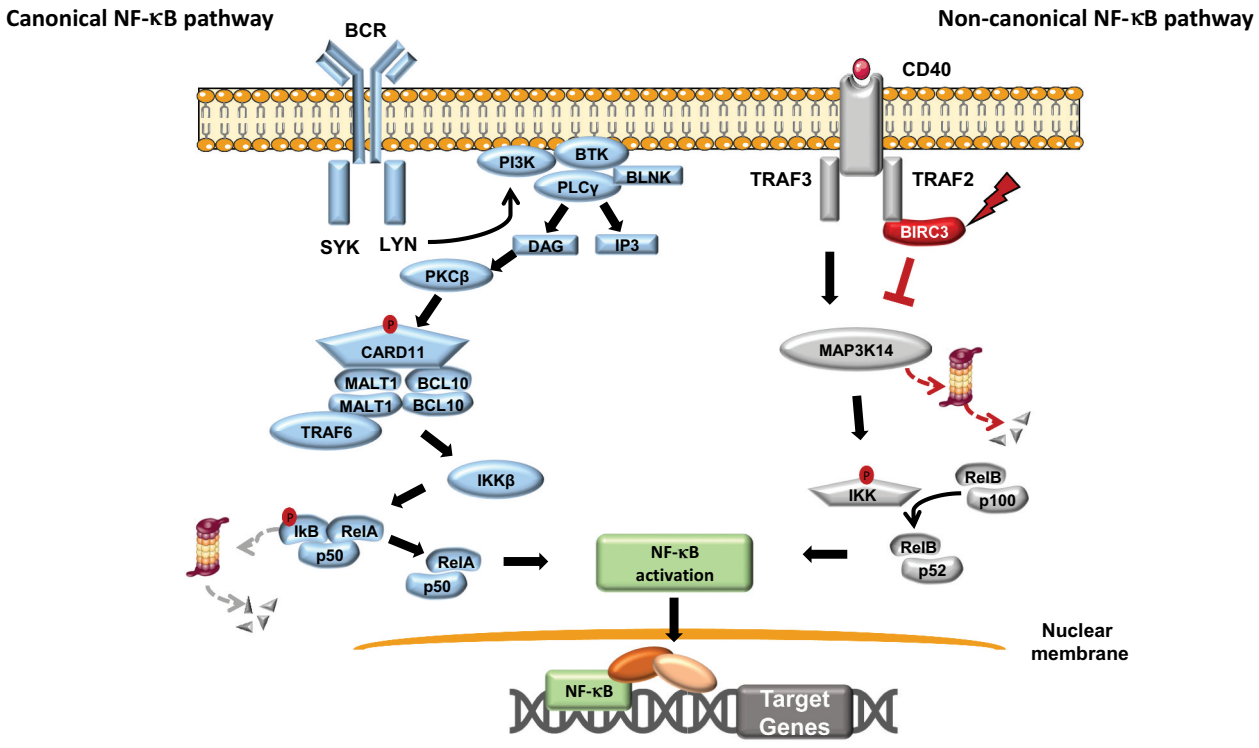

Figure 1. **The role of BIRC3 in the NF-κB pathway**. The NF-κB signaling pathway plays a pivotal role in regulating important cellular processes closely linked to cancer, such as inflammation, cell survival, and proliferation. Two NF-κB pathways exist, namely the canonical pathway (left panel) and the non-canonical pathway (right panel). BIRC3 is a negative regulator of the non-canonical NF-κB pathway. BIRC3 physiologically catalyzes the ubiquitination of MAP3K14 leading to its proteasomal degradation thus rendering the non-canonical NF-κB pathway inactive. In the case of *BIRC3* disrupting mutations, represented by the red arrow, MAP3K14 is no longer ubiquitinated and, therefore, can perform its function of positive signal transducer of the NF-κB pathway. Consistently, MAP3K14 phosphorylates IKK, leading to the processing of p100 to p52. The p52 protein dimerizes with RelB and translocates into the nucleus, where it regulates gene transcription.

impact of *NOTCH1* mutations [73,80,81]. Future studies may further add to a full understanding of the clinical significance of *NOTCH1* mutations in the era of chemo-free therapy for CLL.

NOTCH1 mutational analysis has not entered till now into the clinical practice but might become a predictive biomarker suggesting the use of type 2 anti-CD20 antibodies, i.e. obinutuzumab, in patients carrying these mutations [16,77].

6. Other prognostic biomarkers of asymptomatic CLL patients

Other biomarkers have demonstrated prognostic relevance in CLL but are not currently used in the routine clinical practice [82]. These biological markers include: *i)* expression of intracellular zeta-associated protein of 70 kilo Daltons (ZAP-70), a tyrosine protein kinase belonging to the Src protein kinases family that is not expressed in normal B-cells and whose aberrant expression is an unfavorable prognostic factor [83]; *ii)* surface CD38 expression, that associates with a more aggressive disease, characterized by poor response to therapy and shortened survival compared to patients with low CD38 expression [83]; *iii)* expression of the surface adhesion molecule CD49d, the rate-limiting a-chain of the CD49d/CD29 integrin heterodimer very late antigen-4 (VLA-4), that is an unfavorable prognostic marker and identifies cases characterized by rapid progression and early need of treatment [84,85]; iv) T-cell leukemia-1 oncogene (*TCL1*), that is expressed in almost all CLL and whose protein levels correlate with the aggressive prognostic markers [86]; *v)* del13q14.1, that represents the most frequent genetic abnormalities in CLL. The chromosomal region codes for miR-15/16 that physiologically inhibit the BCL2 antiapoptotic protein [87]. In the presence of of 13q deletion, BCL2 expression is upregulated and CLL cells can proliferate with a greater extent [87]. From a translational point of view, 13q deleted patients are characterized by an indolent disease [47]. *vi)* 11q22.3 deletion, the chromosomal region harboring *ATM*, a pivotal gene in the DNA damage response pathway [22]. Among patients treated with CIT, 11q deletion detected by Fluorescence *In Situ* Hybridization (FISH) identifies cases with an outcome that is intermediate between patients with mutated IGHV genes and patients with *TP53* disruption [47]; *vi*) β2-microglobulin (B2 M) is often elevated in patients with CLL and correlates with stage and tumor burden. Importantly, B2 M baseline level is a variable of the CLL-IPI [14]. Moreover, B2 M should also be used as a dynamic parameter during the course of therapy. Consistently, normalization of B2 M at 6 months during ibrutinib treatment has been associated with improved PFS [88]; *vii) MYD88* mutations, that seem to be associated with younger age at diagnosis, longer time to first treatment (TTFT) and overall survival (OS) [89]; *viii)* Induced myeloid leukemia cell differentiation protein (Mcl-1), an antiapoptotic protein similar to BCL2 and essential during lymphoid development and maintenance of mature T and B lymphocytes. High levels of Mcl-1 protein in CLL correlate with poor *in vitro* response to chemotherapeutic agents and with the failure to respond to fludarabine therapy

[90]. Moreover, higher levels of Mcl-1 might associate with venetoclax resistance [91].

In addition, gene expression profiling studies also may provide insights for CLL prognosis. The microRNA signature associates with known prognostic factors in CLL with disease progression [92]. Further studies have shown that low levels of miR-29 c and miR-223 expression predict treatment free survival and OS [93]. Another microRNA, miR-34a, is the most prominently upregulated miRNA during DDR in CLL cells *in vitro* and *in vivo* during FCR therapy. miR-34a levels can be used as a biomarker of poor outcome, irrespective of *TP53* status [94]. Recently, a reproducible 17-gene signature has been developed and identifies a subset of treatment-naïve patients with IGHV-unmutated CLL who might substantially benefit from treatment with FCR [95].

7. *BTK* and *PLCγ2* mutations

The introduction of BCRi such as ibrutinib has changed the natural history of CLL. Two clinical trials with a 5-year follow-up of single-agent ibrutinib, including both treatment naïve and relapsed/refractory patients, have highlighted the high efficacy of the drug and the long-lasting durability of the response [96,97]. Moreover, phase 3 randomized clinical trials have demonstrated that ibrutinib containing regiments are superior to CIT in different groups of patients, including treatment naïve patients, both fit and unfit, as well relapsed/refractory patients [29–32,98,99].

The molecular mechanisms of refractoriness to ibrutinib have been clarified to a certain extent. In fact, the usage of ibrutinib in CLL faces the emergence of somatically acquired mutations that reduce its efficacy (Figure 2). Similar to the lesson provided by imatinib in chronic myeloid leukemia, in which mutations affecting the binding site of the drug impair imatinib efficiency, mutations in the Bruton Tyrosine Kinase (BTK) binding site also impair ibrutinib efficacy. Ibrutinib exerts its function by binding to a cysteine residue positioned at codon 481 of the *BTK* gene, thus blocking the catalytic site of BTK [100]. Ibrutinib treatment may induce the emergence of *BTK* missense mutations affecting codon 481 (namely p.C481S, leading to a cysteine-to-serine amino acid change), thus altering the binding of the drug to BTK and causing the loss of the ibrutinib therapeutic effect [100]. Functional analysis has documented that the C481S mutation impairs the irreversible covalent binding of ibrutinib to BTK [101]. Somatically acquired mutations of *BTK* have not been identified in pre-treatment samples, thus indicating that they are selected upon exposure to the drug [101].

The BCR signaling cascade encompasses several proteins that have specific characteristics in transducing the signal after ligand binding. Activating mutations of PLCγ2, a protein located downstream to BTK, may constitutively activate BCR signaling even in the presence of BTK inhibition by ibrutinib.

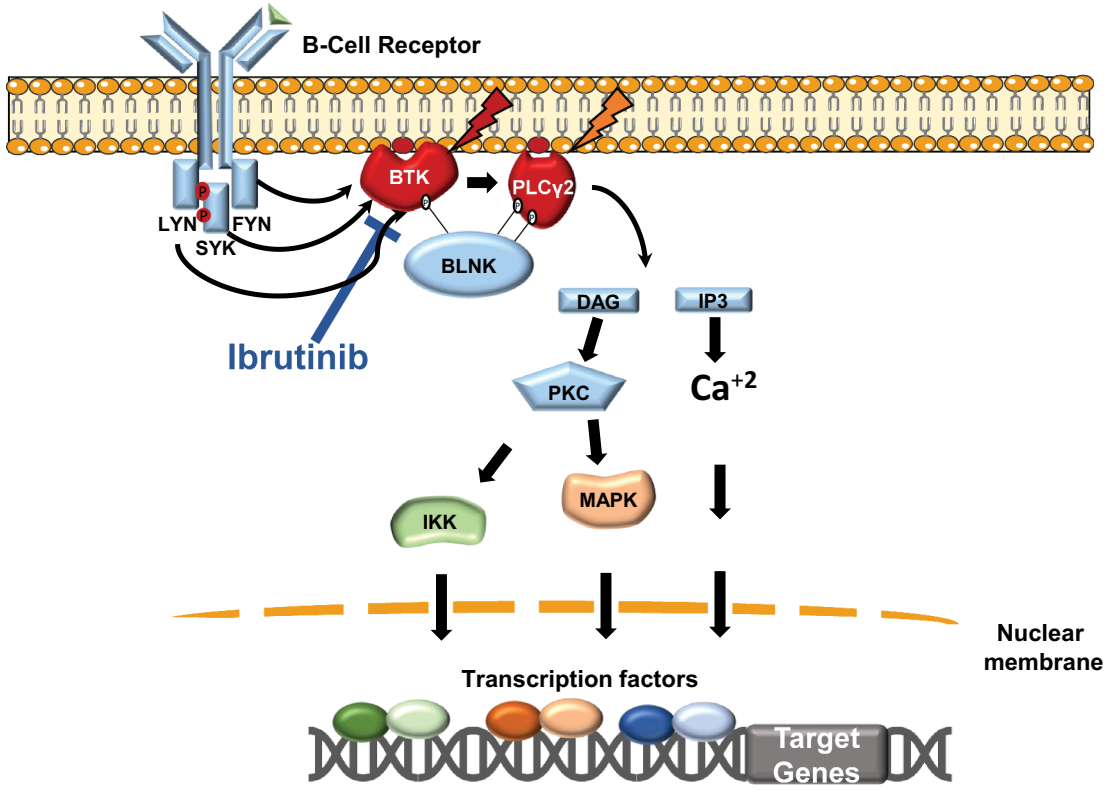

Figure 2. **BTK and PLCγ2 mutations**. The BCR signaling pathway plays a pivotal role in CLL pathogenesis and progression. BCR signaling enhances cell survival through the MAPK pathway. BTK, located downstream to the BCR, is an essential component of BCR signaling and can be targeted by BTKi such as ibrutinib. Mutations of the *BTK* gene (red arrow) inhibit ibrutinib binding to its target and may be acquired during ibrutinib treatment, thus predisposing to drug resistance. Mutations activating the PLCγ2 protein (orange arrow), that is located immediately downstream to BTK, may also be selected during ibrutinib treatment leading to persistent activation of the BCR signaling pathway independent of BTK inhibition.

Consistent with this model, several mutations in the *PLCγ2* gene (e.g. R665W and L845F) are detected in ibrutinib-resistant CLL, leading to autonomous B-cell receptor activity [100–103].

Overall, mutations of *BTK* and of *PLCγ2* have been detected in approximately 85% patients failing ibrutinib treatment. The emergence of *BTK* or *PLCγ2* mutations has been shown to antedate clinical relapse by approximately 9 months [100–104]. Some reports have suggested the use of prospective monitoring of *BTK* or *PLCγ2* mutations during ibrutinib treatment [104]. However, prospective trials are needed to validate the clinical usefulness of mutation monitoring and to assess whether early switch of therapy might be beneficial in CLL patients developing *BTK* or *PLCγ2* mutations but that are still responsive to ibrutinib. At present, the regular monitoring of these two mutations is not recommended by current guidelines [16].

8. *BCL2* mutations

Venetoclax is a BH3-mimetic drug that binds specifically to the hydrophobic groove of BCL2, thereby displacing proapoptotic proteins and rapidly inducing apoptosis in CLL cells that rely on BCL2 for survival [105]. The high efficacy of venetoclax also in *TP53* disrupted patients may rely on the fact that venetoclax acts independent of the DNA repair pathway that is frequently altered in CLL contributing to CIT refractoriness [106–108]. Similar to ibrutinib, also in the case of venetoclax, mutations may target the drug binding domain on the BCL2 protein [105]. These mutations are absent before venetoclax treatment, indicating that they are selected by venetoclax exposure [105]. A pivotal study identified a single heterozygous nucleotide variant in the *BCL2* gene, namely c.302 G > T, p.Gly101Val, in 46% of patients who progressed during venetoclax [105]. By using highly sensitive approaches, the Gly101Val mutation was already detectable, at low frequency, up to 25 months before disease progression [105]. This mutation, confirmed also by independent studies, causes a 30-fold decrease in the binding capacity of venetoclax to the BCL2 protein [105].

Recently, by utilizing more sensitive and specific NGS techniques, a large amount of *BCL2* mutations have been described. Approximately 90% of patients presents at least one additional mutation beside the Gly101Val variant [109]. These mutations, including Asp103Tyr/Glu/Val, Val156Asp, Arg107_Arg110dup, Ala113Gly and Arg129Leu, affect different domains of the BCL2 protein, all of which are involved in venetoclax binding to BCL2, though through different mechanisms. Interestingly, the Asp103Glu variant has demonstrated a reduced binding affinity to venetoclax, while in contrast, this mutation enhances sensitivity to the dual BCL2/BCL-xL inhibitor navitoclax [109]. Importantly, all these novel *BCL2* mutations are not detected prior to venetoclax exposure, suggesting acquisition during the course of treatment [109].

9. Minimal residual disease assessment in CLL

MRD is defined as the number of leukemic cells that can be detected in peripheral blood (PB) or bone marrow (BM) following treatment [14]. Undetectable MRD (uMRD) is currently defined as the presence of less than 1 CLL cell out of 10,000 leukocytes ($<10^{-4}$) [110]. MRD is usually evaluated in the PB, and the ERIC consortium has harmonized a multicolor flow cytometry panel allowing the comparison of MRD results among different studies [110]. More sensitive methods reaching a sensitivity of 10^{-6}, such as high-throughput IGH sequencing (IGH-HTS), have been tested but are not routinely used [111].

From a translational point of view, MRD has been tested in different clinical trials both with CIT and with biological agents [20]. In the CIT era, uMRD has been associated with significantly longer PFS than intermediate ($\geq 10^{-4}$ to $<10^{-2}$) or high MRD ($\geq 10^{-2}$) levels [112–115]. Additionally, uMRD seems to correlate better with PFS than with clinical response assessment. Consistently, patients with uMRD and a partial response due to residual splenomegaly display outcomes similar to patients with uMRD and a complete response [116].

Regarding biological agents, many studies have analyzed MRD in patients treated with BCRi or BCL2i alone or combined with anti-CD20 antibodies [117–119]. In line with the notion that ibrutinib does not induce the direct killing of CLL cells, uMRD is rarely achieved during treatment with single agent ibrutinib [20]. Conversely, when debulking chemotherapy or BCL2i is added, uMRD may be achieved in most of cases [120,121]. The BCL2i venetoclax directly induces apoptosis in CLL, and uMRD can be achieved with venetoclax monotherapy in approximately 25% of patients. Similar to the results obtained in the CIT context, uMRD or intermediate MRD after venetoclax strongly associate with longer PFS [118,119,121,122]. The addition of anti-CD20 antibodies to venetoclax has yielded higher rates of uMRD. In the rituximab-venetoclax arm of the MURANO study, uMRD in PB was achieved in 62% of patients and was strongly associated with longer PFS [118]. Similar results have been also obtained in the obinutuzumab-venetoclax arm of the CLL14 trial [118].

The prognostic value of uMRD and the probability of achieving uMRD are related to different biological factors. For example, after FCR therapy, patients with mutated IGHV genes who are MRD positive have superior outcome compared to patients with uMRD but unmutated IGHV genes [27]. Moreover, in the MURANO study, *TP53* disruption and unmutated IGHV genes predict for lower achievement of uMRD [118]. These observations demonstrate that re-growth of the malignant clone and the subsequent timing of relapse cannot be captured only by MRD detection but need to be complemented by knowledge of other biological features of the disease.

An ongoing area of research concerns the possibility to use MRD to guide decisions of treatment strategies. A retrospective analysis in FCR-treated patients who achieved uMRD after three (of six planned) treatment cycles has demonstrated that the outcome of patients with uMRD who stopped treatment after three cycles is superimposable to the outcome of patients with uMRD who continued to receive the preplanned six FCR courses [116]. These findings suggest that MRD-guided stopping of FCR treatment after three cycles is feasible without affecting long term survival and possibly sparing unnecessary treatment-related toxicities [116]. This strategy needs to be validated in the context of large prospective trials.

Non-randomized MRD-guided approaches are also being tested with biological drugs. One trial is evaluating MRD to guide the duration of ibrutinib, fludarabine, cyclophosphamide and obinutuzumab (iFCG) in first-line treatment of patients with a favorable genetic risk profile (IGHV mutated, no *TP53* disruption) [120]. Patients who achieve complete response with BM uMRD after the first 3 cycles receive 9 additional cycles of ibrutinib with 3 additional cycles of obinutuzumab; all other patients receive 9 additional cycles of ibrutinib and obinutuzumab [120]. Preliminary results have demonstrated that three courses of MRD-guided iFCG are an effective time-limited regimen for young patients with mutated IGHV and without *TP53* abnormalities [120]. Similarly, another individualized approach to MRD guided treatment decisions is currently being explored in the phase II CLARITY trial with ibrutinib and venetoclax [122]. Patients are tested for MRD after 6 and 12 months of combined treatment and continue ibrutinib and venetoclax for the same duration of time that is required to achieve uMRD. For example, patients who achieve uMRD in the BM after 6 months will finish treatment after a total of 12 months of ibrutinib and venetoclax [122].

Overall, these studies are exploring different drug combinations and, based on the MRD level achieved during therapy, aim at defining a fixed duration scheme for patients with uMRD, while offering maintenance therapy or different drugs to patients who do not achieve uMRD.

10. Prognostic biomarkers of asymptomatic CLL patients

Approximately 70% of newly diagnosed CLL patients present in an early stage according to the Binet and Rai staging systems, may never require treatment, and may have a life expectancy similar to the general population [123–125]. Asymptomatic early stage CLL patients are currently managed with a watch and wait strategy, and treatment is started only in case of symptomatic disease including progressive lymphocytosis, enlarged lymph nodes, cytopenia, and systemic symptoms [22]. Three clinical trials comparing either chlorambucil, or fludarabine or FCR *versus* placebo in asymptomatic CLL have not prolonged survival in early stage asymptomatic patients [126–128]. Recently, the phase 3 CLL12 clinical trial of the German CLL Study Group has compared ibrutinib *versus* observation in asymptomatic CLL patients. Preliminary results have demonstrated a higher PFS rate in the ibrutinib arm, compared to placebo. However, survival data are not mature enough to demonstrate a clear advantage of ibrutinib *versus* observation [129]. Therefore, guidelines still recommend a watch and wait strategy for asymptomatic CLL patients [16].

Several studies have searched for clinical and molecular prognosticators that might identify patients with higher risk of early treatment requirement and who might benefit from early intervention [6,7,130–133]. In this context, the combination of simple clinical features, namely lymphocyte count > 15,000/ul and palpable lymph nodes, together with molecular biomarkers, namely unmutated IGHV genes, identifies three different subgroups of treatment naïve Binet A CLL patients with a high risk of early treatment requirement [130]. This risk model, termed IPS-E (International Prognostic Score-Early), has been validated in several independent cohorts and might be useful in the design of early intervention clinical trials [130]. A similar study involving Rai 0 CLL patients has identified five variables, namely 11q deletion, 17p deletion, trisomy 12, unmutated IGHV genes and white blood cells ≥32,000/□l, that are associated with a shorter time to first treatment [131]. Recently, the German CLL Study Group has substantiated that lymphocyte doubling time (LDT) of less than 12 months predicts a shorter TTFT [134]. Notably, half of the patients who required therapy within 12 months from diagnosis had a LDT <12 months. Moreover, the integration of LDT in the CLL-IPI backbone allowed a better stratification of the TTFT in Binet

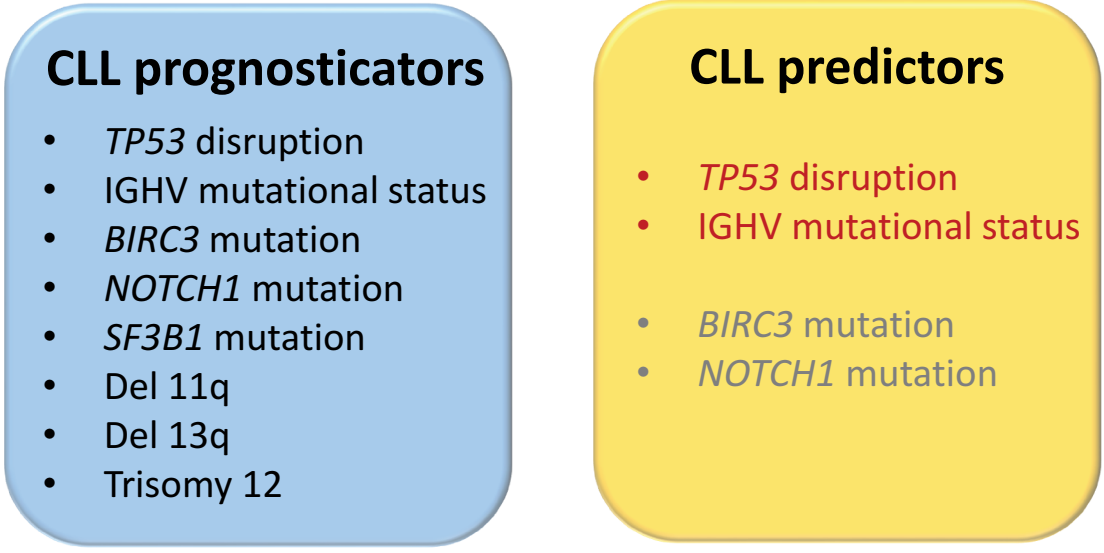

Figure 3. **Molecular prognosticators and predictors in CLL**. Molecular prognosticators are biomarkers that reflect the underlying biology and natural history of the disease, thus defining prognosis in the absence of treatment and independent of treatment received. Conversely, molecular predictors are biomarkers that provide information on the likely benefit from a specific treatment, and thus may allow treatment tailoring in every single patient. Many molecular prognosticators have been identified in CLL (left panel, blue box). However, only a few molecular prognosticators also serve the role of molecular predictors for CLL treatment (right panel, yellow box). In the figure, predictors currently used in the clinical practice are indicated in red, while potential predictors not yet recommended by guidelines are indicated in gray.

A CLL patients enrolled into the CLL1 clinical trials [134]. In addition, the impact of gene mutations has also been tested in this context. Initial results from retrospective studies suggest that *SF3B1, NOTCH1, ATM, U1* and *XPO1* gene mutations might behave as molecular predictors of shorter time to first treatment [7,132,133]. Molecular analysis of prospective, randomized, phase 3 clinical trials of early therapeutic intervention in CLL might contribute to refine the value of molecular features for defining the benefit of early intervention, if any, in specific CLL subgroups.

11. Expert opinion

The dissection of the CLL mutational landscape has allowed a deep understanding of the biology of the disease and has led to the identification of molecular biomarkers with clinical relevance [2]. Acquisition of biological knowledge of CLL has been paralleled by the development of novel medicines targeting the BCR cascade, the *BCL2* pathway or the CD20 surface antigen. The combination of increased molecular understanding of CLL and availability of target therapies has radically changed the risk stratification of CLL and the clinical approach to the disease. For example, IGHV mutational status and *TP53* abnormalities are now routinely used in the clinical practice as robust predictors for treatment decision making (Figure 3) [16].

Novel genetic and immunogenetic biomarkers have the potential to use into the routine clinical practice in the near future. In order to gain solid clinical relevance, a biomarker should also harbor predictive value that informs about the likely benefit from a specific treatment [8]. *BIRC3* mutations are a validated prognostic biomarker since they associate with shorter PFS when patients are treated with CIT [71–73,77]. Recently, *BIRC3* mutations have acquired also a predictive value. In fact, *BIRC3* mutations associate with chemorefractoriness to several CIT regimens, namely FCR and rituximab/obinutuzumab-chlorambucil [72,73,77]. Conversely, the BCL2i venetoclax may overcome the negative prognostic impact of *BIRC3* mutations, therefore indicating BCL2 targeting as an

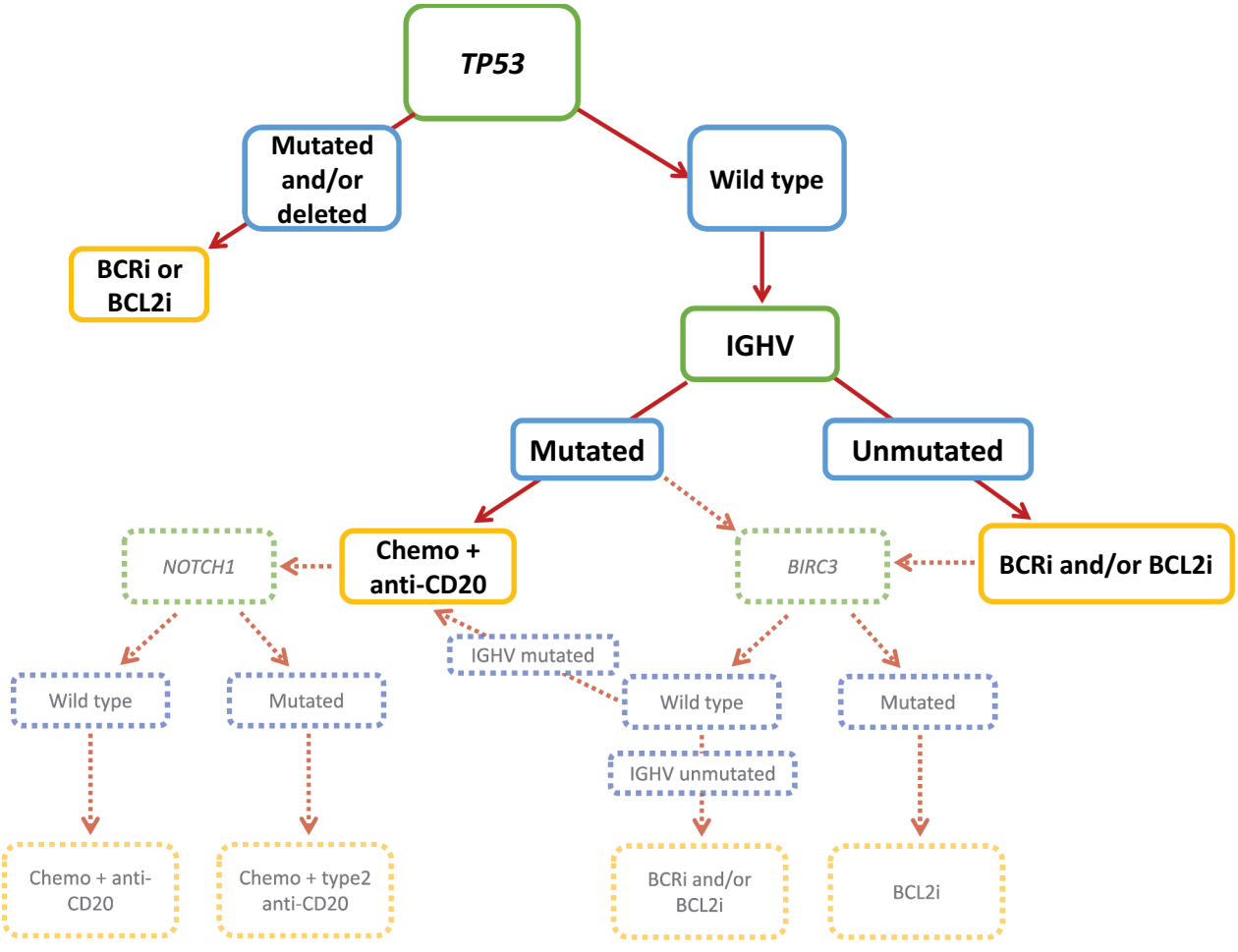

Figure 4. A proposal of a therapeutic algorithm for first line CLL therapy based on molecular predictors. Molecular predictors are represented by green boxes; the genetic status of each predictor is represented in the blue boxes; the suggested therapeutic options based on predictor status are represented by yellow boxes. Whole lines denote the current CLL therapeutic algorithm used in the clinical practice. Dotted lines denote potential novel predictors that may refine the current CLL therapeutic algorithm. *TP53* status is a first decisional node for a precision medicine approach of treatment naïve CLL, since *TP53* disruption mandates first line treatment with BCRi or BCL2i. For *TP53* wild type cases, a second decisional node is represented by IGHV mutation status. IGHV mutated patients may benefit from CIT regimens; conversely, IGHV unmutated patients benefit the most from BCRi or BCL2i. In addition to these established predictors that are recommended by guidelines, other biomarkers are emerging as potential predictors for CLL. Patients carrying *NOTCH1* mutations do not benefit from the addition of type 1 anti-CD20 antibodies (rtituximab and ofatumumab) to chemotherapy, whereas type 2 anti-CD20 antibodies, namely obinutuzumab, provide a benefit. Disruption of *BIRC3* has been shown to predict refractoriness to CIT and may be overcome by the use of the BCL2i venetoclax.

effective strategy for *BIRC3* mutated patients [73]. Also *NOTCH1* mutations are gaining potential predictive value for choosing type-2 over type-1 anti-CD20 antibodies, at least in the context of CIT regimens [78]. The molecular analysis of ongoing phase 3 clinical trials comparing CIT with different biological drugs will conceivably identify other molecular predictors that might help clinicians in a more accurate process of therapeutic decision making (Figure 4).

In recent years, MRD assessment is gaining potential importance for guiding the treatment duration of CLL, a chronic disease that usually responds to therapy but then relapses with higher clinical aggressiveness. In the context of some CIT regimens, namely FCR, patients with uMRD experience long lasting remission posing the question if these patients may be cured [111,116]. MRD monitoring has also been explored in the context of BCRi and BCL2i therapy, alone or combined with different anti-CD20 antibodies [118,119]. Whereas BCRi and BCL2i as single agents are used as continuous treatment until progression, several ongoing MRD-oriented clinical trials are evaluating the possibility of fixed duration therapy in patients who achieve uMRD [120,122]. These trials will also provide information on the usefulness of an early switch of therapy in patients who remain MRD positive [120,122].

The identification of molecular predictors and their introduction in the clinical practice has provided clinicians with robust tools for improving treatment choices for every individual patient. In the coming years, the number and robustness of CLL molecular predictors may further increase the effectiveness of treatment tailoring. An area that is still rather unexplored is the correlation between molecular predictors and patient reported outcomes that may aid in making more informed, individualized treatment decisions in daily practice by obtaining more accurate information on the actual symptom burden experienced by the patient [135]. The optimization of precision medicine thanks to molecular predictors, coupled with response monitoring through MRD, may allow longer survival and improved patient reported outcomes in CLL patients sparing unnecessary clinical and financial toxicities.

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Declaration of interest

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