B cells play a key role in the adaptive immune response, which is the second line of defence against non-self pathogens. B cells form and mature in the bone marrow, and then move to the lymphatic system where they circulate the body. Here, the B cells recognize foreign antigen through their B cell receptor (BCR), leading to maturation of the B cell into either a memory B cell or an effector (plasma) B cell. The BCR is composed of two identical covalently linked immunoglobulin heavy (IgH) chains and a pair of identical immunoglobulin light (IgL) chains connected by disulphide bonds. The variable domains of the IgH and IgL chains result from gene rearrangements at the pro-B (IgH) and pre-B (IgL) cell stages and define the antigen specificity of the BCR. Memory B cells express the same membrane-bound antibody as the parent B cell, while effector B cells secrete it as soluble antibodies. The BCR is anchored to the cell membrane through its transmembrane domain, which is tightly associated with a heterodimer of Igα (CD79A) and Igβ (CD79B) (Figure 1). These membrane-bound proteins each have a cytoplasmic tail that harbours two conserved tyrosine residues as part of their immunoreceptor tyrosine-based activation motif (ITAM), an important signalling component of the BCR. Phosphorylation of these ITAM tyrosine residues by the SRC family kinases Lck/Yes-related novel protein tyrosine kinase (LYN) and FYN as well as B-lymphoid kinase (BLK, Tec...
family) or spleen tyrosine kinase (SYK) is the initial step of signal transduction from the BCR to the nucleus.5,6

BCR signalling can be initiated by two different mechanisms: antigen-induced and antigen-independent (tonic) BCR activation.7 BCR stimulation by antigen results in BCR aggregation, ITAM phosphorylation, and consequently recruitment of SRC-homology 2 (SH2)-domain containing proteins, most often SYK, to the BCR.8,9 SYK is then activated by SRC kinases and autophosphorylation,10 and, together with LYN, phosphorylates the adaptor proteins CD19, B cell adaptor for phosphatidylinositol 3-kinase (BCAP), and B cell linker protein (BLNK). CD19 and BCAP recruit phosphatidylinositol-3 kinase (PI3K) to the plasma membrane. BLNK, together with PI3K, activate Bruton’s tyrosine kinase (BTK) and its downstream target phospholipase Cγ2 (PLCγ2).9 This BCR signalosome generates a wide variety of downstream effects, including activation of the PI3K-AKT-mTOR pathway and the RAS-RAF-MEK-ERK pathway.11 BCR signalling is essential for normal immune function and for survival and proliferation of the B cells.12 B cells that lack a functional BCR rapidly undergo cell death.13

The second mechanism by which BCR signalling can be induced is by tonic BCR activation, a process which is independent of ligand engagement.14 Several mechanisms have been proposed to account for the initiation and regulation of tonic signalling. These include the self-aggregation of BCR molecules, an altered balance between constitutively active protein tyrosine kinases and protein tyrosine phosphatases, or hijacking of the BCR by the B cell activating factor of the tumour necrosis factor (TNF) family (BAFF) receptor.15-17

The critical role of BCR signalling in normal B cell development renders it as no surprise that BCR signalling also supports survival and growth of malignant B cells.18,19 In this review, the focus will be on BCR signalling in chronic lymphocytic leukaemia (CLL), a disease which, due to its relatively indolent nature, has allowed detailed investigation of the tumour cells and their signalling responses over time.20

2 | CHRONIC LYMPHOCYTIC LEUKAEMIA

CLL is the most common form of leukaemia in western countries, with an incidence rate of 5.82/100,000 inhabitants in the United States.21 It is a disease of the elderly, with a median age at diagnosis of 72 years, and more male than female patients are affected (1.7:1).22 CLL is characterized by clonal expansion and accumulation of mature CD5+ B cells in the peripheral blood, bone marrow and lymphoid tissues.23 Survival of the CLL cells relies on signals from the tumour microenvironment,24-26 which is composed of cellular components such as monocyte-derived nurse-like cells (NLC),27 T cells28 and mesenchymal stromal cells.29
Several lines of evidence support the significant role of BCR signalling in the pathophysiology of CLL. Zeta chain–associated protein kinase 70 kDa (ZAP-70, SYK family) is associated with BCR signalling and is a negative prognostic factor in CLL.30-32 Similarly, secretion of the T cell attracting chemokines CCL3 and CCL4 correlates with ZAP-70 expression and responsiveness of the CLL clone to BCR stimulation, and strongly predicts CLL prognosis and time to treatment.33,34 More importantly, the structure of the BCR itself is among the parameters included in the international prognostic index for patients with CLL (CLL-IPI).35 The disease is classified as either mutated or unmutated based on the degree of somatic hypermutation within the BCR antigen-binding site (immunoglobulin heavy chain variable region gene; IGVH) present in the CLL cells. A cut-off at 98% IGHV sequence homology to the germline sequence is used for the classification.36 Although CLL is a heterogeneous disease with high variation in disease course among patients, mutated CLL is typically associated with a more indolent disease progression and better overall survival, whereas unmutated CLL shows a more aggressive course with shorter time to treatment and shorter survival.37,38 CLL cells with an unmutated phenotype are in general more responsive to BCR stimulation than mutated CLL cells, in particular in inducing PI3K signalling.39,40

Evidence exists for both tonic- and antigen-induced BCR activation in CLL. Tonic BCR signalling is supported by studies on primary CLL cells showing constitutive activation of BCR pathway components including LYN,41 SYK,42 ERK1/2,43,44 and STAT3.39 On the other hand, CLL cells express restricted sets of antigen receptors36 giving rise to subsets of cases with almost identical (stereotyped) complementarity-determining region 3 (CDR3) sequences.45,46 This suggests that a particular antigen-binding site may be critical during CLL pathogenesis. CLL cells thus appear to depend on both constitutive and induced BCR signalling that direct cell growth and survival.

3.1 PI3K inhibitors

PI3Ks constitute a family of enzymes that regulate a diverse set of cellular processes, including proliferation, differentiation, survival and intracellular trafficking. The PI3K signalling pathway is one of the most frequently mutated in...
### TABLE 2

Selected ongoing and completed clinical trials with targeted therapies in CLL

| Agent | Class inhibitor | Study | Study phase | R/R-TN | IGVH status (n of mutated/n of unmutated) | TP53/del17p (number of patients) |
|-------|-----------------|-------|-------------|--------|----------------------------------------|----------------------------------|
| Venetoclax + OB vs OB + Chl | BCL-2 | NCT02242942 | 3 | TN | 159/244 | 32 (TP53) 31 (del17) |
| Venetoclax | BCL-2 | NCT01889186 | 2 | R/R | 30/7 | 60 (TP53) 106 (del17p) |
| Venetoclax | BCL-2 | NCT02141282 | 2 | R/R | 67/50 | 28.1 49.5/23.1 84.7/71.3 Not reached 88.2/64.1 Not reached 91.8/93.3 (Fischer K et al, 2019) |
| Duvelisib (R/R vs TN) | PI3K | NCT01476657 | 1 | R/R-TN | 4/0 | 56/83 Not reached (Flinn IW et al, 2018) |
| Duvelisib vs Ofatumumab | PI3K | NCT02158091 | 1 | R/R | 67/50 | 14 9 65 24.7 75 (est. 1 y) nr 91 (est. 1) (Jones JA et al, 2018) |
| Duvelisib + FCR | PI3K/BCL-2 | NCT03534323 | 1/2 | R/R | 67/50 | 14 9 65 24.7 75 (est. 1 y) nr 91 (est. 1) (Jones JA et al, 2018) |
| Duvelisib + Venetoclax | PI3K | NCT01569295 | 3 | R/R | 56/205 | 16.1 1.1/0 75.3/18.4 16.4/8.0 nr Not reached nr (Jones JA et al, 2017) |
| Duvelisib + BR vs Placebo + BR | PI3K | NCT01659021 | 3 | R/R | 50/205 | 16.1 1.1/0 75.3/18.4 16.4/8.0 nr Not reached nr (Jones JA et al, 2017) |
| Duvelisib + Ofatumumab vs Ofatumumab | PI3K | NCT01659021 | 3 | R/R | 56/205 | 16.1 1.1/0 75.3/18.4 16.4/8.0 nr Not reached nr (Jones JA et al, 2017) |
| Ibrutinib | BTK | NCT01744691 | 2 | R/R | 97/19 | 11.5 8 83 not reached 63 not reached 75 (O'Brien S et al, 2016) |
| Ibrutinib (R/R vs TN) | BTK | NCT01105247 | 1/2 | R/R-TN | 38-94 | 60 10/29 89/87 51/(not reached) 44/92 (5 y) not reached 60/92 (5 y) (O'Brien S et al, 2018) |
| Ibrutinib vs Chl | BTK | NCT01722487 | 1 | R/R | 67/50 | 16.1 1.1/0 75.3/18.4 16.4/8.0 nr Not reached nr (Jones JA et al, 2017) |
| Ibrutinib vs Ibrutinib + R vs BR | BTK | NCT01973387 | 3 | R/R | 49/98 | 36 |
| Ibrutinib vs Ibrutinib + R vs BR | BTK | NCT01611090 | 3 | R/R | 101/418 | 0 |
| Ibrutinib vs Ofatumumab | BTK | NCT01578707 | 3 | R/R | 208/182 | 127 (del17) |
| Ibrutinib vs R | BTK | NCT01973387 | 3 | R/R | 49/98 | 36 |
| Ibrutinib + BR vs Placebo + BR | BTK | NCT01292135 | 1 | R/R | 101/418 | 0 |
| Ibrutinib + BR | BTK | NCT01292135 | 1 | R/R | 101/418 | 0 |
| Ibrutinib + OB vs Chl + OB | BTK | NCT02264574 | 3 | TN | 106/123 | 41 |
| Tirabrutinib | BTK | NCT01292135 | 1 | R/R | 101/418 | 0 |
| Tirabrutinib + Ibrutinib | BTK | NCT01292135 | 1 | R/R | 101/418 | 0 |
| Tirabrutinib + Entospletinib | BTK/SYK | NCT02983617 | 2 | R/R | 14 UM-CLL | 18 |
| Zanubrutinib | BTK | NCT02343120 | 1/2 | R/R-TN | 14 UM-CLL | 18 |

Abbreviations: B, bendamustine; BCL-2, B cell lymphoma 2; BTK, Bruton's tyrosine kinase; Chl, chlorambucil; CR, complete response; FCR, fludarabine-cyclophosphamide-rituximab; nr, not reported; OB, obinutuzumab; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PI3K, phosphatidylinositol 3-kinase; R, rituximab; R/R, relapsed/refractory; SYK, spleen tyrosine kinase; TN, treatment naïve.
| Median follow up (months) | CR (%) | ORR (%) | PFS (median in months) | PFS (% at 2 y) | OS (median in months) | OS (% at 2 y) | Reference |
|--------------------------|--------|---------|------------------------|----------------|-----------------------|--------------|-----------|
| 28.1                     | 49.5/23.1 | 84.7/71.3 | Not reached           | 88.2/64.1     | Not reached           | 91.8/93.3   | (Fischer K et al, 2019) |
| 12.1                     | 17     | 79.4    | Not reached           | 72 (est. 1 y) | Not reached           | 86.7 (est. 1 y) | (Stilgenbauer S et al, 2016) |
| 14                       | 9      | 65      | 24.7                  | 75 (est. 1 y) | nr                    | 91 (est. 1)  | (Jones JA et al, 2018) |
| 4/0                      | 56/83  | 15.7    | not reached           | nr            | nr                    | nr           | (Flinn IW et al, 2018a) |
| 22.4                     | 0.6/0.6| 73.8/45.3| 13.3/9.9             | 60/39 (1 y)   | nr                    | nr           | (Flinn IW et al, 2018b) |
| nr                       | nr     | nr      | nr                    | nr            | nr                    | nr           | (Brown JR et al, 2014) |
| 14                       | 3/0    | 70.0/45.5| 20.8/11.1            | nr            | Not reached/31.6      | 79/71 (1 y)  | (Zelenetz AD et al, 2017) |
| 16.1                     | 1.1/0  | 75.3/18.4| 16.4/8.0             | nr            | Not reached           | nr           | (Zelenetz AD et al, 2017) |
| 3.8                      | 0/0    | 81/13   | Not reached/5.5       | 93/46         | Not reached           | 92/80 (1 y)  | (Furman RR et al, 2014) |
| nr                       | n.r    | 90.9/76.9| nr                    | nr            | nr                    | nr           | (Brown JR et al, 2015) |
| 26                       | 27     | 90      | Not reached           | 90            | Not reached           | 95           | (Davids MS et al, 2019) |
| 29                       | nr     | 85/94/79| Not reached/not reached/22.6 | 82/90/34 (30 mo) | Not reached           | 94/95/90 (30 mo) | (Abstract; Sharman JP et al, 2019) |
| 8                        | 25     | 100     | nr                    | nr            | nr                    | nr           | (Abstract; Lampson BL et al, 2010) |
| 11.5                     | 8      | 83      | not reached           | 63            | not reached           | 75           | (O'Brien S et al, 2016) |
| 60                       | 10/29  | 89/87   | 51/(not reached)      | 44/92 (5 y)   | not reached           | 60/92 (5 y)  | (O'Brien S et al, 2018) |
| 18.4                     | 4/2    | 82.4/35.3| (not reached)/18.9    | 90/52 (18 mo) | nr                    | 98/85        | (Burger JA et al, 2015) |
| 30                       | 7/12/26| 93/94/75| (not reached)/(not reached)/43 | 87/88/74     | nr                    | 90/94/95     | (Abstract; Lampson BL et al, 2010) |
| 44                       | nr     | 42.6/4.1| (not reached)/8.1     | 59/3 (3 y)    | not reached           | 74/65 (3 y)  | (Byrd JC et al, 2019) |
| 17.8                     | 3.8/0  | 53.8/7.4| not reached/8.3       | 74/11.9 (18 mo) | not reached/26.1      | nr           | (Huang et al, 2018) |
| 17                       | 10/3   | 83/68   | not reached/13.3      | 79/24 (18 mo) | not reached           | nr           | (Chanan-Khan A et al, 2016) |
| 37.3                     | 40.0   | 93.3    | not reached           | 78.6          | nr                    | nr           | (Brown JR et al, 2015) |
| 31.3                     | 19/8   | 88/73   | (not reached)/19      | 79/31 (30 mo) | not reached           | 86/85 (30 mo) | (Moreno C et al, 2019) |
| 32.5                     | nr     | 96      | 38.5                  | nr            | 44.9                  | n.r          | (Walter HS et al, 2017) |
| nr                       | nr     | nr      | nr                    | nr            | nr                    | nr           | (Danilov AV et al, 2020) |
| 15/34/30                 | 7/7/10 | 83/93/100| not reached/32/not reached | nr            | nr                    | nr           | (Hillmen et al, 2020) |
| nr                       | 0/6.7  | 100/90  | nr                    | nr            | nr                    | nr           | (Abstract; Sharman JP et al, 2019) |
| 13.7                     | 2.6    | 96.2    | not reached           | 100 (est. 1 y) | nr                    | nr           | (Hillmen et al, 2020) |

Abbreviations: B, bendamustine; BCL-2, B cell lymphoma 2; BTK, Bruton’s tyrosine kinase; Chl, chlorambucil; CR, complete response; FCR, fludarabine-cyclophosphamide-rituximab; nr, not reported; OB, obinutuzumab; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PI3K, phosphatidylinositol 3-kinase; R, rituximab; R/R, relapsed/refractory; SYK, spleen tyrosine kinase; TN, treatment naïve.
cancer. Four classes of PI3Ks exist. Class 1 PI3Ks are heterodimers consisting of a p85 regulatory subunit and a p110 catalytic subunit, each of which exists as different isoforms. The catalytic subunits p110α and β are expressed ubiquitously, while p110γ and δ primarily are expressed in leucocytes. The latter subunits were therefore recognized as attractive therapeutic targets in haematological malignancies (Figure 1).

Interest in developing p110δ inhibitors came after it was discovered that mice lacking this isoform did not develop mature B cells. The first drug to obtain FDA approval was the specific p110δ inhibitor idelalisib for relapsed CLL patients who had received at least two prior therapies (Table 1). In the first phase 1 clinical trial (NCT00710528) with heavily pretreated patients and high-risk patients, an overall response rate of 72% and a 15.8-month median progression-free survival were reported (Table 2). 20% of the patients had a grade ≥ 3 pneumonia, and only 1 patient had a grade ≥ 3 alanine transaminase (ALT) or aspartate transaminase (AST) increase. A phase 3 study (NCT01539512) comparing idelalisib and rituximab with placebo and rituximab in relapsed patients who could not undergo chemotherapy showed a markedly improved overall survival at 12 months (92% vs 80%) and overall response (81% vs 13%; Table 2). Serious adverse events occurred in 40% of the patients in the idelalisib arm and 35%
in the placebo arm. These studies showed promising results with acceptable toxicity and idehalisib was therefore moved into first-line clinical trials. However, while idehalisib was highly active also in treatment naïve patients, serious adverse autoimmune events were frequent; 79% of the patients had ALT or AST elevation and 54% experienced transaminitis grade 3 or higher. Therefore, all clinical trials on previously untreated patients were terminated in March 2016, and idehalisib is not given to treatment naïve and younger patients today. Some of the adverse autoimmune effects of idehalisib may be due to inhibition of regulatory T cells.

Duvelisib, a dual inhibitor of both the p110δ and p110γ isoforms (Table 1), received FDA approval in September 2018 for refractory or relapsed CLL patients who have received at least two prior therapies. A phase I study (NCT01476657) showed an overall response rate (ORR) of 56% for patients with relapsed/refractory CLL and 83% for previously untreated CLL patients (Table 2). Colitis and transaminitis were frequently observed in this study as well, with treatment naïve patients being at higher risk. Duvelisib is now being tested in combination with the B cell lymphoma-2 (BCL-2) inhibitor venetoclax for relapsed CLL patients (NCT03534323) (Table 2). Duvelisib has shown synergy with venetoclax in vitro, and duvelisib treatment is associated with changes in apoptotic regulators that may sensitize the CLL cells to venetoclax treatment.

Several additional PI3K inhibitors are currently under investigation in clinical trials. Umbralisib is a next-generation PI3K inhibitor selective for the p110δ isoform (Table 1). In a recent phase 1 study (NCT01767766), this agent showed fewer autoimmune side effects as a monotherapy compared with the PI3K inhibitors idehalisib and duvelisib. In a recent phase 1 study with 21 relapsed/refractory CLL patients (NCT02268851), umbralisib was combined with another drug targeting the BCR pathway, the BTK inhibitor ibrutinib. The combination was well tolerated, and the overall response was 90% with a 2-year overall survival of 95% (Table 2).

3.2 | BTK inhibitors

BTK is a cytoplasmic protein tyrosine kinase that belongs to the tyrosine kinase expressed in hepatocellular carcinoma (Tec) family of non-receptor tyrosine kinases (TFKs) (Figure 1). In 1952, the phenotype of Btk deficiency was first described in a boy who presented with recurrent bacterial infections due to deficiency in humoral immunity. This severe primary immunodeficiency was named X-linked agammaglobulinemia (XLA). In 1993, the causative gene of XLA, Btk, was first identified and isolated. BTK is expressed in all haematopoietic cells except T lymphocytes and plasma cells. BTK regulates cellular processes including survival, proliferation and migration. In addition, BTK is critical to B cell motility and homing, explaining the trafficking of lymphocytes between lymph node and blood, and the lymphocytosis observed in response to treatment with BTK inhibitors.

BTK inhibitors are classified as either irreversible or reversible, referring to their binding to the target. Indeed, most of the small molecule targeted drugs for cancer therapy are irreversible binders (Table 1). These are considered the most potent, but cancer cells acquiring resistance are an increasing issue. Ibrutinib was the first-in-class BTK inhibitor and is an irreversibly acting and orally bioavailable drug. Currently, ibrutinib as a single agent is one of the options as a first-line treatment for CLL patients. When compared to treatment with the anti-CD20 monoclonal antibody ofatumumab in previously untreated patients (NCT01578707), ibrutinib showed significantly improved progression-free survival and 12-month overall survival rate (90% in ibrutinib arm vs 81% in ofatumumab arm) (Table 2).

In a randomized phase 3 trial (RESONATE-2, NCT01722487), ibrutinib also showed superiority over the chemotherapeutic drug chlorambucil in previously untreated CLL or small lymphocytic lymphoma (SLL) patients (Table 2). In general, patients tolerate ibrutinib treatment well. In a 5-year follow-up study, most of the adverse events reported were either grade 1 or 2. Of the grade 3 events reported, infections (9.3%) and atrial fibrillation (5.8%) were most common, but also diarrhoea (3.5%), rash (2.3%) and arthritis (2.3%) were reported. Many of the side effects are believed to be due to off-target effects, and this provides a rationale for developing novel, more selective BTK inhibitors.

Of the novel BTK inhibitors developed so far, acalabrutinib is at the most advanced stage and is approved by the FDA for treatment of adults with CLL. Acalabrutinib is an irreversible inhibitor of BTK, reported to be more selective and to have less off-target effects when compared to ibrutinib. This could mean higher efficacy and fewer side effects. Acalabrutinib is currently tested in two different phase 3 clinical trials for patients with previously
untreated CLL. One is combining acalabrutinib with venetoclax and obinutuzumab compared to chemoimmuno-therapy (NCT03836261) (Table 2). The second study, Elevate-TN, is comparing acalabrutinib alone and in combination with obinutuzumab with chemoimmunotherapy (NCT02475681) (Table 2). Interim results from the Elevate-TN study estimates the 30-month progression-free survival to be 90% in the acalabrutinib + obinutuzumab arm, 82% with acalabrutinib as a single agent and 34% in the obinutuzumab and chlorambucil arm.\(^{75}\) Acalabrutinib is now being compared head-to-head with ibrutinib in a phase 3 study (NCT02477696) (Table 2). Combining targeted therapies with either other targeted therapies, chemotherapy or chemoimmunotherapy is appealing because of the possibility of achieving deeper and long-standing remissions.\(^{52}\)

Zanubrutinib is another potent and highly selective, irreversible next-generation BTK inhibitor, approved by the FDA for treatment of mantle cell lymphoma (MCL) (Table 1). So far, it has shown promising effects on activity and safety (NCT02343120) (Table 2).\(^{76}\) A phase 3 study is currently recruiting 600 patients to compare the overall response rates of zanubrutinib versus ibrutinib in patients with relapsed or refractory CLL or SLL (NCT03734016) (Table 2).\(^{77}\) The selective and irreversible BTK inhibitor tirabrutinib (ONO/GS-4059) (Table 1) has continued to show promising results in a long-term follow-up of relapsed/refractory CLL patients in a phase 1 clinical study (NCT02457559) (Table 2).\(^{78}\) Estimated median PFS was 38.5 months, median overall survival was 44.9 months and the treatment continued to be well tolerated. Because of high efficacy and minimal toxicity, tirabrutinib is now in phase 2 clinical trials (Table 2). One study assesses the effect of tirabrutinib combined with entospletinib and obinutuzumab in relapsed/refractory CLL patients (NCT02983617). Interim results at week 25 showed an overall response rate of 100% in the tirabrutinib and entospletinib arm and 90% in the tirabrutinib, entospletinib and obinutuzumab arm (Table 2). A second phase 2 clinical trial evaluates the effect of tirabrutinib in combination with idelalisib and obinutuzumab in relapsed or refractory CLL patients (NCT02968563) (Table 2). Results from this study are awaited.

Targeted therapies have revolutionized CLL treatment over the last few years. PI3K inhibitors have great potential in CLL treatment and novel PI3K inhibitors are under development and are in different stages of clinical testing. The hope is that some of these drugs can reduce the toxicity associated with idelalisib, and that novel combinations including PI3K inhibitors can increase their efficacy and tolerability. BTK inhibitors have altogether showed positive results in the treatment of CLL, with high efficacy and tolerability also in the elderly patients. Next-generation BTK inhibitors are now becoming available to patients that experiences off-target side effects or resistance to ibrutinib (Table 1). Combining BTK inhibitors with other drugs may also become important to overcome the resistance issues seen with ibrutinib.

### 4. BCR INHIBITORS MAY FACILITATE IMPLEMENTATION OF FUNCTIONAL PRECISION MEDICINE

Despite the recent therapeutic advances, CLL is still considered incurable. The disease is characterized by large inter-patient heterogeneity in both pathologic features and clinical outcome, requiring personalized management approaches. The novel targeted therapies are effective, but only in a subgroup of patients, and the administration is currently indefinite and based on incomplete patient stratification. While gene mutations have been identified in CLL, these are not yet actionable.\(^{79}\) A complementary approach to genomics is therefore needed to introduce precision medicine and improve patient care. We suggest that functional precision medicine, that is the use of functional assays to identify predictive biomarkers, should be considered. Targeted therapies are especially compatible with such high-throughput drug response analyses. The increased clinical relevance of these compounds may therefore accelerate the implementation of functional precision medicine in the clinic. Functional assays have several applications. They are used for drug discovery purposes, that is to identify new candidate drugs. They can serve as companion diagnostics, that is to determine if a certain drug is suitable for a specific patient. And they can be used to guide personalized medicine by screening large numbers of drugs for a single patient. Below, we discuss some of the assays that are currently used in preclinical investigations for the ex vivo determination of responses to cancer treatments, with a focus on application to haematological diseases.

#### 4.1 Measurement of target engagement or target response

To what degree a targeted agent binds to its molecular target may correlate with patient response to that drug. For example, ibrutinib inactivates BTK by irreversibly binding to the amino acid residue Cys481.\(^{80,81}\) The irreversible binding once it has occurred would take the target:drug complex out of the equilibrium with free target and free drug and would mean that it may be possible to dose drug lower or more intermittently and yet achieve the same target occupancy. BTK occupancy in peripheral blood mononuclear cells (PBMCs) reflects drug activity (Figure 2A). While the
recommended daily dose of ibrutinib is 420 mg, a pilot study showed that the dose can be reduced step-wise to 140 mg/d and still occupy almost all BTK molecules in tumour cells from CLL patients.82 A reduction in dose could reduce the inhibition of off-target proteins and consequently lower toxicities. It would be of interest to apply these tests to patient samples before start of treatment to determine the optimal dose for the individual patient. To the best of our knowledge, such studies have not been performed on BTK inhibitors. However, examples of predictive tests exist for other targets in other cancers.83 It is well known that melanoma patients with BRAF mutation have elevated activation of MAPK and that the successful inhibition of this pathway with BRAF or MEK inhibitors correlates with response to therapy. To test the efficacy of the pathway inhibitors, protein phosphorylation can be measured by applying a kinase substrate peptide microarray over which the lysed patient sample is dispensed.84 When measuring kinase activity in melanoma patient samples, no difference in substrate phosphorylation was observed between major genotypes such as mutations in BRAF, NRAS, cyclin-dependent kinase inhibitor 2A (CDKN2A) or TP53. Differences could only be observed when the lysates were exposed to the BTK inhibitor vemurafenib ex vivo.85 This illustrates the value of functional data in patient stratification.

4.2 | Cell signalling analysis by phospho flow

Analysis of several pathways simultaneously may enable better response predictions than analysis of only one pathway. Phospho flow is a powerful technique for this purpose, based on flow cytometry that measures protein phosphorylation events at the cellular level. This feature distinguishes the method from other antibody-based approaches such as Western blots, enzyme-linked immunosorbent assay (ELISA), protein array, and reverse phase protein array (RPPA).86 The method is commonly combined with fluorescent cell barcoding, meaning that each cell sample is stained with a unique set of fluorescent dyes, that is a barcode, to allow for combination and simultaneous analysis of control and test samples (Figure 2B).87 Advantages of barcoding include reduced antibody consumption, increased data robustness and enhanced speed of acquisition. Application of phospho flow has provided valuable information about baseline and drug-induced cell signalling in several haematological malignancies, including CLL,39,43,88,89 acute myeloid leukaemia (AML)90-92 and non-Hodgkin lymphomas,93 as well as in normal haematologic subsets present among the malignant cells.94 The method gives highly reproducible results, and cryopreservation does not affect signalling responses in B lymphocytes.95 By performing high-throughput phospho flow experiments in a 96-well plate format, changes in phosphorylation level of 35 proteins in response to short (20 minutes) treatment with the BTK inhibitor ibrutinib and the PI3K inhibitor idelalisib was systematically analysed in CLL cells.89 Results showed that the targeted agents primarily affect proteins in the BCR signalosome and in the PI3K pathway. In the same study, a method to investigate synergy between ibrutinib and venetoclax, traditionally applied to cell viability data,89,96 was adapted to signalling-response data. This combination of ibrutinib and venetoclax has been used with success in clinical studies on CLL patients.97-99 Based on analyses of phospho flow data, it was shown that synergy between the two drugs can be achieved at doses that are much lower than the recommended daily dose of both ibrutinib or venetoclax. The potency of the targeted agents is supported by retrospective studies on CLL patients that have been treated with reduced doses of ibrutinib or venetoclax in order to limit toxicities.100-105 These studies show that the effect of the treatment is not compromised by lowering the dose, indicating that further dose adjustment studies are warranted.

4.3 | BCL-2 homology domain (BH3) profiling

Proteins of the BCL-2 family control the intrinsic apoptosis pathway by regulating mitochondrial outer-membrane permeabilization (MOMP).106 The BCL-2 family includes both inhibitors and inducers of apoptosis, and, essentially, when the proapoptotic proteins overwhelm the antiapoptotic proteins, MOMP is initialized. While BH3 profiling measures how close the mitochondria are to a threshold of apoptosis, dynamic BH3 profiling estimates the effect an applied drug has on moving the mitochondria closer to this threshold (Figure 2C). BH3 profiling can also identify antiapoptotic vulnerability. The method was useful in the clinical development of venetoclax in CLL and AML as it helped identify these diseases as largely BCL-2 dependent.107,108 The assays are performed by titrating BH3 peptides derived from the α-helical BH3 domains of proapoptotic BCL-2 family proteins and testing how much is required to overwhelm the antiapoptotic proteins and thus induce MOMP.109,110 For cells that are highly primed for apoptosis, little BH3 peptide is required to induce MOMP, and vice versa. Dynamic BH3 profiling can be used in high-throughput analyses of drug sensitivity. The assays are performed in 384-well plate format, and the cells are exposed to drugs for 6-24 hours. Apoptotic priming is then measured by BH3 profiling. A drug-induced increase in BH3 peptide-induced MOMP indicates induction of proapoptotic signalling. Several studies have shown that early measurement of drug-induced proapoptotic signalling predicts the in vivo response to the drug.111-115
4.4 | In vitro drug sensitivity assessment

The ultimate way to use functional tests to guide clinical decisions would be to screen a patient's tumour cells for drug sensitivity in vitro to identify the most effective treatment for that patient (Figure 2D). Over the past decade, such high-throughput assays have been developed. The experiments are typically performed in a 384-well plate format, and the cells are commonly exposed to drugs for 72 hours. Tyner et al have assessed the sensitivity of leukaemic cells from 151 patients to a panel of 66 kinase inhibitors.116 Based on these data, the authors were able to develop an algorithm that correctly predicted pathway dependencies. For example, cells from patients with mutant Fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD)-positive AML were killed by drugs that target FLT3, while cells from patients with BCR-ABL-positive chronic myeloid leukaemia (CML) were killed by drugs targeting ABL. In a proof-of-principle case, the authors showed that in vitro drug sensitivity could predict clinical response as well as development of drug resistance.

Using a similar screening assay, Wennerberg and colleagues developed an individualized systems medicine approach to optimize precision medicine.117 Samples from 28 AML patients were exposed to 187 drugs for 72 hours before cell viability was assessed. Two patients were treated with a combination of dasatinib, sunitinib and temsirolimus in an off-label compassionate use setting based on the results from the drug sensitivity screen. Both patients responded, but the response was short-lived. The drug sensitivity data were used both to suggest the mechanism of resistance, as well as potential ways to counteract it with combinatorial therapies.

Dietrich et al have measured ex vivo drug sensitivity of 246 patient samples from various blood cancers to 63 drugs.118 In addition, they performed analysis of genomics, transcriptomics and DNA methylation status to understand determinants of drug responses. For CLL, they found that the BCR pathway was linked to trisomy 12, an important driver of the disease. Further, they were able to classify the disease into phenotypic subgroups based on dependency of the BCR, mTOR or MEK in association with genomic features as mentioned above. In addition to trisomy 12, the mutation status of IGHV was the most important modulator of drug response. This is in agreement with clinical observations.35 Importantly, the study showed that ex vivo drug responses were associated with disease outcome, underscoring the potential of functional assays as diagnostic tools.

Finally, a study by Schmid and colleagues combined epigenome profiling with single-cell chemosensitivity profiling and bioinformatic data integration in order to identify pharmacologically exploitable vulnerabilities in CLL cells collected from patients before and during ibrutinib treatment.119 By this approach, the authors identified ibrutinib-induced changes which could be used for rationally designing ibrutinib combination therapies.

Taken together, these studies demonstrate that direct assessment of drug sensitivity may give patient benefit even when knowledge about the underlying biology is missing. This approach can therefore complement genomic precision medicine, where a treatment is given based on the presence of a specific gene mutation.120 The clinical utility of drug sensitivity-based treatment decisions is currently investigated in prospective clinical trials on leukaemia and lymphoma (ClinicalTrials.gov identifiers: NCT01620216, NCT03096821).

4.5 | Patient-derived xenografts and other mouse models

In order to elucidate personalized treatment options based on an in vivo setting, patient-derived mouse xenografts (PDXs) can serve as valuable tools.121 The PDXs are models of cancer where human tumour material is grafted into immunodeficient or humanized mice. PDX models are used to determine the contribution of tumour heterogeneity to therapeutic responsiveness, to understand tumour evolution over time and under drug pressure, and to investigate mechanisms leading to resistance to therapy.121 While the models are useful, they also come with challenges. The time it takes to make the PDX, perform in vivo experiments, harvest and analyse data, can be several months. This is a significant amount of time considering that the clinician and patient are waiting for the results to make a treatment decision. Experiments with PDX models are also costly compared to ex vivo functional approaches. At present, these models are therefore less efficient tools with respect to guiding clinical decisions in precision medicine.120

In addition to PDX models, transgenic mouse models of CLL exist.122,123 These models are valuable tools for preclinical studies, and can provide insightful information on pathogenic mechanisms. However, as CLL is heterogeneous in nature, it is important to keep in mind that these models are limited to mimicking one state of the disease or only certain aspects of the disease. Data generated from these models should therefore be interpreted with care. Interestingly, a BTK C481S knock-in mouse model was recently generated.124 This cysteine to serine substitution is the most common mechanism for acquired resistance to BTK inhibitors, and these mice are resistant to irreversible BTK inhibitors. This model may prove useful to identify novel therapeutic targets.124

Together, the available PDX and transgenic mouse models are valuable hypothesis-testing tools and provide novel insights on tumour biology and drug mechanisms. Nevertheless, their use in precision medicine, where
5 | CONCLUSIONS AND OUTLOOK

Signalling through the BCR is essential for B cell survival. Studies on B cell malignancies such as CLL, in which cell signalling is aberrantly regulated, have accelerated our understanding of the cellular and molecular players involved in B cell physiology and pathology. Over the past few years, small molecule inhibitors that target BCR-associated kinases have demonstrated clinical success. The BTK inhibitor ibrutinib and the PI3K inhibitor idelalisib are first-in-class drugs approved for the treatment of CLL, and have revolutionized the management of this disease. Several next-generation agents have since been developed, some of which are already approved for treatment of B cell malignancies (Table 1). Despite novel treatment strategies, we still face the challenge of treatment resistance. A more complete understanding of the in vivo mechanisms of action of these drugs are needed, as well as biomarkers that can predict response to treatment and guide precision medicine. Functional assays are valuable tools that can help reach these goals.

ACKNOWLEDGMENT

Our work on CLL functional precision medicine has been supported by the Norwegian Cancer Society, the Regional Health Authority for South-Eastern Norway, the Research Council of Norway, Stiftelsen Kristian Gerhard Jebsen, and Lilly Constance og Karl Ingolf Larssons stiftelse.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

SSS and LK wrote the manuscript. All authors read, commented and approved the final manuscript.

ORCID

Sigrid S. Skånland https://orcid.org/0000-0003-1630-356X
Kjetil Taskén https://orcid.org/0000-0003-2841-4697

REFERENCES

1. LeBien TW. Fates of human B-cell precursors. Blood. 2000;96:9-23.
2. Schamel WW, Roth M. Monomeric and oligomeric complexes of the B cell antigen receptor. Immunity. 2000;13:5-14.
3. Roth M. Antigen receptor tail clue. Nature. 1989;338:383-384.
4. Papavasiliou F, Jankovic M, Suh H, Nussenzweig MC. The cytoplasmic domains of immunoglobulin (Ig) alpha and Ig beta can independently induce the precursor B cell transition and allelic exclusion. J Exp Med. 1995;182:1389-1394.
5. Johnson SA, Pleiman CM, Pao L, Schneringer J, Hippen K, Cambier JC. Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. J Immunol. 1995;155:4596-4603.
6. Rolli V, Gallwitz M, Wossning T, et al. Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. Mol Cell. 2002;10:1057-1069.
7. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood. 2008;112:1570-1580.
8. Treanor B, Depoil D, Bruckbauer A, Batista FD. Dynamic cortical actin remodeling by ERM proteins controls BCR microcluster organization and integrity. J Exp Med. 2011;208:1055-1068.
9. Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. Mol Immunol. 2004;41:599-613.
10. Rowley RB, Burkhardt AL, Chao HG, Matsueda GR, Bolen JB. Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig alpha/Ig beta immunoreceptor tyrosine activation motif binding and autophosphorylation. J Biol Chem. 1995;270:11590-11594.
11. Zhong Y, Byrd JC, Dubovsky JA. The B-cell receptor pathway: a critical component of healthy and malignant immune biology. Semin Hematol. 2014;51:206-218.
12. Shaffer AL III, Young RM, Staudt LM. Pathogenesis of human B cell lymphomas. Annu Rev Immunol. 2012;30:565-610.
13. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell. 1997;90:1073-1083.
14. Monroe JG. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. Nat Rev Immunol. 2006;6:283-294.
15. Yang J, Roth M. Oligomeric organization of the B-cell antigen receptor on resting cells. Nature. 2010;467:465-469.
16. Pierce SK, Liu W. The tipping points in the initiation of B cell signalling: how small changes make big differences. Nat Rev Immunol. 2010;10:767-777.
17. Schweighoffer E, Vanes L, Nys J, et al. The BAFF receptor transduces survival signals by co-opting the B cell receptor signaling pathway. Immunity. 2013;38:475-488.
18. Koehrer S, Burger JA. B-cell receptor signaling in chronic lymphocytic leukemia and other B-cell malignancies. Clin Adv Hematol Oncol. 2016;14:55-65.
19. Burger JA, Wiestner A. Targeting B cell receptor signalling in cancer: preclinical and clinical advances. Nat Rev Cancer. 2018;18:148-167.
20. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. Blood. 2011;118:4313-4320.
21. Li Y, Wang Y, Wang Z, Yi D, Ma S. Racial differences in three major NHL subtypes: descriptive epidemiology. Cancer Epidemiol. 2015;39:8-13.
22. Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. Am J Hematol. 2019;94:1266-1287.
23. Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med. 1995;333:1052-1057.
24. Reimart N, Nguyen PH, Boucas J, et al. Delayed development of chronic lymphocytic leukemia in the absence of macrophage migration inhibitory factor. Blood. 2013;121:812-821.
25. Pedersen IM, Kitada S, Leoni LM, et al. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. Blood. 2002;100:1795-1801.

26. Tsukada N, Burger JA, Zvaifler NJ, Kipps TJ. Distinctive features of "nurselike" cells that differentiate in the context of chronic lymphocytic leukemia. Blood. 2002;99:1030-1037.

27. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell’Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood. 2000;96:2655-2663.

28. Patten PE, Buggins AG, Richards J, et al. CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. Blood. 2008;111:5173-5181.

29. Ruan J, Hyjek E, Kermani P, et al. Magnitude of stromal hematopoiesis correlates with histologic subtype of non-Hodgkin’s lymphoma. Clin. Cancer Res. 2006;12:5622-5631.

30. Gobessi S, Laurenti L, Longo PG, Sica S, Leone G, Efremov DG. ZAP-70 enhances B-cell-receptor signaling despite absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B cells. Blood. 2007;109:2032-2039.

31. Nolz JC, Tschumper RC, Pittner BT, Darce JR, Kay NE, Jelinek DF. ZAP-70 is expressed by a subset of normal human B-lymphocytes displaying an activated phenotype. Leukemia. 2005;19:1018-1024.

32. Cutrona G, Colombo M, Matis S, et al. B lymphocytes in humans express ZAP-70 when activated in vivo. Eur. J. Immunol. 2006;36:558-569.

33. Sivina M, Hartmann E, Kipps TJ, et al. CCL3 (MIP-1alpha) plasma levels and the risk for disease progression in chronic lymphocytic leukemia. Blood. 2011;117:1662-1669.

34. Yan XJ, Doznomov I, Li W, et al. Identification of outcome-correlated cytokine clusters in chronic lymphocytic leukemia. Blood. 2011;118:5201-5210.

35. International CLL-IPI working group. An international prognostic index for patients with chronic lymphocytic leukaemia (CLL-IPI): a meta-analysis of individual patient data. Lancet Oncol. 2016;17:779-790.

36. 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.

37. Damle RN, Wasił 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.

38. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848-1854.

39. Myhrvold IK, Cremaschi A, Hermansen JU, et al. Single cell profiling of phospho-protein levels in chronic lymphocytic leukemia. Oncotarget. 2018;9:9273-9284.

40. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia. Nat Rev Cancer. 2016;16:145-162.

41. Contri A, Brunati AM, Trentin L, et al. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. J Clin Invest. 2005;115:369-378.

42. Gobessi S, Laurenti L, Longo PG, et al. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. Leukemia. 2009;23:686-697.

43. Blix ES, Irish JM, Husebekk A, et al. Phospho-specific flow cytometry identifies aberrant signaling in indolent B-cell lymphoma. BMC Cancer. 2012;12:478.

44. Muzzio M, Apollonio B, Scielzo C, et al. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. Blood. 2008;112:188-195.

45. 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.

46. 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.

47. Dighiero G, Maloum K, Desablens B, et al. Chlorambucil in indolent chronic lymphocytic leukemia. French Cooperative Group on chronic lymphocytic leukemia. N Engl J Med. 1998;338:1506-1514.

48. Herling CD, Cymbalista F, Groß-Ophoff-Müller C, et al. Early treatment with FCR versus watch and wait in patients with stage Binet A high-risk chronic lymphocytic leukemia (CLL): a randomized phase 3 trial. Leukemia. 2020. https://doi.org/10.1038/s41375-020-0747-7

49. ten Hacken E, Guieze R, Wu CJ. SnapShot: chronic lymphocytic leukemia. Cancer Cell. 2017;32:716.

50. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. Oncogene. 2008;27:5497-5510.

51. Ferrer G, Montserrat E. Critical molecular pathways in CLL therapy. Mol Med. 2018;24:9.

52. Jou ST, Carpino N, Takahashi Y, et al. Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. Mol Cell Biol. 2002;22:8580-8591.

53. Brown JR, Byrd JC, Coutre SE, et al. Idelalisib, an inhibitor of phosphatidylinositol 3-kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. Blood. 2014;123:3390-3397.

54. Furman RR, Shamar JP, Coutre SE, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. N Engl J Med. 2014;370:997-1007.

55. Lampson BL, Kasar SN, Matos TR, et al. Idelalisib given front-line for treatment of chronic lymphocytic leukemia causes frequent immune-mediated hepatotoxicity. Blood. 2016;128:195-203.

56. Brown JR. Phosphatidylinositol 3 Kinase Delta Inhibitors: present and future. Cancer J. 2019;25:394-400.

57. Chellappa S, Kushkekar K, Munteh LA, et al. The PI3K p110delta isoform inhibitor idelalisib preferentially inhibits human regulatory T cell function. J Immunol. 2019;202:1397-1405.

58. Flinn IW, O’Brien S, Kahl B, et al. Duvelisib, a novel oral dual inhibitor of PI3K-delta, gamma, is clinically active in advanced hematologic malignancies. Blood. 2018;131:877-887.

59. Patel VM, Balakrishnan K, Douglas M, et al. Duvelisib treatment is associated with altered expression of apoptotic regulators that helps in sensitization of chronic lymphocytic leukemia cells to venetoclax (ABT-199). Leukemia. 2017;31:1872-1881.

60. Burrel HA III, Flinn IW, Patel MR, et al. Umbralisib, a novel PI3Kdelta and casein kinase-1epsilon inhibitor, in relapsed or refractory chronic lymphocytic leukaemia and lymphoma: an open-label, phase 1, dose-escalation, first-in-human study. Lancet Oncol. 2018;19:486-496.

61. Davids MS, Kim HT, Nicotra A, et al. Umbralisib in combination with ibrutinib in patients with relapsed or refractory chronic

SKÅNLAND ET AL.
lymphocytic leukaemia or mantle cell lymphoma: a multicentre phase 1–1b study. *Lancet Haematol.* 2019;6:e38-e47.

62. Woyach JA, Johnson AJ. Targeted therapies in CLL: mechanisms of resistance and strategies for management. *Blood*. 2015;126:471-477.

63. Choudhary GS, Al-Harbi S, Mazumder S, et al. MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies. *Cell Death Dis.* 2015;6:e1593.

64. Smith CI, Islam TC, Mattsson PT, Mohamed AJ, Nore BF, Vihinen M. The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. *BioEssays.* 2001;23:436-446.

65. Bruton OC. Agammaglobulinemia. *Pediatrics.* 1952;9:722-728.

66. Vetrie D, Vorechovsky I, Sideras P, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature.* 1993;361:226-233.

67. Smith CI, Baskin B, Humire-Greiff P, et al. Expression of Bruton’s agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J Immunol.* 1994;152:557-565.

68. de Gorter DJ, Beuling EA, Kersseboom R, et al. Bruton’s tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity.* 2007;26:93-104.

69. Woyach JA, Smucker K, Smith LL, et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood.* 2014;123:1810-1817.

70. Feng Y, Duan W, Cü X, Liang C, Xin M. Bruton’s tyrosine kinase (BTK) inhibitors in treating cancer: a patent review (2010–2018). *Expert Opin Ther Pat.* 2019;29:217-241.

71. Byrd JC, Brown JR, O’Brien S, et al. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N Engl J Med.* 2014;371:213-223.

72. Burger JA, Tedeschi A, Barr PM, et al. Ibrutinib as initial therapy for patients with chronic lymphocytic leukemia. *N Engl J Med.* 2015;373:2425-2437.

73. Ahn IE, Farooqui MZH, Tian X, et al. Depth and durability of response to ibrutinib in CLL: 5-year follow-up of a phase 2 study. *Blood.* 2018;131:2357-2366.

74. Byrd JC, Harrington B, O’Brien S, et al. Acalabrutinib (ACP-196) in relapsed chronic lymphocytic leukemia. *N Engl J Med.* 2016;374:323-332.

75. Sharan JP, Banerji V, Fogliatto LM, et al. ELEVATE TN: phase 3 study of acalabrutinib combined with obinutuzumab (O) or alone vs o plus chlorambucil (Cib) in patients (Pts) with treatment-naive chronic lymphocytic leukemia (CLL). *Blood.* 2019;134(Supplement 1):31.

76. Tam CS, Trotman J, Opat S, et al. Phase 1 study of the selective BTK inhibitor zanubrutinib in B-cell malignancies and safety and efficacy evaluation in CLL. *Blood.* 2019;134:851-859.

77. Hillmen P, Brown JR, Eichhorst BF, et al. ALPINE: zanubrutinib versus ibrutinib in relapsed/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma. *Future Oncol.* 2020;16:517-523.

78. Walter HS, Jayne S, Rule SA, et al. Long-term follow-up of patients with CLL treated with the selective Bruton’s tyrosine kinase inhibitor ONO/GS-4059. *Blood.* 2017;129:2808-2810.

79. Lazarian G, Guizez R, Wu CJ. Clinical implications of novel genomic discoveries in chronic lymphocytic leukemia. *J Clin Oncol.* 2017;35:984-993.

80. Advani RH, Buggy JJ, Sharan JP, et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J Clin Oncol.* 2013;31:88-94.

81. Pan Z, Scheetens H, Li SJ, et al. Discovery of selective irreversible inhibitors for Bruton’s tyrosine kinase. *ChemMedChem.* 2007;2:58-61.

82. Chen LS, Bose P, Cruz ND, et al. A pilot study of lower doses of ibrutinib in patients with chronic lymphocytic leukemia. *Blood.* 2018;132:2249-2259.

83. Friedman AA, Letai A, Fisher DE, Flaherty KT. Precision medicine for cancer with next-generation functional diagnostics. *Nat Rev Cancer.* 2015;15:747-756.

84. Hilhorst R, Houkes L, Mommersteeg M, Musch J, van den Berg A, Ruijtenbeek R. Peptide microarrays for profiling of serine/threonine kinase activity of recombinant kinases and lyses of cells and tissue samples. *Methods Mol Biol.* 2013;977:259-271.

85. Tahiri A, Roe K, Ree AH, et al. Differential inhibition of ex vivo tumor kinase activity by vemurafenib in BRAF(V600E) and BRAF wild-type metastatic malignant melanoma. *PLoS One.* 2013;8:e72692.

86. Skånland SS. Phospho flow cytometry with fluorescent cell barcoding for single cell signaling analysis and biomarker discovery. *J Vis Exp.* 2018;140:e58386.

87. Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods.* 2006;3:361-368.

88. Parente-Ribes A, Skånland SS, Burgler S, et al. Spleen tyrosine kinase inhibitors reduce CD40L-induced proliferation of chronic lymphocytic leukemia cells but not normal B cells. *Haematologica.* 2016;101:e59-e62.

89. Skånland SS, Cremaschi A, Bendiksen H, et al. An in vitro assay for biomarker discovery and dose prediction applied to ibrutinib plus venetoclax treatment of CLL. *Leukemia.* 2020;34:478-487.

90. Irish JM, Hovland R, Krutzik PO, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell.* 2004;118:217-228.

91. Irish JM, Anensen N, Hovland R, et al. FLT3 Y591 duplication and Bcl-2 overexpression are detected in acute myeloid leukemia cells with high levels of phosphorylated wild-type p53. *Blood.* 2007;109:2589-2596.

92. Schumich A, Prchal-Murphy M, Maurer-Granofszky M, et al. Phospho-profiling linking biology and clinics in pediatric acute myeloid leukemia. *Hemasphere.* 2020;4:e312.

93. Myklebust JH, Brody J, Kohrt HE, et al. Distinct patterns of B-cell receptor signaling in non-Hodgkin lymphomas identified by single-cell profiling. *Blood.* 2017;129:759-770.

94. Majumder MM, Leppa AM, Hellesoy M, et al. Multi-parametric single cell evaluation defines distinct drug responses in healthy hematologic cells that are retained in corresponding malignant cell types. *Haematologica.* 2020;105:1527-1538.

95. Hermansen UJ, Tjømmford GE, Munthe LA, Taskén K, Skånland SS. Cryopreservation of primary B cells minimally influences their signaling responses. *Sci Rep.* 2018;8:17651.

96. Ianevski A, He L, Aittokallio T, Tang J. SynergyFinder: a web application for analyzing drug combination dose-response matrix data. *Bioinformatics.* 2017;33:2413-2415.

97. Jain N, Keating M, Thompson P, et al. Ibrutinib and venetoclax for first-line treatment of CLL. *N Engl J Med.* 2019;380:2095-2103.
98. Hillmen P, Rawstron AC, Brock K, et al. Ibrutinib plus venetoclax in relapsed/refractory chronic lymphocytic leukemia: the CLARITY Study. *J Clin Oncol.* 2019;37:2722-2729.

99. Kater AP, Levin MD, Niemann CU. Ibrutinib and venetoclax for first-line treatment of CLL. *N Engl J Med.* 2019;381:788-789.

100. Akhtar OS, Attwood K, Lund I, Hare R, Hernandez-Illaliturri FJ, Torka P. Dose reductions in ibrutinib therapy are not associated with inferior outcomes in patients with chronic lymphocytic leukemia (CLL). *Leuk Lymphoma.* 2019;60:1650-1655.

101. Ahn IE, Basumallik N, Tian X, Soto S, Wiestner A. Clinically indicated ibrutinib dose interruptions and reductions do not compromise long-term outcomes in CLL. *Blood.* 2019;133:2452-2455.

102. Mato AR, Timlin C, Ujiani C, et al. Comparable outcomes in chronic lymphocytic leukaemia (CLL) patients treated with reduced-dose ibrutinib: results from a multi-centre study. *Br J Haematol.* 2018;181:259-261.

103. Mato AR, Nabhan C, Thompson MC, et al. Toxicities and outcomes of 616 ibrutinib-treated patients in the United States: a real-world analysis. *Haematologica.* 2018;103:874-879.

104. Mato AR, Thompson M, Allan JN, et al. Real-world outcomes and management strategies for venetoclax-treated chronic lymphocytic leukemia patients in the United States. *Haematologica.* 2018;103:1511-1517.

105. Roeker LE, Fox CP, Eyer TA, et al. Tumor lysis, adverse events, and dose adjustments in 297 venetoclax-treated CLL patients in routine clinical practice. *Clin Cancer Res.* 2019;25:4264-4270.

106. Bhola PD, Letai A. Mitochondria-judges and executioners of cell death sentences. *Mol Cell.* 2016;61:695-704.

107. Deng J, Isik E, Fernandes SM, Brown JR, Letai A, Davids MS. Bruton’s tyrosine kinase inhibition increases BCL-2 dependence and enhances sensitivity to venetoclax in chronic lymphocytic leukemia. *Leukemia.* 2017;31:2075-2084.

108. Pallis M, Burrows F, Ryan J, et al. Complementary dynamic BH3 profiles predict co-operativity between the multi-kinase inhibitor TG02 and the BH3 mimetic ABT-199 in acute myeloid leukaemia cells. *Oncotarget.* 2017;8:16220-16232.

109. Vo TT, Ryan J, Carrasco R, et al. Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. *Cell.* 2012;151:344-355.

110. Ni CT, Sarosiek KA, Vo TT, et al. Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science.* 2011;334:1129-1133.

111. Ryan J, Montero J, Rocco J, Letai A. iBH3: simple, fixable BH3 profiling to determine apoptotic priming in primary tissue by flow cytometry. *Biol Chem.* 2016;397:671-678.

112. Townsend EC, Murakami MA, Christodoulou A, et al. The public repository of xenografts enables discovery and randomized Phase II-like trials in mice. *Cancer Cell.* 2016;30:183.

113. Montero J, Sarosiek KA, DeAngelo JD, et al. Drug-induced death signaling strategy rapidly predicts cancer response to chemotherapy. *Cell.* 2015;160:977-989.

114. Etchin J, Montero J, Berezovskaya A, et al. Activity of a selective inhibitor of nuclear export, selinexor (KPT-330), against AML-initiating cells engrafted into immunosuppressed NSG mice. *Leukemia.* 2016;30:190-199.

115. Wu SC, Li LS, Kopp N, et al. Activity of the Type II JAK2 Inhibitor CHZ868 in B Cell Acute Lymphoblastic Leukemia. *Cancer Cell.* 2015;28:29-41.

116. Tyner JW, Yang WF, Bankhead A III, et al. Kinase pathway dependency in primary human leukemias determined by rapid inhibitor screening. *Cancer Res.* 2013;73:285-296.

117. Pemovska T, Kontro M, Yadav B, et al. Individualized systems medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. *Cancer Discov.* 2013;3:1416-1429.

118. Dietrich S, Oles M, Lu J, et al. Drug-perturbation-based stratification of blood cancer. *J Clin Invest.* 2018;128:427-445.

119. Schmid C, Vladimir GI, Rendeiro AF, et al. Combined chemosensitivity and chromatin profiling prioritizes drug combinations in CLL. *Nat Chem Biol.* 2019;15:232-240.

120. Letai A. Functional precision cancer medicine-moving beyond pure genomics. *Nat Med.* 2017;23:1028-1035.

121. Byrne AT, Alferz DG, Amant F, et al. Interrogating open issues in cancer precision medicine with patient-derived xenografts. *Nat Rev Cancer.* 2017;17:254-268.

122. Chen SS, Chiorazzi N. Murine genetically engineered and human xenograft models of chronic lymphocytic leukemia. *Semin Hematol.* 2014;51:188-205.

123. Simonetti G, Bertilacci MT, Ghia P, Klein U. Mouse models in the study of chronic lymphocytic leukemia pathogenesis and therapy. *Biol. 2014;124:1010-1019.

124. Estupinan HY, Bouderlique T, He C, et al. Novel mouse model resistant to irreversible BTK inhibitors: a tool identifying new therapeutic targets and side effects. *Blood Adv.* 2020;4:2439-2450.

125. 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(23):2225-2236.

126. Stilgenbauer S, Eichhorst B, Schetelig J, et al. Venetoclax in relapsed or refractory chronic lymphocytic leukaemia with 17p deletion: a multicentre, open-label, phase 2 study. *Lancet Oncol.* 2016;17(6):768-778.

127. Jones JA, Mato AR, Wierda WG, et al. Venetoclax for chronic lymphocytic leukaemia progressing after ibrutinib: an interim analysis of a multicentre, open-label, phase 2 trial. *Lancet Oncol.* 2019;19(1):65-75.

128. Flinn IW, Hillmen P, Montillo M, et al. The phase 3 DUO trial: duvelisib vs obinutuzumab in relapsed and refractory CLL/SLL. *Blood.* 2018;132(3):2446-2455.

129. Zelenetz AD, Barrientos JC, Brown JR, et al. Idelalisib or placebo in combination with bendamustine and rituximab in patients with relapsed or refractory chronic lymphocytic leukaemia with 17p deletion: a multicentre, open-label, phase 2 trial. *Lancet Oncol.* 2017;18(3):297-311.

130. Jones JA, Robak T, Brown JR, et al. Efficacy and safety of idelalisib in combination with obinutuzumab for previously treated chronic lymphocytic leukaemia: an open-label, randomised phase 3 trial. *Lancet Oncol.* 2013;14(Supplement_1):32.

131. Lampson BL, Tyekucheva S, Crombie JL, et al. Preliminary safety and efficacy results from a phase 2 study of acalabrutinib, venetoclax and obinutuzumab in patients with previously untreated chronic lymphocytic leukemia (CLL). *Blood.* 2019;134(4):e114-e126.

132. O’Brien S, Jones NA, Coutre SE, et al. Ibrutinib for patients with relapsed or refractory chronic lymphocytic leukaemia with 17p deletion (RESONATE-17): a phase 2, open-label, multicentre study. *Lancet Oncol.* 2016;17(10):1409-1418.
133. O’Brien S, Furman RR, Coutre S, et al. Single-agent ibrutinib in treatment-naïve and relapsed/refractory chronic lymphocytic leukemia: a 5-year experience. Blood. 2018;131(17):1910-1919.

134. Byrd JC, Hillmen P, O’Brien S, et al. Long-term follow-up of the RESONATE phase 3 trial of ibrutinib vs ofatumumab. Blood. 2019;133(19):2031-2042.

135. Huang X, Qiu L, Jin J, et al. Ibrutinib versus rituximab in relapsed or refractory chronic lymphocytic leukemia or small lymphocytic lymphoma: a randomized, open-label phase 3 study. Cancer Med. 2018;7(4):1043-1055.

136. Chanan-Khan A, Cramer P, Demirkan F, et al. Ibrutinib combined with bendamustine and rituximab compared with placebo, bendamustine, and rituximab for previously treated chronic lymphocytic leukaemia or small lymphocytic lymphoma (HELIOS): a randomised, double-blind, phase 3 study. Lancet Oncol. 2016;17(2):200-211.

137. Brown JR, Barrington JC, Barr PM, et al. The Bruton tyrosine kinase inhibitor ibrutinib with chemoimmunotherapy in patients with chronic lymphocytic leukemia. Blood. 2015;125(19):2915-2922.

138. Moreno C, Greil R, Demirkan F, et al. Ibrutinib plus obinutuzumab versus chlorambucil plus obinutuzumab in first-line treatment of chronic lymphocytic leukaemia (iLLUMINATE): a multicentre, randomised, open-label, phase 3 trial. Lancet Oncol. 2019;20(1):43-56.

139. Danilov AV, Herbaux C, Walter HS, et al. Phase Ib study of tirabrutinib in combination with idelalisib or entospletinib in previously treated chronic lymphocytic leukaemia. Clin Cancer Res. 2020;26(12):2810-2818.

How to cite this article: Skånland SS, Karlsen L, Taskén K. B cell signalling pathways—New targets for precision medicine in chronic lymphocytic leukaemia. Scand J Immunol. 2020;92:e12931. https://doi.org/10.1111/sji.12931