

# Mechanisms of resistance to venetoclax in hematologic malignancies

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;

D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online)

*Adv Clin Exp Med.* 2024;33(12):1421–1433

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## Funding sources

None declared

## Conflict of interest

Klaudia Zielonka: None declared

Krzysztof Jamrozik: Research support from AbbVie, Janssen; Consulting role for AbbVie, AstraZeneca, Janssen, Beigene.

Received on November 14, 2023

Reviewed on November 26, 2023

Accepted on January 13, 2024

Published online on March 1, 2024

## Abstract

Venetoclax, a BH3 mimetic, is a novel targeted anti-cancer drug with a unique mechanism of action leading to the execution of apoptosis through inhibition of the Bcl-2 protein. The development of venetoclax has revolutionized the treatment paradigm of several hematologic malignancies, including treatment-naïve and relapsed or refractory chronic lymphocytic leukemia (CLL) as well as acute myeloid leukemia (AML) in unfit patients. However, despite the high effectiveness of venetoclax in these diseases, some patients, as in the case with other targeted therapies, develop primary or secondary resistance to the drug. Various mechanisms contributing to the resistance to venetoclax have been elucidated, including selection of mutations in the BCL-2 binding groove which decrease affinity to venetoclax, or compensatory overexpression of anti-apoptotic proteins such as MCL-1. Moreover, alterations in cell metabolism and signaling pathways like MAPK or ERK activation have also been reported, suggesting the resistance to venetoclax is highly complex and involves multiple pathways. This review aimed to describe the mechanisms of resistance to venetoclax in AML, CLL, multiple myeloma, and other hematologic malignancies, as well as to propose a perspective to circumvent it.

**Key words:** acute myeloid leukemia, resistance, chronic lymphocytic leukemia, apoptosis, venetoclax

## Cite as

Zielonka K, Jamrozik K. Mechanisms of resistance to venetoclax in hematologic malignancies.

*Adv Clin Exp Med.* 2024;33(12):1421–1433.

doi:10.17219/acem/181145

## DOI

10.17219/acem/181145

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## Introduction

Venetoclax is a novel targeted anti-cancer drug with a unique mechanism of action involving the execution of apoptosis of malignant cells through the selective inhibition of the B-cell lymphoma-2 (BCL-2) protein.<sup>1</sup> The BCL-2 family proteins are a pivotal point in the intrinsic pathway of programmed cell death (apoptosis).<sup>2</sup> This heterogeneous group of proteins regulates apoptosis by promoting either the cell's survival or its death.

The BCL-2 family proteins contain specific regions of homology called B-cell 2 homology (BH) domains, including BH1, BH2, BH3, and BH4 domains, which implicate the BCL-2 protein's functions in cells.<sup>3</sup> According to the function and number of BH domains, BCL-2 family proteins may be subclassified as proapoptotic and anti-apoptotic proteins.<sup>3</sup> BH3-only proteins (BID, BIM, PUMA, Noxa, HRK, BIK, BME, and BAD) are proapoptotic proteins that share only a short BH3 region with other groups of the BCL-2 family proteins.<sup>3</sup> Proapoptotic BH3-only proteins may exhibit their proapoptotic function as activators by directly activating BAX or BAK, or as sensitizers by neutralizing the anti-apoptotic function of BCL-2 proteins.<sup>3</sup> BAX and BAK are proapoptotic effectors that have multiple BH domains. In contrast, BCL-2, B-cell lymphoma-extra-large (BCL-xL), mantle cell lymphoma-1 (MCL-1), A1, BCL-B, and BCL-w are anti-apoptotic proteins and contain 4 domains, BH1–BH4.<sup>3</sup> The BH1, BH2 and BH3 domains of anti-apoptotic proteins form a hydrophobic cleft that can interact with

the BH3 domain of proapoptotic proteins.<sup>3</sup> This interaction is an important regulatory mechanism of apoptosis (Fig. 1).

Various stimuli such as oncogenic stress, DNA damage or uncontrolled proliferation lead to the upregulation of proapoptotic BH3-only proteins, which contribute to the oligomerization of the effector proteins, BAX and BAK, in the mitochondrial outer membrane. Permeabilization of the mitochondrial membrane leads to leakage of cytochrome c, which binds to the adaptor molecule apoptotic protease activating factor 1 (APAF-1) in the cytosol. APAF-1 oligomerizes, forming an apoptosome that initiates the cascade of caspases. Finally, caspase proteases cleave dozens of proteins, resulting in rapid cell death.<sup>3,4</sup> In cancer cells, the balance between survival and cell death is disrupted. Resistance to apoptosis and persistent viability of malignant cells is one of the hallmarks of cancer.<sup>5</sup> Overexpression of BCL-2 was recognized in multiple hematologic malignancies, including chronic lymphocytic leukemia (CLL), follicular lymphoma (FL) and Waldenström macroglobulinemia.<sup>6–8</sup> Thus, BCL-2 was suggested to be a rational and potent target of novel therapies. Overexpression of anti-apoptotic BCL-2 proteins prevents malignant cells from undergoing apoptosis by blocking mitochondrial outer membrane permeabilization.<sup>3</sup> BH3 mimetics constitute a novel class of drugs that act as proapoptotic BH3 sensitizers. By binding to the BH3 domain groove of anti-apoptotic proteins such as BCL-2 or MCL-1, they enable the sequestration of BH3-only activator proteins, like BIM, and trigger apoptosis.<sup>9</sup>

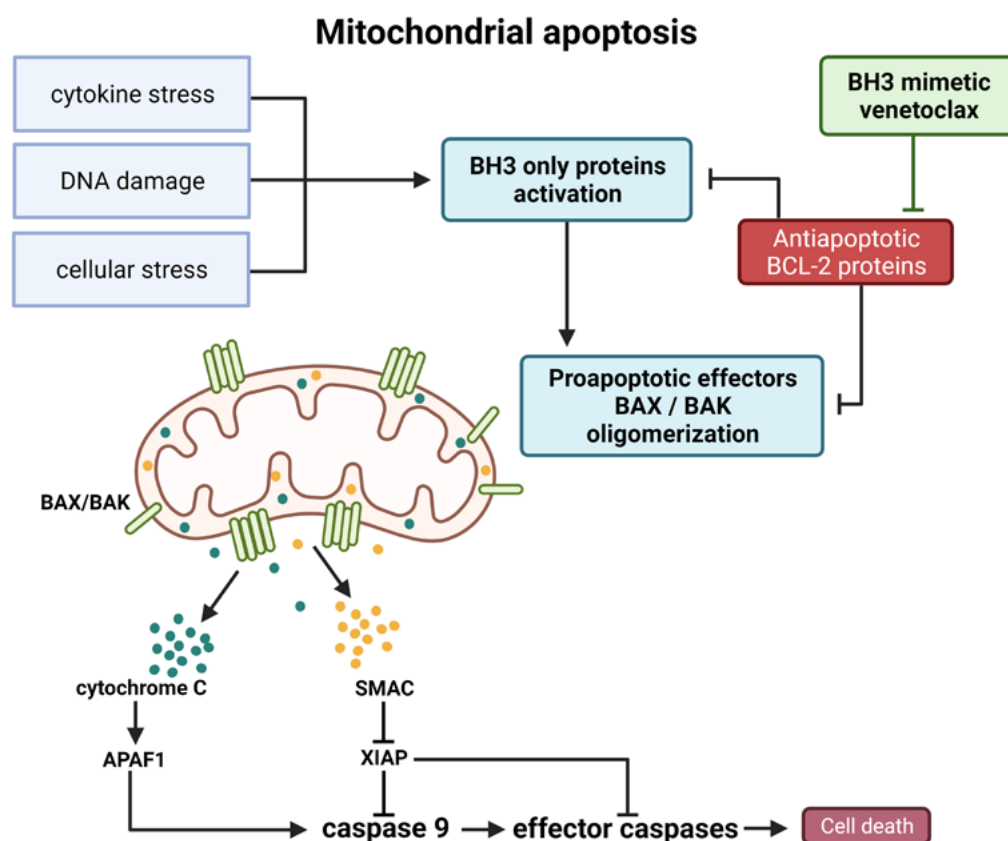


Fig. 1. Intrinsic apoptosis and venetoclax's mechanism of action. Created with BioRender.com

APAF1 – apoptotic peptidase activating factor 1; BCL-2 – B-cell lymphoma 2; BH3 – B-cell homology domain 3; XIAP – X-linked inhibitor of apoptosis protein.

The introduction of BH3 mimetics has enabled significant progress in the development of targeted therapies for several hematological tumors.<sup>9–13</sup> Despite initial difficulties with the first-in-class BH3 mimetic drug, navitoclax, which bound to both BCL-2 and MCL-1 and resulted in severe thrombocytopenia,<sup>10,11</sup> a highly selective BCL-2 inhibitor, venetoclax (ABT-199) has revolutionized current therapies in hematologic malignancies.<sup>9</sup> Based on the high efficacy shown in the MURANO trial investigating venetoclax in combination with anti-CD20 monoclonal antibody rituximab, the drug was approved for the treatment of relapsed or refractory (RR) CLL. The cohort treated with venetoclax had a 2-year rate of progression-free survival (PFS) of 84.9% (95% confidence interval (95% CI): 79.1–90.6) and an overall response rate of 92.3% (compared to 72.3% in the control bendamustine-rituximab treatment arm by a difference between the groups of 20.0 percentage points; 95% CI: 12.4–27.6).<sup>12</sup> In the VIALE-A study,<sup>13</sup> untreated AML patients were randomized to receive azacitidine with venetoclax or in monotherapy. Patients receiving venetoclax had a median overall survival (OS) of 14.7 months compared to 9.6 months in the control group (hazard ratio (HR) for death: 0.66; 95% CI: 0.52–0.85;  $p < 0.001$ ). In this group, complete remission (CR) was obtained in 36.7% of patients (compared to 17.0% in the control group;  $p < 0.001$ ).<sup>13</sup>

However, it has been noted that treatment with venetoclax does not lead to a cure for AML or CLL, and gradual resistance to the drug develops in most patients.<sup>13,14</sup> Pathways underlying the mechanism of intrinsic primary resistance of malignant cells to different anti-cancer therapies, including venetoclax, are complex and not well understood. When it comes to long-term exposure to venetoclax, the majority of patients would become refractory despite an initial response (secondary or acquired resistance).<sup>15</sup> Importantly, the main determinants of venetoclax resistance recognized in the literature are mutations in the BCL-2 binding groove and the upregulation of other anti-apoptotic proteins such as BCL-XL and MCL-1.<sup>16,17</sup> However, the mechanisms responsible for resistance to venetoclax are more complex and comprise changes in the metabolism of leukemic cells or the tumor microenvironment.<sup>18–21</sup> A better understanding of these mechanisms may allow pre-treatment identification of patients with a high probability of primary resistance to venetoclax or to develop venetoclax combinations preventing secondary resistance. Moreover, the prediction of sensitivity to venetoclax and risk stratification by assessment of potential risk factors such as the Gly101Val mutation may be crucial to address other tailored therapies for patients who do not respond to venetoclax.<sup>22,23</sup>

## Objectives

This review aims to summarize mechanisms of resistance to venetoclax in hematologic malignancies and propose possible ways to counteract them.

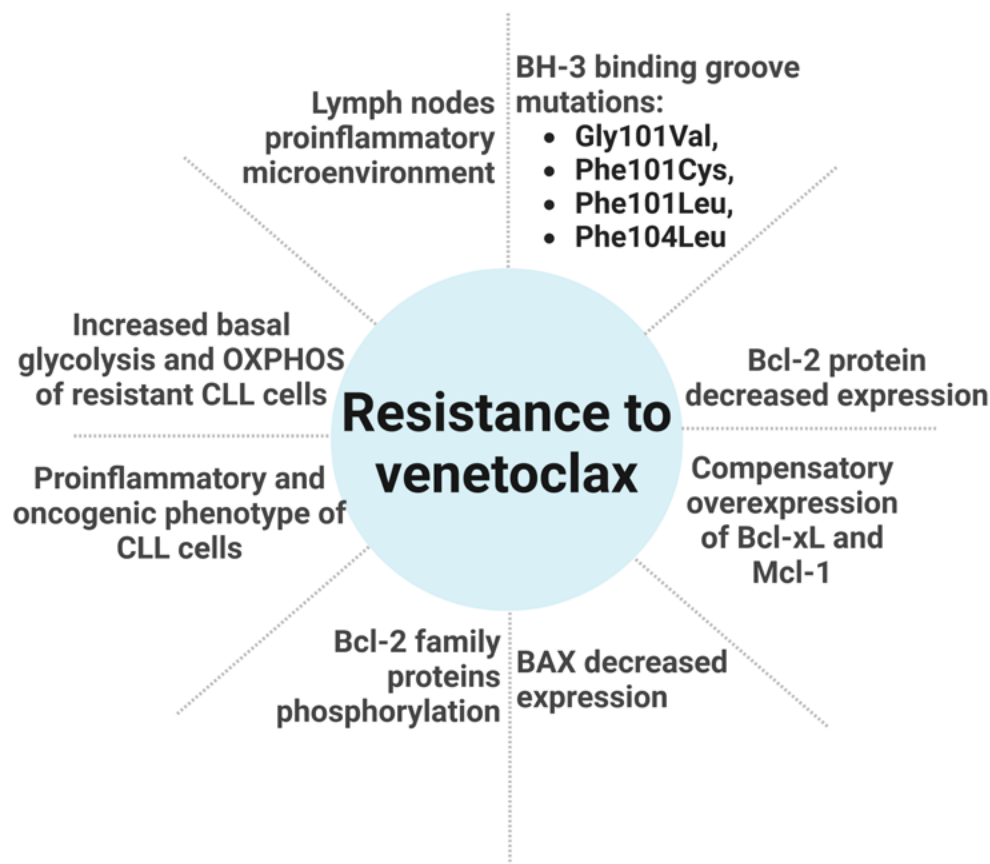
## Chronic lymphocytic leukemia/small lymphocytic lymphoma

Chronic lymphocytic leukemia is the most prevalent leukemia in the adult population in the Western world. It is characterized by the clonal expansion and accumulation of typically CD5-positive B-cells within the blood, bone marrow, lymph nodes, and spleen.<sup>24</sup> Long-lasting survival of malignant cells, which are highly dependent on BCL-2 and in the majority arrested in the G0/G1 cell cycle, is the key element in the pathophysiology of CLL.<sup>25,26</sup> In CLL, loss of miR-15a/16-1 results in overexpression of anti-apoptotic BCL-2 proteins and allows leukemic cells to escape apoptosis.<sup>8</sup> This entity remains incurable. However, significant progress in the prognosis of CLL has been achieved during the last decade due to the development of novel targeted therapies.<sup>24</sup> This included venetoclax, which showed high efficacy even in patients with poor risk due to genetic alterations, including *TP53* and *del(17p)*, or with comorbidities who did not qualify for the usual chemoimmunotherapy regimens.<sup>14</sup>

In CLL, venetoclax is administered in fixed-duration regimens when combined with anti-CD20 antibodies or Bruton tyrosine kinase (BTK) inhibitors or continuously in monotherapy. Despite the high effectiveness of the drug in general patient populations, a minority of patients experience primary or secondary resistance. Moreover, some patients may develop adverse events such as autoimmune cytopenias, which may result in venetoclax suspension or dose reduction and, thus, decreased drug efficacy, leading to CLL progression.<sup>27–30</sup>

Multiple studies have been conducted so far that identified a variety of mechanisms leading to venetoclax resistance in CLL (Fig. 2). One of the first reports concerning mechanisms of venetoclax resistance was performed by Tahir et al.<sup>4</sup> Analysis of venetoclax-resistant cell lines comprising activated B-cell-like (ABC) subtype diffuse large B-cell lymphoma (DLBCL) cell lines (HBL1 and U2932), 2 follicular/germinal center B-cell (GCB) lymphoma cell lines (OCI-Ly1 and SC-1), 2 mantle cell lymphoma (MCL) cell lines (HBL2 and Granta-519), as well as 1 leukemia line (RS4;11) revealed that there are multiple alterations in gene expression levels or in post-translational modifications that contribute to venetoclax resistance. This involved an increase in the anti-apoptotic proteins BCL-XL or MCL-1.<sup>4</sup> Interestingly, it was associated with a significant decrease in proapoptotic proteins such as BAX in the venetoclax-resistant leukemia cell line (RS4;11).<sup>4</sup> This suggests that the process is more complex and involves various pathways.<sup>4</sup>

Chronic lymphocytic leukemia is a genetically heterogeneous disease; thus, identification of secondary mutations driving resistance to venetoclax before clinical relapse may be practical to assess predictive factors. Interestingly, in a study performed by Herling et al.,<sup>31</sup> a group of 9 patients with either *del(17p)* or *TP53* mutations who clinically



**Fig. 2.** Mechanisms of resistance to venetoclax in chronic lymphocytic leukemia. Created with BioRender.com

Bcl-2 protein – B-cell lymphoma-2; Bcl-xL – B-cell lymphoma-extra large; BH-3 – B-cell 2 homology 3; CLL – chronic lymphocytic leukemia; Mcl-1 – mantle cell lymphoma-1; OXPHOS – oxidative phosphorylation.

progressed or relapsed after treatment with venetoclax (median time 15.4 months), the accumulation of genome alterations was revealed. By performing whole-exon sequencing (WES), the authors showed multiple recurrent mutations that were not seen before treatment commencement.<sup>31</sup> In the majority of samples, alterations in genes associated with oncogenesis, such as *CD274* (*PD-L1*), *NOTCH1*, *RBI*, *SF3B1*, and *TP53*, were detected. Interestingly, in 2 patients, missense mutations of B-cell translocation gene 1 (*BTG1*) emerged during venetoclax treatment.<sup>31</sup> This phenomenon was reported to drive lymphomagenesis in Bcl-2 overexpression models of aggressive lymphomas.<sup>32</sup> In a singular case, a *BRAF* mutation was also revealed.<sup>31</sup> Additionally, *BRAF* mutations were detected in 2 RR CLL patients in a similar study.<sup>33</sup> Supposedly, *BRAF* inhibitors may be used along with venetoclax to postpone the time to resistance.

Interestingly, a study performed in RR CLL/SLL patients treated with venetoclax as monotherapy (median time on therapy 20.1 months (range: 1.2–60.3)) revealed genetic abnormalities associated with refractoriness to venetoclax that were not reported before.<sup>33</sup> Samples of patients were collected prior to and after discontinuation of venetoclax due to an unacceptable toxicity or disease progression. Of the 43 patients, 15 acquired mutations in BCL-2 and progression of the disease was observed in 14 patients.<sup>33</sup> Multiple novel BCL-2 mutations were obtained, including the most common Asp103 (11 occasions) and Gly101

(8 occasions). Interestingly, WES and targeting sequencing revealed a significant increase in expression of other anti-apoptotic protein genes comprising MCL1, BCL-XL and BCL2A1 (BFL-1, false discovery rate (FDR) <0.2), which are known for apoptosis inhibition in the presence of venetoclax.<sup>33</sup> Furthermore, loss-of-function mutations in the *PMAIP1*-encoding NOXA regulatory protein were identified in patients who developed resistance to venetoclax monotherapy with a variant allele frequency (VAF) >20% in 3 of 4 cases.<sup>33</sup> Hypothetically, *PMAIP1* may be a key driver of venetoclax resistance.<sup>33</sup>

Mutations of genes encoding protein binding sites of drugs are well-known mechanisms of resistance to any drug. Similarly, BCL-2 binding groove mutations, which decreased affinity to venetoclax, were also observed. For example, CLL cells cultured to become refractory to venetoclax had alterations in the BH3 binding groove, such as Phe101Cys, Phe101Leu and Phe104Leu, which contributed to venetoclax-binding disruption of BCL-2.<sup>4,34</sup> One of the first mutations discovered clinically in RR CLL at disease progression was Gly101Val, which was detected after the median time of treatment with venetoclax 36 months (range; 6.5–73 months).<sup>17</sup> Substitution of glycine to valine at amino-acid-position 101 of the BCL-2 protein resulted in poor binding of venetoclax to the binding groove.<sup>17</sup> Recent studies have also reported the presence of Gly101Val mutations in patients who became refractory to venetoclax, supporting this hypothesis.<sup>22,23,35,36</sup> A study



performed outside clinical trials revealed that Gly101Val and/or Asp103Tyr mutations were present in 16.7% of patients treated with venetoclax as monotherapy or combined with rituximab.<sup>37</sup> Ninety percent of them further relapsed (median time of follow-up of 26 months (7–32 months)).<sup>37</sup> All of the changes were detected using standard-of-care droplet digital polymerase chain reaction (ddPCR) tests. Hypothetically, screening for Gly101Val and Asp103Tyr mutations may be implemented in daily practice to identify resistance before clinical progression.<sup>37</sup> Interestingly, Gly101Val was present after cessation of venetoclax treatment for over 6 months<sup>17</sup> and in 3 out of 7 patients with CLL who failed to clear minimal residual disease (MRD) after 1 year of treatment with venetoclax.<sup>35</sup> Consequently, further studies are essential to evaluate Gly101Val in the context of re-treatment with venetoclax. Furthermore, in 10 out of 11 RR CLL patients resistant to venetoclax with the Gly101Val mutation, additional mutations were harbored.<sup>22</sup> The most common was the Asp103 codon with a substitution of amino acids (6/11 patients); however, Val156Asp and in-frame insertion (Arg107\_Arg110dup) were also reported.<sup>22</sup> Therefore, many BCL-2 gene protein mutations may develop during venetoclax administration.<sup>22</sup>

As presented, there is no single mutation that drives resistance to venetoclax in CLL cells. Interestingly, this issue was investigated by Thomalla et al.<sup>38</sup> A study performed on high-risk RR CLL patients revealed methylation of the PUMA promoter, which resulted in the downregulation of PUMA.<sup>38</sup> Importantly, an increase in DNA methylation from 10% to 30% was observed clinically in 5 out of 6 patients who acquired resistance toward venetoclax.<sup>38</sup>

In some cases, despite refractoriness to venetoclax, BCL-2 mutations are undetectable, and a novel mechanism was investigated comprising the overproduction of anti-apoptotic proteins such as BCL-XL or MCL-1.<sup>17,38</sup> This was a compensatory way to overcome BCL-2 blockade favoring CLL survival, and was confirmed in vitro and further elucidated in clinical practice.<sup>4,17</sup> Interestingly, much attention has been drawn to the mechanisms of the overproduction of MCL-1 and BCL-XL. A few heterogeneous metabolic pathways were identified which contribute to the process.

Interestingly, BCL-2 family proteins may undergo post-translational modifications, including phosphorylation, which alter their activity and may influence mitochondrial apoptosis.<sup>39</sup> Importantly, in the study performed by Guieze et al.,<sup>37</sup> patients refractory to venetoclax had detected growing subclones of CLL cells with an amplified region on chromosome 1q23.<sup>40</sup> This region encodes MCL-1 protein and PRKAB2 with the regulatory subunit of AMP-activated protein kinase (AMPK) genes.<sup>40</sup> Further studies performed on the samples revealed that MCL-1 and AMPK were expressed at higher levels. Overexpression of AMPK correlated with relapse ( $p \leq 0.0062$ ), and a high-pretreatment MCL-1 expression (>10% of positive cells) was associated with shorter PFS ( $p = 0.017$ ).<sup>40</sup> However, this was

studied in a small number of patients and requires a larger cohort to confirm. Importantly, Chong et al.<sup>41</sup> also analyzed the impact of phosphorylation in regard to intrinsic or acquired resistance to venetoclax in lymphoid malignancies. They demonstrated that hyperphosphorylation of BCL-2 family proteins resulted in MCL-1 dependence instead of BCL-2 dependence in cells. Moreover, hyperphosphorylation of BCL-2 led to a stronger connection between BCL-2 and BAX, whose separation is crucial to initiate apoptosis.<sup>41</sup> Chronic lymphocytic leukemia samples at the time of progression during venetoclax treatment had increased hyperphosphorylated BCL-2, MCL-1 and BAD proteins compared to pre-treatment levels.<sup>41</sup> In this process, a few kinases were involved, comprising AKT, extracellular-signal-regulated kinase (ERK) and GSK3b kinases, suggesting a distinct pattern of kinase activity in resistance to venetoclax.<sup>41</sup> Importantly, the use of fingolimod, a protein phosphatase 2A-activating drug, reversed the resistance by switching the dependence from MCL-1 to BCL-2.<sup>41</sup>

Increased expression of receptor tyrosine kinase-like orphan receptor (ROR1) was detected in multiple hematologic malignancies, including CLL. Receptor tyrosine kinase-like orphan receptor activates the non-canonical WNT signaling pathway and, by activating cell survival signaling events, leads to oncogenesis.<sup>42</sup> Moreover, ROR1 expression was elucidated as a prognostic factor in CLL.<sup>43</sup> Receptor tyrosine kinase-like orphan receptor was investigated in terms of resistance to venetoclax. Ghia et al.<sup>35</sup> reported a significant rise in ROR1 expression in CLL patients after 1 year of treatment with venetoclax compared to values prior to treatment. In their study, samples of patients with CLL who did not eradicate MRD during continuous treatment with venetoclax were compared before and 1 year after therapy.<sup>35</sup> A significant correlation between the high level of expression of ROR1 prior to treatment and further failure to eradicate MRD was noted ( $p = 0.00006$ ).<sup>35</sup> Importantly, the levels of ROR1 were higher after 1 year of the therapy. Furthermore, ROR1 induced the WNT pathway and, consequently, *BCL2L1* gene expression encoding the BCL-XL protein.<sup>35</sup> Interestingly, WNT5a is a ligand of ROR1, which is present in higher levels in CLL patients, contrary to the general population.<sup>43</sup> WNT5a may activate the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway in CLL cells and, therefore, upregulate BCL-XL and MCL-1.<sup>43</sup>

Moreover, MCL-1 overproduction as a way of induction of resistance to venetoclax was associated with higher levels of NF- $\kappa$ B.<sup>44</sup> The enhanced activity of NF- $\kappa$ B was present in CLL cells after venetoclax therapy.<sup>44</sup> Supposedly, monitoring of NF- $\kappa$ B may serve as a biomarker indicative of relapse after venetoclax and the need for venetoclax re-administration or initiation of other drugs.<sup>44</sup> Moreover, elevated levels of protein kinase RNA-like endoplasmic reticulum kinase (pERK) were also observed in CLL cells resistant to venetoclax.<sup>45</sup> Other studies demonstrated that overexpression of MCL1 was a result of altered

phospho-p38 signaling.<sup>38</sup> Interestingly, WES in patients who relapsed to venetoclax revealed acquired loss of (8p), which affects the *MCL-1* gene.<sup>45</sup>

Cytogenetic abnormalities of CLL are important prognostic factors, and the trisomy 12 mutation chromosome, which often harbors Notch1, was linked to more rapid progression, bulky lymphadenopathy and increased risk of Richter transformation.<sup>46,47</sup> The CLL cells with trisomy 12 are characterized by reduced interferon regulatory factor 4 (IRF4) compared to other cytogenetic groups.<sup>48</sup> Importantly, a study performed by Fiorcari et al.<sup>49</sup> presented decreased sensitivity to venetoclax in CLL cells with trisomy 12.<sup>49</sup> The possible mechanism involved a reduction in the expression of IRF4. This significantly affects the functions of immune cells associated with the increased activity of the Notch2 protein, a part of the Notch signaling pathway.<sup>49</sup> Moreover, Notch-signaling exerts pro-survival effects on CLL cells due to the enhancement of MCL-1.<sup>49</sup>

Under normal “therapeutic” conditions, venetoclax affects the metabolism of CLL cells. Chronic lymphocytic leukemia cells resistant to venetoclax have higher basal levels of glycolysis.<sup>40</sup> Moreover, the loss of PUMA resulted in higher oxidative phosphorylation (OXPHOS) and adenosine triphosphate (ATP) production in mouse models.<sup>38</sup> Chronic lymphocytic leukemia cells in lymph nodes are more resistant to venetoclax and have increased expression of genes linked to glycolysis, OXPHOS, the tricarboxylic acid cycle, and amino acid metabolism.<sup>50</sup> The main substrate for the tricarboxylic acid cycle in lymph nodes for CLL cells was glutamine.<sup>50</sup> The BCR stimulation induced resistance to venetoclax, and the refractoriness was attenuated by inhibition of glutamine uptake.<sup>50</sup> Hypothetically, changes in metabolic pathways and blockade of glutamine import may decrease the resistance to venetoclax in lymph nodes.

The CLL microenvironment, which serves as an important site of activation and proliferation of CLL cells, was proven to drive resistance to venetoclax.<sup>25</sup> Tumor microenvironments, especially in lymph nodes, are suggested to maintain higher expression of Notch2 and MCL-1, and, therefore, may additionally contribute to venetoclax resistance.<sup>49</sup> Additionally, a higher concentration of anti-apoptotic BCL-XL in the lymph node environment was also present. This may explain the low efficacy of venetoclax in clearing nodal disease.<sup>51</sup> Interestingly, ibrutinib, a first-generation BTK inhibitor that mobilizes CLL cells from lymph nodes and other lymphoid niches, enhanced the cytotoxic activity of venetoclax in preclinical models.<sup>52</sup> Moreover, the synergistic feature of combining venetoclax and ibrutinib was assessed clinically in the CAPTIVATE study, which presented CR rates of 55% (95% CI: 48–63) in the overall population and of 31% (95% CI: 18–44) in patients with bulky disease >5 cm.<sup>53</sup> Nevertheless, cases of resistance to both venetoclax and ibrutinib were demonstrated in the literature with the presence of BCL-2, BTK and phospholipase C $\gamma$ 2 (PLCG2) mutations. Interestingly, aside from the well-described Gly101Val mutation, novel

genetic BCL-2 abnormalities were detected comprising the variant Ala113Gly (VAF, 31.7%) and in-frame mutations resulting in p.Arg107\_Arg110dup detected in 3 patients (VAFs 0.4% and 5%).<sup>54</sup>

A study performed by Thijssen et al.<sup>55</sup> highlighted that pro-survival BCL-2 family members, which were aforementioned as leading to resistance to venetoclax, were upregulated by environmental signals. Stimulation of CLL cells with CD40 ligand in vitro, which mimicked activated T-cell-mediated signaling, contributed to an increase in BCL-2 family members: MCL-1, BCL-XL and Bfl-1. This resulted in a complete resistance to venetoclax.<sup>55</sup> Interestingly, the resistance was altered not only by rituximab, an anti-CD20 antibody, but also by c-Abl tyrosine kinase inhibitors imatinib and dasatinib.<sup>55</sup> Furthermore, CD40 stimulation led to the activation of the non-canonical NF- $\kappa$ B pathway in both normal B-cells and CLL and correlated with the higher expression of BCL-XL.<sup>21</sup> Nevertheless, an association of MCL-1 and NF- $\kappa$ B pathway remains contradictory in the literature. Upregulation of MCL-1 induced by CD40 was suggested to be dependent on PI3K-AKT-mTOR, not on NF- $\kappa$ B activation.<sup>56</sup>

Interestingly, overexpression of anti-apoptotic proteins, such as BCL-2, BCL-XL and MCL-1, associated with the lymph node environment, was assessed in the study performed by Jayappa et al.<sup>20</sup> Chronic lymphocytic leukemia cells from the peripheral blood and lymph nodes of both naïve and refractory to multiple drug treatment patients were compared. Chronic lymphocytic leukemia cells recently activated in lymph nodes, which were characterized with the same phenotype as resident cells (CD69<sup>+</sup>/CXCR4<sup>Low</sup>), presented increased expression of anti-apoptotic proteins comprising of MCL-1, BCL-XL and BCL-2 induced by the NF- $\kappa$ B pathway.<sup>20</sup> Moreover, this drug-resistant phenotype was present in the treatment-naïve patients' CLL cells.<sup>20</sup> Furthermore, venetoclax-resistant CLL cells expressed the phenotype CD69<sup>+</sup> Ki67 CXCR4<sup>+</sup>, which was the same pattern as present in lymph nodes resident cells.<sup>20</sup> Importantly, previous studies substantiated the belief that the CD69<sup>+</sup> CD38<sup>+</sup> CD49d<sup>+</sup> phenotype was associated with refractoriness to venetoclax and demonstrated its linkage with unfavorable outcomes.<sup>20,57</sup> This indicates the possibility of intrinsic refractoriness to venetoclax.<sup>20</sup> Hypothetically, this may be a reason for the low OS in this group of patients.<sup>20</sup> Thus, the CD69<sup>+</sup> CD38<sup>+</sup> CD49d<sup>+</sup> phenotypes may be used in clinical practice to find patients who may relapse on therapy with venetoclax. Nevertheless, further studies are essential.<sup>20</sup> Moreover, CLL cells from lymph nodes expressed higher Ki-67.<sup>58</sup> Interestingly, an assessment of RR CLL cells revealed that survival in venetoclax positively correlated with Ki-67 expression, suggesting that proliferative potential is linked to resistance to venetoclax.<sup>59</sup> Importantly, the suggestion was made that resistance to venetoclax selects the most activated CLL cells with the highest proliferative capacity.<sup>59</sup>

Nurse-like cells (NLCs) are significant players in the leukemic environment. They produce chemokines such as CXCL12 and CXCL13 as well as secrete proteins, such as B-cell activating factor (BAFF) and proliferation-inducing ligand (APRIL), therefore being responsible for CLL cell survival.<sup>60</sup> WNT5a was presented in higher levels in CLL patients contrary to general populations.<sup>61</sup> However, a minority of CLL cells produce WNT5a, suggesting other sources of WNT5a production.<sup>61</sup> A study performed by Guo et al.<sup>62</sup> revealed that NLCs may serve as a source of WNT5a in patients with CLL. Furthermore, WNT5a induced survival and migration of CLL cells. Interestingly, further research revealed that CLL cells cultured with both NLCs and venetoclax were less susceptible to venetoclax compared to a group devoid of NLCs.<sup>62</sup> The postulated mechanism of NLCs-induced resistance is the activation of the NF- $\kappa$ B pathway in CLL cells and, subsequently, upregulation of MCL1 and BCL-XL.<sup>62</sup> Importantly, the upregulation of immune pathways, which are linked to resistance to venetoclax, was described in a recently published study with the 5-year results of the MURANO clinical trial.<sup>63</sup> The transcriptomic profile was evaluated prior to re-treatment and after therapy. Differences in transcriptomic profiles according to MRD status were revealed.<sup>63</sup> In non-responders, patients with detectable MRD and patients who relapsed after time, the overexpression of the *ABCB1* gene was associated with resistance and inflammatory genes, such as an inflammatory response, IFN $\gamma$  response and IL2/STAT5, were detected.<sup>63</sup>

Multiple mechanisms leading to resistance to venetoclax have been described; however, more studies are essential to improve the outcomes of refractory to venetoclax patients with CLL.

## Acute myeloid leukemia

Acute myeloid leukemia (AML) is the most common acute leukemia in the elderly, as the median age at diagnosis is 70 years.<sup>64</sup> Unfortunately, due to comorbidities and advanced age, this subset of patients usually does not qualify for intensive induction chemotherapy, which is the standard of care in younger AML patients.<sup>64</sup> Therefore, venetoclax is a breakthrough in the therapy for patients ineligible for chemotherapy with a dismal prognosis. In clinical trials, venetoclax, especially combined with hypomethylating agents, such as azacitidine or low-dose cytarabine, presented a high overall response rate with an acceptable toxicity profile.<sup>13,65</sup>

Despite promising results in clinical trials, 24.7% of patients do not respond to the upfront treatment with venetoclax.<sup>66</sup> A retrospective analysis performed by Stevens et al.<sup>18</sup> outlined previous therapies in RR AML correlated with resistance and were predictive of response to venetoclax ( $p = 0.0036$ ). Niu et al.<sup>67</sup> conducted a study on AML cell lines with intrinsic resistance to venetoclax in which

treatment with venetoclax resulted in increased stability of the MCL-1 protein. Moreover, the increased stability of the MCL-1 protein contributed to the sequestration of BIM, which is an apoptosis-regulating protein.<sup>67</sup> One of the proposed theories that explain this event involves the displacement of BIM from the BCL-2 protein by venetoclax and, consequently, its sequestration by MCL-1.<sup>67</sup> Furthermore, the enhanced stability of MCL-1 may be an effect of ubiquitin ligase displacement or deubiquitinases; however, this requires further research to assess the mechanism.<sup>67</sup> A retrospective analysis performed by Zhang et al.<sup>68</sup> revealed that in the cohort of patients with AML refractory to venetoclax, none of the BCL-2 mutations were detected. Hypothetically, this may be due to the short time of exposure to venetoclax in this group (median time of exposure: 5 months (3–9 months)).<sup>68</sup>

Indeed, clonal mutations such as *FLT3-ITD*, one of the most common genetic alterations, or the newly emerged *TP53* mutation, were reported to be hypothetically associated with resistance to venetoclax.<sup>68</sup> Interestingly, this becomes coherent with other studies. Both *FLT3-ITD* and *PTPN11* mutations, which are associated with a substantially poorer prognosis in AML, were recognized before treatment in patients who did not respond to venetoclax therapy.<sup>66,69–71</sup> Supposedly, this may be an intrinsic mechanism of resistance to venetoclax caused by the enhanced expression of BCL-XL and MCL-1.<sup>66</sup> Studies performed in vitro presented that the FLT3-ITD inhibitor, quizartinib, together with venetoclax yielded durable remissions.<sup>69</sup> Nevertheless, further studies performed on larger cohorts are essential to evaluate the combined therapy of venetoclax and FLT3 inhibitors in clinical practice to overcome primary resistance.

Furthermore, sequencing of RNA derived from AML patients revealed additional genetic mutations associated with the immune system and inflammation-related responses, which were considered to be associated with primary resistance to venetoclax.<sup>72</sup> A strong correlation with resistance to venetoclax was seen with the overexpression of *s100* family genes: *s100a6*, *s100a8* and *s100a9* (false discover rate <0.05), suggesting *s100a8* and *s100a9* genes as potential predictors of venetoclax resistance ( $p < 0.05$ ).<sup>72</sup> Moreover, mutations of genes associated with the spliceosome *sf3b1* were correlated with lower response rates to venetoclax-based therapy and a lower OS (HR: 2.5; 95% CI: 1.1–5.65;  $p = 0.02$ ).<sup>65</sup> Prior investigations have reported the detection of the *SF3B1* mutation in RR CLL patients resistant to venetoclax.<sup>31</sup> Interestingly, well-known mutations in *IDH1/2* or *NPM1*, which are both associated with a good prognosis in AML, were detected in a cohort of patients treated with venetoclax and correlated with favorable responses.<sup>65,66,73</sup>

Over the decades, the French-American-British (FAB) classification was used to classify AML, but nowadays, it does not have any clinical significance. This classification distinguishes blasts blocked at various differential stages



according to their morphologic similarity to hematopoietic cells.<sup>74</sup> Interestingly, in a study performed by Pei et al.,<sup>75</sup> 62% of patients with mature AML cells of the monocytic subtype FAB-M5 were refractory to venetoclax in combination with azacitidine.<sup>75</sup> This may be due to the loss of BCL-2 during the development of M5 cells. Moreover, a monocytic subtype of AML has an increased level of MCL1, with a lower BCL2/MCL1 ratio, as well as a higher expression of BCL2A1, BCL2L11 (BIM), BID, and JAK2.<sup>75,76</sup>

Interestingly, primitive AML displays a more prevalent regulation of OXPHOS through BCL-2.<sup>75</sup> Significantly, FAB-M5 was a predictor of response to venetoclax ( $p = 0.0066$ ) with a median OS of 89 days compared to the OS of 518 days for non-FAB-M5 patients ( $p = 0.0039$ ).<sup>75</sup> Moreover, further studies demonstrate the importance of JAK/STAT and/or MAPK pathways in blasts that are resistant to venetoclax.<sup>76</sup> The use of JAK/STAT and/or MAPK inhibitors, ruxolitinib or trametinib, respectively, may overcome resistance to venetoclax.<sup>76</sup>

Leukemic stem cells (LSCs) play a central role in the pathogenesis of AML.<sup>77</sup> Interestingly, LSCs have an altered baseline energy metabolism and cannot use basic sources such as glucose or fatty acids.<sup>78</sup> Thus, the metabolism of amino acids is an essential way to maintain OXPHOS and is the most important mechanism of energy to maintain the viability of LSCs.<sup>78</sup> Moreover, OXPHOS depends on BCL-2.<sup>79</sup> Inhibition of OXPHOS emerged as a mechanism of action for venetoclax.<sup>79</sup> The combination of venetoclax with azacitidine affects the metabolism of LSCs by disrupting the tricarboxylic acid cycle, decreasing the catabolism of amino acids, and consequently decreasing OXPHOS.<sup>19,80</sup> However, RR LSCs are metabolically distinct from de novo LSCs.<sup>19</sup> Contrary to de novo LSCs, RR LSCs differ by compensatory upregulation of additional metabolic pathways that exploit fatty acids. Subsequently, this may lead to resistance to venetoclax with azacitidine.<sup>19</sup> Interestingly, RR LSCs had enhanced nicotinamide metabolism and consequently increased metabolism of nicotinamide adenine dinucleotide (NAD<sup>+</sup>), which is a crucial component in energy metabolism pathways. Increased energy metabolism is one of the mechanisms associated with resistance to venetoclax in RR AML and may explain the poor response to venetoclax in AML RR patients.<sup>78</sup>

Not only is the amino acid metabolism altered in refractory to venetoclax AML cells – fatty acids catabolism is also altered.<sup>18</sup> Additionally, resistant to venetoclax AML cell lines relied on glycolysis through upregulation of the PI3K/AKT pathway.<sup>81</sup> Interestingly, the *TP53* mutation, which correlates with a worse prognosis in AML, resulted in the upregulation of fatty acids in AML cell lines, which drives resistance to venetoclax.<sup>66,82</sup> Additionally, the upregulation of fatty acids was a result of MCL-1 overexpression through the RAS/MAPK pathway.<sup>18</sup> Furthermore, targeting fatty acid metabolism may be a potent way to circumvent resistance to venetoclax.<sup>18</sup>

Aside from alteration in BCL-2 or MCL-1 expression, a mutation in proapoptotic BAX was also reported as carrying resistance to venetoclax in AML.<sup>83</sup> Seventeen percent of patients who relapsed after venetoclax were carrying BAX variants, which were not reported before in patients with AML treated with chemoimmunotherapy.<sup>83</sup> The mutations of BAX, such as frameshift abnormalities or missense variants, disrupted BAX protein expression. This resulted in a deficiency of the proapoptotic function of BAX and was associated with resistance to venetoclax.<sup>83</sup> Moreover, preclinical studies revealed that the upregulation of MCL-1 is due to RAS/MAPK pathway activation.<sup>84</sup> Coherently, clinical data reports that patients with AML refractory to venetoclax harbor a *PTPN11* mutation which is one of the components of the RAS/MAPK signaling pathway.<sup>69</sup> Interestingly, a *PTPN11* mutation was strongly associated with FAB-M5,<sup>85</sup> which was mentioned as expressing lower levels of BCL-2 and being more resistant to venetoclax.<sup>75</sup> This finding was supported by another study performed by Pollea et al.<sup>80</sup> in which the presence of the *PTPN11* mutation along with other RAS pathway components was a predictor of shorter responses ( $p = 0.0019$ ) to venetoclax.<sup>80</sup>

## Multiple myeloma

Multiple myeloma (MM) is a plasma cell dyscrasia in which cytogenetic heterogeneity dictates prognosis. Of all the translocations in MM, t(11;14) is the most common and is regarded as a standard-risk cytogenetic aberration.<sup>86</sup> Multiple myeloma cells with t(11;14) have higher expression of the anti-apoptotic protein BCL-2 and lower expression of MCL-1 compared to normal MM cells. Higher expression of BCL-2 was demonstrated in MM cell lines, which was associated with resistance to dexamethasone.<sup>87</sup> Moreover, in RR MM with t(11;14) treated with venetoclax in combination or as monotherapy, favorable outcomes were observed.<sup>88</sup> Supporting studies presented initial promising results of the treatment of RR MM harboring t(11;14) with venetoclax combined with carfilzomib and dexamethasone.<sup>89</sup> Additionally, a combination of venetoclax with bortezomib was also presented.<sup>90</sup> The newly developed targeted therapies of BCL-2 inhibitors identify t(11;14) as the first predictive marker in MM.<sup>91</sup> Nevertheless, due to the increased risk of fatal infections and, therefore increased overall mortality,<sup>90</sup> venetoclax was not approved in the therapy of MM by the U.S. Food and Drug Administration (FDA).<sup>92</sup> Interestingly, a study performed by Leblay et al.<sup>93</sup> revealed that MM cells with t(11;14) have upregulation of B cell markers such as PTPRC (CD45), PIK3AP1, MS4A1 (CD20), CD79A, CCR7, IRF8, CIITA, CXCR4, BEND5, and VPREB3, whereas MM cells without t(11;14) have a “plasma cell” phenotype. Loss of the B-cell phenotype and selection of the plasma cell phenotype were associated with acquiring resistance to venetoclax.<sup>93</sup> Moreover, the resistant MM samples had decreased



expression of the regulatory protein NOXA, which was expressed in sensitive t(11;14) patients.<sup>93</sup> The loss of function mutation in NOXA was also observed in CLL resistant to venetoclax.<sup>33</sup>

Other possible mechanisms of resistance were studied by Chakraborty et al.<sup>94</sup> In MM cell lines resistant to venetoclax, a significant increase in the anti-apoptotic proteins MCL1 and BCL-XL was observed. Moreover, resistant cells had significantly upregulated PKA-ERK-CREB pathways and downregulated apoptotic genes compared to parental cells.<sup>94</sup> As mentioned above, CLL is characterized by overexpression of BCL-2. Therefore, CLL cells are very sensitive to highly selective BCL-2 inhibitors such as venetoclax.<sup>25</sup> In comparison to MM, the expression of anti-apoptotic proteins is heterogeneous, and MM is dependent on MCL-1 or BCL-2 or co-dependent on either BCL-2 MCL-1 or BCL-XL/MCL-1.<sup>95</sup> However, the majority of patients are MCL-1-dependent, and therefore, in this subset of patients, the MCL-1 inhibitor S63845 would be more efficacious. The use of venetoclax should be limited only to the minority of patients who are dependent on BCL-2.<sup>96</sup>

The bone marrow microenvironment plays a significant role in the pathogenesis of MM.<sup>97</sup> For example, mesenchymal stromal cells interact with MM cells through cell-cell adhesion, soluble factors and extracellular vesicles, leading to dysregulation of key metabolic pathways in MM cells and ultimately to drug resistance.<sup>98</sup> Interestingly, preclinical studies performed by Algarin et al.<sup>99</sup> demonstrated that resistance to venetoclax in MM was an effect of the enhanced interactions of MCL-1 and BIM by mesenchymal stromal cells. Moreover, this was avoided through combined use of venetoclax with an MCL-1 inhibitor (S63845), suggesting the possible role of double blockade of anti-apoptotic proteins to avoid resistance in MM.<sup>99</sup> The important role of the bone marrow microenvironment in MM in resistance to drugs was demonstrated in another study performed by Gupta et al.<sup>96</sup> They revealed that in the bone marrow microenvironment, interleukin (IL)-6 signaling and the Ras/MAPK pathway led to greater dependence on MCL1 rather than BCL-2 by MM cells.<sup>96</sup> This implies another possible reason for resistance to venetoclax in MM.

## Other lymphoproliferative disorders

Due to its high efficacy in CLL and AML, venetoclax was studied in vitro to evaluate its effects on other hematologic malignancies. Follicular lymphoma is characterized by t(14;18), which juxtaposes the *BCL-2* gene to the immunoglobulin heavy chain gene promoter region, resulting in overexpression of BCL-2 and making venetoclax a rational target.<sup>6</sup> Nevertheless, venetoclax in FL was not as effective as it was reported in CLL.<sup>100</sup> Moreover, data regarding the mechanisms of resistance to venetoclax in FL remain scarce. Interestingly, a single case with the novel mutation *Phe104Ile* was reported in a sample

of patients who developed resistance to venetoclax.<sup>101</sup> The mutation contributed to decreased affinity of the binding site of the BCL-2 protein to venetoclax and resistance to venetoclax in FL.<sup>101</sup> The mutations in the binding site of the BCL-2 are similar to those observed in the case of *Gly101Val* mutations in resistant CLL.<sup>22,101</sup> Moreover, preclinical studies suggested that resistance to venetoclax in FL may be due to the activation of ERK1/2 and AKT pathways, resulting in lower levels of BIM.<sup>102</sup> Nevertheless, further studies on this matter are essential to elucidate the cause of venetoclax failure in FL.

Resistance of MCL to venetoclax is linked to non-BCL-2 genes.<sup>103</sup> The genetic characteristics of patients' refractory to venetoclax were proposed and did not encompass mutations in *BCL-2* genes. Mutations that were suggested to be responsible for resistance to venetoclax comprised of *TP53*, *SMARCA4*, *CELSR3*, *CCND1*, and *KMT2D*.<sup>103</sup> However, due to the small number of patients, the data are limited, and study outcomes should be taken with caution. Similar to CLL, CD40 stimulation in lymph nodes resulted in the activation of the NF- $\kappa$ B pathway, a rise in BCL-XL expression, and, consequently, resistance of MCL cells to venetoclax.<sup>104</sup>

Considering the overexpression of BCL-2 in hairy cell leukemia (HCL), venetoclax was studied in vitro to analyze its efficacy toward HCL cells.<sup>105</sup> Venetoclax leads to apoptosis of HCL cells. The same study indicated the role of microenvironmental signals in the resistance to venetoclax in HCL.<sup>105</sup> Moreover, similarly to other presented studies, resistance to venetoclax in DLBCL was also demonstrated to be mediated by an increase in the BCL-XL protein.<sup>106</sup> Venetoclax may be a potent drug in other lymphoid malignancies such as DLBCL or HCL, but further studies, particularly clinical trials, are needed.

## Future perspectives

Until now, resistance to venetoclax in hematological cancers has been considered an inevitable event involving multiple processes. The academic community is extensively exploring possible ways to overcome this problem. In line with this, identification of sensitivity biomarkers prior to starting venetoclax may be theoretically useful for the individualization of treatment with BH3 mimetics in clinical practice. One such biomarker may be the presence of an IDH1/2 mutation in AML, which was correlated with better response to venetoclax and was even a predictor for longer remission duration ( $p = 0.042$ ).<sup>69,73,80</sup> Additionally, the identification of biomarkers for resistance during therapy with venetoclax, such as NF- $\kappa$ B in CLL or CD14 and/or CLEC7A in AML, may be a practical tool to elucidate the subset of patients who are refractory to venetoclax prior to clinical signs.<sup>44,107</sup>

An additional way to overcome resistance to venetoclax may be the administration of venetoclax in combination with active drugs having different mechanisms of action,

such as combination therapy with the BTK inhibitor, ibrutinib, for CLL.<sup>104</sup> In the treatment of CLL following resistance to venetoclax, administration of a BTK inhibitor may be recommended.<sup>108</sup> Interestingly, the blockade of the JAK1/2 pathway by ruxolitinib may also overcome resistance to venetoclax.<sup>109</sup> Moreover, pairing with other BH3 mimetics, such as MCL-1 inhibitors, or alvocidib, a cyclin-dependent kinase 9 (CDK9) inhibitor that down-regulates MCL-1, may also be a possible way to counteract resistance.<sup>110–112</sup> Other combinations presented in vitro are comprised of high activity of SYK inhibition by entospletinib, as well as the phosphoinositide 3-kinase delta (PI3K $\delta$ ) inhibitor, idelalisib, in refractory to venetoclax CLL cells.<sup>59</sup> Eventually, novel BCL-2 inhibitors such as lisafoclax may also be administered in patients with a secondary resistance to venetoclax.<sup>113</sup> Finally, cell lines resistant to venetoclax were sensitive to extrinsic apoptosis.<sup>38</sup> Drugs that could influence tumor necrosis factor alpha (TNF- $\alpha$ ) or TNF-related apoptosis-inducing ligand (TRAIL) that modify external apoptosis may potentially be used in case of resistance to venetoclax, independently of intrinsic apoptosis.<sup>38</sup> More options to counteract resistance to venetoclax are suggested in the literature; nevertheless, their description is beyond the scope of this paper.

## Limitations

This study may have several limitations, which are typical of literature review methodology. The review comprises only papers which were published in English. Therefore, the data may be incomplete. Moreover, due to the novelty of the topic, the mechanisms of resistance to venetoclax are now being studied extensively, and original papers are being published frequently. Therefore, it is not possible to review all of the newly published research.

## Conclusions

Resistance to venetoclax in hematologic malignancies is a complex process that comprises plenty of mechanisms, including alterations in cellular metabolism and genetic mutations. Novel agents in the class of BH3 mimetics may be more effective in patients who developed resistance to venetoclax. Moreover, the administration of venetoclax combined with other agents may prevent or delay the development of resistance. Finally, further research is essential to identify and evaluate plausible targeted drugs that could reverse the resistance to venetoclax or novel BCL-2 inhibitors.

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