**Abstract**

The challenge in classical Hodgkin Lymphoma (cHL) management is the 30–40% of refractory/relapsed cases.

**Aims:** The aim of this work was to determine whether NIK and BCL-2 could be useful as prognosis biomarkers in cHL. In addition, we evaluated BCL-2 as a directed-therapy in cHL cell lines using venetoclax.

**Main Methods:** We evaluated NIK and BCL-2 expression in 112 untreated cHL patients' lymph-node biopsies by immunohistochemistry. cHL cell lines were treated with venetoclax alone or combined with vincristine or doxorubicin. Cell viability, metabolic activity and cell death were analyzed by trypan-blue exclusion method, MTS assay and FDA/IP staining respectively.

**Key Findings:** No correlation between NIK or BCL-2 expression and the majority of the clinical parameters was found. Patients with $\geq 60\%$ BCL-2+ HRS-cells had a shorter disease-free survival (DFS) and overall survival (OS) ($p=0.002, p=0.02$ respectively). A decision tree analysis, in a 30 patients subgroup, showed that patients with $<60\%$ NIK+ HRS-cells but with $\geq 60\%$ BCL-2+ HRS-cells had a worse outcome in terms of DFS and OS. These parameters performed better as prognosis indicators as compared to the diagnosis bone marrow status. Human cHL cell lines U-H01, KM-H2, L1236, SUPHD1,
L540 showed sensitivity to venetoclax. The co-treatment effect of venetoclax and vincristine or doxorubicin on cell viability was diverse depending on the cell line evaluated.

**Significance:** BCL-2 should be considered as a prognosis biomarker as well as a potential new therapeutic target in cHL. We report for the first time the cytotoxic effect of venetoclax in human cHL cell lines.

**Keywords:** Hodgkin Lymphoma; BCL-2; Prognosis; Refractory Disease; Relapsed Disease; Venetoclax

### 1 Introduction

Classical Hodgkin Lymphoma (cHL) represents 95% of all HL diagnosed, being the remaining 5% nodular lymphocyte-predominant HL (NLPHL). cHL is further divided into four histological subtypes: nodular sclerosis, mixed cellularity, lymphocyte-rich and lymphocyte-depleted [1]. All of these entities are characterized by a low percentage of malignant cells (1–5%) embedded in a milieu of inflammatory cells [2,3]. The malignant fraction of the cHL consists of a spectrum of neoplastic cells including mononuclear cells known as Hodgkin (H) cells, multinucleated ones named as Reed-Sternberg (RS) cells and apoptotic mummified cells. Usually the malignant cell is identified as HRS (Hodgkin-Reed Sternberg) cell [4]. Approximately 8,500–9,000 new cases are diagnosed in the US per year [5,6]. cHL shows a bimodal distribution affecting young patients (15–30 years old) and adults older than 55 years of age [5,6].

The disease is curable and a long-term overall survival rate of 70–80% at five years can be achieved after the first line therapy that combines doxorubicin, bleomycin, vinblastine and dacarbazine (ABVD). However primary refractory disease and relapses occur in 10–20% of early stage patients with favorable features and in 30–40% of advanced stage patients. The standard treatment for relapsed/refractory cHL is salvage high-dose chemotherapy and autologous stem cell transplantation followed by optional consolidation in high risk patients. Approximately 55% of refractory patients persist with no response after salvage therapies and among relapsed patients, 40% of them do not reach a complete response [7–10].

Single-cell techniques have proven that HRS cells arise from germinal center (GC) B-cells with a defective B-cell receptor and loss of the characteristic B-cell genes expression profile due to epigenetic modifications [4,11–14]. Although normal GC B-cells that lack functional high affinity antibody undergo apoptosis within the GC, HRS cells escape this programmed cell death due to still unknown mechanisms. One possible explanation is the alteration of apoptosis regulators expression in HRS cells which may prevent cell death caused by the absence of the functional B-cell receptor and may account for treatment resistance [15–17].

The challenge in cHL management is the refractory/relapsed disease but little is known regarding the molecular biology underlying these conditions. We have previously reported that the alternative NfκB pathway plays a role in human cHL cells survival, being the mitogen-activated protein kinase kinase kinase 14 (NIK) stabilization responsible for its constitutive signaling [18]. We recently described by ChIP-Seq and expression arrays that the anti-apoptotic molecule BCL-2 was one of the target genes only controlled by the RelB-p52 dimer in cHL, among all NfκB dimers [19].

In this work, we aimed to determine whether mediators of the alternative NfκB signaling such as NIK and its target gene BCL-2 could be useful as prognosis biomarkers in cHL. We have previously reported that cHL cell lines were sensitive to NIK inhibition [18]. In the current work we evaluated BCL-2 as a directed-therapy in cHL cell lines, using a small-molecule drug that mimics the function of the BH3-only protein and selectively inhibits BCL-2, known as venetoclax [20].

### 2 Material and Methods

#### 2.1 Patient samples

Paraffin embedded cHL lymph node biopsies of 112 untreated patients [61 male median age and (range) 40.7 (9–78); 51 female 36 (6–88)], diagnosed during the 2007–2017 period in the Italian Hospital of Buenos Aires, were analyzed. The studied population features are summarized in the Tables 1 and 2.
Analysis of the association between BCL-2 or NIK expression and the main clinical, laboratory parameters, PET-CT Scan score performed at the end of the first line treatment and therapy received at diagnosis of the primary disease.

<table>
<thead>
<tr>
<th>Feature</th>
<th>N° of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>61 (54.5)</td>
</tr>
<tr>
<td>Female</td>
<td>51 (45.5)</td>
</tr>
<tr>
<td>Age group (years)</td>
<td></td>
</tr>
<tr>
<td>1–29</td>
<td>44 (39.3)</td>
</tr>
<tr>
<td>30–49</td>
<td>33 (29.5)</td>
</tr>
<tr>
<td>50–79</td>
<td>33 (29.5)</td>
</tr>
<tr>
<td>≥80</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>IPS</td>
<td></td>
</tr>
<tr>
<td>1 or 2</td>
<td>106 (94.6)</td>
</tr>
<tr>
<td>3 or more</td>
<td>6 (5.4)</td>
</tr>
<tr>
<td>Hb (g/L)*</td>
<td></td>
</tr>
<tr>
<td>≤10.5</td>
<td>49 (43.8)</td>
</tr>
<tr>
<td>&gt;10</td>
<td>51 (45.5)</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>24 (21.4)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>72 (64.3)</td>
</tr>
<tr>
<td>Erythrocyte sedimentation (mm/h)</td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>41 (36.6)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>25 (22.3)</td>
</tr>
<tr>
<td>Leucocytes mm(^3)</td>
<td></td>
</tr>
<tr>
<td>≤15,000</td>
<td>86 (76.8)</td>
</tr>
<tr>
<td>&gt;15,000</td>
<td>15 (13.4)</td>
</tr>
<tr>
<td>Lymphocytes mm(^3)*</td>
<td></td>
</tr>
<tr>
<td>1.500–4000</td>
<td>6 (5.3)</td>
</tr>
<tr>
<td>≤1.500</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>≥4000</td>
<td>92 (82.1)</td>
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<tr>
<td>Serum LDH levels (UI/L)</td>
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</tr>
<tr>
<td>Normal</td>
<td>62 (55.3)</td>
</tr>
<tr>
<td>Elevated</td>
<td>26 (23.2)</td>
</tr>
<tr>
<td>PET-CT Scan end of treatment score</td>
<td></td>
</tr>
<tr>
<td>Deauville 1</td>
<td>23 (20.5)</td>
</tr>
<tr>
<td>Deauville 2</td>
<td>30 (26.8)</td>
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<tr>
<td>Deauville 3</td>
<td>10 (8.9)</td>
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<tr>
<td>Deauville 4</td>
<td>3 (2.7)</td>
</tr>
<tr>
<td>Deauville 5</td>
<td>5 (4.5)</td>
</tr>
<tr>
<td>First line treatment</td>
<td></td>
</tr>
<tr>
<td>Only chemotherapy</td>
<td>70 (62.5)</td>
</tr>
<tr>
<td>Chemo + radiotherapy</td>
<td>42 (37.5)</td>
</tr>
<tr>
<td>First line chemotherapy</td>
<td></td>
</tr>
<tr>
<td>ABVD</td>
<td>107 (95.5)</td>
</tr>
<tr>
<td>AVD</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>Vincristine, etoposide, doxorubicin</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>BEACOPP</td>
<td>2 (1.8)</td>
</tr>
</tbody>
</table>

Note 1: When columns do not sum to the total, data were missing or unknown.
The average follow-up period was 51.2 (±136) months. The disease-free survival (DFS) was considered the length of time between absence of disease determined by imaging and the reappearance of signs or symptoms. The overall

Note 2: Relapsed patients treatments was not analyzed. BCL-2 expression was evaluated in untreated patients.

Only the hemoglobin concentration (*) , $p_{\text{adj}}=0.019$ and the number of lymphocytes per mm$^3$ (**) , $p_{\text{adj}}=0.008$ showed a statistical significant correlation with NIK expression.

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Analysis of the BCL-2 or NIK expression correlation with the main pathological parameters.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Axillary</th>
<th>Mediatinum</th>
<th>Ingual</th>
<th>Head and neck</th>
<th>Multiple</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphadenopathy topography</td>
<td>7 (6.2)</td>
<td>10 (9)</td>
<td>4 (3.6)</td>
<td>30 (26.8)</td>
<td>41 (36.6)</td>
</tr>
<tr>
<td>Histological subtype of cHL*</td>
<td>Nodular sclerosis</td>
<td>81 (72.3)</td>
<td>Mixed cellularity</td>
<td>25 (22.3)</td>
<td>Lymphocyte-rich</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte-depleted</td>
<td>2 (1.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>I</td>
<td>19 (17)</td>
<td>II</td>
<td>50 (45)</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>15 (13.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extra nodal involvement at diagnosis</td>
<td>Yes</td>
<td>11 (10)</td>
<td>No</td>
<td>64 (57.2)</td>
<td></td>
</tr>
<tr>
<td>Bone marrow at diagnosis</td>
<td>Infiltrated</td>
<td>9 (8)</td>
<td>Free</td>
<td>77 (69)</td>
<td></td>
</tr>
<tr>
<td>CD3 expression in HRS-cells</td>
<td>Positive</td>
<td>10 (9)</td>
<td>Negative</td>
<td>77 (69)</td>
<td></td>
</tr>
<tr>
<td>CD15 expression in HRS-cells</td>
<td>Positive</td>
<td>59 (53)</td>
<td>Negative</td>
<td>37 (33)</td>
<td></td>
</tr>
<tr>
<td>CD20 expression in HRS-cells</td>
<td>Positive</td>
<td>18 (16)</td>
<td>Negative</td>
<td>87 (77.7)</td>
<td></td>
</tr>
<tr>
<td>CD30 expression in HRS-cells</td>
<td>Positive</td>
<td>105 (94)</td>
<td>Negative</td>
<td>2 (1.8)</td>
<td></td>
</tr>
</tbody>
</table>

Note: when columns do not sum to the total, data were missing or unknown.

Only the histology sub-type showed a statistical significant association with BCL-2 expression (*) , $p_{\text{adj}}=0.001$.

The average follow-up period was 51.2 (±136) months. The disease free survival (DFS) was considered the length of time between absence of disease determined by imaging and the reappearance of signs or symptoms. The overall
All patients included but 5 received ABVD as first line therapy [in 2/5 bleomycin was excluded due to toxicity, 1/5 received vincristine, doxorubicin, etoposide and 2/5 BEACOPP (Bleomycin, Etoposide, Doxorubicin, Cyclophosphamide, Vincristine, Procarbazine, Prednisone)].

The investigation has been conducted according to the principles expressed in the Helsinki Declaration and after the Ethics Committee on Research Protocols approval of the Italian Hospital of Buenos Aires.

### 2.2.2.2 Immunohistochemistry

The NIK (αNIK ab7204, Abcam, United Kingdom), BCL-2 [αBCL-2 (C-2) sc-7382, Santa Cruz Biotech, USA] and serum control staining was performed on formalin-fixed, paraffin-embedded sections of cHL lymph node biopsies and processed as described elsewhere [13]. Both primary antibodies were used at a 1:200 dilution. Positivity was defined as HRS cytoplasmic granular bright brownish staining and was analyzed by three independent oncohematopathologists who also reviewed all the cases analyzed. The labeling index for each antibody was calculated as the percentage of labeled malignant cells out of the total number of HRS cells counted. BCL-2 and NIK staining was analyzed in all HRS cells identified in each biopsy. A ROC (Receiver Operating Characteristic) curve determined a cut-off point value of 60% to define a biopsy as positive or negative for BCL-2 or NIK expression. Those cases that expressed BCL-2 or NIK in ≥60% of the HRS cells were classified as positive. Surrounding cells were considered positive even when a single BCL-2 positive T-cell was observed.

### 2.3.2.3 Cell lines

Human cHL cell lines U-H01, L1236, KM-H2, SUPHD1, L540 y HDLM-2, with a doubling time of 4 days, 2.2 days, 2.5 days, 3-4 days, 70-100 hours and 3-5 days respectively [22-27], grown in RPMI-1640 ( Gibco, USA) supplemented with 20% bovine fetal serum (Internegocios S.A., Argentina) and 1% antibiotics (GIBCO), incubated at 37°C with 5% CO2. They were authenticated and routinely tested for mycoplasma contamination. U-H01, L1236, KM-H2 and SUPHD1 are of B-cell origin, meanwhile L540 and HDLM-2 cell lines are described as of T-cell origin [27,28].

### 2.4.2.4 Western blot

Cells were lysed in a solution containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 50 mM β-glycerophosphate, 1% Tween-20, 0.2% Nonidet P-40 and protease inhibitors. Proteins were resolved in the same conditions that were previously reported [18,19].

### 2.5.2.5 Cell treatments

cHL cell lines were plated in triplicate and treated with 1 μM venetoclax (MedKoo Biosciences, USA) or DMSO as a control (0.5 μl DMSO/ml culture medium) each 24 hours during 10 days. This period was selected in order to allow at least 1 cycle of cell division for all the cell lines included. A dose-response curve was performed for venetoclax (100 nM – 5 μM).

In drug combination experiments cells were first treated with 1 μM of venetoclax for seven days followed by 1 μM of doxorubicin (DOX) (Sigma-Aldrich, USA) or vincristine (VCR) (Filaxis, Argentina) for 72 hours. A dose-response curve was performed for VCR (100 nM – 1 μM) and DOX (100 nM – 1 μM) treatments.

### 2.6.2.6 Cell growth analysis

Total number of viable cells was determined every 24 hours using the trypan blue (GIBCO) exclusion method.

### 2.7.2.7 MTS assay

Cell general metabolic state was assayed by MTS (Cell Proliferation Assay Kit ab197010 Abcam, UK) according to the manufacturer instructions. Cells were analyzed in a microplate reader (Multiscan Ex, Absorbance Microplate Reader, Thermo Electron Corporation, China) at a 490 and 620 nm OD. Results were calculated as follows: OD ratio = (OD490-OD620 sample) – (OD490-OD620 media).
Cell death was determined using fluorescein diacetate (FDA) (Thermo Fisher Scientific, USA) and propidium iodide (PI) (Thermo Fisher, USA) staining. 5×10^5 cells were seeded and treated as mentioned above. Cells were then stained with 1 μM of FDA for 20 min at 37°C with 5% CO₂. Then cells were incubated with 1μg/ml of PI for 5 min to determine the percentage of viable and dead cells respectively. Stained cells were acquired on a CyFlow Space (Sysmex-Partec) and analyzed with the Flowing 2.1.5 software (Scripps Institute, La Jolla, USA).

### 2.9.2.9 Statistical analysis

Bivariate relationships between the BCL-2 and NIK expression and the clinical and pathological parameters were evaluated using the Spearman's Rho Test. A difference of p<0.05 was considered to be significant. The Kaplan-Meier method was used to estimate the DFS and the OS. In univariate survival analysis, two sided log-rank test for equality of survivor functions was used to assess the prognostic significance of different parameters on NIK and BCL-2 positivity. Multivariate analysis was performed using the Cox proportional model to evaluate the predictive power of each variable independently of the others (p<0.05). SPCCPC+ (version10) software was used for the analyses.

The statistical significance of living cells determined by the trypan blue staining exclusion method was determined applying the 2-way ANOVA Test using the GRAPH PAD PRISM software. A difference of alive and dead cells between vehicle and venetoclax treated cells with p≤0.05 was considered statistically significant with a confidence interval of 95%. The same analysis was performed in the drug combining experiments. The Bliss independence method was performed to evaluate drug combination effects [29].

### 2.10.2.10 Classification or decision trees

We performed an additional multivariate analysis, the Classification Regression Trees or Decision Trees to assess the effect of specific variables on survival status, including known predictors of cHL prognosis and the variables BCL-2 and NIK expression [21].

This method is named classification tree as the original method of data presentation is as a binary tree. The method consists in the data set partitioning. Initially all objects are considered as belonging to the same group. The group is split into two subgroups from one of the regressive variables so that the heterogeneity at the level of the dependent variable is minimal. The two subgroups [nodes] formed are separated again if sufficient heterogeneity to produce a partition observation and/or if the size of the node is greater than the minimum established to continue the algorithm. The process ends when any of these conditions is met [21].

### 3.3 Results

#### 3.1.3.1 Expression of BCL-2 but not NIK predicted outcome in cHL patients

We evaluated NIK and BCL-2 HRS-cells cytoplasmic expression by immunohistochemistry in 112 cHL lymph-node biopsies (Fig. 1A-B). BCL-2 was also detected in the HRS surrounding T-cells (Fig. 1B). There was a positive correlation between NIK and BCL-2 HRS-cells expression (p≤0.001).
The ROC curve defined a cut-off point of ≥60% HRS+ cells to define NIK+ or BCL-2+ cases. Out of the 112 cHL patients, 25 relapsed [17/25 (68%) expressed BCL-2 in ≥60% of HRS-cells]. As shown by the Kaplan-Meier curves, BCL-2 expression correlated with shorter DFS and OS [Long Rank Test (Mantle Cox) $p=0.02$, $p=0.002$ respectively] (Fig. 2A-2B). The statistical significance remained in the multivariate analysis [Cox Regression and the Linear Regression ($p=0.01$)]. The BCL-2+ microenvironment cells showed no prognosis correlation (Fig. 2C-2D).
BCL-2 as a prognosis biomarker in eHL patients. The expression of BCL-2, determined by immunohistochemistry, in the malignant cells of lymph node biopsies correlated with poor prognosis. BCL-2 expression in ≥60% of HRS cells associated with shorter overall survival (A) and shorter disease free survival (B) [≥60% and <60% indicate HRS+ cells]. C-D. BCL-2 expression assayed by immunohistochemistry in HRS cells’ surrounding T lymphocytes showed no association with prognosis.

The HRS-cells NIK expression did not associate either with DFS or OS (Fig. 3A-3B). Out of the 112 patients, 25 relapsed and 19/25 (76%) of them expressed NIK in ≥60% of HRS-cells.
No association between BCL-2+ HRS-cells and the majority of the clinical-pathological parameters was found (Tables 1-2) (Spearman’s Rho Test $p$ = NS). BCL-2 expression showed a positive correlation with the histological subtype ($p$ = 0.001) (Table 2). NIK expression associated with the hemoglobin concentration ($p$ = 0.019) and the lymphocytes/mm$^3$ ($p$ = 0.00) (Table 1).

In a 30 patients subgroup, NIK expression was the main feature to predict DFS-status and OS-status using a Decision Tree analysis (Fig. 4A-B). Patients with $\geq$60% HRS NIK+ cells, had a worse outcome. Patients with a better outcome (NIK < 60%) were further dichotomized by the BCL-2+ HRS-cells. Patients with NIK expression <60% but $\geq$60% BCL-2+ HRS-cells had a shorter DFS and OS. BCL-2 expression was a better indicator of the OS than the bone marrow status in the group of patients that expressed NIK in <60% of HRS cells (Fig. 4A-B).
A Decision Tree evaluated the OS-status in 36 patients (Fig. 4C), being lymphocytes/mm$^3$ the main feature to predict OS-status. Patients with a worse outcome (lymphocytes ≤1500 or ≥4000 mm$^3$) were further dichotomized by the bone marrow status (disease free or infiltrated) at diagnosis. BCL-2+ HRS cells percentage at diagnosis was able to predict a worse outcome among patients that had a disease-free bone marrow. NI-BM: non-infiltrated bone marrow. I-BM: infiltrated bone marrow.

Venicelox induced cell death in human cHL cell lines

3.2.3.2 Venetoclax induced cell death in human cHL cell lines
Since BCL-2 correlated with worse prognosis in cHL in terms of DFS and OS we decided to explore its potential benefit as a therapeutic target.

We evaluated BCL-2 expression in U-H01, L1236, KM-H2, SUPHD1, L540 and HDLM-2 human cHL cell lines by western blot. Results showed that all of them expressed BCL-2 (Fig. 5A). Then cells were treated with 1 μM of the selective BCL-2 inhibitor venetoclax or its vehicle DMSO each 24 hours during 10 days (Fig. 5B). All cell lines, except HDLM-2, were sensitive to venetoclax as was shown by a decrease in the number of living cells after trypan blue exclusion assay ($p < 0.0001$) (Fig. 5C).
Human cHL cell lines are sensitive to venetoclax. A. All cHL cell lines tested, KM-H2, SUPHD1, HDLM-2, L540, L1236 and U-H01 expressed BCL-2 determined by western blot. B. The venetoclax chemical structure and the treatment scheme applied are depicted. C. A decrease in the number of living cells was determined by the trypan blue exclusion method for five out of the six human cHL cell
The decrease in the number of living cells may be due to a reduced metabolic activity that could be explained either by a cytostatic or a cytotoxic effect. To further evaluate this, we choose the two B-cell derived cHL cell lines U-H01 and KM-H2. After double staining with FDA and PI, both cell lines were analyzed by flow cytometry and results showed that venetoclax was able to increase significantly the PI+ events \(23,38\% \pm 2,856\%\) in U-H01 cells \((p = 0,0002)\) and \(49,14\% \pm 7,293\%\) PI+ events in KM-H2 cells \((p = 0,0005)\). These results suggested that venetoclax induced cell death in cHL cell lines (Fig. 5D-5E).

### 3.3.3 Venetoclax combination with cytotoxic drugs

It has been reported that high BCL-2 expression levels confer resistance to CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) proapoptotic activities and are associated with poor outcome in DLBCL patients \([30]\). Thus, we decided to treat KM-H2 and U-H01 cell lines, combining venetoclax with VCR or DOX. We selected VCR and DOX since both are included in the CHOP regimen to which BCL-2 expression induced resistance to apoptotic cell death in DLBCL patients and because both cytotoxic drugs are used in cHL patients treatment \([7,30,31]\).

### 3.4.3.4 Venetoclax sensitized KM-H2 cells but not U-H01 cells to the effect of vincristine

The drug combination effect of venetoclax + VCR on cell viability, their metabolic activity as well as the induction of cell death were analyzed at day 10 of treatment (Fig. 6A).
Venetoclax sensitized KM-H2 cells but not U-H01 cells to the effect of vincristine. A. The vincristine chemical structure and the combined vincristine and venetoclax treatment scheme applied are depicted. B. Venetoclax monotherapy sensitized KM-H2 cells to vincristine effect as shown by the trypan blue exclusion method. C. MTS and D. PI staining. Error bars represent the mean of three experiments performed in triplicate for each condition for each time point ± normalized SEM. E-G. U-H01 cells showed sensitivity to venetoclax and vincristine treatments determined by trypan blue exclusion method, MTS and PI staining. No significant difference was obtained in the combined venetoclax and vincristine treatment. H. Dot plot of a representative FDA+ PI staining assay, acquired by flow cytometry, showing a significant difference in the percentage of KM-H2 dead cells when treated with venetoclax and vincristine as compared to single therapy with each drug. I. For the induction of cell death, the effect of venetoclax and VCR combination was analyzed by the effect-based strategy through Bliss independence model calculating the Combination Index (CI). Dotted lines represent the value of the expected additive effect calculated as: Expected additive effect = (venetoclax effect + VCR effect) × 100 (where 0 ≤ venetoclax effect ≤ 1 and 0 ≤ VCR effect ≤ 1). The resulting CI was calculated as: CI = (venetoclax effect + VCR effect - venetoclax effect × VCR effect)/Combination effect. CI < 1 indicated a synergistic effect of venetoclax + VCR treatment in KM-H2 cells, meanwhile an antagonistic effect was determined in U-H01 cells (CI > 1).

The combined treatment of venetoclax and VCR showed a different outcome in KM-H2 and U-H01 cells. Venetoclax + VCR treatment decreased the number of alive cells in KM-H2 cell line and this effect was accompanied by a metabolic activity reduction as compared to each drug alone (Fig. 6B-6C). A 87% ± 2% of PI+ cells was observed after the combination of venetoclax with VCR while a 70% ± 2% PI+ and a 39% ± 4% PI+ cells was observed after
Venetoclax monotherapy and VCR single therapy, respectively (Fig. 6D-H). These results indicated that venetoclax sensitized KM-H2 cells to the induction of cell death by VCR treatment. The Bliss independence model [22] showed a combinatory index (CI) <1 in the KM-H2 cells which indicated a synergistic effect between venetoclax and VCR (Fig. 6I).

Venetoclax did not sensitize U-H01 cells to the effect of VCR (Fig. 6E-G). Moreover, after performing the Bliss independence model, the CI value was higher than 1 indicating that these drugs would act antagonistically (Fig. 6I).

3.5.3.5 The combination of venetoclax with doxorubicin showed an additive effect in U-H01 cells

Similar to the venetoclax + VCR therapy outcome, the cell lines developed a different response to the combined venetoclax + DOX treatment (Fig. 7A). No significant differences were observed between venetoclax + DOX treatment as compared to the effect of DOX alone for both trypan blue exclusion method and MTS assay in KM-H2 cell line (Fig. 7B-7C). However, a higher percentage of PI+ cells was shown after the combined treatment as compared to single venetoclax ($p<0.0001$) and DOX ($p=0.0002$) therapies (Fig. 7D). The effect of the combined treatment upon the induction of cell death was lower than the expected by the Bliss independence model (89% PI+ cells and 82% PI+ cells, respectively), showing an antagonistic effect between venetoclax and DOX (CI >1) in KM-H2 cells (Fig. 7I).
The combination of venetoclax with doxorubicin showed an additive effect on U-H01 cells. A. The doxorubicin chemical structure and the combined doxorubicin and venetoclax treatment scheme applied are depicted. B-G. KM-H2 and U-H01 cells showed sensitivity to venetoclax and doxorubicin treatments determined by trypan blue exclusion method, MTS and PI staining. The combined venetoclax and doxorubicin treatment showed an enhanced number of dead cells in U-H01 cells as compared to single drug therapies. Error bars represent the mean of three independent experiments ± normalized SEM. H. Dot plot of a representative FDA/PI staining assay, acquired by flow cytometry, showing a significant difference in the percentage of U-H01 dead cells when treated with venetoclax and doxorubicin as compared to single therapy with each drug as monotherapy. I. For the induction of cell death, the effect of venetoclax and DOX combination was analyzed by the effect-based strategy through Bliss independence model calculating the Combination Index (CI). Dotted lines represent the value of the expected additive effect calculated as: Expected additive effect = (venetoclax effect + DOX effect - venetoclax effect × DOX effect) × 100 (where 0 ≤ venetoclax effect ≤ 1 and 0 ≤ DOX effect ≤ 1). The resulting CI was calculated as: CI = (venetoclax effect + DOX effect - venetoclax effect × DOX effect)/Combination effect. CI > 1 indicated an antagonistic effect of venetoclax + DOX treatment in KM-H2 cells, meanwhile an additive effect was determined in U-H01 cells (CI = 1).
The number of alive cells determined by trypan blue and the metabolic activity showed similar results in response to venetoclax + DOX treatment as compared to single drugs treatment in U-H01 cell line (Fig. 7E-F). In this regard, a 74%±2% of PI+ cells was observed in response to venetoclax + DOX while a 44%±2% of PI+ cells and a 54%±3% PI+ cells was observed after venetoclax monotherapy and DOX single therapy, respectively (Fig. 7G-H). The Bliss independence model analysis showed a CI=1 indicating an additive effect between venetoclax and DOX in these cells (Fig. 7I).

Discussions

cHL is a clonal B cell lymphoid malignancy that accounts for 15-20% of all lymphomas in many Western countries [4]. The current challenge in the clinical management of cHL is the group of primary refractory and relapsed patients. The early detection of refractoriness and risk of relapse is imperative for an adequate therapeutic decision [32].

NIK is a positive regulator of the alternative NFκB arm and we and others have previously reported its role in human cHL survival [18,33,34]. However, to date there are no reports that establish a relationship between the expression of this kinase and the cHL patients’ prognosis. In this work, we showed that although there was a trend towards a worse evolution of the disease in those patients who expressed NIK in ≥60% of HRS cells, there was no association with DFS or OS. Despite this, the percentage of NIK+ HRS cells emerged as a novel indicator of both DFS and OS status in a subgroup of 30 patients subjected to a specific multivariate analysis known as classification tree (Fig. 4A-B).

NIK stabilization led to the constitutive alternative NFκB signaling pathway [18]. We have previously shown that BCL-2 sustained expression is partially a consequence of this mechanism [19]. In addition, in this work we demonstrated a positive correlation between the expression of this anti-apoptotic protein and NIK in HRS cells of cHL patient samples. Moreover, we showed that BCL-2 expression in cHL lymph node biopsies at diagnosis provided relevant prognosis information, since its expression in ≥60% HRS-cells was associated with worse prognosis in terms of DFS and OS in cHL (Fig. 2A-B).

BCL-2 did not correlate with the majority of the clinical and pathological features analyzed, including the PET-CT Scan performed at the end of the first line treatment, which nowadays has become the gold standard in remission assessment in cHL [35]. Furthermore, BCL-2 expression in the surrounding lymphocytes did not correlate with prognosis (Fig. 2C-D).

Smolewski et al. analyzed HRS cells BCL-2 expression in 194 patients with HL including the NLPHL histology. Using a 10% cut-off for positivity, BCL-2 was expressed in 47% of tumors [36]. If we apply this cut-off to our cohort, 69/112 (61,6%) cHL patients were positive.

Several studies have reported that high expression of BCL-2 and p53 in HRS cells conferred a worse prognosis, but their independent predictive value has varied [37-44]. Rassidakis et al. reported BCL-2 expression in 412 cHL patients treated with ABVD or equivalent regimens associated with an inferior DFS and OS. For their statistical analysis they considered tumors with any HRS cell that expressed BCL-2 to be positive. They concluded that BCL-2 was expressed in 61% of patients with cHL [41]. If we considered tumors with any BCL-2 expressing HRS-cell as positive, 72/112 (64,3%) cases were positive.

The Canioni et al. study investigated HRS cells expression of BCL-2, CD20 and ki-67 in 18 refractory or early relapsed and in 41 responder patients. They found a median value of BCL-2+ HRS cells of 51% in refractory cases and of 12% of BCL-2+ HRS cells in responders [42]. The Benharroch study analyzed 209 lymph-node biopsies, including those used by Canioni, showing that BCL-2 was less expressed in primary-refractory as compared to early-relapsed [43].

As described above, the majority of the cHL BCL-2 expression studies reported a correlation with poor prognosis. In our case, we found that BCL-2 was an independent factor that may contribute to the failure of primary therapy and also to the inability to save these patients after relapse. Furthermore, BCL-2 expression was a better indicator of the OS status even before the bone marrow infiltration at diagnosis, in the subgroup of patients with less than 60% of NIK+ HRS cells (Fig. 4B). Applying a different classification tree analysis in a subgroup of 36 cHL patients, BCL-2 expression in ≥60% of HRS cells, indicated a worse outcome in terms of the OS among the group of patients that had a disease-free bone marrow at diagnosis (Fig. 4C).
Despite the directed therapies already available, which include brentuximab-vedotin and anti-PD1 blockade [45,47], relapsed/refractory cHL patients still need more specific therapies capable of interfering other oncogenic pathways playing a role in the cHL pathogenesis such as the alternative NFκB signaling arm.

BCL-2 mRNA and protein expression in HRS cells has been shown [37–44]. This expression may explain resistance to treatment-induced apoptosis of HRS cells and eventual clinical failure. In line with this, we previously showed that BCL-2 exogenous expression could partially rescue cHL cells from the death induced by Rel-B depletion [19]. Moreover, in this work we reinforced the fact that BCL-2 expression was associated with a poor outcome in cHL patients. Considering this background, we decided to evaluate venetoclax effect in human cHL cell lines. This selective BCL-2 inhibitor has already been approved for other onco-hematological malignancies [48–50].

Small-molecule modulators and mimetics have been developed in attempts to inhibit BCL-2 family proteins. Obatoclax (GX15-070) and navitoclax (ABT-263) are pan BCL-2 family inhibitors, and venetoclax is a BCL-2 selective inhibitor [51]. However, a clinical trial (NCT00359892) in thirteen cHL patients showed that Obatoclax had limited clinical activity in heavily pretreated patients [52]. Venetoclax has shown to be effective in certain B-cell lymphomas with good tolerance and in four different relapsed and refractory Non-Hodgkin lymphomas [52,53].

The current research is the first work showing human cHL cell lines response to venetoclax monotherapy. All human cHL cell lines tested, established from primary refractory patients or patients with progressive disease, showed sensitivity to venetoclax except HDLM-2 (Fig. 5C). Moreover, venetoclax induced cell death in KM-H2 and U-H01 cell lines. Previous results in CLL patients carrying a p53 mutation or a 17p deletion developed a better response to venetoclax [54]. Like L540 cells, HDLM-2 is a T-cell derived Hodgkin lymphoma cell line. The latter has a single p53 gene copy and an exon 8-11 deletion meanwhile L540 has wild-type p53 [55–58]. It may be possible that p53 status in HDLM-2 cells explains the different outcome.

Since venetoclax resistance has already emerged in patients [59,60], we decided to examine the effect of different combinatorial strategies in KM-H2 and U-H01 cells. Venetoclax sensitized KM-H2 cells to the effect of vincristine and the combination of venetoclax and DOX had an additive effect on the induction of U-H01 cell death. Thus the effect of the combination of venetoclax with VCR or DOX on cell death induction depended on the cell line evaluated. Further studies should be performed to address the mechanisms that could better explain these observations.

Albeit BCL-2 expression has been associated to bad prognosis before as we have discussed in this work, the availability of venetoclax nowadays highlights the need to revisit this finding. The BCL-2 blockade along with the conventional therapy could potentially anticipate patients’ poor outcome. Research should move forward to investigate the venetoclax effect in the context of lymphoma microenvironment such as in vitro co-cultivation studies between human cHL cell lines-stroma cell lines and in vivo assays in mice. Furthermore, clinical trials need to be carried out in order to determine the efficacy of venetoclax alone or combined with cytotoxic drugs in cHL patients.

## Conclusions

Our work aimed to contribute to a still unsolved need in the cHL patients clinical management. It is necessary the accurate early identification of patients at risk of relapse as well as the first line therapy refractory patients. In addition, the analysis of molecular factors involved in the biology of these conditions as novel therapeutic targets would potentially benefit this subgroup of patients.

The HRS-cells BCL-2 expression correlated with shorter DFS and OS, contributing prognosis information. Human cHL cell lines showed sensitivity to venetoclax monotherapy, a BCL-2 selective inhibitor. Venetoclax sensitized KM-H2 cells to vincristine and had an antagonistic effect in U-H01. Combined venetoclax and doxorubicin showed an antagonistic effect in KM-H2 cells and an additive effect in U-H01. Our findings suggest that BCL-2 expression could provide relevant prognosis data at diagnosis in cHL. Patients with this disease could benefit from BCL-2 blockade probably in combination with the current first line therapy.

## Uncited reference

[46]

## Declaration of competing interest
AMGC, MD, NC, VO, NS, DF, SC, MZ, ERB, FJ, HGR, MN and SMR declare that they have no conflict of interests.

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Ethics approval

This research was approved by the Ethics Committee on Research Protocols of the Italian Hospital of Buenos Aires (HIBA).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Data generated or analyzed during this study are available from the corresponding author on reasonable request.

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DLBCL cells with acquired resistance to venetoclax are not sensitized to BIRD-2 but can be resensitized to venetoclax through Bcl-XL inhibition. Biomolecules. 10 (7) (2020) 1081, doi:10.3390/biom10071081.


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