Seeking for possible prognostic and predictive factors in chronic lymphocytic leukaemia (CLL), with a focus on the main role of the CD86 molecule

PhD Thesis

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LIST OF ABBREVATIONS:

AgPC: Antigen presenting cell

APC: Allophycocyanin

APC-AF: APC- Alexa Fluor™

BAK: Bcl-2 homologous killer

BAX: Bcl-2 associated X

BC: Beckman Coulter

Bcl-2: B-cell lymphoma 2

BCR: B-cell receptor

BD: Becton Dickinson

BL: Biolegend

BM: Bone marrow

BMSC: bone marrow stromal cells

BTK: Bruton's tyrosine kinase

BV: Brilliant Violet™

CBC: complete blood count

CD: cluster of differentiation

CLL: Chronic lymphocytic leukaemia

Co: treatment-naïve

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

DAB: Diamino-benzidine

DMEM: Dulbecco's modified Eagle's medium

ECD: Electron coupled dye

ERIC: European Research Initiative on CLL

FC: Fludarabine-Cyclophosphamide

FITC: Fluorescein isothiocyanate

FCR: Fludarabine-Cyclophosphamide-Rituximab

G/l: giga/liter, $x10^9$/liter

IBR: Ibrutinib

IgHV: immunoglobulin heavy chain variable region genes

IHC: immunohistochemistry

ImS: immunological synapse

IS: ibrutinib sensitive

IR: ibrutinib-resistant

ITP: immune thrombocytopenic purpura

LDH: lactate dehydrogenase

MFC: multicolour flow cytometry

MFI: median fluorescence intensity

mg/dl: milligram/deciliter

MRD: measurable residual disease

NGS: next- generation sequencing

NIC: National Cancer Institute

OS: overall survival

PB: peripheral blood

PC5.5: Phycoerythrin-cyanine 5.5

PC7: Phycoerythrin-cyanine 7

PE: Phycoerythrin

PERCP-Cy5.5: Peridinin-chlorophyll-Cyanine 5.5

PFS: progression free survival

PI: Propidium iodide

R/R CLL: relapsed/refractory CLL

R-Benda: Rituximab-Bendamustine

R-CVP: Rituximab-Cyclophosphamide Vincristine Prednisone

TTT: time to treatment

U/l: unit/liter

VEN: Venetoclax

WHO: World Health Organization

ZAP70: Zeta Chain of T Cell Receptor Associated Protein Kinase 70

1. INTRODUCTION

1.1. Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia (CLL) is the most common adult leukaemia in developed countries (1,2). CLL affects mainly the elderly people and according to the World Health Organization (WHO) classification of haematological tumours it is a malignancy of mature B-cells (3). Besides the peripheral blood (PB), other lymphoid tissues (i.e. lymph nodes, spleen, liver, skin) and the bone marrow (BM) may also be affected; however, the disease is most commonly diagnosed from the PB. The diagnosis of CLL is based on the complete blood count (CBC) and the immunophenotype of the circulating B-cells (4, 5). Elevated lymphocyte count is commonly seen in the laboratory results and more than 5 G/l absolute aberrant lymphocyte number is needed to establish the diagnosis. CLL cells usually show light chain restriction (kappa or lambda) and co-expression of CD19, CD23 and CD5 cell surface antigens. This characteristic feature is most commonly measured by multicolour flow cytometry (MFC)(3).

The clinical presentation of the disease varies greatly (6-9). In some instances, CLL is discovered as an "accidental diagnosis", meaning that the patient does not present any symptoms apart from elevated absolute lymphocyte count, while in other cases a wide range of symptoms are observed. The main clinical symptoms include B symptoms (fever higher than 38 °C, night sweats, weight loss exceeding 10% of the whole body mass in the last six months), but lymphadenopathy, hepatosplenomegaly and elevated LDH (more than 250 U/l) and β2 microglobulin (more than 2.4 mg/dl) levels are also commonly observed (10-12). In addition, opportunistic infections and autoimmune disorders may also occur due to the immune dysfunction associated with presence of the CLL cells (13-15). Although the aforementioned clinical symptoms are not essential diagnostic criteria for CLL, some of them are utilized in prognostic score systems (i.e CLL-IPI score) (5), therefore act as prognostic markers.

1.2. Prognostic Factors

Since CLL is a clinically heterogenous disease, there is great need to find factors that correlate with its clinical behaviour. These factors are called prognostic factors. According to the National Cancer Institute (NCI) definition, a prognostic factor is: "A

situation or condition, or a characteristic of a patient that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring" (16).

Although several clinical parameters have important prognostic values, currently the best prognostic value is attributed to the mutational status of B-cell receptor's (BCR) immunoglobulin heavy chain variable region genes (IgHV). BCR is a protein complex belonging to the immunoglobulin superfamily consisting of two light and two heavy chains. The light and the heavy chains each contain one variable region, that are responsible for the antigen recognition (17). These variable regions undergo somatic hypermutation during B-cell development to increase their affinity to the antigen (18). CLL can be classified into mutated (IgHV-M) and un-mutated (IgHV-U) categories based on the IgHV mutation status. In the case of IgHV-U the IgHV region shows more than 98% homology with the germline DNA sequence, whereas if the DNA homology is less than 97% the sample is classified as - IgHV-M. There is an intermediate, so-called borderline (IgHV-B) category when the DNA homology stands between 97 and 98%. The IgHV-U status usually associates with an unfavourable disease course; therefore, it should be tested at time of the diagnosis (5, 19).

Another important indicator of CLL prognosis is the TP53 mutation status of the tumour cells. It is well-known that the p53 protein plays a central role in regulating the apoptotic processes, and its loss of function leads to unfavourable disease outcomes in many different malignancies (20-22), including CLL. Patients with TP53 deficiency (caused by del17p and/or TP53 mutation) have worse survival rates than the patients with wild-type TP53 (23), thus the detection of TP53 aberrations is a key component of molecular diagnostic workup of CLL (24-27).

Although these molecular methods are excellent tools for outlining the various prognostic groups of CLL patients, they are time-consuming and costly, so there has been a demand for faster and less expensive approaches. Certain cell surface markers identified by MFC prooved to be equally reliable prognostic factors (28); moreover, the availability of MFC is generally wider than the molecular methods. Perhaps for this reason MFC has emerged as a potential tool for identifying relevant prognostic factors. CD38 was one of the first cell surface markers that was thought to possess a prognostic value. The CD38 positive CLL cells were shown to be more responsive to BCR signaling, which provides them with a proliferative advantage, translating into a worse overall prognosis for patients

with CD38 positive clones (29-31). Another marker that may indicate a more aggressive disease course in CLL is the CD49d molecule. CD49d is a cell adhesion molecule that belongs to the integrin superfamily. As an integrin, its increased expression likely provides better adhesion of the neoplastic B-cells to their protective micro-environment in the lymph nodes or bone marrow, resulting in longer tumour cell survival (32). Possibly for this reason, the CD49d positive CLL patients may expect a less favorable prognosis (33, 34). By flow cytometric evaluation of these CD markers CLL patients can be stratified into either favourable or unfavourable prognostic groups, which facilitates the selection of the most appropriate treatment option.

1.3. Predictive Factors

In the past, CLL used to be an incurable disease with an unpredictable disease-course. The majority of patients do not require immediate therapy at diagnosis, and in their case close monitoring and "watch and wait" strategy is sufficient, whereas other less fortunate patients require prompt therapeutic intervention due to the rapid progression of the disease (35, 36). Since the 1950s chemotherapy has been an important component of the therapeutic regimen. Initially, only alkylating agents (i.e. chlorambucile, cyclophosphamide) were used as therapy, but later chlorambucile was replaced by fludarabine, resulting in a more effective fludarabine-cyclophosphamide (FC) combination (37). The discovery of the therapeutic benefits of monoclonal antibodies had a strong impact on the treatment of CLL, resulting in the introduction of the anti CD20 antibody Rituximab into the daily practice. The combination of Fludarabine Cyclophosphamyde, and Rituximab (FCR) significantly improved the survival of CLL patients (38-40). While this chemoimmunotherapy prooved to be effective in several disease subgroups, some patients do not respond adequately to this treatment option (41). These patients are identified as relapsed/refractory CLL (R/R CLL) cases, and the frequent appearence of therapeutical failure in this subpopulation underlines the importance of prediction-based treatment selection.

According to NCI definition a predictive factor is: "A condition or finding that can be used to help predict whether a person's cancer will respond to a specific treatment. Predictive factor may also describe something that increases a person's risk of developing a condition or disease" (16). The CLL8 trial demonstrated that the TP53 mutation status has a considerable influence on the overall survival (OS) of CLL patients that is

independent of the therapy used. Patients harbouring wild type TP53 gene can expect a longer survival compared to patients with mutated TP53 when treated with FC or FCR therapy (42). In addition, Malcikova et al. suggested that TP53 mutation promotes the clonal evolution of the CLL cells, often correlating with increased resistance to chemotherapy (43).

Visentin et al. demonstrated that IgHV mutational status could be considered not only as a prognostic, but also as a predictive factor in CLL (44). According to their study, CLL patients with IgHV-U mutation status showed earlier progression under FCR treatment compared to patients with IgHV-M.

Concerning the common flow cytometric markers, CD38 was found to be an exellent prognostic factor, but its treatment predictive power has not yet been clearly demonstrated (30, 45). Conversely, CD49d has been recently integrated into the FCR-treatement decision-making algorithm (41). Lately, the detection of the measurable residual disease (MRD) by MFC in CLL has proved to be yet another tool to predict the survival (46). According to the NCI the MRD means the following: „A term used to describe a very small number of cancer cells that remain in the body during or after treatment. MRD can be found only by highly sensitive laboratory methods that are able to find one cancer cell among one million normal cells. Checking to see if there is MRD may help plan treatment, find out how well treatment is working or if cancer has come back, or make a prognosis" (16). While MRD determination has been a widely used method for risk-stratification in several haematological malignancies (47), its introduction into clinical use in CLL had to wait until the anti-CD52 antibody, alemtuzumab has became widely avaliable (48).

The measurement of MRD was not performed routinely in CLL in the past since alemtuzumab – the only treatment option considered to achieve MRD negativity - was rarely used in CLL (49, 50). Alemtuzumab is commonly used as a conditioning therapy prior to bone marrow transplantation, but this treatment modality is rarely performed in CLL patients (51, 52). FCR combination is a far more ubiquitously used treatment option in CLL. Therefore, the door to the routine use of MRD detection in CLL was opened in parallel to the discovery that MRD negativity is also achievable by FCR treatment (53). Moreover, the CLL8 trial suggested the MRD level might be an independent predictor of the progression free survival (PFS) and overall survival (54).

1.4. The Era of Targeted Therapies

As a result of intensive research in recent years, the B-cell receptor (BCR) signaling pathway and the Bcl-2 antiapoptotic pathway have been revealed as key factors in the survival of CLL cells. The BCR signaling pathway plays a crucial role in the activation of immature B-cells and this pathway is also used by neoplastic B-cells (55). For this reason, several members of this pathway have been considered as potential targets for inhibition in B-cell malignancies and Bruton's tyrosine kinase (BTK) receptor appeared as the most appealing candidate (56). It can firmly be stated that the inhibition of BTK has revolutionized CLL's treatment (57). The first BTK inhibitors were non-selective, therefore they had many side effects, but since the introduction of the highly selective BTK inhibitor ibrutinib (IBR), the treatment of CLL has drastically changed (58). IBR binds to the 481-cysteine amino acid, which is located in the active center of the BTK receptor, resulting in the inhibition of BTK signal transduction (43, 59).

Ibrutinib has proved to be highly effective even among high-risk and R/R CLL patients and also among patients with unfavourable prognosis (del17p, TP53 mutation, IgHV-U) (60, 61). Although ibrutinib is a highly effective drug, continuous treatment is required to maintain a stable remission status, which may in turn lead to drug-resistance due to progressively accumulating mutations in the CLL cells (62). For this reason, the identification of reliable predictive factors has become more and more important in patients treated with ibrutinib. MRD measurement seemed to be an appealing approach to forecast disease progression, but unfortunately it is not suitable to predict an imminent relapse during ibrutinib treatment (63). A point mutation of the BTK gene affecting the 481-cysteine locus can interfere with the binding of ibrutinib to the active center of the receptor, resulting in ibrutinib resistance (64). Consequently, the C481S missense mutation of the BTK gene is considered to be a potentially useful predictor of ibrutinib resistance (65-67).

It is widely accepted that Bcl-2 protein plays a key role in the survival of malignant B-cells including CLL cells (68). The Bcl-2 protein inhibits the oligomerization of the BAK and BAX proteins thus suspends the formation of a pore in the outer mitochondrial membrane, resulting in the suppression of apoptosis (69). Thus, the overexpression of the Bcl-2 protein is a convinient way for CLL cells to circumvent cell death (70). This observation has led to the development of Bcl-2 inhibitors, especially the highly selective

venetoclax (VEN) in recent years. VEN binding to the Bcl-2 protein suspends its inhibitory effect, making it possible for the BAK and BAX proteins to form a pore in the outer mitochondrial membrane, releasing cytochrome c and other pro-apoptotic factors into the cytosol (71). VEN has proven to be an effective treatment option both in monotherapy and in combination with rituximab, for R/R patients and patients with unfavourable prognosis (72-74). Beyond prolonging the OS, VEN is able to achieve MRD negativity regardless of the prior treatments or prognostic factors (72, 75).

Although venetoclax is an appealing treatment option for a wide range of CLL patients, circumventing the development of drug resistance can be challenging. The most frequent cause of venetoclax-resistance are Bcl-2 gene mutations that interfere with the binding of venetoclax to bcl-2 (76). The D103Y and G101V mutations of the Bcl-2 gene are the most reliable molecular predictors of the venetoclax resistance to date. However, none of them have a 100% penetrance, which means they are unable to predict the disease progression in all cases (77, 78). A novel promising predictive factor is MRD. It has been convincingly proven by several studies that the deeper MRD levels achieved by venetoclax associate with more durable remissions (73, 75), thus suggesting that MRD negativity could be a predictive factor of the disease outcome.

1.5. Flow Cytometry as a Potentially Suitable Tool for Revealing Prognostic and Predictive Factors

Having reviewed the available prognostic and predictive factors of CLL, two methodologies have been mentioned several times: multicolour flow-cytometry and molecular methods. Since my work is focused on the utility of flow cytometry in CLL treatment, I am going to briefly introduce its advantages in discovering novel prognostic and predictive factors. MFC and immunohistochemistry (IHC) are frequently refered to as cellular technologies. This technology allows the simultaneous monitoring of expression of multiple markers and comparison of their relative expression levels. For this reason, MFC has become a more and more appealing tool for cell analysis.

There are two cells' attribution phenomena which can be investigated by MFC. First, the co-expression of multiple markers, and secondly their expression level. Although the WHO diagnosis of CLL is based on the expression of certain surface markers (3), but the investigation of just a few markers can sometimes be insufficient to make a differential diagnosis. During MRD analysis, we are searching for rare events, so the specificity and

the sensitivity of the used method has prime importance. For this reason, at least five more markers (ROR1, CD81, CD20, CD43, CD79b) should be detected on a single cell at the same time in order to minimize the amount of the false positive or false negative events (79).

Determining the expression levels of different markers is also a potential application of MFC, but in some cases MFC results should be validated by other protein-based techniques, for instance, western blot. While the absolute value of the protein expression level is best determined by using western blot, MFC is more suitable to investigate the relative expression values (compared to the isotype or internal positive or negative controls). Knowledge of the relative expression level of surface markers (e.g. CD49d, CD38) is often sufficient to predict disease progression in routine diagnostic settings (80, 81).

1.6. CLL surface markers and the microenvironment

The search for new prognostic and predictive factors is a hotspot in CLL research. Yet, there are CLL patients whose treatment outcomes are not satisfactory, therefore finding novel markers is greatly needed and some markers associated with the microenvironmental interactions of the B-cells seem to be a promising candidate for this goal.

The CD27 molecule is a member of the tumour necrosis factor superfamily (82). It is primarily located on the surface of the B-cells and plays a pivotal role in the B-cell development (83). Moreover, CD27 has recently appeared as a novel target in cancer immunotherapy (84). In addition, an important connection was revealed between the CD27 expression and the IgHV mutation status of the B-cells. The expression level of CD27 was elevated among B-cells with mutated IgHV compared to B-cells with unmutated IgHV (85). Based on this observation CD27 might have an important prognostic value in CLL because the IgHV mutation status is a known prognostic factor in CLL.

The CD86 molecule (B7-2), as a member of the B-7 family, belongs to the immunoglobulin protein superfamily (86). It has a monomeric structure and is expressed on the surface of the antigen-presenting cells (AgPC) (dendritic cells, macrophages, B cells), together with CD80 molecule (B7-1) (87, 88). It has two known ligands: the CD28

and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). CD86 functions as a co-stimulatory molecule in the formation of the immunological synapse (ImS), and also plays an important role in its regulation (89-91). As a component of the ImS, CD86 regulates the cell-cell interactions between the subunits of the ImS, allowing T-cell activation or inhibition, depending on its ligand (91, 92). The expression of CD86 is lower on CLL cells than on non-malignant B-cells (93), but its expression can be induced by T-cells or other participants in the microenvironment (94, 95). According to Huemer et al. patients required earlier therapy when CD86 expression on CLL cells was high (96).

The background literature suggests that the CD27 and the CD86 molecules may interact with the neoplastic cells, so in my opinion their role in terms of CLL progression may be an important question to be addressed.

2. OBJECTIVES

Against this background, we wanted to address the following questions:

The prognostic value of the CD86 molecule in CLL

- Is there any connection between CD86 expression of CLL cells and disease outcome?

The influence of the venetoclax resistance on the phenotype of CLL cells

- Is there any connection between the venetoclax treatment and the phenotype of CLL cells?
- Is there any connection among the resistance mechanisms of CLL cells and their immunophenotype?

The effect of the ibrutinib and its resistance on the phenotype of CLL cells

- Is there any connection between the microenvironment related surface markers of CLL cells and the ibrutinib treatment?
- Is there any correlation between the expression of the microenvironment related surface markers on the CLL cells and the presence of the BTK^{C481S} mutation of the CLL cells?

3. METHODS

3.1. Patients' Clinical Characteristics

In our first study, we collected peripheral blood (PB) samples from 50 patients with CLL. The diagnosis of CLL was based on the WHO classification of tumours of lymphoid tissue(3). Only blood samples in which the proportion of CLL cells ratio among the lymphocytes exceeded 85% based on flow cytometry measurement (in the lymphocyte gate $CD19^+$ and $CD5^+$ cells were considered as CLL cells) were used, resulting in 49 samples (female/male ratio was 22/27 with a median age of 67 years (40–87)). For the analysis of the time to treatment values (the time from the diagnosis to the first treatment) (TTT), the clinical data were only available for 18 patients (7 from the $CD86^{low}$ group and 11 from the $CD86^{high}$ group).

In our second study, we obtained PB samples from CLL patients who were treated with VEN monotherapy (400 mg daily dose). We were able to enrol 4 patients in our study (named V1-V4, female/male ratio 1/3; median age 70 years (49-76)). The patients' medical histories were unclear, except for V2. Further investigation revealed that patients V1, V3 and V4 patients had received chemoimmuno therapy prior to venetoclax monotherapy. Patient V2 attended regular follow-up visits, and his samples were analysed by flow cytometry on days 0, 180, 270, 360, and 450 of the treatment. Patient V2 lost his clinical response to VEN at month of treatment. At this time, a BM sample was taken to exclude the presence of Richter's transformation. PB samples from patient V2 were also used later when this patient was treated with ibrutinib. The detailed medical history of patient V2 is shown on **Figure 1**.

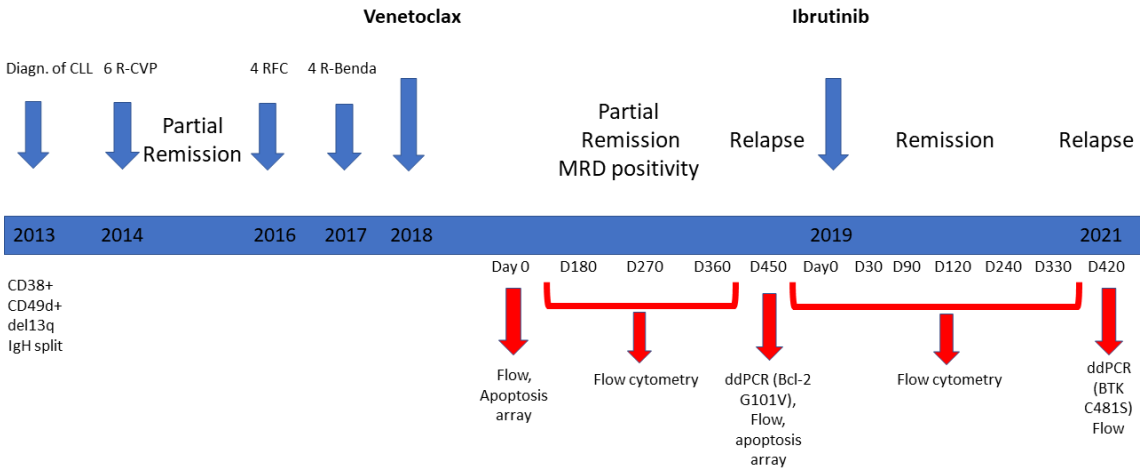

Figure 1. The V2's patient medical history is summarized on this figure.

In our third study, PB samples were collected from treatment-naïve (Co) (n=10, female/male ratio 3/7; median age 69 years (55-83)), ibrutinib-sensitive (IS) (n=7, female/male ratio 5/2; median age 72 years (63-86)) and clinically ibrutinib-resistant (IR) (n=11, female/male ratio 2/9; median age 70 years (56-87)) CLL patients in six Hungarian oncohaematology centres. CLL was diagnosed according to current WHO guidelines (97). Treatment-naïve patients were selected from those patients' samples whose samples arrived at our laboratory during the third study's investigational period. All patients treated with ibrutinib received the drug as a singleagent at a daily dose of 420 mg. Patients were followed according to the the institutional protocols of the participating centers. Patients in the IS cohort were treated with IBR for exactly one year, and PB samples were collected after one year of IBR treatment. We wanted to exclude potentially resistant samples from this cohort, so BTK^{C481S} mutation status analysis was performed. And all IS samples were found to be negative for this mutation. Patients in the IR group patients were treated with ibrutinib for at least 4 months (median 28.5 months, range: 4-57 months) and the PB samples were collected when they were considered ibrutinib resistant. The clinical characteristics of the patients are summarised in **Table 1**.

Table 1. Patients' clinical characteristic of our third study. (F:female, M: male, NA: data not avaliable, NP: not performed, U: unmutated, MU: mutated, B: borderline, del: deletion, TN: tretament naïve)

Cohorts	Patient ID	Prior chemo-immunotherapy	Sex	Age at taking the sample (year)	IgHV mutation status	TP53 status	Duration of the therapy (months)	CD49d expression at taking the sample	CD38 expression at taking the sample	BTK^{C481S} mutation status at taking the sample
Tretament naïve	Co1	no	F	74	NA	del 17p	TN	NP	negative	NP
	Co2	no	M	83	NP	NP	TN	negative	negative	NP
	Co3	no	M	66	NP	NP	TN	positive	negative	NP
	Co4	no	M	72	NP	NP	TN	dim	negative	NP
	Co5	no	M	55	U	wild type	TN	negative	negative	NP
	Co6	no	F	63	NP	NP	TN	negative	negative	NP
	Co7	no	M	68	U	wild type	TN	NP	positive	NP
	Co8	no	M	56	U	wild type	TN	negative	positive	NP
	Co9	no	M	70	MU	wild type	TN	NP	negative	NP
	Co10	no	F	80	NP	NP	TN	negative	negative	NP
Ibrutinib-sensitive	IS1	yes	F	86	NA	NA	12	negative	negative	wild type
	IS2	yes	F	76	NA	NA	12	negative	negative	wild type
	IS3	yes	M	63	NA	NA	12	positive	positive	wild type
	IS4	yes	M	63	U	NA	12	negative	positive	wild type
	IS5	yes	F	70	U	MU	12	negative	positive	wild type
	IS6	yes	F	74	U	MU	12	positive	positive	wild type
	IS7	yes	F	72	NA	MU	12	negative	positive	wild type
Clinically ibrutinib-resistant	IR1	yes	M	77	U	wild type	21	negative	negative	wild type
	IR2	yes	M	71	U	wild type	4	negative	positive	wild type
	IR3	yes	F	74	NA	wild type	16	negative	negative	wild type
	IR4	yes	F	87	NA.	NA.	6	negative	positive	wild type
	IR5	yes	M	62	B	MU	25	positive	positive	MU
	IR6	yes	M	74	NA	wild type	32	negative	positive	MU
	IR7	yes	M	61	U	MU	57	negative	negative	MU
	IR8	yes	M	56	U	wild type	36	negative	positive	MU
	IR9	yes	M	70	NA.	wild type	49	positive	positive	MU
	IR10	yes	M	69	U	NA	57	positive	negative	MU
	IR11	NA	M	70	U	NA	NA	positive	positive	MU

In all of our trials, the clinical response to the treatment was defined according to the iwCLL guideline (10). A clinically significant response was defined as a complete or partial remission. The loss of the best response achieved was defined as relapse and therefore as resistance to the treatment.

3.2. Measurement of the Surface Markers Expression by Flow Cytometry

We used flow cytometry to determine the relative expression level of surface markers in all three studies. Briefly, the stain-lyse-wash procedure was used to prepare the samples for measurement in all three studies. Where the procedure differed in studies, we describe the differences. The 50 µl PB or BM samples were incubated with the antibodies against surface epitopes for 13 minutes at 4°C. The antibodies used in our studies are listed in **Table 2,3**.

Table 2. Overview table of antibodies used in our first study.

Laser	***Blue*** *488nm, 40mW*		
Fluorescent channels	FL1	FL2	FL3
Panel 1	**CD5** **FITC** (DK23, Dako)	**CD86** **PE** (2331, BD)	**CD19** **PC5.5** (J3-119, BC)

BC: Beckman Coulter, Brea California USA, BD: Becton Dickinson Biosciences, New Jersey, USA, Dako: Dako, Glostrup, Denmark, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, PC5.5: Phycoerythrin-cyanine 5.5.

Table 3. Overview table of antibodies used in our second and third study.

Laser	***Blue*** *488nm, 40mW*				***Red*** *640nm, 40mW*	
Fluorescent channels	FL1	FL2	FL3	FL4	FL5	FL6
Panel 1	**ROR1 FITC** (2A2, Milteny)	**CD49d PE** (L25,BD)	**CD5 ECD** (BL1a, BC)	**CD45 PC7** (J33, BC)	**CD3 APC** (UCHT1, BC)	**CD19 APC-AF700** (J3-119, BC)
Panel 2	**CD69 FITC** (FN50, BD)	**CD184 PE** (12G5,BD)	**CD27 PC5.5** (1A4CD27,BC)	**CD38 ECD** (LS198-4-3, BC)	**CD86 APC** (2331, BD)	**CD19 APC-AF700** (J3-119, BC)
Panel 3	**CD44 FITC** (DJ18, BL)	**CD40 PE** (5C3, BL)	**CD185 PercpCy5.5** (J252D4, BL)	**CD45 PC7** (J33, BC)	**CD197 AF647** (G043H7, BL)	**CD19 APC-AF750** (J3-119, BC)

Milteny: MACS Milteny Biotech, Bergisch Gladbach, Germany, FITC: Fluorescein isothiocyanate, PE: *Phycoerythrin, ECD: Electron coupled dye, PC7: Phycoerythrin-cyanine-7, APC: Allophycocyanin, APC-AF700: APC- Alexa Fluor™ 700, PC5.5: Phycoerythrin-cyanine 5.5, BL: Biolegend San Diego, California, USA, Percp-Cy5.5: Peridinin-chlorophyll-Cyanine 5.5, AF647: Alexa Fluor™ 647, APC-AF750: APC-Alexa Fluor™ 750.*

Samples were then lysed using BD FACS™ Lysing Solution (BD) for 10 minutes. Prior to measurement, all samples were washed twice (5 min, 400 g, room temperature) with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH = 7.4). All antibodies used were pre-titrated, fluorescence staining was performed in the dark, and stained samples were protected from light.

In our first study, samples were measured by using a 4-colour FACSCalibur flow cytometer (BD), the instrument settings of which were regularly checked using BD Calibrite Beads (BD). In this study, a minimum of least 20,000 events were measured from each sample and flow data were analysed using CellQuest Pro software (BD).

In our second and third studies, an 8-colour Navios flow cytometer was used (BC), and instrument settings were regularly checked using Flow-Set Pro and Flow-Check Pro QC beads (BC). A minimum of 50,000 events were measured from each sample to obtain the

sufficient numbers of CLL cells. Kaluza 2.1.1 software (BC) was used to analyse the flow data. Results were based on the median of median fluorescence intensity (MFI).

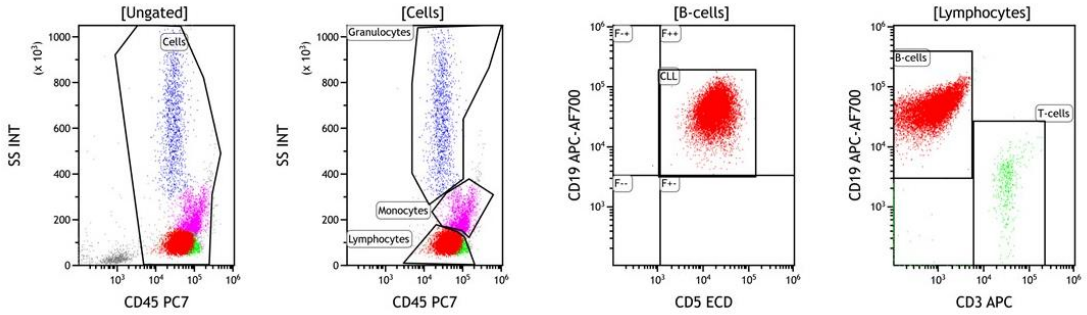

Figure 2. Determination of the proportion of CLL cells.. The proportion of CLL cells was assessed by using anti-CD45, anti-CD19, anti-CD5, and anti-CD3 antibodies. Live cells (Cells), lymphocytes, monocytes and granulocytes were identified using side scatter (SSC) and CD45 dot plots.B-cells (B-cells) were then identified based on their high CD19 and low CD3 expression. Finally, the proportion of CLL cells among CD19 positive lymphocytes was assessed by CD5 expression (CLL). The proportion of CLL cells among B-cells was over 98% in each sample, so CLL cells were considered to be B-cells.

In addition to CD86, several other markers were examined in our second and third studies. These were such surface markers whose expression might influence the course of the disease (see **Table 3**). In these studies, the proportion of CLL cells was assessed by using anti-CD45, anti-CD19, anti-CD5, and anti-CD3 antibodies. Lymphocytes, monocytes, and granulocytes were identified using side scatter (SSC) and CD45 dot plots. B-cell and T-cell proportions among lymphocytes were calculated based on CD19 and CD3 expression. Finally, the proportion of CLL cells among CD19 positive lymphocytes was assessed by CD5 expression. The ratio of CLL cells to B-cells was over 98% in each sample, therefore B-cells were considered as CLL cells in the subsequent analysis **(Figure 2)**.

Due to the large number of markers studied and the small number of samples available, it was not possible to perform measurements with isotype controls from each sample; however, the feasibility and specificity of the antibodies were tested. Therefore, we had to establish a novel gating strategy using internal controls to calculate the MFI value. According to our measurements, the CD19 negative lymphocyte population was suitable as an internal negative control for CD69, CD86 CD184, and CD185. For CD27,

the granulocyte population was used as an internal negative control. To calculate the relative MFI value, the MFI value of the internal controls was subtracted from the MFI value of B-cells to determine the relative expression of specific markers **(Figure 3)**.

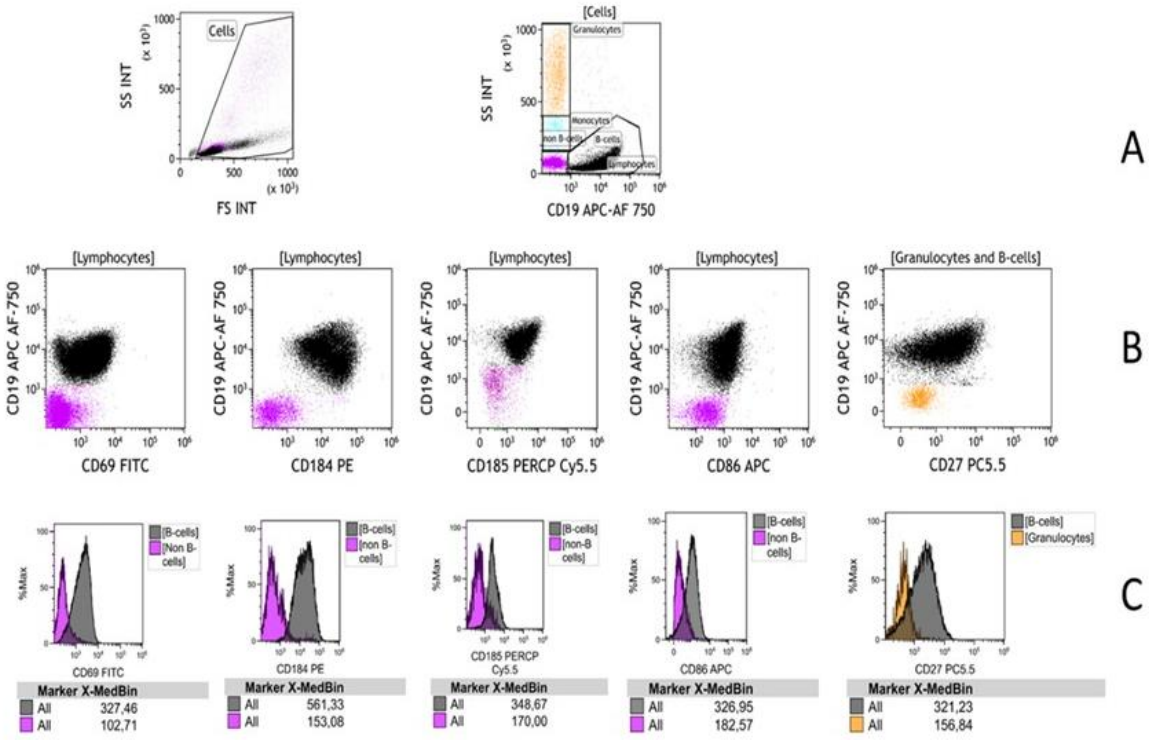

Figure 3. Gating strategy and determination of MFI values. Live cells (Cells), lymphocytes, monocytes, granulocytes were identified by their forward scatter (FSC) and side scatter (SSC) properties, and B-cells by CD19 expression (B-cells) (A). According to our measurements, the CD19 negative lymphocyte population (purple) was suitable as an internal negative control for CD69, CD184, CD185, and CD86. For CD27, the granulocyte population (orange) was used as an internal negative control (B). To calculate the relative MFI value, the MFI value of CD19 negative lymphocytes was subtracted from the MFI value of B-cells (black) to determine the relative expression of CD184, CD185, CD69, and CD86. In the case of CD27, the MFI value was calculated by subtracting the MFI value of granulocytes from the MFI value of B-cells (C).

3.3. CLL MRD Measurement by Flow Cytometry

In our second study, by the 12th month of venetoclax treatment, the number of CLL cells in the V1, V3, V4 patients had decreased dramatically, so we were no longer able to determine surface marker expression and had to start measuring MRD. The stain-lyse-wash technique was also used to prepare the samples for MRD measurement, but the initial sample volume was 300 µl. The 300 µl PB samples were incubated with the antibodies against surface epitopes for 13 minutes at 4°C (the antibodies are listed in **Table 4)**.

Table 4. Overview table of antibodies used in our MRD measurements.

Laser	***Violet*** *405nm, 40mW*			***Blue*** *488nm, 40mW*				***Red*** *640nm, 40mW*	
Fluorescent channels	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9
Panel 1	**Syto40** (Invitrogen)	**CD5 BV510** (L17F12, Sony)	**CD3 BV605** (UCHT1,Sony)	**CD81 FITC** (JS-81, BD)	**ROR1 PE** (2A2,BC)	**CD79b PERCP-Cy5.5** (3A2-2E7,BD)	**CD19 PC7** (J3-119, BC)	**CD43 APC** (1G10, BD)	**CD20 APC-AF750** (B9E9, BC)

Invitrogen: Invitrogen, Waltham, MA, USA, BV510: Brilliant Violet™ 510, Sony: Sony, Minato City Tokyo, Japan, BV605: Brilliant Violet™ 605, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, *PERCP-Cy5.5: Peridinin-chlorophyll-Cyanine5.5, PC7: Phycoerythrin cyanine 7, APC: Allophycocyanin, APC-AF750: APC- Alexa Fluor™ 750.*

The MRD panel (ROR1, CD81, CD5, CD20, CD43, CD79b) was designed according to the ERIC (European Research Initiative on CLL) guidelines (79). Samples were then lysed using BD FACS™ Lysing Solution (BD) for 15 minutes. Prior to measurement all samples were washed twice (10 min, 400 g, room temperature) with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH = 7.4). All antibodies used were pre-titrated, fluorescence staining was performed in the dark, and stained samples were protected from light. Finally, samples

were measured using a 10-colour FACSLyric^{TM} flow cytometer (BD). Instrument settings were checked regularly using BD^{TM} CS&T Beads (BD). A minimum of 1,000,000 events were measured from each sample to achieve the desiredlevel of sensitivity. The data were analysed using Kaluza 2.1.1 software (BC), and a gating strategy was established to determine the MRD level **(Figure 4)**.

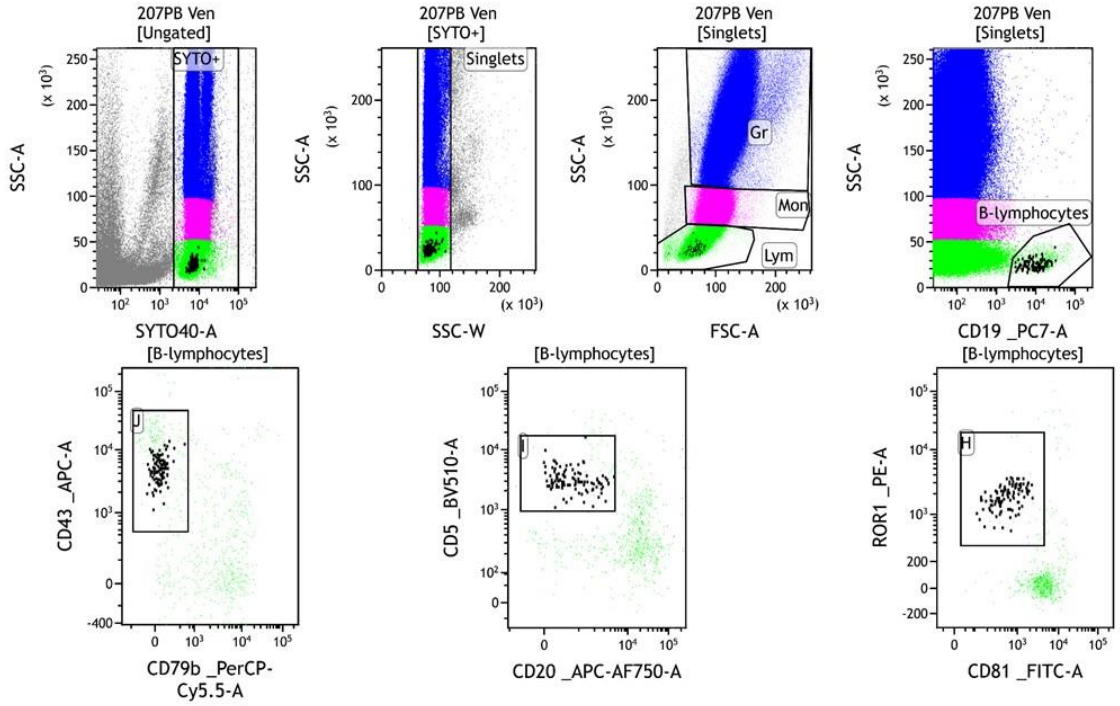

Figure 4. The flowchart shows the gating strategy used to determine CLL MRD. First, the SYTO-positive events were gated from the more than one million events measured. Then, the singlets were selected from the SYTO-positive events. Next, the granulocyte (blue), monocyte (purple) and lymphocyte (green) populations were gated based on their forward scatter. Next, the B- and T-lymphocyte populations were gated based on CD19 or CD3 expression. Finally, the CD5-positive CD81-negative CD43-positive CD79b-negative and CD5-positive CD20- negative cells were considered as CLL cells (marked black).

3.4. Apoptosis Array

In our second study, we wanted to know if there was a change in the expression of the apoptotic or anti-apoptotic proteins in the case of VEN resistance in our second study, so we performed an apoptosis array on the original PB of the V2 patient and the resistant PB and BM samples to address this question. The proportion of CLL cells among the mononuclear cells was assessed by flow cytometry and was greater than 85% in each sample. CLL cells were isolated by density gradient centrifugation using Ficoll-Histopaque-1077 (Sigma Aldrich, Saint Louis, Missouri, USA). Apoptosis array (R&D Systems Minneapolis, Minnesota, USA, Human apoptosis antibody array kit) was performed on the mononuclear cell fraction of the samples according to the manufacturer's instructions. The Apoptosis array kit used was a membrane-based sandwich immunoassay. Antibodies were spotted onto a nitrocellulose membrane to bind to the specific target proteins. The captured proteins were detected using biotinylated detection antibodies and visualised using chemiluminescent detection reagents. The signal generated was proportional to the amount of the protein. Analysis was performed using ImageJ software version 1.50d (National Institutes of Health, Bethesda, Maryland, USA), and the pixel density was calculated by the software. The value of the pixel density correlated with the the level of proteins in the cells. SigmaPlot software was used for data visualisation.

3.5. Molecular Methods

The presence of the Bcl-2^{D103Y} resistance mutation was tested in the venetoclax-resistant peripheral blood and bone marrow samples of the V2 patient. The *BTK*^{C481S} resistance mutation was detected in the IS and IR samples by high-sensitivity digital droplet PCR (ddPCR) (Bio-Rad Laboratories, Hercules, California, USA). In each sample, 100ng of input DNA was used, and all reactions were carried out according to the manufacturer's instructions. Droplets were generated using the QX200 Automated Droplet Generator (Bio-Rad Laboratories), followed by fluorescence signal detection using the QX200 Droplet Reader System (Bio-Rad Laboratories). Results were evaluated and quantified using the Bio-Rad QuantaSoft software (version 1.7) **(Figure 5.)** (66, 67, 98).

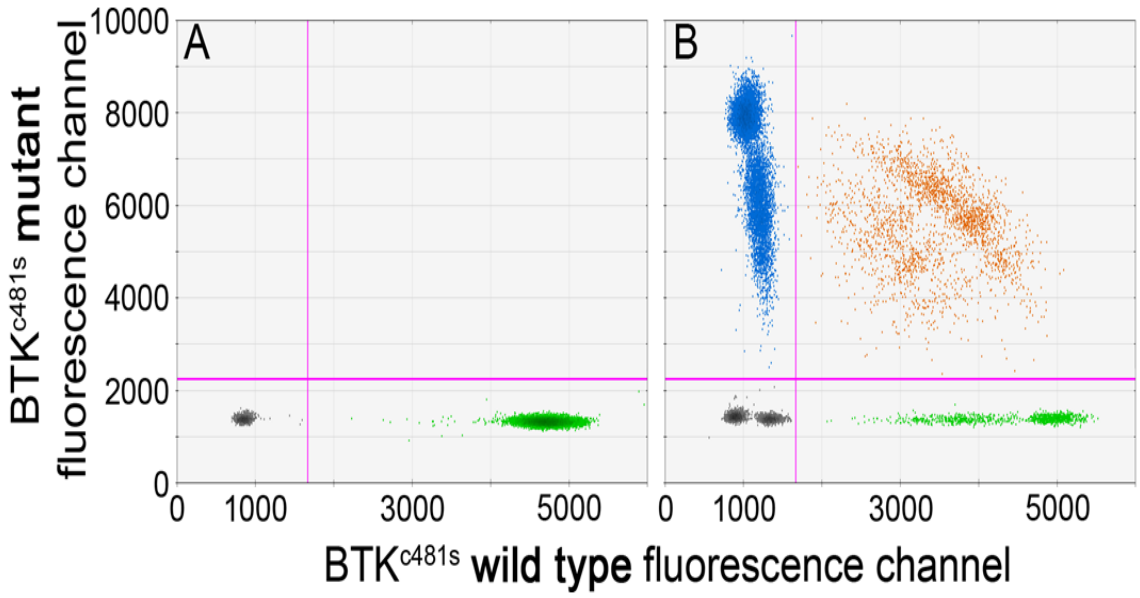

Figure 5. Detection of the BTKC481S and Bcl-2G101V mutations by digital droplet PCR. The BTK^{C481S} mutation negative (A) and BTK^{C481S} positive (B) cases are shown in these representative dot plots. The variant allele frequency (VAF) cut-off value was 0%. Samples were considered BTK^{C481S} positive if the detected VAF was greater than 0%. The green dot population represents the droplets containing only wild-type DNA. Orange dots represent droplets containing both wild-type and mutant DNA, while the blue dot population represents droplets containing mutant DNA only. Grey dots represent empty droplets containing water. VAF was calculated in each sample as the ratio of droplets containing mutant DNA (blue) to droplets containing wild-type DNA molecules (green). The same method was used to calculate the Bcl-2^{G101V} VAF.

The variant allele frequency (VAF) cut-off was 0%. Samples were considered positive for the mutation if it was detected at a VAF higher than the 0% cut-off. The *BTK*^{C481S} mutation status of all patients in the IR group was published previously by Bödör et al. (98).

3.6. Statistical Analysis

SigmaPlot 12.5 (Systat Software Inc. San Jose, CA, USA) was used for plotting and statistical analysis. All variables were tested for normal distribution in order to select the appropriate parametric or non-parametric statistical test. Normality (Shapiro-Wilk) and equal variance tests were performed respectively, followed by Kruskal-Wallis test, one way ANOVA test, Tukay test, and Mann–Whitney U test based on their results. Differences were considered statistically significant at $p<0.05$.

3.7. Ethical Statement

The studies were conducted in accordance with the Declaration of Helsinki and were approved by the local ethics committee of Semmelweis University (TUKEB 7/2006) and the Hungarian Medical Research Council (ID:45371-2/2016/EKU). Patients enroled in our second and third studies were informed in writing and understood the details of the studies; in addition, voluntary written informed consent was obtained from all participants.

4. RESULTS

4.1. The effect of CD86 on CLL cells

In our first study, we wanted to clarify Huemers' findings (96), that CLL patients with a high CD86 expression have a worse disease outcome than patients with a low CD86 expression. To do this, we collected samples from 49 CLL patients. The samples were divided into a $CD86^{high}$ (14 samples) and a $CD86^{low}$ (35 samples) expression group based on the median fluorescence of the samples (the cut-off value was set at 7.9 MFI). We chose the patients' TTT value to estimate progression. Unfortunately, clinical data were only available for 18 patients (7 from the $CD86^{low}$ group and 11 from the $CD86^{high}$ group). We found that the mean TTT time was not significant ($p = 0.37$), but it was remarkably shorter in the $CD86^{high}$ group (714 days) than in the $CD86^{low}$ group (1325 days). Perhaps, the cohort of our patients was not large enough to obtain results with sufficient statistical power.

To summarise our results, we obtained a similar result to Huemer et al., namely that in our study the TTT was remarkably shorter in the $CD86^{high}$ cohort. This observation suggests that the increased CD86 expression on CLL cells may lead to an unfavourable disease outcome. For this reason, we have continued on our study to elucidate the potential effect of CD86 on the disease course of patients treated with novel targeted therapies.

4.2. A novel resistance mechanism in venetoclax treatment and its prediction

In our second study, we enrolled 4 venetoclax-treated patients (V1, V2, V3, V4) to investigate how the immunophenotype of CLL cells changes during the treatment. The VEN treatment of V1, V3, V4 patients proved to be so effective that they achieved the MRD negativity by the end of the first year, and in their case the CD86 expression level could no longer be measured **(Figure 6.)**.

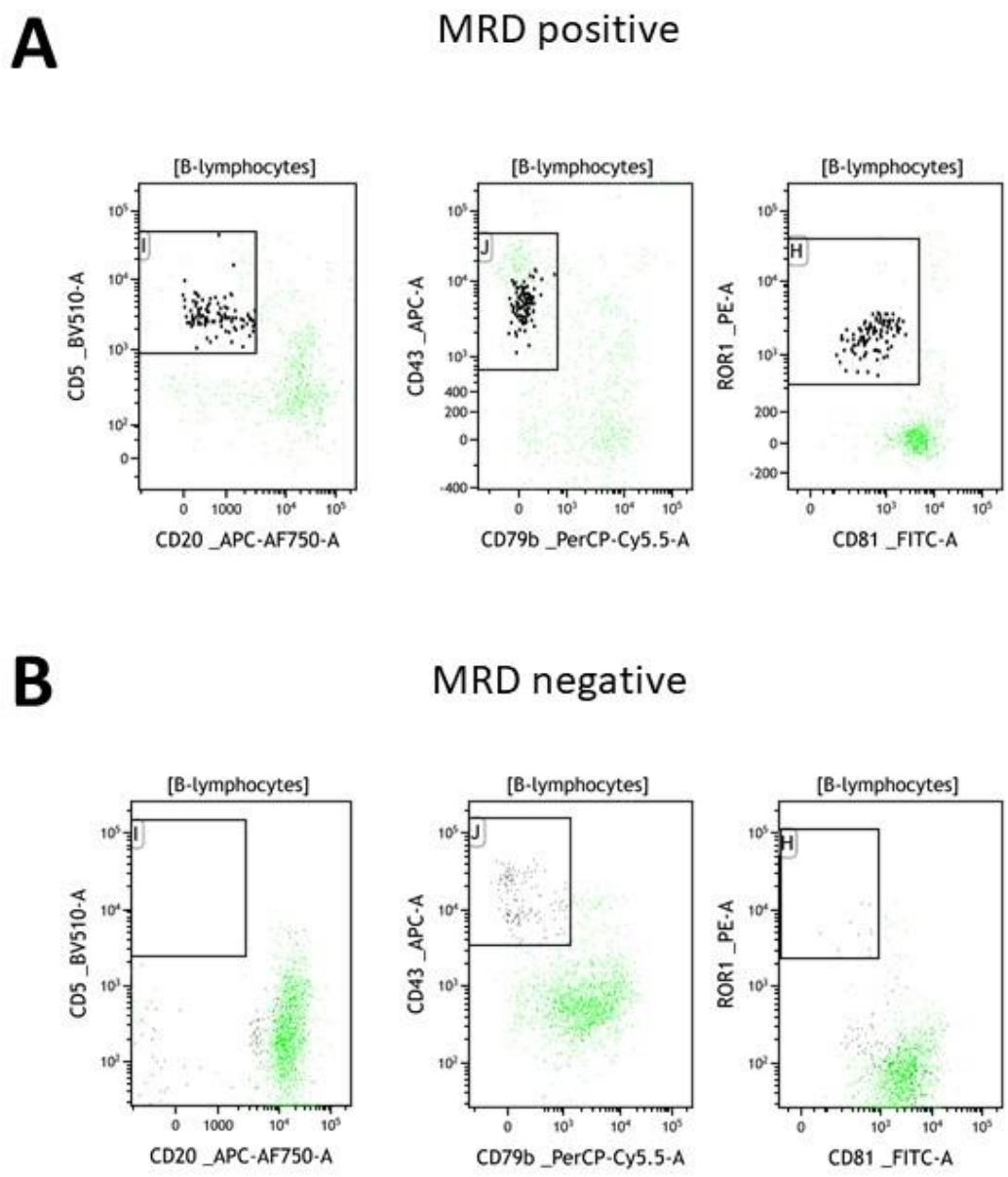

Figure 6. MRD detection in Venetoclax treated samples. The difference of the MRD positive and MRD negative samples is seen from two representative samples on this figure. Part A represents the MRD positive sample (CLL cells in black) while part B the MRD negative one.

The V2 patient had never achieved MRD negativity, so we were able to measure CD86 expression levels on days 0, 180, 270, 360 and 450, when VEN resistance occurred.

We observed that CD86 expression did not change remarkably until day 270 of treatment (day 0 159.38, day 180 150.85, day 270 153.65 MFI values), but then began to increase (day 360 208.07 MFI value), reaching its maximum level at day 450, when signs of clinical resistance occurred (day 450 250.83 MFI value). We also compared the CD86 expression of the CLL cells in the resistant PB and BM samples, but it was not notably different in these two compartments (PB 250.83 vs BM 253.26 MFI value) **(Figure 7.)**.

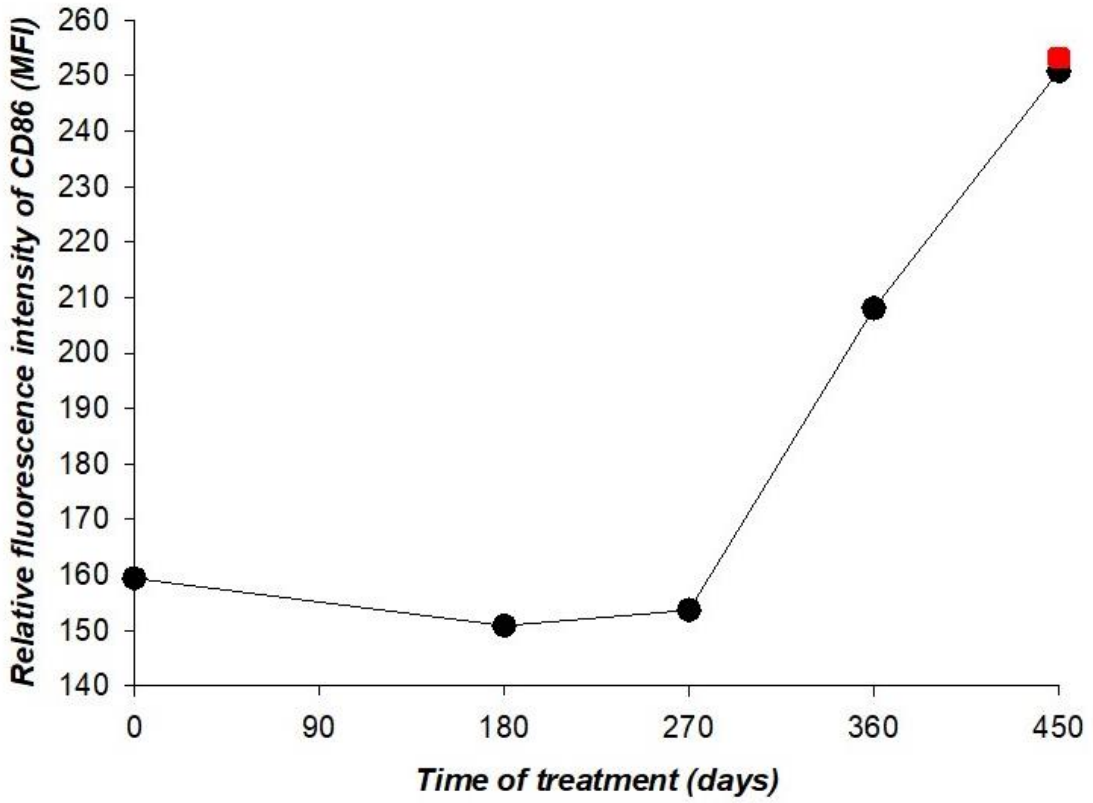

Figure 7. Changes in CD86 expression during venetoclax treatment. The CD86 expression of CLL cells from peripheral blood (black circle) or bone marrow (red square) of a follow-up CLL patient during venetoclax therapy was determined by flow cytometry.

We assumed that the change in CD86 expression was only a sign of emerging resistance, so we wanted to determine what factors might be involved in the emergence of clinical resistance. To this end, we examined the presence of the Bcl-2^{D103Y} resistance mutation, but this mutation was only identified at a very low variant allele frequency level in bone marrow (0.19%), and peripheral blood (0.53%) samples at the time of the resistance (on day 450 of VEN treatment). We assumed that if this mutation had caused the resistance in this case, the VAF level would have been much higher due to the

expansion of the dominant clones, and therefore we suggested that this mutation could not clearly explain the resistance.

We also examined the expression of several proteins involved in the apoptotic cascade to identify alternative resistance mechanisms. An apoptosis array was used to determine the levels of 33 apoptotic proteins in patient samples before the VEN treatment (day 0) and at the time of clinical resistance (day 450). We also compared the expression pattern of these proteins in the peripheral blood and bone marrow samples at the time of VEN resistance to detect potential compartmental differences. We found a transient difference in the expression of the anti-apoptotic Bcl-XL (day 0 PB 176.66 vs. day 450 PB 148.36 vs. day 450 BM 219.78 pixel density), but an increased expression of Bcl-2 was detected in the resistant samples (day 0 PB 1797.82 vs. day 450 PB 3566.64 vs. day 450 BM 2943.55 pixel density). In peripheral blood and bone marrow samples, there was a slight decrease in the level of proapoptotic BAD (day 0 PB 589.51 vs. day 450 PB 521.89 vs. day 450 BM 498.39 pixel density) and also in the level of BAX (day 0 PB 850.70 vs. day 450 PB 716.08 vs. day 450 BM 843.28 pixel density).

Interestingly, the expression of the anti-apoptotic XIAP (day 0 PB 2411.33 vs. day 450 BM 3687.27-pixel density) increased (1.5-fold) in the bone marrow compared to the initial peripheral blood sample. We also found that there was a 1.9-fold difference in expression of XIAP between the resistant BM and PB samples (day 450 PB 1940.43 vs. day 450 BM 3687.27 pixel density) **(Figure 8.)**.

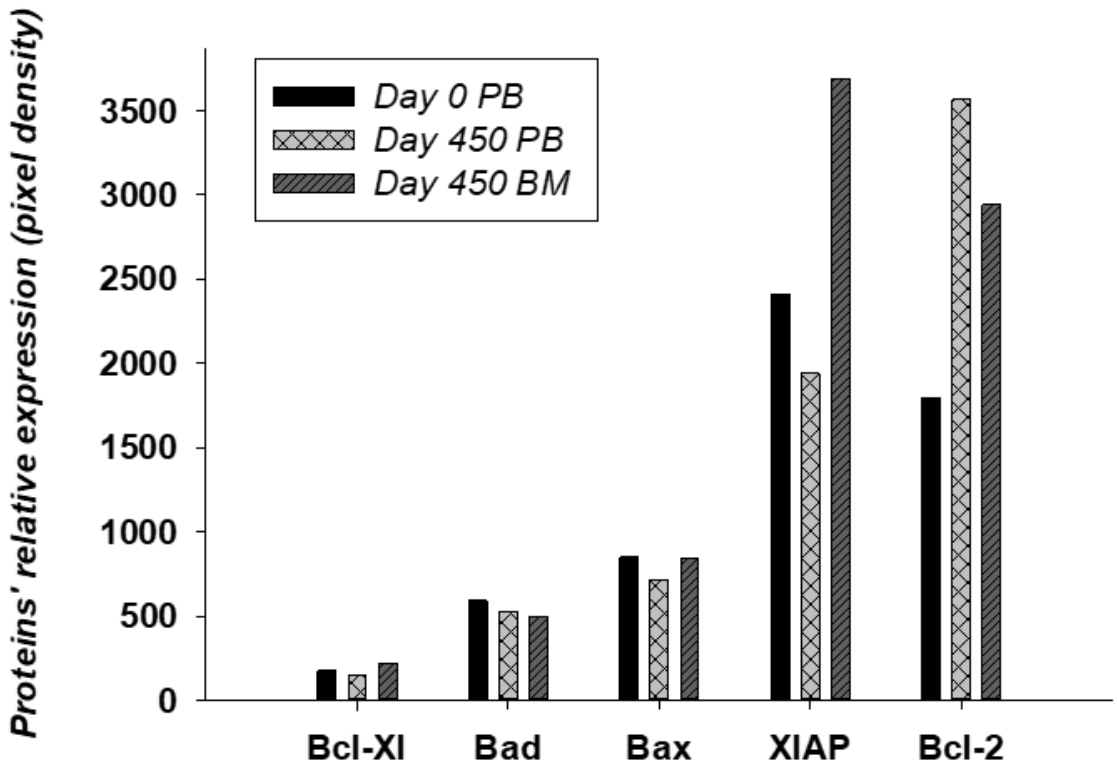

Figure 8. The levels of apoptotic proteins changed at the onset of venetoclax resistance. The chart shows the difference in protein levels in the samples from the V2 patient as measured by apoptosis arrays. Day 0 PB represents the peripheral blood (PB) sample at the start of venetoclax treatment, while day 450 PB and BM represent peripheral blood and bone marrow (BM) samples at the time of clinical resistance.

To summarise the results of our case-study, although several different resistance mechanisms may have evolved in CLL cells to evade the effect of venetoclax, CD86 may be able to detect them and predict resistance several months before it occurs clinically.

4.3. Revealing a Phenotypical Appearance of Ibrutinib Resistance

In our third study, we wanted to determine whether CD86 and several other surface markers have an impact on the disease outcome of CLL patients treated with ibrutinib. In this study, we compared the immunophenotypes of treatment-naïve (Co), ibrutinib-sensitive (IS) and clinically ibrutinib-resistant (IR) cohorts of CLL patients. We observed that CD27 and CD86 showed significant differences in some cohorts. CD27 expression was significantly lower in the IS group compared to both the Co group (IS vs. Co: 51.136 vs. 172.709 MFI values, $p = 0.020$) and the IR group (IS vs. IR: 51.136 vs. 156.341 MFI

values, p = 0.030). When comparing the expression levels of CD86 between the IS and Co groups (IS vs. Co 27.23 vs. −29.308 MFI values, p = 0.052), the detected values tended to be significant. In addition, CD86 expression was significantly higher in the IR group compared to the IS group (IR vs. IS 97.788 vs. 27.23 MFI values, p = 0.031) **(Figure 9.)**.

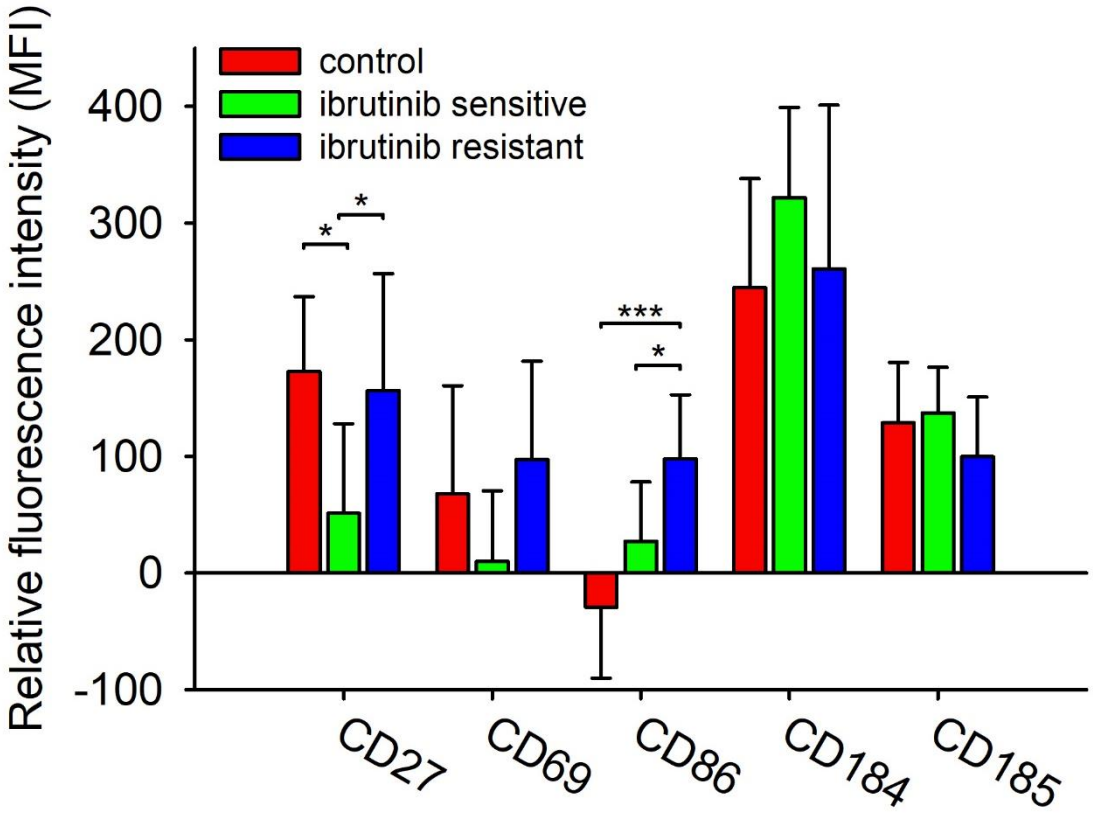

*Figure 9. Immunophenotype of CLL cells in different treatment cohorts. The expression level of five different surface markers (CD69, CD184, CD27, CD86, CD185) in three different cohorts (treatment-naïve n = 10, ibrutinib-sensitive n = 7, ibrutinib-resistant n = 11) was measured by flow cytometry. Relative median fluorescence intensity (MFI) values were calculated as the difference between the MFI value of internal negative controls and B-cells. ANOVA or Kruskal-Wallis test with Holm-Sidak post hoc test was used for statistical evaluation. *p < 0.05; ***p < 0.01.*

After the venetoclax resistance was confirmed in the V2 patient, treatment with ibrutinib was started. We continued to follow this patient to observe the potential change

in the expression pattern of the CD27 and CD86 markers. We used the initial expression level (day 0) of the CD27 and CD86 as a benchmark. Initially, clinical remission was observed, and the expression of both markers decreased, reaching its lowest level on day 120. From then on, the expression of CD27 and CD86 tended to increase, exceeding the benchmark on day 330 (MFI values on day 330 vs. day 0: CD27: 218.26 vs. 200.42, CD86: 213.85 vs. 192.56). The sign of the clinical resistance, appeared on day 420, and the expression of CD27 and CD86 reached its maximum level on day 420 (MFI values on day 420: CD27: 240.57, CD86: 304.41) **(Figure 10.)**.

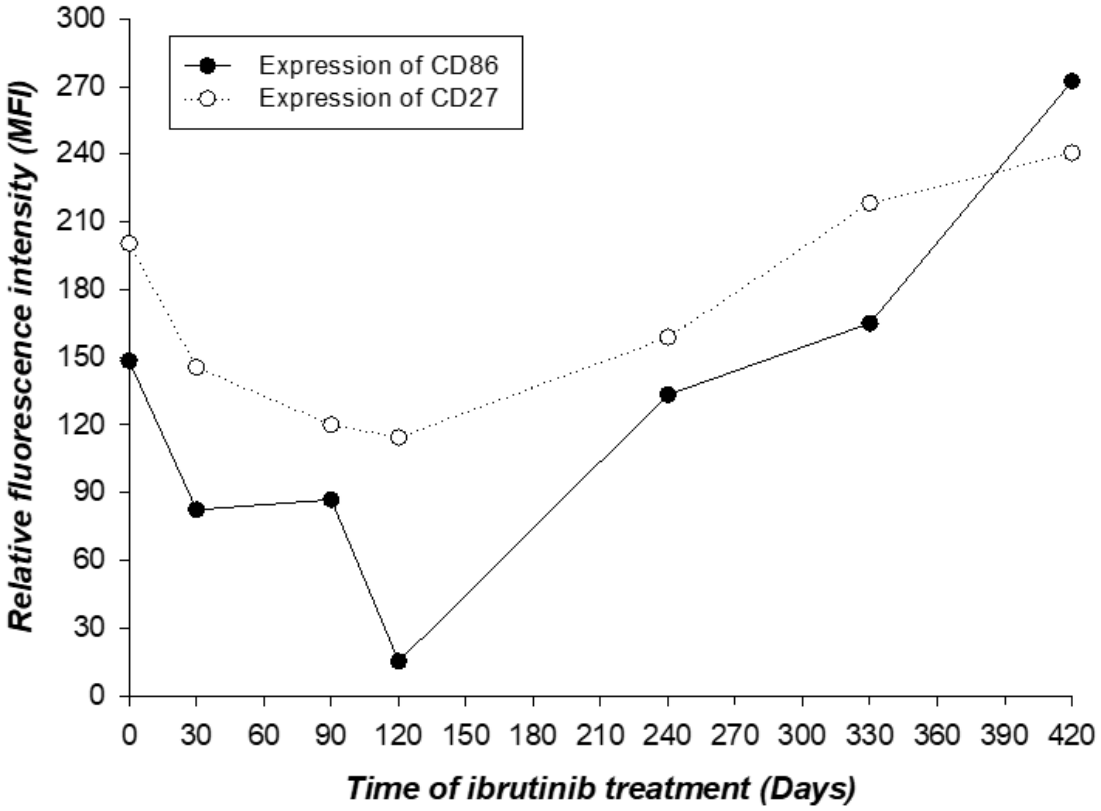

Figure 10. Changes in CD27 and CD86 expression on CLL cells during ibrutinib monotherapy. Relative expression levels of CD27 (dotted line) and CD86 (black line) were determined during ibrutinib treatment in a peripheral blood sample from a patient who became resistant on day 420 of tretament.

We reasoned that if the elevated CD86 expression level is indicative of drug resistance, similar to venetoclax treatment, and the the molecular background of CLL cells may also be involved in emergence of resistance, then there may be a relationship between the CD86 expression and the BTK^{C481S} resistance mutation of the CLL cells. Therefore, we assessed

the BTK^{C481S} mutation status of the clinically ibrutinib-resistant patients and compared it with the expression levels. We found that 64% of ibrutinib-resistant cases (7/11) harboured the BTK^{C481S} mutation, and CD86 expression was significantly higher in the BTK^{C481S} mutant samples than in the wild-type samples (BTK^{C481S} mutant vs. BTK^{C481S} wild-type 134.28 vs. 33.92 MFI values, $p < 0.001$). The CD27 expression level of resistant cases was also associated with the BTK^{C481S} mutation status of the CLL cells. We found that CD27 expression was also significantly higher in cases with the BTK^{C481S} mutation. However, the data showed a rather large scatter (CD27 expression of BTK^{C481S} mutant vs. BTK^{C481S} wild-type cases 205.283 vs. 70.692 MFI values, $p = 0.011$) **(Figure 11.)**.

Analyzing the results of our third study, we concluded that both CD86 and CD27 may be suitable markers of ibrutinib resistance.

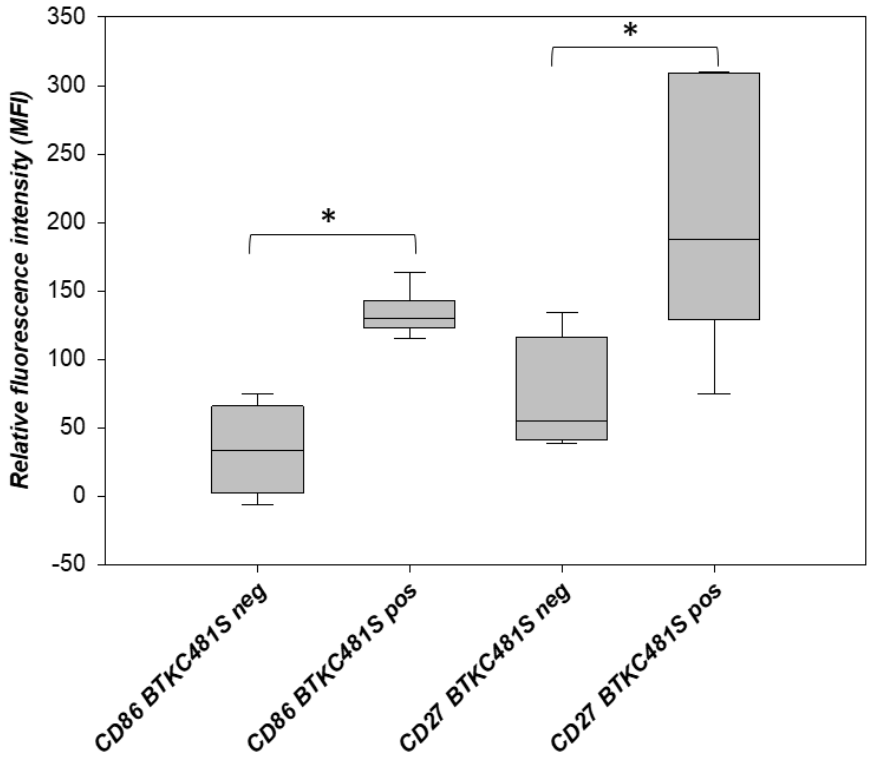

*Figure 11. The phenotypic difference between CLL samples with wild-type or mutated BTK gene. Relative expression of CD86 and CD27 surface markers and the BTK^{C481S} mutation status in the clinically ibrutinib-resistant patients (n = 11) were confered. T-test or Mann-Whitney-test was used for statistical analysis. *p < 0.05.*

5. DISCUSSION

In the 21st century, every area of human life has become increasingly personalised and customised, and oncology is no exception. The scientific community has long dreamed of personalised therapy. Predicting disease progression and finding the right treatment for the right patient can take us a step closer to that goal. For this reason, the search for prognostic and predictive factors has recently become a focus of research interest. Although several genetic lesions have been described by characterising the CLL genome, leading to the discovery of novel prognostic and predictive factors, several institutions are not equipped to detect them. Therefore, the search for more easily detectable markers remains an important task. For this reason, we attempted to discover or rediscover prognostic or predictive markers in CLL that could be investigated by flow cytometry and could also be used as surrogate for molecular markers.

Based on the findings of Huemer et al., CD86 seemed to be a promising target of interest to us. According to their results, high CD86 expression on CLL cells did not only correlate with a higher proliferation rate but it was also associated with an unfavourable disease outcome. They also showed that CD86-positive CLL cells are enriched for DNA double-strand brakes (96). Regarding the prognostic value of CD86, we agree with Huemer et al., because according to our results, patients with CD86-positive CLL cells required earlier treatment than patients with CD86-negative CLL cells. Although, we did not find statistically significant difference, this may be due to the small number of patients. On the other hand, the difference between TTT of the $CD86^{high}$ and $CD86^{low}$ cohorts was remarkably high, suggesting that CD86 may have a prognostic value.

After evaluating the results of our first study, our interest turned to finding surface markers of CLL cells that could be widely used as predictive factors for treatment with ibrutinib or venetoclax. Our candidates included known prognostic factors (CD49d, CD38), potential prognostic factors (CD86, CD69), and other microenvironmental factors (CD184, CD197, CD44, CD40, CD185, CD27, ROR1). After some preliminary experiments failed due to a lack of reliable internal controls, we had to narrow down our candidate list to CD69, CD184, CD185, CD27 and CD86. CD184 and CD185 are known as chemokine receptors that are highly involved in the regulation of B-cells migration (99, 100). The prognosis of CLL can be predicted by measuring the expression of CD184 (101-103), and CD185 can intervene in BCR signalling (104), so they seemed to be

promising targets for investigation. However, our studies did not show any significant correlation between their expression and IBR or VEN treatments. The expression of CD27 may reflect the activation status of B-cells and could therefore be considered as an activation marker (83, 105). The question may arise: if this marker behaves as an activation marker, could it influence the effect of ibrutinib through BCR signalling? Gobessi et al. showed that BCR pathway activity can be increased in Zeta Chain of T Cell Receptor Associated Protein Kinase 70 (ZAP70)-positive CLL cells (106). Furthermore, Lafarge et al. observed that the CD27 expression was increased in ZAP70-positive CLL cells (107). These observations may suggest that increased CD27 expression on CLL cells may be associated with increased BCR activity, making CD27 a potentially useful marker of ibrutinib efficacy. However, this hypothesis was not easily put into practice as the results seemed to be contradictory. Shen et al. observed that CD27 expression on CLL cells increased after in vitro treatment with ibrutinib (108), whereas Rendeiro et al. found that CD27 expression decreased after the same treatment (109). We found the same results as Rendeiro et al., but the conditions were different. CD27 expression on CLL cells was significantly lower in the ibrutinib-treated cohort than in the treatment-naïve cohort in our investigated clinical samples. In addition, we compared CD27 expression on CLL cells in the ibrutinib-resistant and the ibrutinib-sensitive cohorts and found a significantly higher level of CD27 in the ibrutinib-resistant cohort. This observation is consistent with the results of our molecular study, as the CLL cells with high CD27 expression are more likely to harbor the BTK^{C481S} resistance mutation. Another important question is whether or not CD27 expression is able to predict the treatment failure. In general, a decision to change treatment is not based on the change in expression of one marker at one point in time. Clinicians usually look at the trend of the marker's expression level alongside the patients's clinical status. And if one or the other changes, it may trigger a change in treatment. Therefore, it is very important to consider the tendency of a marker's expression to change the treatment. Our case study showed that the tendency of CD27 expression on CLL cells changes along with the patient's clinical status. Its expression decreased when ibrutinib treatment seemed to be effective and increased when the clinical resistance emerged, suggesting that CD27 may work well as a predictive marker for ibrutinib treatment.

Based on our observations, CD86 may also be a promising candidate as a predictive factor for ibrutinib treatment. Its expression on CLL cells is significantly lower than on non-malignant B-cells (93), whereas it is increased on activated B-cells (110), suggesting that CD86 may be a marker of CLL cell activation (92, 111). According to Huemer et al., higher CD86 expression may be associated with worse disease outcome (96), and this was also observed in our study. Similar to CD27, the question may arise as to whether a so-called activation marker could interfere with the effect of ibrutinib or not. Herman et al. showed that the expression of CD86 was reduced by ibrutinib treatment (112). We measured that CD86 was almost statistically significantly higher in the ibrutinib-sensitive group than in the control group. Regarding Herman's work, our results may be controversial, but it must be considered that our control cases were only samples of treatment-naïve patients, whereas Herman's cohort was mixed, as it included treatment-naïve and pre-treated patients. Therefore, chemo-immunotherapy could explain the contradiction between our results. In addition, we observed in our case study that the ibrutinib decreased the expression level of CD86 during the first 3 months of treatment in a patient who had been pretreated with chemotherapy, which tends to confirm Herman's observation. Another question that may be worth considering is whether CD86 is able to predict not only the effect of ibrutinib but also the resistance. We found that the expression of CD86 on CLL cells was higher in ibrutinib-resistant samples compared to ibrutinib-sensitive patient samples, and this result suggests that the higher CD86 expression could be a sign of ibrutinib resistance. As we mentioned earlier, a decision to change treatment is not usually based on a single measurement of a marker, so we also needed to look at how CD86 expression changes during treatment. Our case study showed that CD86 expression started to increase continuously after reaching its lowest level. It then peaked at the onset of clinical resistance. Furthermore, similar to CD27, CD86 expression levels exceeded baseline levels as early as 3 months before the onset of the clinical resistance. When we looked at the result of the BTK^{C481S} mutation study, we came to the same conclusion as with CD27. In ibrutinib-resistant patients, CD86 expression was significantly higher in BTK^{C481S} mutant samples than in the wild-type samples. This suggests that CD86 may also be a predictive marker for ibrutinib treatment.

At this point we have to mention some limitations of our studies. From my point of view, the biggest weakness of the trials is the small number of patients. In fact, ibrutinib

has become so successful in the treatment of CLL with an overall responserate of more than 85% (113) that it was difficult to find such patients who did not respond to the treatment. This was particularly the case during the period when ibrutinib was not widely used in Hungary. On the other hand, the study period was overshadowed by the COVID pandemic, and we did not want to enrol patients whose ongoing COVID infection could interact with ibrutinib treatment, as ibrutinib treatment can interact with COVID infection (114-116). Finally, the COVID pandemic has kept many clinicians away from providing us with clinical data.

Now, that we may have two equally useful markers for predicting the ibrutinib resistance, another question may arise. Can either CD27 or CD86 be used more widely as a predictive marker for venetoclax? In fact, CD86 was a better predictor factor of venetoclax treatment than CD27. We measured the expression of several surface markers, including CD27, during the venetoclax monotherapy, and only the change in CD86 expression was remarkable. Up to day 270 of treatment CD86 expression did not change remarkably but after reaching its lowest level (similar to ibrutinib treatment) it started to increase steadily, reaching its maximum level at the onset of clinical resistance (**Figure 12.)**.

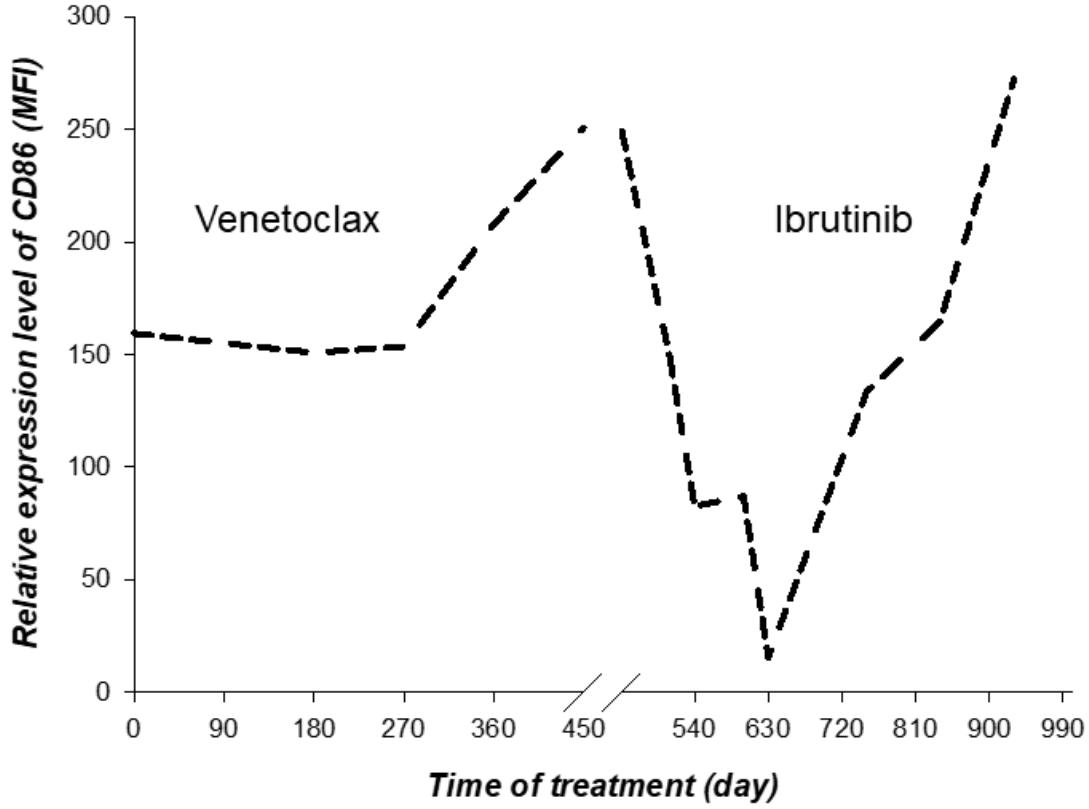

Figure 12. Changes in CD86 expression during venetoclax and ibrutinib monotherapy. The relative expression levels of CD86 were determined in the patient's peripheral blood sample during treatment. The patient became resistant to venetoclax on day 450 of treatment, necessitating the initiation of ibrutinib monotherapy. After 420 days of treatment, the patient became resistant to ibrutinib, which was accompanied by increasing CD86 expression.

In this case, we were able to address the question of whether CD86 expression differed between the resistant peripheral blood and bone marrow samples. We did not find any notable expression differences between the two compartments. This observation may seem marginal because bone marrow samples are not often tested in CLL. This is mostly done to exclude Richter's transformation of the disease or as part of the clinical trials (10). Nevertheless, it may be useful to have such a marker whose PB expression relates to what is happening in the BM. Based on the results of our apoptosis array, CLL cells may evade the effect of venetoclax in different ways in different compartments. While XIAP had a more pronounced effect on resistance in the BM, Bcl-2 was more effective in the PB. However, the expression of Bcl-2 increased in both compartments compared

to the first sample. Our observation could be considered limited as it is based on only one case study, but the results of Elias et al. confirmed our observation as they found that venetoclax-resistant CLL cells can be characterised by their increased CD86 expression and in these cells increased Bcl-2 levels can also be shown (117). Although Smith et al. mentioned that measuring Bcl-2 family member proteins by flow cytometry could be a useful tool to tailor antiapoptotic treatment (118), Czeti et al aptly pointed out that there are numerous pitfalls in measuring an intracellular marker by flow cytometry (119). Therefore, we believe that CD86 as a surface marker may become sooner a predictive marker of venetoclax treatment than Bcl-2 as an intracellular protein. Regarding our MRD results, 3 out of 4 patients achieved MRD negativity with venetoclax treatment during our observation period. This is a remarkably higher rate than what we found in the literature for venetoclax monotherapy (74). In addition, we observed, in line with the literature, that MRD negativity conferred a higher chance of achieving durable remission (46, 120, 121). However, it should be noted that our cohort was extremely small, so it is not possible to draw any significant conclusions regarding these results.

Finally, we would like to draw attention to our other interesting observation. We found that increased CD86 expression is often associated with resistance mutations, such as BTK^{C481S} and $Bcl\text{-}2^{D103Y}$. A plausible explanation could be that the CD86-positive CLL has a higher incidence of DNA damage due to high AID activity (96), and this could lead to an increased mutational burden, which could explain why these mutations occur more frequently in CD86-positive CLL cells.

In the field of oncology, the 21st century has so far been the era of molecular biology. An enormous number of gene mutations have been discovered, which may bring mankind one step closer to personalised medicine. Most notably, human cancer genes have been sequenced (122, 123), next-generation sequencing (NGS) has become more widely available and less expensive worldwide (124), and CLL has become a potentially curable disease (120, 125, 126). But are we there yet? Unfortunately, not. NGS is not omnipotent. It has weaknesses, one of which is its massive demand on bioinformatics resources (127). On the other hand, tumour cells have not stopped evolving, and are still developing new mechanisms to evade the effects of drugs. The key to overcoming these mechanisms is not always readily apparent from the genes (128, 129). As we have shown, venetoclax resistance may be associated not only with the Bcl-2^{D103Y} mutation but also with increased expression of anti-apoptotic proteins. On the other hand, the detection of the BTK^{C481S} mutation can predict the occurrence of ibrutinib resistance, but there are patients who show progression despite the absence of this mutation (62, 98, 130). So we can say that detecting a mutation is not always enough to detect resistance and to prevent relapsing.The solution may be to use different methods at the same time, as VEN-IBR combination therapy seems to be more effective in overcoming CLL than either therapy alone (131, 132). It could be tested whether a combination of flow cytometry and molecular techniques could provide earlier results in emerging resistance in a less costly way. For this purpose, we have established a potentially useful algorithm below (**Figure 13.**).

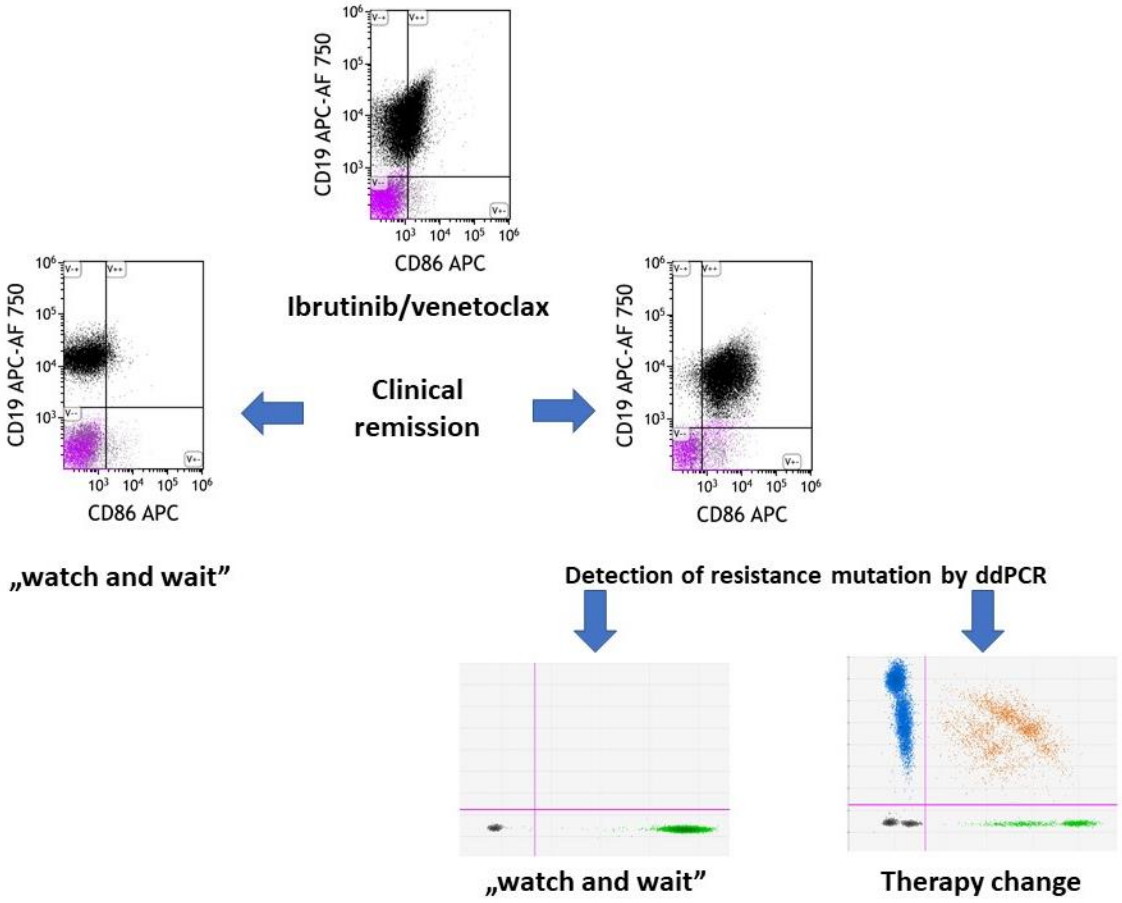

Figure 13. A potentially useful decision algorithm for the early detection of ibrutinib or venetoclax resistance by using flow cytometry and digital droplet PCR. During the treatment with ibrutinib or venetoclax, monitoring CD86 expression could guide the treatment-making decision in the absence of clinical resistance. If CD86 expression is decreasing and there is no resistance mutation, a "watch and wait" approach can be used. Then, if CD86 expression increases, resistance mutation detection should be performed. If the mutation status is negative, the "watch and wait" approach can be used, whereas if it is positive, the therapy should be changed.

6. CONCLUSIONS

As a conclusion, we can make the following new statements:

1. The expression of CD86 is increased in the case of venetoclax resistance.
2. There is connection between the increased Bcl-2 protein level and increased CD86 expression of the venetoclax resistant CLL cells.
3. The expression of CD27 and CD86 is decreased due to ibrutinib but both expressions are increased in the case of ibrutinib resistance.
4. The expression of CD27 and CD86 is increased in case of the occuring of BTK^{C481S} resistance mutation in CLL cells.
5. CD86 seems to be a suitable predictive marker of ibrutinib and venetoclax treatment in CLL, since its increased expression can foreshadow a potential ibrutinib and venetoclax treatment failure. Moreover, measuring its expression during ibrutinib or venetoclax treatment can be a surrogate method of revealing the resistance in case of the lack of the appropriate molecular tests.

7. SUMMARY

CLL is an indolent and incurable disease; however, the life expectancy of patients has increased dramatically in recent years due to the development of more accurate diagnostic procedures and novel therapeutic agents. However, finding the right treatment for the right patient is difficult without prognostic or predictive factors. Therefore, our studies aimed to find novel prognostic or predictive factors, focusing on the major role of CD86. In our studies, we wanted to investigate whether the CD86 expression in CLL has any influence on the course of the disease and whether the expression of CD86 or other microenvironmental markers can predict drug resistance in the case of ibrutinib or venetoclax treatment or not.

Our most important discovery was undoubtedly the discovery of a link between the resistance to two very commonly used and highly effective drugs and some surface markers of CLL cells. Although the Bruton's tyrosine kinase inhibitor ibrutinib and the Bcl-2 inhibitor venetoclax act on different targets, both have been proved to be effective in treating CLL. In both cases, drug resistance can occur, leading to treatment failure. Our studies have shown that the expression of CD27 and CD86 is reduced by ibrutinib treatment, and the expression of both markers increases in the case of ibrutinib resistance. In addition, venetoclax decreases CD86 expression in CLL cells, and venetoclax resistance increases CD86 expression. In addition, we found an association between ibrutinib resistance mutation (BTK^{C481S}) and the CD86 expression of CLL cells; namely, its expression is increased in the presence of BTK^{C481S}.

In conclusion, CD86 appears to be a suitable predictive marker in CLL, as its increased expression may predict potential treatment failure with ibrutinib and venetoclax. Furthermore, measuring its expression during ibrutinib or venetoclax treatment may be a surrogate method to detect resistance in the absence of appropriate molecular tests.

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9. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Thesis is based on the following publication:

Revealing a phenotypical appearance of Ibrutinib Resistance in Patients with Chronic Lymphocytic Leukaemia by Flow Cytometry

***Ferenc Takács**, Lili Kotmayer, Ágnes Czeti, Gábor Szalóki, Tamás László, Gábor Mikala, Ágnes Márk, András Masszi, Péter Farkas, Márk Plander, Júlia Weisinger, Judit Demeter, Sándor Fekete, László Szerafin, Beáta Margit Deák, Erika Szaleczky, Adrienn Sulák, Zita Borbényi, Gábor Barna*

Pathology & Oncology Research DOI: 10.3389/pore.2022.1610659 (2022)

Identification of a novel resistance mechanism in venetoclax treatment and its prediction in chronic lymphocytic leukemia

***Ferenc Takács**, Gábor Mikala, Noémi Nagy, Andrea Reszegi, Ágnes Czeti, Gábor Szalóki, Gábor Barna*

Acta Oncologica DOI: 10.1080/0284186X.2021.1878388 (2021)

The role of minimal residual disease in chronic lymphocytic leukemia - a review (Hungarian)

***Ferenc Takács**, Ilona Kardos, Ágnes Czeti, Dóra Aczél, Sarolta Illés, Alexandra Balogh, Júlia Gaál-Weisinger, Gábor Szalóki, Gábor Barna*

Hematológia és Transzfuziológia DOI:10.1556/2068.2020.53.1.4 (2020)

The Effect of CD86 Expression on the Proliferation and the Survival of CLL Cells

***Ferenc Takács**, Csilla Tolnai-Kriston, Márk Hernádfői, Orsolya Szabó, Gábor Szalóki, Ágota Szepesi, Ágnes Czeti, András Matolcsy, Gábor Barna*

Pathology & Oncology Research DOI: 10.1007/s12253-018-0512-7 (2019

THE AUTHOR'S OTHER PUBLICATION

Successful thrombolytic therapy is associated with increased granulocyte CD15 expression and reduced stroke-induced immunosupression

Katalin Anna Béres-Molnár, Ágnes Czeti, ***Ferenc Takács****, Gábor Barna, Dániel Kis, Gabriella Róka, András Folyovich, Gergely Toldi*

Brain and Behavior DOI: 10.1002/brb3.2732 (2022)

First-in-human study of WT1 recombinant protein vaccination in elderly patients with AML in remission: a single-center experience

Stefanie Kreutmair, Dietmar Pfeifer, Miguel Waterhouse, ***Ferenc Takács****, Linda Graessel, Konstanze Döhner, Justus Duyster, Anna Lena Illert, Anna-Verena Frey, Michael Schmitt, Michael Lübbert*

Cancer Immunology, Immunotherapy DOI: 10.1007/s00262-022-03202-8 (2022)

Limitations of VS38c labeling in the detection of plasma cell myeloma by flow cytometry

Ágnes Czeti, Gábor Szalóki, Gergely Varga, Virág Réka Szita, Zsolt István Komlósi, ***Ferenc Takács****, Ágnes Márk, Botond Timár, András Matolcsy, Gábor Barna*

Cytometry part A DOI: 10.1002/cyto.a.24488 (2021)

Lenalidomide abrogates the survival effect of bone marrow stromal cells in chronic lymphocytic leukemia

Csilla Kriston, Márk Hernádfői, Márk Plander, Ágnes Márk, ***Ferenc Takács****, Ágnes Czeti, Gábor Szalóki, Orsolya Szabó, András Matolcsy, Gábor Barna*

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Screening and monitoring of BTK C481S mutation in a real-world cohort of patients with relapsed/refractory chronic lymphocytic leukemia during ibrutinib therapy

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