Original Article
Running Title: Expression Modulation of CTLA-4, STAT3 and LAG-3 by Ibrutinib and Idelalisib in CLL
Received: February 15, 2025; Accepted: December 7, 2025

Modulation of CTLA-4, STAT3 and LAG-3 Expression by Ibrutinib and Idelalisib in Leukemic Cells of Patients with Chronic Lymphocytic Leukemia

Fatemeh Mousavi-Mirkalaei[*], MSc, Saeid Taghiloo[*], PhD, Ali-Asghar Ghasemi[*], MD, Ehsan Zaboli[**,***], MD, Mohammad Eslami-Jouybari[**,***], MD, Ramin Shekarriz[**,***], MD, Leila Mirzakhani[**,***], MD, Zohreh Ehsani[**,***], MD, Maryam Alizadeh-Foroutan[**,***], MD, Hossein Asgarian-Omran[*,**♦], PhD

[*]*Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*
[**]*Gastrointestinal Cancer Research Center, Non-Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran*
[***]*Department of Hematology and Oncology, Imam Khomeini Hospital, Mazandaran University of Medical Science, Sari, Iran*

♦Corresponding Author
Hossein Asgarian-Omran, PhD
Gastrointestinal Cancer Research Center,
Non-Communicable Diseases Institute,
Mazandaran University of Medical Sciences,
Sari, Iran
Email: asgarianhossein@yahoo.com
hasgarian@mazums.ac.ir

Abstract

Background: Small molecule inhibitors are the new therapeutic approaches for many cancers, but their exact mechanisms in tumor evasion and expansion remains unknown. The present study aimed to evaluate the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3) and signal transducers and activators of transcription 3 (STAT3) in isolated leukemic cells from patients with chronic lymphocytic leukemia (CLL) following treatment with ibrutinib and idelalisib.

Method: In this in-vitro experimental study, leukemic B cells were isolated from CLL patients, cultured and treated with ibrutinib and idelalisib for 72 hours. The optimal IC50 values of 5 and 15 µM were determined for ibrutinib and idelalisib, respectively. Following treatment, the mRNA expression of these genes was measured by Real-time polymerase chain reaction assay using β-actin as a housekeeping control. One-way analysis of variance (ANOVA) test was employed for multiple comparisons. *P*-values of <0.05 were considered to be statistically significant.

Results: Isolation of CLL cells showed the purity of >97% as confirmed by flow cytometry. Leukemic cells indicated a significance reduction in cell viability following treatment with applied drugs compared with the untreated group. Treatment with ibrutinib showed a relative decrease in CTLA-4 (P = 0.09) and a relative increase in the LAG-3 and the STAT3 expression. Idelalisib indicated a significant increase in the STAT3 expression (P = 0.04) and also modulation in the CTLA-4 and LAG-3 expression (P > 0.05).
Conclusion: These data show that ibrutinib and idelalisib not only serve as cytotoxic drugs, but also influence the immune escape mechanisms of CLL cells by disrupting the signaling pathways which should be considered for further treatment approaches, especially for combinational strategies.

Keywords: Chronic lymphocytic leukemia, ibrutinib, idelalisib, CTLA-4, LAG-3

Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult lymphocytic leukemia caused by the clonal expansion of dysfunctional mature $CD19^+/CD5^+/CD23^+$ B lymphocytes in the peripheral blood, bone marrow, lymph nodes and spleen.[1] CLL usually affects elderly people with an average age of 70 years and is more common in men.[2,3] According to the American Cancer Society in 2020, about 21040 new cases of CLL and 4060 mortality were reported from the disease.[4] Although detailed information on the prevalence of CLL in Iran is not available, preliminary investigations indicate a relatively high prevalence of this disease in Iran. About 30%-40% of CLL patients show non-aggressive form of the disease with poor clinical course that never reach the criteria for starting treatment, while about 60% of all patients have an aggressive form of the disease and then require immediate treatment.[5] Combination chemo-immunotherapy with fludarabine/pentostatine, cyclophosphamide, and rituximab (FCR/PCR) is the current standard therapy for CLL patients with side-effects and risk of secondary cancers.[6-8] Therefore, to tackle these problems, targeted therapies are promoting using small molecule inhibitors (SMIs) which inhibit B cell receptor (BCR) signaling pathways such as bruton's tyrosine kinase (BTK), phosphoinositide 3-kinase (PI3K) and B-cell lymphoma 2 (BCL-2), with very promising results in this malignancy.[9] BTK is a non-receptor cytoplasmic tyrosine kinase which connects BCR, toll-like receptors (TLRs) and chemokine receptor signaling pathways and therefore plays an important role in cell processes including proliferation, differentiation, survival, apoptosis and migration [10]. In addition, excessive activation of PI3K due to dysregulation of BCR signaling pathway plays an important role in the proliferation and survival of CLL leukemic cells.[10] Blocking agents for BCR and PI3K signaling pathways, ibrutinib and idelalisib, has resulted in improved overall survival and progression-free survival of CLL patients.[10]

Despite the known benefits and antitumor effects of small molecule inhibitors, many of their detailed molecular mechanisms and interactions in the tumor microenvironment remain unclear. It is well established that tumor cells evade from the host immune responses by interaction with other cells in the tumor microenvironment and subsequently upregulation of immune checkpoint molecules in their surfaces which interact with their corresponding ligands on the surrounding immune cells. Therefore, more precise identification and understanding of the mechanisms and signaling pathways involved in different

tumors, could help to improve the efficacy of treatment strategies.[11-14] In this regard, CLL leukemic cells interact with bystander immune and non-immune cells in their tumor microenvironment and are able to escape from the immune system by using their surface molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-ligand 1 (PD-L1), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and T-cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT).[15-18] CTLA-4 is an inhibitory immune checkpoint expressed on the surface of T-cells in tumor microenvironment and suppresses the immune system by modulation of CD80/CD86 costimulatory molecules. This inhibitory molecule is also expressed on the surface of CLL leukemic cells.[19,20] On the other hand, the communication between CTLA-4 and CD80 leads to upregulation and phosphorylation of signal transducers and activators of transcription 3 (STAT3) resulting in increasing leukemic cells proliferation, survival and disease progression.[16,21,22] Upregulation in the LAG-3 protein levels both in the serum of CLL patients and on the surface of the leukemic and bystander T cells has been indicated in the tumor microenvironment of CLL, which leads to the leukemic cells activation through the stimulation of mitogen-activated protein kinase/extracellular signal kinase (MAPK/ERK) and PI3K/AKT (protein kinase B) pathways, bystander T cells inhibition and activation of anti-apoptotic pathways in the CLL cells.[18]

Therefore, activation of multiple signaling pathways in CLL leukemic cells and the subsequent increased expression of immune inhibitory molecules can lead to the immune system suppression and proliferation of CLL cells. In the present study, the expression of CTLA-4, LAG-3 and STAT3 was investigated in CLL leukemic cells following treatment with ibrutinib and idelalisib to more understand the mechanisms of SMI drugs on survival of leukemic cells and their immune evasion.

Materials and Methods

Sample collection

This in-vitro experimental study was done on the peripheral blood samples of 15 patients with CLL who attended Imam Khomeini Hospital in Sari affiliated to Mazandaran University of Medical Sciences from 2023-2024. All samples were from newly diagnosed CLL patients according to the World Health Organization (WHO) criteria, who did not receive any chemotherapy or immunosuppressive drugs for at least 6 months before sampling. Written informed consent was achieved from all patients based on the Declaration of Helsinki and ethical standards of Mazandaran University of Medical Sciences with the registration number (IR.MAZUMS.IMAMHOSPITAL.REC.1401.037).

Isolation of CLL leukemic cells

First, 4-5 ml of heparinized peripheral blood samples were obtained from all 15 CLL patients and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Histopaque (Biowest, USA) solution. Then, $CD19^+$ B-cells were isolated by Magnetic-Activated Cell Sorting (MACS) method in a positive selection system based on the instruction manual (MiltenyiBiotec company, Germany). To do so, isolated PBMCs from the previous step were diluted with prepared MACS buffer (PBS 0.15 M containing EDTA, BSA) and the cell suspension was incubated with microbead anti-CD19 for 45 min at 4°C (MilteneyiBiotec, Germany). Then, the MACS separation LS column was placed in a laminar hood and activated with 500 µl of MACS buffer. The 70 µm pre-separation

filter was also placed on the LS column and the cell suspension was added to the filter and washed three times with 500 µl of MACS buffer to remove the unbounded cells. In the last step, antibody-bounded cells were collected in a microtube by fast passing of 500 µl of MACS buffer into the column. Two-color flow cytometry method with PE-conjugated anti-CD3 and FITC-conjugated anti-CD5 monoclonal antibodies (Biolegend, USA) was applied to determine the cell purity. Corresponding isotype-matched controls antibodies were added to the related microtubes. After 45 min of incubation in the dark at 4°C, the cells were washed and then read by Partec PAS flow cytometer. The purity of leukemic cells isolated by MACS assay was checked by flow cytometry.

IC50 determination and treatment of CLL leukemic cells with SMIs

Two SMIs including ibrutinib (BTK inhibitor) and idelalisib (PI3K δ inhibitor) (Cayman, USA) were used in this study. These chemical reagents were dissolved in DMSO (Merck, Germany), and aliquots stored in -20°C freezer. The number of 3×10^5 leukemic cells were seeded in each well of 96-well culture plates (SPL, South Korea) in 200 µl of complete medium containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 µg/mL streptomycin (Biowest, USA). Different concentrations of ibrutinib and idelalisib were added to the culture plates and incubated for 72 hours in a 37°C incubator containing 5% CO_2 (Binder, Germany). Subsequently, 20 µL of freshly prepared 5 mg/mL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor) solution (Sigma-Aldrich, USA) was added to each well and incubated at 37°C for 4 h. Following incubation, the culture plate was centrifuged for 10 min at 850 g and then, the medium was carefully removed and the formazan crystals were dissolved in 150 µL DMSO by incubating at 37 °C for 40 min in the dark at room temperature. All samples were performed in triplicates and optical density (OD) values were read at 570 nm and 720 nm by an ELISA plate reader (Synergy H1 BioTek, USA). To determine the IC50 values of applied SMIs, isolated leukemic cells from 5 CLL patients were treated with increasing concentrations of ibrutinib and idelalisib and then the cell viability was monitored by MTT assay. Each experiment was run in triplicate format. After determining the specific IC50 of each applied SMIs, the samples were treated with the optimal dose of each SMIs. The CLL leukemic cells without treatment were considered as the control group. For RNA extraction and expression analysis, the number of 3×10^6 leukemic cells were seeded in 6-well culture plates and incubated with the optimal dose of each SMIs for 72 hours in a 37°C incubator containing 5% CO_2.[23]

RNA extraction and cDNA synthesis

Following culture of CLL leukemic cells with applied SMIs in this study, total RNA was extracted using the RNeasy kit (Denazist, Iran) according to the manufacturer's instructions. The quality and quantity analysis of the extracted RNA was confirmed by agarose gel-electrophoresis (Bio-Rad, UK) and nano-spectrophotometer (WPA, England), respectively. Complementary DNA (cDNA) synthesis was done using 1 µg of total RNA and 1 µl of random-hexamer primer using a cDNA synthesis kit (Yektatajhiz, Iran) according to the manufacturer's protocol. The final cDNA samples were frozen at -70°C until the Real-time PCR test was performed.[23]

Semi-quantitative Real-time polymerase chain reaction (RT-PCR)

Gene expression analysis was performed using a Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (Amplicon, Denmark) reagent in an ABI Step-One Real-Time PCR platform (ABI system, USA) with the following primers: β-actin, forward: CCT

TCC TGG GCA TGG AGT CCT, reverse: TGG GTG CCA GGG CAG TGAT;[24] CTLA-4, forward: TTCTTCTCTTCATCCCTGTC, reverse: CACAGACTTCAGTCACCT; LAG-3, forward: CACCTGCCATATCCATCT, reverse: CAAAGCGTTCTTGTCCAG and STAT3, forward: GAGTCAAGATTGGGCATA, reverse: TTGGCTTCTCAAGATACC.[25] The LAG-3 and CTLA-4 primers were designed by AllelID software and the specificity was confirmed with the online software Primer-Blast. No template control was also considered for all genes and reactions. First, real-time PCR reaction tubes were placed in ABI Step-One system at 95°C for 10 min as Hot-start point and then, were amplified in 45 cycles at 94°C for 30 seconds as initial denaturation and 60°C (all genes) for 30 sec and as extension step at 72°C for 30 sec. The PCR amplicon sizes were 177 bp, 149 bp, 179 bp and 174 bp for CTLA-4, LAG-3, STAT3 and β-actin, respectively. All runs were completed with a melting curve analysis which done by Linreg software to confirm the specificity of the amplification curves and the absence of primer dimers. The relative mRNA level expression was estimated by normalizing the target gene's fluorescence data to the housekeeping gene β-actin ($2^{-\Delta\Delta Ct}$).[23]

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software, USA) was used for data statistical analysis. Quantitative data are expressed as mean ± SEM. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine the normality distribution of the obtained data. One-way analysis of variance (ANOVA) test was employed for multiple comparisons. *P*-values <0.05 were considered to be statistically significant.

Results

Inhibition of CLL leukemic cells proliferation by BTK and PI3K signaling pathway inhibitors

Following isolation of $CD19^+$ B-cells by MACS method, two-color flow cytometric staining with anti-CD5-FITC and anti-CD3-PE fluorochrome-conjugated antibodies was performed to measure the purity of positively isolated leukemic CLL cells. The purity of isolated cells was more than 97% as indicated in Figure 1. In order to determine the optimum concentration of ibrutinib and idelalisib for in vitro experiments, isolated CLL leukemic cells were cultured with increasing concentrations of mentioned SMIs for 72 h and the cell viability was monitored by MTT assay. After that, IC50 values were calculated for both drugs which were determined 5 and 15 µM for ibrutinib and idelalisib, respectively.[26] Based on IC50 results, the CLL cells proliferation was evaluated in comparison with the untreated group. As expected, CLL leukemic cells indicated a significance reduction ($P < 0.05$) in cell viability following 72 h treatment with ibrutinib and idelalisib compared to the untreated group as control (Figure 2).

Evaluation of CTLA-4, LAG-3 and STAT3 expression following exposure of CLL leukemic cells to ibrutinib and idelalisib

After measurement the cell viability of CLL leukemic cells following treatment with ibrutinib and idelalisib, the mRNA expression of CTLA-4, LAG-3 and STAT3 was also monitored. As illustrated in Figure 3A, treatment of isolated leukemic cells with ibrutinib resulted a decreasing in the expression of CTLA-4 ($P = 0.09$) which indicated that inhibition of BTK signaling pathway could affect the expression of this inhibitory molecule in CLL leukemic cells, while treatment with idelalisib showed a non-significant increasing in CTLA-4 expression ($P > 0.05$). Regarding the LAG-3 gene expression, idelalisib caused a downregulation in its expression ($P > 0.05$),

while ibrutinib almost showed no effects (Figure 3B). On the other hand, the expression level of STAT3 was increased in both group following treatment with BTK and PI3K δ inhibitors, which the difference was significant for idelalisib treatment (P = 0.04, Figure 3C).

Correlation analysis of various immune checkpoint molecules in CLL leukemic cells following exposure to ibrutinib and idelalisib

Pearson correlation analysis was applied to evaluate the association between CTLA-4, LAG-3, STAT3 gene expression with other genes investigated in our previous research following CLL leukemic cells treatment with ibrutinib and idelalisib.[26] As shown in Figure 4A, following treatment of CLL leukemic cells by ibrutinib, a positive significant correlation was observed between STAT3 and PD-L1 expression (r= 0.66, P = 0.03) as well as between CTLA-4 and CD200 expression (r= 0.79, P < 0.01). In addition, a significant negative correlation was observed between LAG-3 and PD-L1 genes expression (r= -0.80, P = 0.01) and also between CD155 and CD200 expression (r= -0.68, P = 0.04) in CLL leukemic cells after treatment with ibrutinib. Regarding the idelalisib, a significant positive correlation was found between Gal-9 and CD200 genes expression (r= 0.86, P < 0.01) and between CD155 and PD-L1 expression (r= 0.87, P < 0.01) in CLL cells treated by this SMI (Figure 4A). The heat map analysis of all investigated genes in this study and our previous research is also represented in Figure 4B.

Discussion

In this study, CTLA-4, LAG-3, and STAT3 expression was assessed after in-vitro treatment of CLL leukemic cells with ibrutinib and idelalisib to clarify the role of these SMIs in leukemic cell survival and immune evasion. Our findings demonstrate that treatment with ibrutinib and idelalisib significantly reduced CLL cell viability, confirming the pivotal role of BCR-related signaling pathways in the survival and proliferation of CLL cells. Our results also indicate that ibrutinib by inhibiting the BTK signaling pathway leads to a downregulation in CTLA-4 gene expression and an upregulation in LAG-3 and STAT3 genes expression in isolated CLL leukemic cells. In addition, the PI3K δ signaling pathway inhibition by idelalisib leads to a significant increase in the expression of STAT3 gene in the CLL leukemic cells and showed a relative downregulation in the suppressor LAG-3 gene which can be effective in CLL disease therapeutic approaches.

The observed reduction in cell viability following treatment with ibrutinib and idelalisib is consistent with previous studies highlighting the therapeutic efficacy of BTK and PI3Kδ inhibitors in CLL through disruption of survival signals mediated by the tumor microenvironment.[27] These results further support the notion that targeting BCR signaling pathways not only suppresses leukemic cell growth but may also modulate immune-related mechanisms involved in disease progression. Previous studies have indicated that BTK pathway has an essential role in the CLL leukemic cells survival, and BTK inhibitor drugs like ibrutinib can inhibit these signaling pathways by forming a covalent bond in its enzyme site.[27] Also, it was found that the overexpression of the PI3K δ isoform leads to B cell malignancies. Thus, the selective inhibition of this signaling pathway by idelalisib results in apoptosis of leukemic cells in CLL patients.[28] Regarding other studies in this field, it has been shown that treatment of CLL patients with idelalisib leads to a reduction in CTLA-4, LAG-3 and PD-1 genes expression on their T helper and Treg cells.[29] Although, our in-vitro study on isolated CLL leukemic cells indicated a decrease in LAG-3 gene expression following treatment with idelalisib, a

negative correlation was also found between LAG-3 with both CTLA-4 and PD-L1 expression following CLL cells exposure to idelalisib and ibrutinib, respectively. Mhibik et al. indicated in their study that BTK inhibitor therapy with ibrutinib leads to a decreased expression in immunosuppressive CTLA-4 and CD200 inhibitory immune checkpoint molecules by CLL leukemic cells.[30] A similar result was also obtained in the present study, which showed that treatment with ibrutinib led to a decrease in the expression of CTLA-4 by leukemic cells, while idelalisib caused a relative increasing in CTLA-4 expression. Additionally, correlation analysis of our current results showed a significant positive correlation between CTLA-4 and CD200 genes expression by CLL leukemic cells following exposure to ibrutinib which confirm the results published by Mhibik et al. cells.[30]

CTLA-4/CD86 axis leads to tyrosine phosphorylation of STAT3 and binding of STAT3 to corresponding DNA, and subsequently leads to the activation of downstream immunosuppressive genes including IL-10 and IL-6, leukemic cells proliferation and tumor survival.[17] Moreover, ibrutinib was shown to inhibit the STAT3 pathway in human glioblastoma, breast cancer and CLL leukemic cells.[19,31,32] Contrary to other studies, our results indicate the increased expression of STAT3 in isolated CLL leukemic cells following treatment by both ibrutinib and idelalisib inhibitory drugs, with statistically significant results in the idelalisib group. This finding may reflect a compensatory activation of alternative survival and transcriptional pathways in response to pharmacological inhibition of BCR signaling. STAT3 activation has been previously implicated in treatment resistance and immune escape in CLL,[32] suggesting that its upregulation may represent an adaptive response of leukemic cells to targeted therapies. Overall, these differences could be due to several reasons, including the tumor microenvironment in various cancers and different methods used. This study examined the direct effect of the SMI drugs on isolated CLL cells, while some other studies were clinical trials and examined the therapeutic effects of the drugs on patients and measured the related molecular changes. However, combining these studies and gaining a deeper understanding of the molecular mechanisms of the drugs could be very helpful in explaining and designing of new therapeutic strategies.

Interestingly, LAG-3 expression was downregulated following idelalisib treatment, while ibrutinib had minimal impact on its expression. Given the established role of LAG-3 in promoting leukemic cell survival and inhibiting bystander T-cell function through activation of MAPK/ERK and PI3K/AKT pathways,[33] the observed reduction in LAG-3 expression following PI3Kδ inhibition may represent an additional immunomodulatory effect of idelalisib beyond its direct anti-proliferative activity. The upregulation of LAG-3 was indicated in previous studies in CLL patients, which binds to MHC-II molecules on the surface of both immune and malignant cells and causes CLL cells activation and stimulation of MAPK/ERK, PI3K/AKT. These events led to the induction of anti-apoptotic pathways and prevent the spontaneous apoptosis of leukemic cells.[33] In accordance with these results, our findings indicated a relative downregulation in LAG-3 expression in CLL leukemic cells following treatment with PI3K inhibitor, idelalisib, which confirms that this pathway is involved in the modulation of LAG-3.

One of the main strengths of the present study is the use of primary CLL leukemic cells isolated from newly diagnosed, treatment-naïve patients, which closely reflects the biological characteristics of the disease in-

vivo. In addition, the high purity of isolated $CD19^+$ leukemic cells and the simultaneous evaluation of multiple immune checkpoint molecules and signaling pathways provide a comprehensive insight into the immunomodulatory effects of BTK and PI3Kδ inhibitors. However, this study has certain limitations. The relatively small sample size may have limited the statistical power to detect significant differences in gene expression. Moreover, the in-vitro nature of the study does not fully recapitulate the complex interactions between CLL cells and the tumor microenvironment in-vivo. In addition, gene expression analysis was limited to the mRNA level, and further studies evaluating protein expression and functional immune assays are warranted. Future investigations with larger cohorts, in-vivo models, and combination treatment approaches are required to validate and extend these findings.

Conclusion

Our study indicates that the BTK and PI3K δ signaling pathways play significant roles in CLL leukemic cells expansion. Therefore, a detailed understanding of their mechanisms can be useful in the diagnosis and treatment of the CLL disease. Although both ibrutinib and idelalisib drugs have received FDA approval for the treatment of CLL, there is still no complete and accurate information about the detailed molecular mechanisms of these drugs and how they affect the tumor microenvironment, especially on the mechanisms of tumor cell escape from the host immune response. More precise identification of these mechanisms can be very helpful in improving treatment strategies, especially in combination therapy approaches.

Acknowledgments

The authors thank all CLL patients who participated in this research project.

Funding

This study was financially supported by Mazandaran University of Medical Sciences (Project number: 9266).

Authors' Contribution

Fatemeh Mousavi-Mirkalaei performed the experiments and wrote the initial draft of the manuscript. Saeid Taghiloo participated in the final data analysis. Alia-Asghar Ghasemi helped in performing experimental procedures. Maryam Alizadeh-Foroutan, Leila Mirzakhani, Zohreh Ehsani, Ehsan Zaboli, Mohammad Eslami-Jouybari and Ramin Shekarriz participated in the diagnosis and collection of peripheral blood samples of CLL patients. Hossein Asgarian-Omran conceived the original idea, designed the experiments, edited and approved the final manuscript. All authors read and approved the final manuscript.

Conflict of Interest

None declared.

References

1. Grywalska E, Bartkowiak-Emeryk M, Pasiarski M, Olszewska-Bożek K, Mielnik M, Podgajna M, et al. Relationship between the expression of CD25 and CD69 on the surface of lymphocytes T and B from peripheral blood and bone marrow of patients with chronic lymphocytic leukemia and established prognostic factors of this disease. *Adv Clin Exp Med.* 2018;27(7):987-99. doi: 10.17219/acem/74437. PMID: 29893517.
2. Braish J, Cerchione C, Ferrajoli A. An overview of prognostic markers in patients with CLL. *Front Oncol.* 2024;14:1371057. doi: 10.3389/fonc.2024.1371057. PMID: 38817892; PMCID: PMC11137234.

3. Cantú ES, McGill JR, Stephenson CF, Hoffmann HM, Tang L, Yan J, et al. Male-to-female sex ratios of abnormalities detected by fluorescence in situ hybridization in a population of chronic lymphocytic leukemia patients. *Hematol Rep.* 2013;5(1):13-7. doi: 10.4081/hr.2013.e4. PMID: 23888240; PMCID: PMC3719107.
4. Strati P, Shanafelt TD. Monoclonal B-cell lymphocytosis and early-stage chronic lymphocytic leukemia: diagnosis, natural history, and risk stratification. *Blood.* 2015;126(4):454-62. doi: 10.1182/blood-2015-02-585059. PMID: 26065657; PMCID: PMC4624440.
5. Jakšić B, Pejša V, Ostojić-Kolonić S, Kardum-Skelin I, Bašić-Kinda S, Coha B, et al. Guidelines for diagnosis and treatment of chronic lymphocytic leukemia. Krohem B-Cll 2017. *Acta Clin Croat.* 2018;57(1):190-215. doi: 10.20471/acc.2018.57.01.27. PMID: 30256032; PMCID: PMC6400341.
6. Hampel PJ, Parikh SA. Chronic lymphocytic leukemia treatment algorithm 2022. *Blood Cancer J.* 2022;12(11):161. doi: 10.1038/s41408-022-00756-9. PMID: 36446777; PMCID: PMC9708674.
7. Kay NE, LaPlant BR, Pettinger AM, Call TG, Leis JF, Ding W, et al. Cumulative experience and long term follow-up of pentostatin-based chemoimmunotherapy trials for patients with chronic lymphocytic leukemia. *Expert Rev Hematol.* 2018;11(4):337-49. doi: 10.1080/17474086.2018.1442716. P MID: 29460654.
8. Kuss B, Nagarajan C, Hsieh WS, Cheah CY. Practical management of chronic lymphocytic leukemia with acalabrutinib. *Leuk Lymphoma.* 2022;63(12):2785-94. doi: 10.1080/10428194.2022.2098289. PMID: 35852229.
9. Jain N, Thompson P, Ferrajoli A, Nabhan C, Mato AR, O'Brien S. Approaches to chronic lymphocytic leukemia therapy in the era of new agents: The conundrum of many options. *Am Soc Clin Oncol Educ Book.* 2018(38):580-91. doi: 10.1200/EDBK_200691. PMID: 30231393.
10. Smolewski P, Robak T. Current Treatment of refractory/relapsed chronic lymphocytic leukemia: A focus on novel drugs. *Acta Haematol.* 2021;144(4):365-79. doi: 10.1159/000510768. PMID: 33238270.
11. Bedard PL, Hyman DM, Davids MS, Siu LL. Small molecules, big impact: 20 years of targeted therapy in oncology. *Lancet.* 2020;395(10229):1078-88. doi: 10.1016/S0140-6736(20)30164-1. PMID: 32222192.
12. Pastorczak A, Domka K, Fidyt K, Poprzeczko M, Firczuk M. Mechanisms of immune evasion in acute lymphoblastic leukemia. *Cancers.* 2021;13(7):1536. doi: 10.3390/cancers13071536. PMID: 33810515; PMCID: PMC8037152.
13. Taghiloo S, Norozi S, Asgarian-Omran H. The effects of PI3K/Akt/mTOR signaling pathway inhibitors on the expression of immune checkpoint ligands in acute myeloid leukemia cell line. *Iran J Allergy Asthma Immunol.* 2022;21(2):178-88. doi:

10.18502/ijaai.v21i2.9225. PMID: 35490271.
14. Ranjbar A, Taghiloo S, Nozari P, Hedayatizadeh-Omran A, Asgarian-Omran H. Effects of c-Kit receptor, AKT, and NF-κB inhibitors on immune evasion in multiple myeloma cells. *Iran J Allergy Asthma Immunol.* 2025;24(1):89-99. doi: 10.18502/ijaai.v24i1.18024. PMID: 40052893.
15. Forconi F, Moss P. Perturbation of the normal immune system in patients with CLL. *Blood.* 2015;126(5):573-81. doi: 10.1182/blood-2015-03-567388. PMID: 26084672.
16. Severin F, Frezzato F, Visentin A, Martini V, Trimarco V, Carraro S, et al. In chronic lymphocytic leukemia the JAK2/STAT3 pathway is constitutively activated and its inhibition leads to CLL cell death unaffected by the protective bone marrow microenvironment. *Cancers.* 2019;11(12):1939. doi: 10.3390/cancers11121939. PMID: 31817171; PMCID: PMC6966457.
17. Asgarian-Omran H, Forghani P, Hojjat-Farsangi M, Roohi A, Sharifian RA, Razavi SM, et al. Expression profile of galectin-1 and galectin-3 molecules in different subtypes of chronic lymphocytic leukemia. *Cancer Invest*. 2010;28(7):717-25. doi:10.3109/07357907.2010.494319. PMID: 20590446.
18. Shapiro M, Herishanu Y, Katz BZ, Dezorella N, Sun C, Kay S, et al. Lymphocyte activation gene 3: a novel therapeutic target in chronic lymphocytic leukemia. *Haematologica.* 2017;102(5):874. doi: 10.3324/haematol.2016.148965. PMID: 28154084; PMCID: PMC5477606.
19. Ciszak L, Frydecka I, Wolowiec D, Szteblich A, Kosmaczewska A. CTLA-4 affects expression of key cell cycle regulators of G0/G1 phase in neoplastic lymphocytes from patients with chronic lymphocytic leukaemia. *Clin Exp Med.* 2016;16(3):317-32. doi: 10.1007/s10238-015-0360-7. PMID: 26003188; PMCID: PMC4969362.
20. Do P, Beckwith KA, Cheney C, Tran M, Beaver L, Griffin BG, et al. Leukemic B cell CTLA-4 suppresses costimulation of T cells. *J Immunol.* 2019;202(9):2806-16. doi: 10.4049/jimmunol.1801359. PMID: 30910862; PMCID: PMC6478536.
21. Herrmann A, Lahtz C, Nagao T, Song JY, Chan WC, Lee H, et al. CTLA4 Promotes Tyk2-STAT3–Dependent B-cell Oncogenicity. *Can Res.* 2017;77(18):5118-28. doi: 10.1158/0008-5472.CAN-16-0342. PMID: 28716895; PMCID: PMC5600851.
22. Ma W, Zhang Y, Qi Y, Guo S. STAT3 promotes chronic lymphocytic leukemia progression through upregulating SMYD3 expression. *Arch Med Sci.* 2019;15(5):1163-75. doi: 10.5114/aoms.2018.77733. PMID: 31572461; PMCID: PMC6764298.
23. Soltanshahi M, Taghiloo S, Asgarian-Omran H. Expression modulation of immune checkpoint molecules by ibrutinib and everolimus through STAT3 in MCF-7 breast cancer cells. *Iran J Pharm Res.* 2022;21(1):e127352. doi: 10.5812/ijpr-127352. PMID: 35873012; PMCID: PMC9293249.
24. Ranjbar A, Soltanshahi M, Taghiloo S, Asgarian-Omran H. Glucose metabolism in acute myeloid leukemia cell line is regulated via

combinational PI3K/AKT/mTOR pathway inhibitors. *Iran J Pharm Res.* 2023;22(1):e140507. doi: 10.5812/ijpr-140507. PMID: 38435444; PMCID: PMC10909123.

25. Taghiloo S, Ajami A, Alizadeh-Navaei R, Zaboli E, Asgarian-Omran H. Treatment by PI3K/mTOR inhibitor BEZ235 combined with TLR-7/8 agonist interfere with immune evasion mechanisms of WEHI-3 mouse leukemia cells. *Iran J Immunol.* 2022;19(1):6. doi: 10.22034/IJI.2022.92576.2155. PMID: 35293347.
26. Mousavi-Mirkalaei F, Taghiloo S, Alizadeh-Foroutan M, Zaboli E, Eslami-Jouybari M, Shekarriz R, et al. Expression modulation of immune inhibitory molecules by small molecule inhibitor drugs in leukemic cells of chronic lymphocytic leukemia. *Iran J Pharm Res.* 2025;12;24(1):e159353. doi: 10.5812/ijpr-159353. PMID: 41104252; PMCID: PMC12523818.
27. Wen T, Wang J, Shi Y, Qian H, Liu P. Inhibitors targeting Bruton's tyrosine kinase in cancers: drug development advances. *Leukemia.* 2021;35(2):312-32. doi: 10.1038/s41375-020-01072-6. PMID: 33122850; PMCID: PMC7862069.
28. Sharma S. Signaling pathways and novel inhibitors in chronic lymphocytic leukemia. *Federal Practitioner*. 2014;31(8):18-22.
29. Rohrbacher L, Brauchle B, Ogrinc Wagner A, von Bergwelt-Baildon M, Bücklein VL, Subklewe M. The PI3K∂-selective inhibitor idelalisib induces T-and NK-cell dysfunction independently of B-cell malignancy-associated immunosuppression. *Front Immunol.* 2021;12:557. doi: 10.3389/fimmu.2021.608625. PMID: 33790890; PMCID: PMC8005712.
30. Mhibik M, Gaglione EM, Eik D, Kendall EK, Blackburn A, Keyvanfar K, et al. BTK inhibitors, irrespective of ITK inhibition, increase efficacy of a CD19/CD3-bispecific antibody in CLL. *Blood.* 2021;138(19):1843-54. doi: 10.1182/blood.2020009686. PMID: 34046681; PMCID: PMC8586964.
31. Boudny M, Trbusek M. The important role of STAT3 in chronic lymphocytic leukaemia biology. *Klin Onkol.* 2020;33:32-8. doi: 10.14735/amko202032. PMID: 32075387.
32. Shi Y, Guryanova OA, Zhou W, Liu C, Huang Z, Fang X, et al. Ibrutinib inactivates BMX-STAT3 in glioma stem cells to impair malignant growth and radioresistance. *Sci Ttransl Med.* 2018;10(443). eaah6816. doi: 10.1126/scitranslmed.aah6816. PMID: 29848664; PMCID: PMC6431250.
33. Shapiro M, Herishanu Y, Katz BZ, Dezorella N, Sun C, Kay S, et al. Lymphocyte activation gene 3: a novel therapeutic target in chronic lymphocytic leukemia. *Haematologica.* 2017;102(5):874-82. doi: 10.3324/haematol.2016.148965. PMID: 28154084; PMCID: PMC5477606.

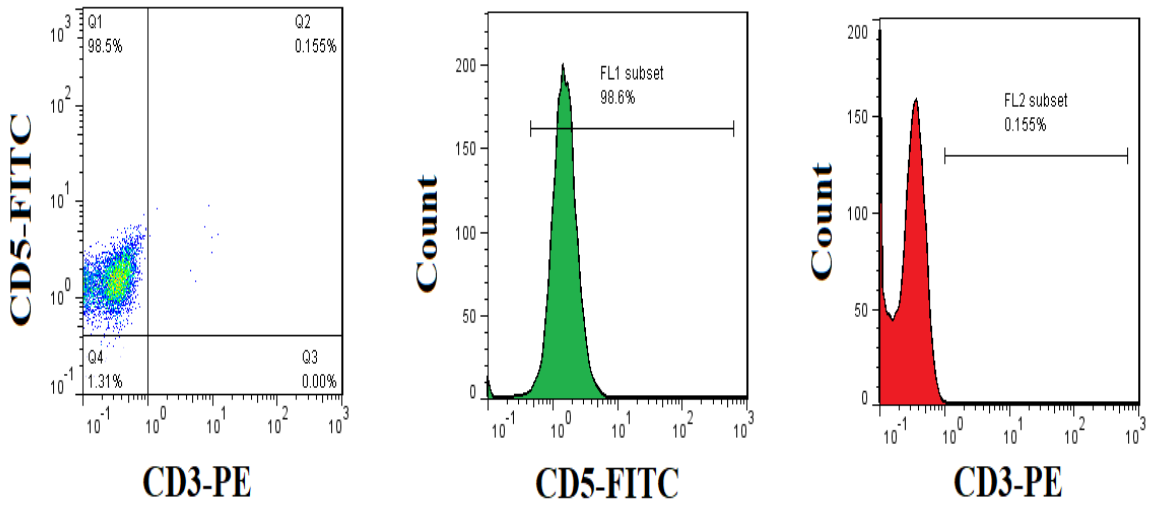


Figure 1. Purity analysis of isolated CLL leukemic cells by flow cytometry: Two-color flow cytometry method was used to determine the purity of CLL cells isolated by magnetic activated cell sorting method. The purity of isolated leukemic cells was determined to be more than 97%. Representative data for a CLL patient is shown.
CLL: Chronic lymphocytic leukemia

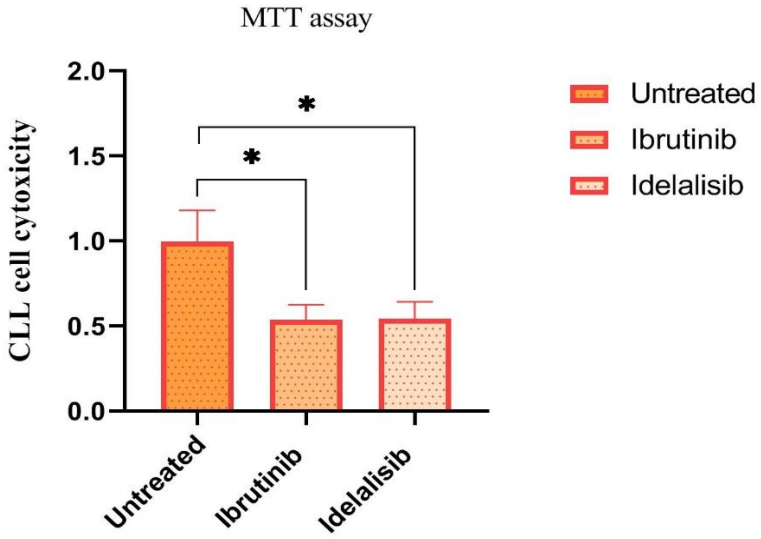
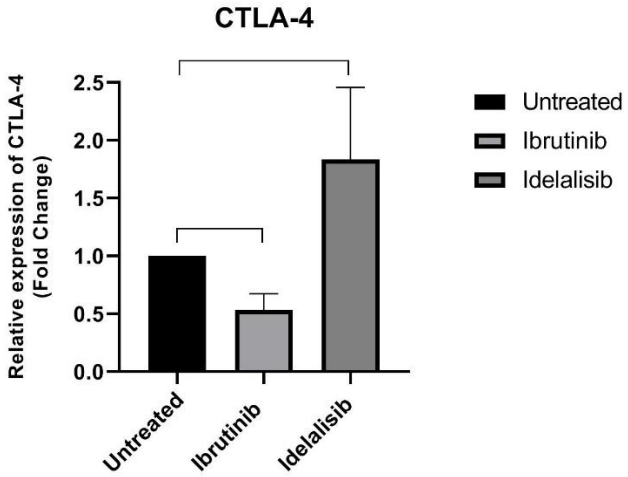
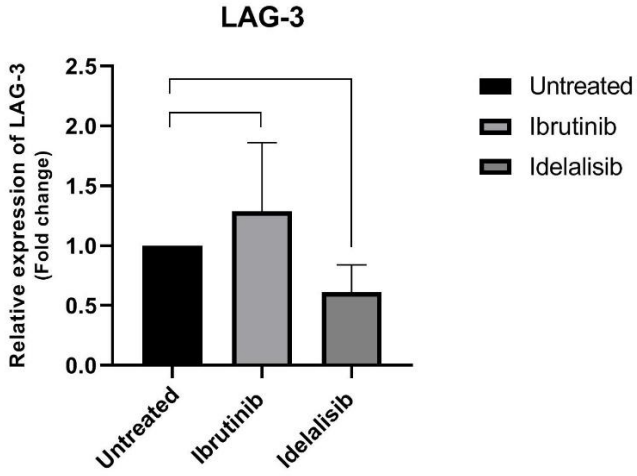


Figure 2. The viability of CLL leukemic cells after treatment with ibrutinib and idelalisib: Isolated CLL Leukemic cells indicated a significant reduction in viability following in-vitro treatment with ibrutinib and idelalisib as demonstrated by MTT assay. *P*-values <0.05 were considered to be statistically significant.
CLL: Chronic lymphocytic leukemia

A



B



C

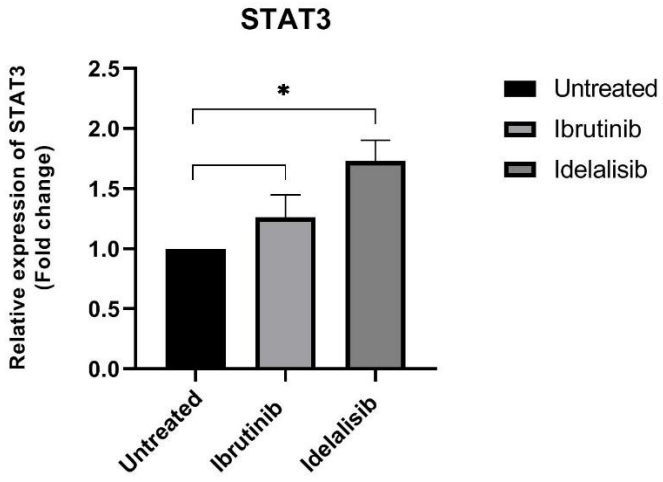
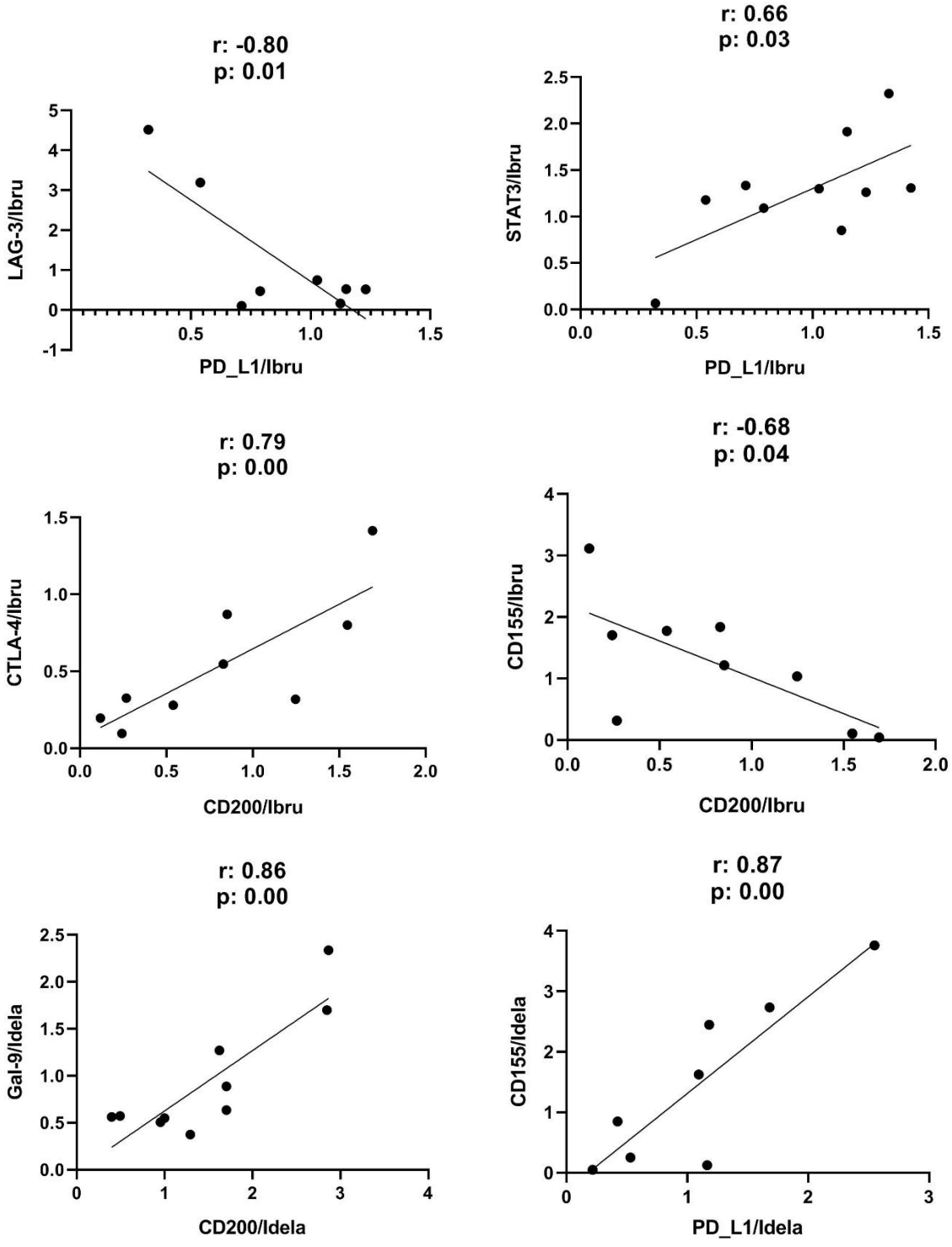


Figure 3. Evaluation of the CTLA-4, LAG-3, STAT3 genes expression in isolated leukemic cells from patients with CLL following treatment with optimal concentrations of ibrutinib and idelalisib:

A, B and C indicate fold changes in CTLA-4, LAG-3 and STAT3 mRNA expression following treatment of CLL leukemic cells with applied small molecule inhibitors. The mRNA expression results were deducted by Real-time polymerase chain reaction technique using β-actin as a housekeeping control. The results were reported as mean ± SEM. * $P < 0.05$.

CTLA-4: Cytotoxic T-lymphocyte associated protein 4; LAG-3: Lymphocyte-activation gene 3; STAT3: Signal transducer and activator of transcription 3; CLL: Chronic lymphocytic leukemia.

A


r: -0.80
p: 0.01
LAG-3/Ibru
PD_L1/Ibru
r: 0.66
p: 0.03
STAT3/Ibru
PD_L1/Ibru
r: 0.79
p: 0.00
CTLA-4/Ibru
CD200/Ibru
r: -0.68
p: 0.04
CD155/Ibru
CD200/Ibru
r: 0.86
p: 0.00
Gal-9/Idela
CD200/Idela
r: 0.87
p: 0.00
CD155/Idela
PD_L1/Idela

B

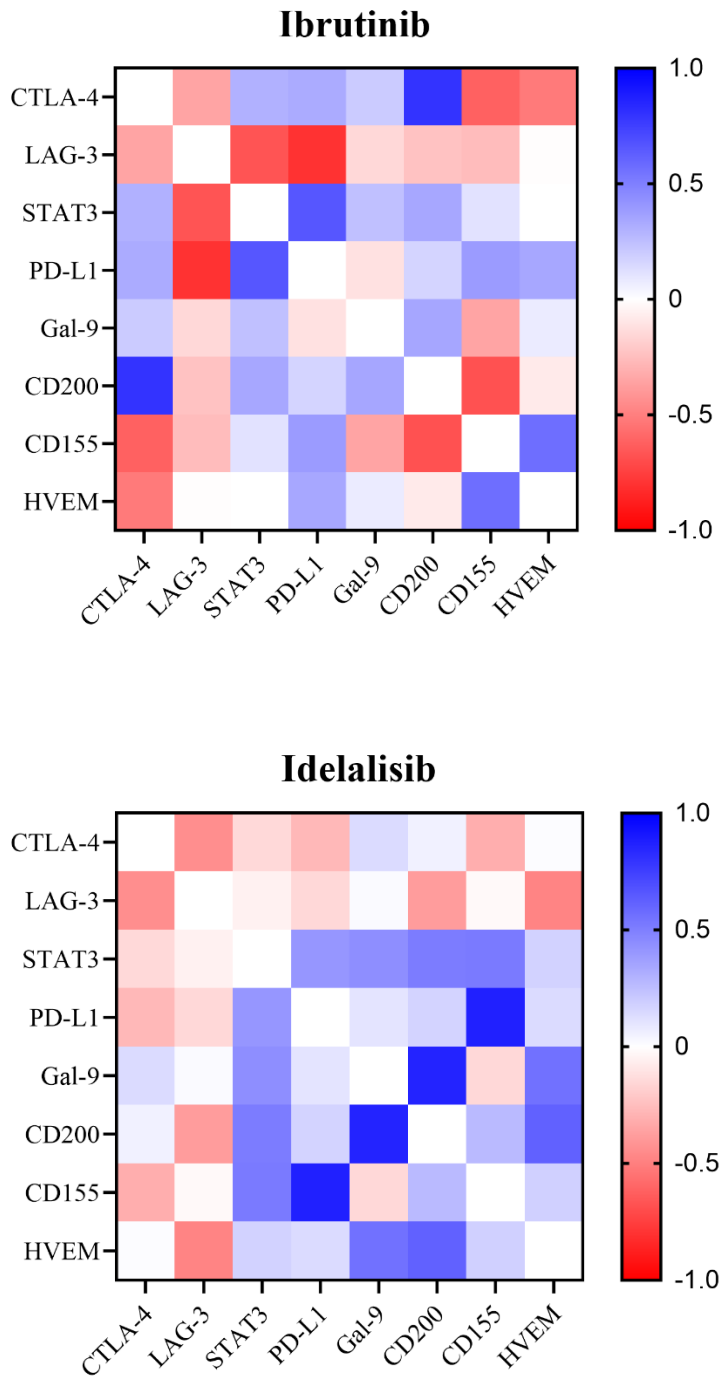


Figure 4. Pearson correlation coefficient analysis between CTLA-4, LAG-3, STAT3, PD-L1, Gal-9, CD200, CD155 and HVEM genes expression in leukemic cells from patients with CLL treated by ibrutinib and idelalisib drugs: A. Positive significant correlation was observed between STAT3 and PD-L1 expression as well as between CTLA-4 and CD200 expression following treatment of CLL leukemic cells by ibrutinib. A significant negative correlation was observed between LAG-3 and PD-L1 genes expression and also between CD155 and CD200 expression in CLL leukemic cells after treatment with ibrutinib. A significant positive correlation was found between Gal-9 and

CD200 genes expression and between CD155 and PD-L1 expression in CLL cells treated by idelalisib. B. The heat map analysis of all investigated genes is represented. CTLA-4, LAG-3 and STAT3 expression data was from the present study, while the expression data for PD-L1, Gal-9, CD200, CD155 and HVEM was prepared from our previous research study.

CTLA-4: Cytotoxic T-lymphocyte associated protein 4; LAG-3: Lymphocyte-activation gene 3; STAT3: Signal transducer and activator of transcription 3; PD-L1: Programmed death-ligand 1; Gal-9: Galectin-9; HVEM: Herpesvirus entry mediator; CLL: Chronic lymphocytic leukemia.