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Gene Expression Analysis of Galectin-9, MALAT1, and ZAP-70 in Chronic Lymphocytic Leukemia and Their Diagnostic Potential: Association with Clinical Laboratory Features

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous B-cell malignancy where emerging molecular diagnostics offer potential improvements in diagnosis and prognostication. Recent evidence implicates Galectin-9, the long non-coding RNA MALAT1, and the tyrosine kinase-associated adaptor ZAP-70 in the pathophysiology and progression of CLL. This study aimed to quantitatively assess the transcriptional expression of these candidate biomarkers using quantitative real-time PCR (qRT-PCR) across three groups: treatment-naïve CLL, treated CLL, and healthy controls. A total of 59 Kurdish adults were enrolled and categorized into three groups: 12 newly diagnosed, treatment-naïve CLL patients; 27 CLL patients who had completed one line of therapy; and 20 healthy controls. Demographic and clinical data, including CBC parameters, flow cytometric immunophenotyping reports, in addition to cytological evaluation results that were performed using Leishman-stained peripheral blood smears, were collected. Total RNA was extracted from whole blood collected into EDTA tubes within 3–4 hours post-collection, followed by complementary DNA (cDNA) synthesis. Quantitative real-time PCR (qRT-PCR) was conducted to assess the transcriptional expression of Galectin-9, MALAT1, and ZAP-70, normalized to β-actin, across all samples.

All three targets were significantly overexpressed in treated and Naïve CLL patients versus controls: Galectin-9 ΔCt: 5.31 ± 0.29 vs. 6.33 ± 0.11 ($p = 0.0035$); MALAT1 ΔCt: 1.50 ± 0.59 vs. 3.31 ± 0.14 ($p = 0.0025$); ZAP-70 ΔCt: 4.92 ± 0.66 vs. 7.52 ± 0.13 ($p < 0.0001$). ROC analysis showed strong discriminatory power for ZAP-70 (AUC = 0.89, $p < 0.0001$) and MALAT1 (AUC = 0.73, $p = 0.003$), with Galectin-9 demonstrating marginal significance (AUC = 0.64, $p = 0.07$). MALAT1 and ZAP-70 showed strong co-regulation ($r = 0.64$, $p < 10^{-5}$) and were associated with several hematological parameters. These findings support the use of MALAT1 and ZAP-70 as minimally invasive biomarkers for CLL diagnosis and risk stratification, while Galectin-9 may reflect immune dysregulation.

1.Introduction

Chronic lymphocytic leukemia (CLL) is a mature B-cell tumor distinguished by the gradual buildup of monoclonal B cells (Hallek and Al-Sawaf, 2021;El Said, 2019). The clinical presentation of CLL is varied, ranging from those with severe disease to others with an indolent course and no symptoms who do not require treatment (Catovsky et al., 2019). According to GLOBOCAN, the global cancer database, the predominant leukemia in adults globally is AML, followed by CLL (males incidence: 28%; females incidence: 24%) (Daltveit et al., 2025). An epidemiological profile of CLL finds that the condition is more prevalent in men, with a male-to-female ratio ranging from around 1.2:1 to 1.7:1 (Ou et al., 2022). The CLL cells' survival is predicated upon a conducive microenvironment comprising cellular elements like T cells, macrophages, and stromal follicular dendritic cells (Arruga et al., 2020). This microenvironment generates numerous vital proteins (cytokines, angiogenic factors, and chemokines) that engage with leukemic cells through specific adhesion molecules or surface receptors to facilitate the survival of CLL cells (Cerreto et al., 2023).

At the molecular level, disruptions in immune-checkpoint pathways, dysregulation of long non-coding RNAs, and alteration of tyrosine-kinase signaling have surfaced as important elements in the pathogenesis and progression of CLL (Pepe and Balatti, 2020;Maher et al., 2025). Galectin-9 (LGALS9 gene), an immunomodulatory lectin associated with T-cell exhaustion and tumor immune evasion, is overexpressed in aggressive CLL and corresponds with unfavorable clinical indicators (Yıldırım, 2024).

Galectin-9 (Gal-9), encoded by the LGALS9 gene, is a tandem-repeat β-galactoside–binding lectin characterized by two carbohydrate-recognition domains (CRDs) joined by a linker peptide. Gal-9 modulates cell–cell and cell–matrix interactions and plays significant roles in immune regulation (John and Mishra, 2016). Altered expression of Gal-9 correlates with the development, progression, and metastasis of many malignancies (Wang et al., 2018;Zhou et al., 2018). The prognostic role of Gal-9 was also examined in CLL. Elevated amounts of Gal-9 correlated with clinical progression and unfavorable prognosis in CLL patients (Bojarska-Junak et al., 2023). Gal-9 is an essential inhibitor of the immune system (Cao et al., 2024). It fosters the advancement of regulatory T-cells (Tregs) and inhibits the differentiation of Th17 cells (Oomizu et al., 2012). Furthermore, Gal-9 has the ability to trigger the apoptosis of Th1 cells and CD8+ cytotoxic T-cells via T-cell immunoglobulin and mucin domain 3 (TIM-3), leading to the reduction of severe inflammation (Yıldırım, 2024).

Long non-coding RNAs (lncRNAs) are crucial in various biological activities, including DNA repair, gene expression regulation, transcription, and translation. MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), often referred to as NEAT2 (nuclear enriched abundant transcripts), is an 8.7 kb carcinogenic lncRNA situated on chromosome 11 (Li et al., 2021;Fu et al., 2020). It plays a crucial function in alternative splicing and the regulation of gene expression. In vitro studies indicate that the deregulation of TP53 by oncoviruses results in elevated expression of MALAT1, while the overexpression of TP53 correlates with the depletion of MALAT1 (Jeffers et al., 2013). The long non-coding RNA MALAT1 has been linked to the enhancement of CLL cell survival and proliferation by influencing epigenetic and splicing mechanisms (Zhang et al., 2024). Elevated MALAT1 levels correlated with unfavorable cytogenetic subgroups such as 13q14 deletion and 11q22 deletion (Ahmadi et al., 2018).

Moreover, zeta chain of T cell receptor associated protein kinase 70 (ZAP-70), a tyrosine-kinase-associated adaptor protein often lacking in healthy B cells, is inappropriately produced in CLL and reflects the unfavorable-prognosis IgVH-unmutated phenotype, indicating shorter treatment-free survival (Chen et al., 2020). ZAP-70 undergoes tyrosine phosphorylation and interacts with the B-cell receptor (BCR) in activated CLL cells. This indicates that ZAP-70 may participate in BCR signal transduction even in the presence of Spleen tyrosine kinase (SYK), the structurally similar protein typically seen in B cells (Ghergus, 2015).

The present study explores the expression profiles of Galectin-9, MALAT1, and ZAP-70 in chronic lymphocytic leukemia (CLL) using real-time PCR, with the aim of establishing their utility as novel independent diagnostic biomarkers. We further correlate their transcriptional signatures with complete blood count (CBC) parameters to uncover clinically relevant associations. By integrating molecular and hematological insights, this research aspires to refine diagnostic accuracy and lay the groundwork for more personalized therapeutic approaches in CLL.

2. Materials and methods

2.1 Ethics approval

The Human Ethics Committee of Technical Health and Medical College, Polytechnic University – Erbil examined and approved this study protocol under order number (25/0069 HRE).

2.2 Participants and Samples Collection:

In this study, 59 individuals aged 35–84 years were recruited from Nanakali Hospital in Erbil, Kurdistan Region of Iraq, from August 2024 till March 2025. Participants were divided into three groups: a healthy control group (n = 20) comprising volunteers with no hematologic disorders; an untreated CLL group (n = 12) of newly diagnosed, treatment-naïve patients; and a treated CLL group (n = 27) of patients who had completed one line of therapy with different therapeutic regimens **(Table 1)**. One group was treated with a combination of Rituximab and Bendamustine (BR protocol). Another group received Rituximab and Bendamustine in combination with the Bruton's tyrosine kinase inhibitor Ibrutinib (BRI protocol). The third group was managed with Rituximab, Fludarabine, and Cyclophosphamide (FCR protocol), which represents a standard chemo-immunotherapy regimen.

From each patient, 2 mL of venous blood was collected into EDTA tubes under standardized conditions, and RNA extraction was processed within 3–4 hours. All CLL diagnoses were confirmed by flow cytometry, and the reports for all included patients were retrospectively retrieved from the hospital's medical records database (BD FACSCanto™ Ⅱ, US). Additional demographic and clinical data, such as age, gender, ethnicity, complete blood counts (Medonic, Sweden), were prospectively collected. These clinical test results were utilized for further analysis and correlation with gene expression findings.

Table 1: Demographic characteristics (total number, age, and gender) of CLL patients and healthy participants.

Characteristic	Value
Total CLL Patients number	39
Median Age at Diagnosis (Years)	60
Patients Gender	Male 51.2%, Female 48.7%
Total Healthy Persons Number	20
Median Age (Years)	50.5
Healthy Persons Gender	Male 45%, female 55%

2.3 Flow Cytometry Protocol Used at Nanakaly Hospital

At Nanakaly Hospital, flow cytometry immunophenotyping was performed using peripheral blood samples (2 ml) collected in EDTA tubes or bone marrow aspiration, and performed within 2-3 hours after sample collection. Samples were processed using a standard stain-lyse-wash protocol (Jeffery, 2022). A multi-parameter antibody panel was employed to identify clonal B-cell populations characteristic of CLL. The panel included fluorochrome-conjugated monoclonal antibodies against **CD5, CD10, CD19, CD23, CD20, CD25, CD79b, CD38, CD45, CD123, CD138**, **CD200, BCL2, IgM, FMC7** and surface **kappa and lambda light chains** (BD FACSCanto™ Ⅱ, US). Red blood cells were lysed using commercial lysing solution (BD Biosciences, US), followed by incubation with antibodies at room temperature in the dark for 15–20 minutes. After staining, cells were washed with phosphate-buffered saline (PBS) and analyzed using a flow cytometer (BD FACSCanto™ Ⅱ, US) with standardized compensation and gating strategies. Lymphocytes were gated based on forward and side scatter properties.

2.4 Cytological Staining Protocol for Peripheral Blood Smear

A peripheral blood smear was prepared by placing a small drop of the blood at the end of a clean glass slide and using a second slide at a 30–45° angle to create a thin monolayer smear,

which was then air-dried at room temperature to ensure proper cell adherence. Smear was fixed by covering it with methanol 99.85% (Atom Scientific, Manchester) for 2–3 minutes to preserve cellular structure and adhere the sample to the slide. A thin blood film was covered with approximately 1 mL of Leishman stain (Anamol, India), incubation for 1 minute, followed by the addition of 1 mL of Leishman buffer, incubation for 6 minutes to ensure optimal staining. Finally, the slides were rinsed under a slow stream of distilled water until the runoff was clear, then air-dried completely and observed under a light microscope for detailed examination of cell morphology **(Figure 6)**. A 40× objective was used. Final identification of fine nuclear and cytoplasmic features requires a 100× objective with immersion oil (Chonat et al.).

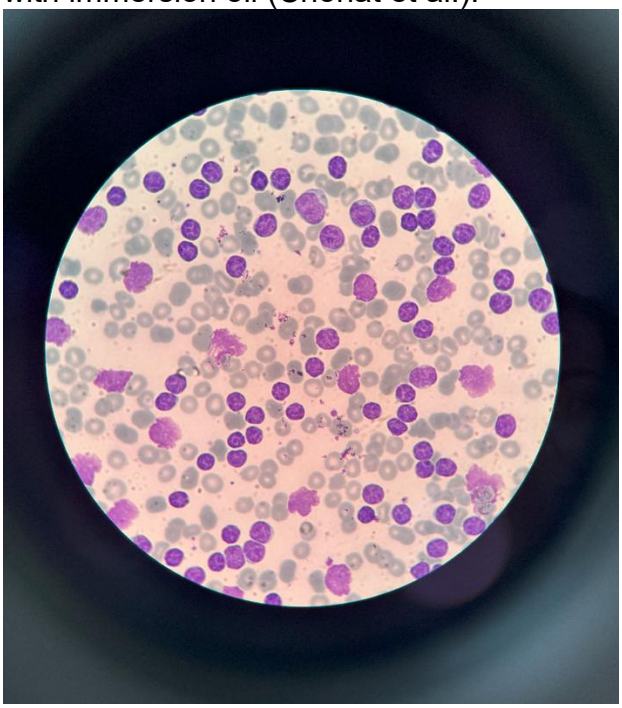

Figure 6: Peripheral Blood Smear Showing Lymphocytic Predominance in a Patient with Chronic Lymphocytic Leukemia, stained with Leishman Stain and Observed Under 100× Oil Immersion.

2.5 RNA extraction, cDNA synthesis, and qRT-PCR analysis

2.5.1 The procedure of RNA extraction from blood samples.

Total RNA was extracted from EDTA-treated whole blood with the use of a QIAamp RNA Blood Mini Kit from (QIAGEN, Germany) following the manufacturer's protocol. Red blood cells were lysed using Buffer EL, and the mixture was incubated on ice. Leukocytes were pelleted by centrifugation, followed by cell lysis with Buffer RLT containing β-mercaptoethanol. The homogenized lysate was passed through QIAshredder spin columns, and RNA binding was facilitated with ethanol addition. After washing with RW1 and RPE buffers, RNA was eluted in RNase-free water. The concentration and purity of RNA were measured by OneDrop TOUCH NanoDrop Spectrophotometer (Biometrics, USA).

2.5.2 Complementary DNA (cDNA)

cDNA was synthesized with cDNA Synthesis UltraScript® Reverse Transcriptase kit from (PCRBIOSYSTEM) by using SimpliAmp™ Thermal Cycler 96-WELL (Applied Biosystems), in a 20 µL reaction volume containing 4 µL of 5× cDNA synthesis mix, 1 µL of UltraScript for cDNA synthesis, 10 µL of PCR-grade water, and 5 µL of extracted RNA.

2.5.3 Quantitative real-time PCR (qRT-PCR)

The transcript levels of three different target genes (Galectin-9, Zap-70, and MALAT1) were assessed by operating the reactions in the Bio-Rad (CFX 96) Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The 20 µL reaction mixture was prepared by the addition 10µl of 2× SyGreen Blue mix (PCRBIOSYSTEMS), 1µl of each forward and reverse specific primers (the stock solution concentration for each primer was 100 picomole/µl, prepared directly within its supplied vial by reconstituting the lyophilized oligonucleotide, the vial contained 30,000 picomoles of primer, to which 300 µL of nuclease-free water was added), 5.5µl of nuclease-free water (dH_2O), and 2.5µl of cDNA template. A two-replicate qPCR method was used to amplify the target genes.

2.5.4 Primer sequences

For detection of Gal-9, ZAP-70, and MALAT1, primer pairs were purchased from (Humanizing Genomics macrogene, Korea) as follows: MALAT-1 forward, 5'-GACTTCAGGTCTGTCTGTTCT-3'; MALAT-1 reverse, 5'-CAACAATCACTACTCCAAGC-3' (Chen et al., 2015), ZAP-70 forward, 5′-

CGCTGCACAAGTTCCTGGT-3′; ZAP-70 reverse, 5′-GACACCTGGTGCAGCAGCT-3′ (Wiestner et al., 2003) and Gal-9 forward, 5'-GGACGGACTTCAGATCACTGT-3'; Gal-9 reverse, 5'-CCATCTTCAAACCGAGGGTTG-3' (Wang et al., 2021). The human β-actin was used as the internal control for normalization, and the primers were as follows:β-actin forward, 5'-TCGAGCAATCTCAACTCGG-3'; β-actin reverse, 5'-TGAAGGTAGTTTCGTTGGATG-3' (Awla and Othman, 2023).

2.5.5 Thermocycler conditions of Quantitative real-time PCR

Four-step cycling protocol. The reaction was initiated by a pre-denaturation step at 95 °C for 2 minutes (1 cycle) to activate the DNA polymerase and ensure complete denaturation of the template strands. This was followed by 40 cycles of denaturation at 95 °C for 5 seconds and annealing/extension at 60 °C for 30 seconds, allowing primer binding and DNA synthesis. After amplification, a melting curve analysis was carried out by heating the reaction to 65 °C for 5 seconds, followed by a brief final extension at 95 °C for 5 seconds to verify the specificity of the amplified products. All samples were analyzed in two replicates alongside No-Template Controls (NTC) to exclude contamination. Relative gene expression was calculated using the ΔCt and 2^−ΔΔCt method for normalization using β-actin as a reference gene.

2.6 Statistical Analysis

Data analysis was conducted using GraphPad Prism version 10.4 (GraphPad Software, USA). To evaluate the quantitative data between CLL patients and healthy controls, we employed Normality and lognormality test, ONE WAY ANOVA with Tukey's post hoc test, the receiver operating characteristic (ROC) curve, and Pearson correlation analysis.

3. Result

3.1 Demographic of all participants

Only the gender, age, and some clinical data of the 39 CLL patients were analyzed due to the available information about their characteristics. It appeared that 19 (48.7%) out of the total (39) patients were female, with median age (62 years). Furthermore, the males constituted 20 (51.2%) of the CLL patients with median age (61 years). The healthy control group in this study consisted of 20 individuals, including (11) females and (9) males, with ages ranging (41-66) years. The average age of the female participants was 50.4 years, while the average age of the male participants was (50.7) years. All controls were clinically evaluated to confirm the absence of hematological or systemic disorders, ensuring their suitability for comparison with the CLL patient groups **(Table 1)**.

3.2 Diagnostic Immunophenotyping of Peripheral Blood in CLL

Flow cytometric immunophenotyping confirmed the diagnosis of CLL in all (39) patient samples, revealing characteristic antigenic patterns consistent with clonal B-cell proliferation. The majority of cases demonstrated a positive expression of CD23 (87%) and CD5 (87.1%), which are hallmark features of CLL immunophenotype. Additionally, CD5 expression was observed in most cases, further supporting the CLL diagnosis. Lambda shows clonal predominance in this cohort, in which the data indicates a slight predominance of lambda-positive cells (38.4%) over kappa-positive cells (35.8%), which contradicts the normal 2:1 kappa dominance, indicating monoclonal proliferation of one type of light chains (either kappa or lambda). Notably, CD10, CD38, CD123, and CD138 were consistently negative in all patients, effectively excluding other B-cell malignancies such as follicular lymphoma or plasma cell neoplasms. Other markers, such as CD20 and CD79b, displayed heterogeneous and negative or dim expression patterns, respectively, consistent with the immunophenotypic profile of CLL **(Table 2)**.

Table 2: Diagnostic immunophenotyping markers in CLL patients by flow cytometry (n = 39).

Marker	Negative (%)	Dim (%)	Dim–Moderate (%)	Moderate (%)	Bright (%)	Heterogeneous (%)
Kappa	64.1	33.3	–	2.5	–	–
Lambda	61.5	23.0	7.6	7.6	–	–
CD5	12.8	17.9	20.5	43.5	5.1	–
CD10	100	–	–	–	–	–
CD19	12.8	23.0	10.2	53.7	–	–
CD20	25.6	20.5	17.9	12.8	–	23.0
CD23	12.8	43.5	17.9	25.6	–	–
CD25	69.2	25.6	–	5.1	–	–
CD38	100.00	–	–	–	–	–
CD45	58.9	2.5	–	28.2	10.2	–
BCL2	94.8	5.1	–	–	–	–
IgM	66.6	33.3	–	–	–	–
CD79b	76.9	17.9	2.5	–	–	–
FMC7	87.1	2.5	–	10.2	–	–
CD123	100	–	–	–	–	–
CD138	100	–	–	–	–	–
CD200	23.0	25.6	10.2	41.0	–	–

3.3 Comparative Analysis of CBC Parameters in Treated vs. Untreated CLL Cases

Analysis of complete blood count (CBC) parameters revealed substantial differences between treated and untreated CLL patients, reflecting both disease activity and therapeutic response **(Table 3)**. Untreated patients exhibited a markedly elevated white blood cell (WBC) count, with (50%) showing “very high” values and an additional (41.6%) classified as “high,” indicating significant lymphoproliferation. In contrast, only (25.9%) of treated patients had very high WBC counts, and the majority (55.5%) fell within normal limits, suggesting hematologic normalization following therapy. Similarly, lymphocyte counts were disproportionately elevated in the untreated group, with (91.6%) showing high and very high values, compared to (51.8%) among treated patients. Red blood cell (RBC) indices showed a pronounced shift toward anemia in the treated group (25.9%) had low RBCs and (33.3%) had low hemoglobin, likely due to therapy-induced marrow suppression. Conversely, all untreated patients maintained normal RBC and hemoglobin levels. Platelet counts were reduced in a notable subset of treated patients (25.9%), while thrombocytopenia was less frequent among the untreated (16.6%).

Table 3: Comparative Analysis of main CBC parameters in untreated and treated CLL patients.

Parameter	Treated (n = 27)	Untreated (n= 12)
WBC	Low: 3.7% Normal: 55.5% High: 14.8% Very High: 25.9%	Low: 0% Normal: 8.3% High: 41.6% Very High: 50.0%
LYM	Low: 7.4% Normal: 40.7% High: 25.9% Very High: 25.9%	Low: 0% Normal: 8.3% High: 33.3% Very High: 58.3%
RBC	Low: 25.9% Normal: 62.9% High: 3.7%	Normal: 100.0%
HGB	Low: 33.3% Normal: 66.66%	Normal: 100.0%
PLT	Low: 25.9% Normal: 74.0%	Low: 16.6% Normal: 83.3%

3.4 Normality Assessment of Gene Expression Data

Before conducting comparative statistical analyses, the distribution of gene expression data for Galectin-9, MALAT1, and ZAP-70 was examined to verify the assumption of normality required for parametric testing. The evaluation employed four complementary statistical tests: D’Agostino-Pearson omnibus, Anderson-Darling, Shapiro-Wilk, and Kolmogorov-Smirnov. The results demonstrated that all three genes displayed normally distributed expression values across the control, untreated, and treated CLL

patient groups, with *p*-values exceeding the (0.05) significance threshold in nearly all tests.

3.5 Chronic lymphocytic leukemia-related gene expression

Galectin-9 mRNA levels, expressed as ΔCt values, differed significantly across untreated, treated CLL patients, and healthy controls. The untreated group (n = 12) exhibited a mean ΔCt of (5.3 ± 0.29) SEM, the treated group (n = 27) 6.07 ± 0.17, and controls (n = 20) 6.3 ± 0.1 **(Figure 1a)**. One-way ANOVA revealed a significant effect of group on Galectin-9 expression (F = 6.285, p = 0.0035, R^2 = 0.186). Post-hoc tests showed that the untreated group had a significantly lower ΔCt than both the treated group (mean diff. -0.75, *p* =0.02) and controls (mean diff. -1.01, *p*=0.002), indicating higher Galectin-9 expression in treatment-naïve patients; no significant difference was observed between treated patients and controls (*p* =0.5). These results demonstrate that Galectin-9 is markedly upregulated in untreated CLL and partially normalized following therapy. MALAT1 transcript levels, expressed as ΔCt values where a lower ΔCt indicates higher gene expression, varied significantly among the three groups **(Figure 1b)**. The untreated CLL cohort (n = 12) showed a mean ΔCt of (1.5 ± 0.59) SEM, the treated patients (n = 27) 2. ± 0.28, and healthy controls (n = 20) 3.31 ± 0.14. One-way ANOVA demonstrated a significant overall effect of group on MALAT1 expression (F = 6.732, p = 0.0025, R^2 = 0.20). Post-hoc Tukey's tests revealed that both untreated and treated CLL patients exhibited significantly lower ΔCt values—and thus higher MALAT1 expression than controls (untreated vs. control: mean diff. –1.817, p = 0.0024; treated vs. control: mean diff. –1.069, p = 0.0366), whereas the difference between untreated and treated patients did not reach significance (mean diff. –0.748, p = 0.2820). These results indicate that MALAT1 is upregulated in CLL regardless of treatment status, with a trend toward greater expression in treatment-naïve patients.

ZAP-70 transcript levels, expressed as ΔCt values (where a lower ΔCt indicates higher gene expression), differed markedly among untreated CLL patients, treated patients, and healthy controls **(Figure 1c)**. One-way ANOVA demonstrated a significant overall effect of group on ZAP-70 expression (F = 13.72, p < 0.0001, R^2 = 0.33). Descriptively, the untreated cohort (n = 12) had a mean ΔCt of 4.92 ± 0.66 SEM, the treated group (n = 27) 5.30 ± 0.36, and controls (n = 20) 7.52 ± 0.13. Post-hoc Tukey's tests revealed no significant difference between untreated and treated patients (mean diff. −0.38, p = 0.79), whereas both CLL groups showed significantly lower ΔCt values and thus higher ZAP-70 expression than controls (untreated vs. control: mean diff. −2.60, p = 0.0002; treated vs. control: mean diff. −2.22, p < 0.0001). These results indicate that ZAP-70 is substantially upregulated in CLL regardless of treatment status.

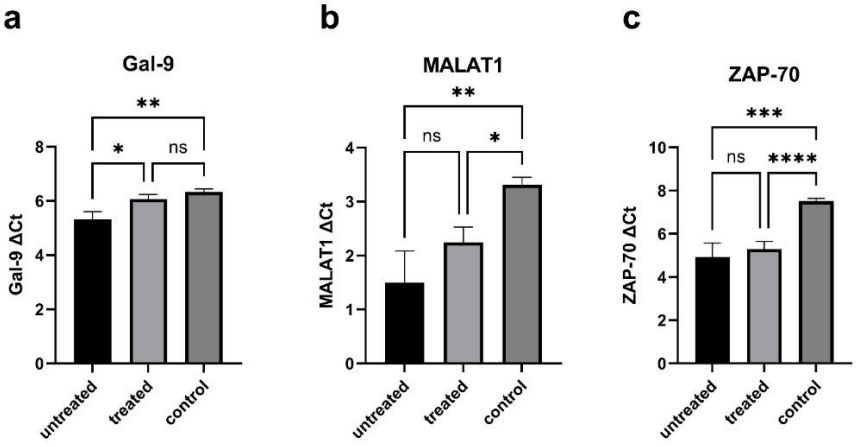

Figure 1: One-way ANOVA with Tukey's post hoc test: Relative mRNA expression(ΔCt) of (a) Galectin-9, (b) MALAT1, (c) ZAP-70, in untreated, treated, and control groups.

3.6 Gene Expression Analysis

Quantitative PCR analysis revealed marked upregulation of Galectin-9, MALAT1, and ZAP-70 in CLL patients, with expression partially attenuated following treatment. Galectin-9 levels were elevated by 1.74-fold (173.9%) in untreated patients compared to 1.30-fold (129.5%) in treated patients. MALAT1 exhibited a 3.37-fold increase (337.1%) in the untreated cohort compared to a 2.40-fold rise (239.7%) post-therapy. ZAP-70 showed the greatest dysregulation, rising 7.90-fold (789.8%) in treatment-naïve individuals and 4.64-fold (464.4%) in those who received treatment **(Figure 2)**. These findings underscore the pronounced overexpression of these biomarkers in CLL and the modulatory impact of therapy on their transcriptional profiles.

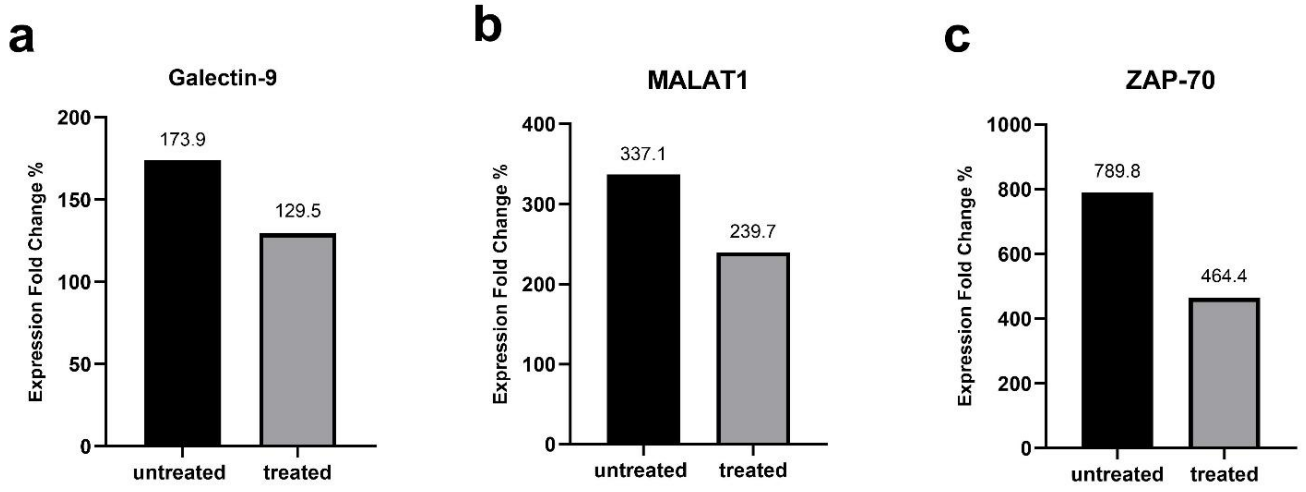

Figure 2: Fold change in mRNA expression of Galectin-9, MALAT1, and ZAP-70 in untreated and treated CLL patients (a) Galectin-9, (b) MALAT1, (c) ZAP-70.

3.7 Gene Expression Markers

Analysis of ΔCt-based ROC curves evaluated the diagnostic performance of Galectin-9, MALAT1, and ZAP-70 expression in distinguishing CLL patients from healthy controls. ΔCt Galectin-9 showed limited discriminative ability, with an AUC of 0.64 (p = 0.07) **(Figure 3a)**. In contrast, ΔCt MALAT1 provided fair diagnostic accuracy (AUC = 0.73, p = 0.003) **(Figure 3b)**, while ΔCt ZAP-70 achieved excellent discrimination between CLL and control groups, yielding an AUC of 0.89 (p < 0.0001) **(Figure 3c)**.

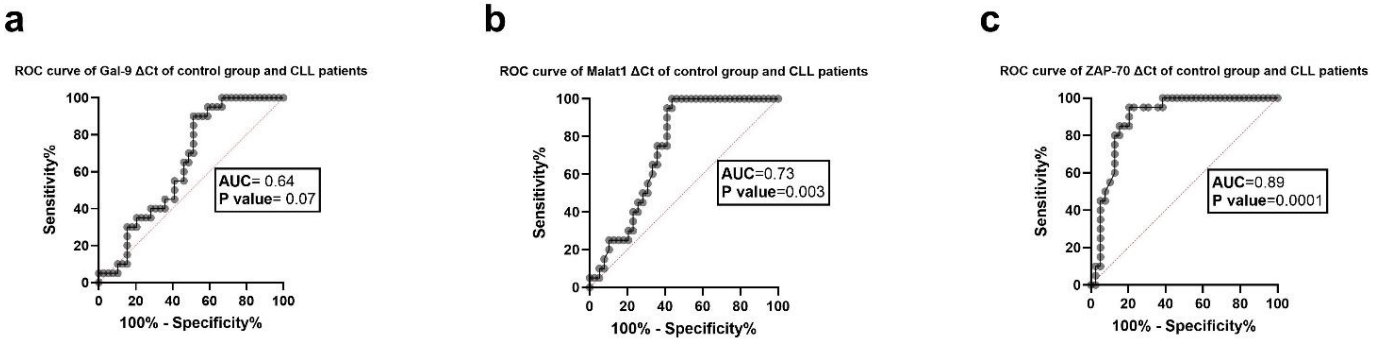

Figure 3: ROC analysis for gene expression markers in the CLL patient group and control groups. Panel (a) Galectin-9, (b) MALAT1, and (c) ZAP-70.

3.8 Inter-Marker Expression Correlations in CLL

Pearson correlation analysis of ΔCt values across all CLL patients treated and untreated together (n = 39) revealed a moderate, statistically significant positive association between Galectin-9 and MALAT1 expression (r = 0.472, 95% CI 0.184–0.686, p = 0.0024), and a strong, highly significant correlation between MALAT1 and ZAP-70 (r = 0.639, 95% CI 0.405–0.794, $p < 1\times10^{-5}$). In contrast, Galectin-9 and ZAP-70 displayed only a weak, non-significant relationship (r = 0.145, 95% CI –0.179–0.440, p = 0.38) **(Figure 4)**.

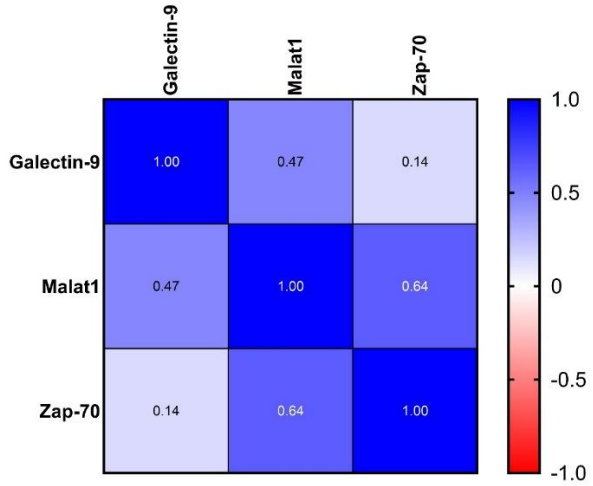

Figure 4: Heatmap of Pearson correlations among Galectin-9, MALAT1, and ZAP-70 ΔCt values in all CLL patients.

3.9 Correlation analysis among Gene Expression with Hematological parameters in Treatment-Naïve CLL Patients

As illustrated in the treatment-naïve CLL cohort (n = 12), Pearson correlation analysis of ΔCt values revealed distinct relationships between each biomarker and hematologic indices **(Figure 5)**.

Galectin-9 ΔCt showed weak-to-moderate associations with blood counts, correlating negatively with lymphocyte percentage (r = –0.25) and MID-cell population (r = –0.39), and positively with the platelet-to-lymphocyte ratio (r = 0.36), while its relationship with total WBC was modest (r = –0.21). On the red-cell side, Galectin-9 ΔCt correlated positively with RBC count (r = 0.36), hemoglobin (r = 0.13), and hematocrit (r = 0.16), but inversely with mean corpuscular hemoglobin (r = –0.28), MCV (r = –0.24), and RDW (r = –0.06). Among platelet indices, correlations were uniformly low (PLT r = 0.18, MPV r = 0.17, PDW r = –0.13, PCT r = 0.25).

MALAT1 ΔCt exhibited stronger linkage to both hematological parameters. It correlated negatively with lymphocytes (r = –0.39) and MID cells (r = –0.40), and positively with PLT/LYM ratio (r = 0.55). In the RBC panel, MALAT1 ΔCt correlated with RBC (r = 0.48), hemoglobin (r = 0.29), hematocrit (r = 0.40), and, to a lesser extent, MCHC (r = –0.24) and MCV (r = –0.10). Among platelet metrics, MALAT1 ΔCt correlations were modest (PLT r = 0.26; MPV r = 0.13; PDW r = –0.26; PCT r = 0.28).

ZAP-70 ΔCt was most closely associated with red-cell indices, correlating with hematocrit (r = 0.61), MCH (r = 0.47), MCV (r = 0.37), and RBC count (r = 0.12), while its relationships to lymphoid and platelet parameters were minimal (e.g., LYM r = 0.01; PLT r = –0.03; MPV r = 0.36).

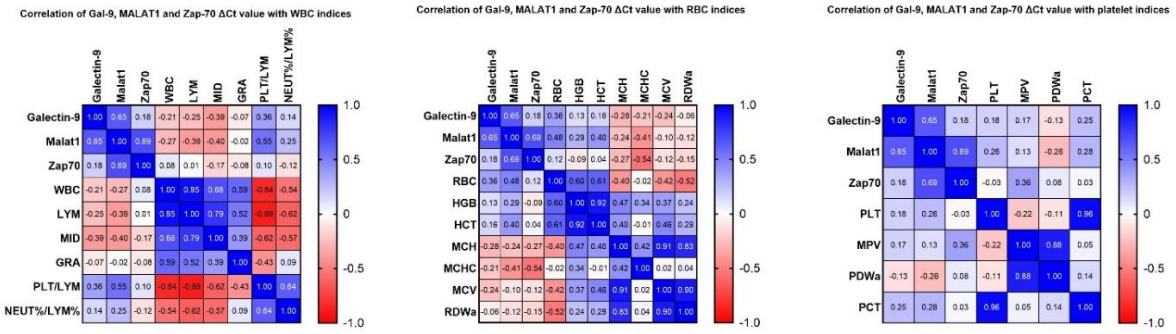

Figure 5: Heatmap of Pearson correlation coefficient among Galectin-9, MALAT1, and ZAP-70 expression and Hematological parameters in Treatment-Naïve CLL Patients.

4. Discussion

The demographic profile of the CLL cohort revealed a predominance of cases in older adults. This supports previous studies indicating that CLL primarily affects elderly populations, particularly males, due to gender-based immunogenetic and hormonal factors (Ou et al., 2022).

The flow cytometric findings confirmed classical CLL immunophenotypes, with co-expression of CD19 and CD5 observed in the majority of patients. Dim expression of CD20 and CD79b, along with lambda predominance, aligns with the diagnostic criteria defined by ERIC and iwCLL (Shi et al., 2024). The universal negativity for CD10, CD38, CD123, and CD138 helped rule out other lymphoproliferative disorders and plasma cell neoplasms (Roshal et al., 2024). Overall, these flow cytometry findings reinforce the diagnosis of CLL and highlight the value of multiparametric immunophenotyping in characterizing disease phenotype and excluding differential diagnoses.

CBC data revealed profound leukocytosis and lymphocytosis in untreated CLL patients, reflective of active disease. Treated patients showed relatively normalized WBC and lymphocyte counts but a higher incidence of anemia and thrombocytopenia, possibly due to bone marrow suppression from therapy. These findings are in agreement with previous reports that therapy in CLL often reduces disease burden but can result in cytopenias, which require careful monitoring during treatment (Hodgson et al., 2011).

In this study, we demonstrate that Galectin-9, MALAT1, and ZAP-70 are markedly upregulated at the mRNA level in treatment-naïve CLL patients and that their expression partially normalizes following therapy. Moreover, MALAT1 and ZAP-70 ΔCt values yielded fair to excellent diagnostic performance (AUCs of 0.73 and 0.89, respectively), while Galectin-9 alone was insufficient as a standalone marker (AUC 0.64).

4.1 Galectin-9 as an Immune-Regulatory Marker in CLL

Our finding of elevated Galectin-9 expression in untreated CLL in comparison to controls aligns with recent reports implicating Galectin-9 in tumor immune evasion and disease progression (Yıldırım, 2024). Bojarska-Junak et al, observed that high serum Galectin-9 concentrations correlate with worse clinical outcomes in CLL patients (Bojarska-Junak et al., 2023). Consistent with our findings, another study has demonstrated that Galectin-9 mRNA expression is significantly elevated in CLL mononuclear cells: 25 CLL patients exhibited higher Gal-9 transcript levels than 15 healthy controls (Taghiloo et al., 2017).

Mechanistically, Galectin-9 binding to TIM-3 on T cells induces T cell exhaustion and promotes regulatory T cell expansion, thereby blunting anti-tumor immunity. Correlation analysis demonstrated that Galectin-9 expression (ΔCt) exhibits minimal association with most standard hematological parameters in CLL. Specifically, very weak, non-significant correlations were observed with neutrophil-to-lymphocyte ratio, hemoglobin, hematocrit, platelet count, and mean platelet volume. Although trends toward weak-to-moderate correlations were noted for platelet/lymphocyte ratio, red blood cell count, and PCT, none of these relationships reached statistical significance. Taken together, these data suggest that while Galectin-9 is upregulated in CLL, its expression level is largely independent of conventional CBC indices(Alimu et al., 2023).

4.2 Potential MALAT1–Galectin-9 Regulatory Axis in CLL Pathophysiology

Statistically significant moderate positive correlation was observed between MALAT1 and Galectin-9 expression (ΔCT), with a ($r = 0.47$, p-value = 0.002), indicating a potential regulatory relationship between these two molecules in the pathophysiology of disease. Alnajjar Also noted a significant correlation between MALAT1 and Galectin-9 in bone tissue (Alnajjar, 2022). This MALAT1–Galectin-9 axis may contribute to immune escape mechanisms in hematological malignancies by promoting an immunosuppressive tumor microenvironment. This mechanistic link warrants further investigation through functional assays to determine whether MALAT1 silencing affects Galectin-9 expression and its downstream immunomodulatory effects.

4.3 MALAT1 Dysregulation and Diagnostic Utility

We reported significantly higher MALAT1 expression in both untreated and treated CLL patients compared to controls. MALAT1 has been shown to promote CLL cell survival via splicing-factor scaffolding and microRNA sponging (e.g., miR-363-3p), driving proliferation and apoptosis resistance. Importantly, ROC analysis indicates that MALAT1 ΔCt can discriminate CLL from healthy individuals with an AUC of 0.73 ($p = 0.003$), suggesting potential as a minimally invasive diagnostic biomarker. The strong correlation between MALAT1 and ZAP-70 ($r = 0.64$, $p < 1\times10^{-5}$) further supports a coordinated role in aggressive CLL phenotypes, likely reflecting shared reliance on BCR-associated signaling and epigenetic reprogramming (Tomic Vujovic et al., 2024;Chen et al., 2024).

Also, we noticed that MALAT1 expression demonstrated notable associations with selected hematological parameters, suggesting a potential role in systemic immune regulation. A moderate positive correlation was observed between MALAT1 expression and platelet-to-lymphocyte ratio (PLR), indicating a possible link between MALAT1 activity and inflammatory or immune response status.

4.4 Potential MALAT1–ZAP-70 Regulatory Axis in CLL Pathophysiology

The strong positive correlation between MALAT1 and ZAP-70 suggests these molecules functionally converge on B-cell receptor (BCR)–associated signaling and epigenetic reprogramming in aggressive CLL. In CLL cells, ZAP-70 acts as a critical adaptor that amplifies BCR signal transduction by promoting phosphorylation of downstream kinases such as SYK, ERK, and AKT, thereby driving proliferation and survival (Leveille et al., 2022;Turk et al., 2024). Concurrently, MALAT1, a nuclear-enriched lncRNA, modulates the transcriptome and chromatin landscape: it scaffolds splicing factors and recruits epigenetic modifiers, altering alternative splicing and histone marks at loci encoding BCR pathway components and pro-survival genes (El Said, 2019;Fernández-Garnacho et al., 2023). Moreover, MALAT1 can act as a competitive endogenous RNA, sequestering microRNAs that would otherwise repress key BCR signaling mediators, thus further enhancing signal strength (Chen et al., 2025). Together, these mechanisms, ZAP-70's direct potentiation of BCR cascades and MALAT1's epigenetic and post-transcriptional reinforcement of the same pathways, create a positive feedback loop that sustains robust BCR signaling.

4.5 ZAP-70 Expression and Prognostic Implications

Consistent with prior studies, ZAP-70 mRNA was significantly overexpressed in CLL patients (ΔCt 4.92 ± 0.66 untreated; 5.30 ± 0.36 treated vs. 7.52 ± 0.13 controls; $p < 0.0001$). ZAP-70 positivity is a well-established surrogate for unmutated IgVH status and inferior treatment-free survival (Ghergus, 2015;Chen et al., 2020). Our ROC data (AUC 0.89, $p < 0.0001$) underscore its high diagnostic accuracy.

5. Conclusion

CLL shows marked upregulation of MALAT1, Galectin-9, and ZAP-70, especially in untreated patients. MALAT1 and ZAP-70 demonstrate strong diagnostic value and correlated expression, indicating linked roles in CLL aggressiveness. MALAT1 also aligns with inflammatory status. Combined transcript analysis may enhance patient risk stratification and therapeutic planning. Validation in larger, longitudinal studies is needed.

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Conflict of Interest

The authors declare no conflict of interest

References

AHMADI, A., KAVIANI, S., YAGHMAIE, M., PASHAIEFAR, H., AHMADVAND, M., JALILI, M., ALIMOGHADDAM, K., ESLAMIJOUYBARI, M. & GHAVAMZADEH, A. 2018. Altered expression of MALAT1 lncRNA in chronic lymphocytic leukemia patients, correlation with cytogenetic findings. *Blood research* 53**,** 320

ALIMU, X., ZHANG, J., PANG, N., ZHANG, R., CHEN, R., ZENG, X., TUDAHONG, S., CHEN, G., MUHASHI, M. & ZHAO, F. 2023. Galectin-9 and myeloid-derived suppressor cell as prognostic indicators for chronic lymphocytic leukemia. *Immunity, Inflammation and Disease* 11**,** e853

ALNAJJAR, F. A. 2022. *The role of the long non-coding RNA MALAT1 in mediating inflammation in the human*

osteoarthritis joint. PhD Dissertation, University of Birmingham.
ARRUGA, F., GYAU, B. B., IANNELLO, A., VITALE, N., VAISITTI, T. & DEAGLIO, S. 2020. Immune response dysfunction in chronic lymphocytic leukemia: dissecting molecular mechanisms and microenvironmental conditions. *International Journal of Molecular Sciences* 21**,** 1825
AWLA, F. & OTHMAN, G. 2023. Association of Orai1 and Peizo1 genes expression with cellular proliferation, metastasis and angiogenesis biomarkers in colon tumor biopsies of CRC patients. *Cellular and Molecular Biology* 69**,** 232-236
BOJARSKA-JUNAK, A., KOWALSKA, W., CHOCHOLSKA, S., SZYMAŃSKA, A., TOMCZAK, W., ZAROBKIEWICZ, M. K. & ROLIŃSKI, J. 2023. Prognostic Potential of Galectin-9 mRNA Expression in Chronic Lymphocytic Leukemia. *Cancers* 15**,** 5370
CAO, Z., LENG, P., XU, H. & LI, X. 2024. The regulating role of galectin-9 in immune cell populations. *Frontiers in Pharmacology* 15**,** 1462061
CATOVSKY, D., ELSE, M. & OSCIER, D. 2019. The clinical presentation of CLL. In: Hallek M, Eichhorst B, Catovsky D, eds. *Chronic Lymphocytic Leukemia***.** Hematologic Malignancies. Springer, Cham.
CERRETO, M., FOÀ, R. & NATONI, A. 2023. The role of the microenvironment and cell adhesion molecules in chronic lymphocytic leukemia. *Cancers* 15**,** 5160
CHEN, H., MENG, T., LIU, X., SUN, M., TONG, C., LIU, J., WANG, H. & DU, J. 2015. Long non-coding RNA MALAT-1 is downregulated in preeclampsia and regulates proliferation, apoptosis, migration and invasion of JEG-3 trophoblast cells. *International journal of clinical and experimental pathology* 8**,** 12718
CHEN, J., MOORE, A. & RINGSHAUSEN, I. 2020. ZAP-70 shapes the immune microenvironment in B cell malignancies. *Frontiers in Oncology* 10**,** 595832
CHEN, J., SATHIASEELAN, V., REDDY CHILAMAKURI, C. S., ROAMIO FRANKLIN, V. N., JAKWERTH, C. A., D'SANTOS, C. & RINGSHAUSEN, I. 2024. ZAP-70 augments tonic B-cell receptor and CCR7 signaling in IGHV–unmutated chronic lymphocytic leukemia. *Blood Advances* 8**,** 1167-1178
CHEN, N., ZHANG, Y., JIANG, Y., GAO, G., LI, J. & SHI, W. 2025. The Role of MicroRNAs, CircRNAs, and LncRNAs in Chronic Lymphocytic Leukemia. *Current Pharmaceutical Design*.https://doi.org/10.2174/0113816128361848250401200001.
CHONAT, S., LOCKHART, B., GRACIAA, S. & PAESSLER, M. E. Peripheral Blood Smear Review.
DALTVEIT, D. S., MORGAN, E., COLOMBET, M., STELIAROVA-FOUCHER, E., BENDAHHOU, K., MARCOS-GRAGERA, R., RONGSHOU, Z., SMITH, A., WEI, H. & SOERJOMATARAM, I. 2025. Global patterns of leukemia by subtype, age, and sex in 185 countries in 2022. *Leukemia* 39**,** 412-419
EL SAID, N. H. 2019. *Identification of MALAT1 as a PRC2-Ezh1 Associated lncRNA Essential for Epigenetic Control of Skeletal Muscle Adaptation and Plasticity.* PhD Dissertation, King Abdullah University of Science and Technology
FERNÁNDEZ-GARNACHO, E. M., NADEU, F., MARTÍN, S., MOZAS, P., RIVERO, A., DELGADO, J., GINÉ, E., LÓPEZ-GUILLERMO, A., DURAN-FERRER, M. & SALAVERRIA, I. 2023. MALAT1 expression is associated with aggressive behavior in indolent B-cell neoplasms. *Scientific Reports* 13**,** 16839
FU, S., WANG, Y., LI, H., CHEN, L. & LIU, Q. 2020. Regulatory networks of LncRNA MALAT-1 in cancer. *Cancer management and research***,** 10181-10198
GHERGUS, D. 2015. *Autoimmune phenomena pathogenesis in chronic lymphocytic leukemia: the role of ZAP-70.* Université de Strasbourg.
HALLEK, M. & AL-SAWAF, O. 2021. Chronic lymphocytic leukemia: 2022 update on diagnostic and therapeutic procedures. *American journal of hematology* 96**,** 1679-1705
HODGSON, K., FERRER, G., PEREIRA, A., MORENO, C. & MONTSERRAT, E. 2011. Autoimmune cytopenia in chronic lymphocytic leukaemia: diagnosis and treatment. *British journal of haematology* 154**,** 14-22
JEFFERS, L. K., DUAN, K., ELLIES, L. G., SEAMAN, W. T., BURGER-CALDERON, R. A., DIATCHENKO, L. B. & WEBSTER-CYRIAQUE, J. 2013. Correlation of transcription of MALAT-1, a novel noncoding RNA, with deregulated expression of tumor suppressor p53 in small DNA tumor virus models. *Journal of cancer therapy* 4**,** 10.4236/jct. 2013.43094
JEFFERY, U. 2022. Flow Cytometry.In: W. Weiss and C. Wardrop, eds. *Schalm's Veterinary Hematology***,** 7th ed. Hoboken: Wiley-Blackwell, pp.1295-1310
JOHN, S. & MISHRA, R. 2016. Galectin-9: From cell biology to complex disease dynamics. *Journal of biosciences* 41**,** 507-534
LEVEILLE, E., CHAN, L. N., MIRZA, A.-S., KUME, K. & MÜSCHEN, M. 2022. SYK and ZAP70 kinases in autoimmunity and lymphoid malignancies. *Cellular signalling* 94**,** 110331
LI, G., DENG, L., HUANG, N. & SUN, F. 2021. The biological roles of lncRNAs and future prospects in clinical application. *Diseases* 9**,** 8
MAHER, N., MOUHSSINE, S., MATTI, B. F., ALWAN, A. F. & GAIDANO, G. 2025. Molecular Mechanisms in the Transformation from Indolent to Aggressive B Cell Malignancies. *Cancers* 17**,** 907
OOMIZU, S., ARIKAWA, T., NIKI, T., KADOWAKI, T., UENO, M., NISHI, N., YAMAUCHI, A., HATTORI, T., MASAKI, T. & HIRASHIMA, M. 2012. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. *PloS one* 7**,** e48574
OU, Y., LONG, Y., JI, L., ZHAN, Y., QIAO, T., WANG, X., CHEN, H. & CHENG, Y. 2022. Trends in disease burden of chronic lymphocytic leukemia at the global, regional, and national levels from 1990 to 2019, and projections until 2030: a population-based epidemiologic study. *Frontiers in oncology* 12**,** 840616
PEPE, F. & BALATTI, V. 2020. Role of non-coding RNAs in the development of targeted therapy and immunotherapy

approaches for chronic lymphocytic leukemia. *Journal of Clinical Medicine* 9**,** 593

ROSHAL, M., GAO, Q., HUTCHERSON, S., THOREN, K., ZHU, M. & MURATA, K. 2024. Evaluation of Plasma Cell Neoplasms. *Manual of Molecular and Clinical Laboratory Immunology* 2**,** 1206-1223

SHI, M., JEVREMOVIC, D., HANSON, C. A. & OLTEANU, H. 2024. Chronic Lymphocytic Leukemia: Diagnosis and Minimal Residual Disease Detection. *Manual of Molecular and Clinical Laboratory Immunology* 2**,** 1193-1205

TAGHILOO, S., ALLAHMORADI, E., EBADI, R., TEHRANI, M., HOSSEINI-KHAH, Z., JANBABAEI, G., SHEKARRIZ, R. & ASGARIAN-OMRAN, H. 2017. Upregulation of Galectin-9 and PD-L1 immune checkpoints molecules in patients with chronic lymphocytic leukemia. *Asian Pacific journal of cancer prevention: APJCP* 18**,** 2269

TOMIC VUJOVIC, K., UGRIN, M., TOSIC, N., VUKOVIC, V., MARJANOVIC, I., KOSTIC, T., STANKOVIC, S., OTASEVIC, V., SARAC, S. & ANTIC, D. 2024. Expression Pattern and Prognostic Significance of the Long Non-Coding RNA Metastasis-Associated Lung Adenocarcinoma Transcript 1 in Chronic Lymphocytic Leukemia. *International Journal of Molecular Sciences* 25**,** 922

TURK, A., ČEH, E., CALIN, G. A. & KUNEJ, T. 2024. Multiple omics levels of chronic lymphocytic leukemia. *Cell death discovery* 10**,** 293

WANG, H., ZHAO, Y., REN, B., QIN, Y., LI, G., KONG, D., QIN, H., HAO, J., SUN, D. & WANG, H. 2021. Endometrial regenerative cells with galectin-9 high-expression attenuate experimental autoimmune hepatitis. *Stem Cell Research & Therapy* 12**,** 1-17

WANG, K., CHEN, Z., WU, R., YIN, J., FAN, M. & XU, X. 2018. Prognostic role of high gal-9 expression in solid tumours: a meta-analysis. *Cellular Physiology and Biochemistry* 45**,** 993-1002.

WIESTNER, A., ROSENWALD, A., BARRY, T. S., WRIGHT, G., DAVIS, R. E., HENRICKSON, S. E., ZHAO, H., IBBOTSON, R. E., ORCHARD, J. A. & DAVIS, Z. 2003. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood* 101**,** 4944-4951

YILDIRIM, C. 2024. Galectin-9, a pro-survival factor inducing immunosuppression, leukemic cell transformation and expansion. *Molecular biology reports* 51**,** 571

ZHANG, C., QIN, Y., WU, Y., XU, H. & SHU, Y. 2024. Long non-coding RNA MALAT1 in hematological malignancies and its clinical applications. *Chinese Medical Journal* 137**,** 1151-1159

ZHOU, X., SUN, L., JING, D., XU, G., ZHANG, J., LIN, L., ZHAO, J., YAO, Z. & LIN, H. 2018. Galectin-9 expression predicts favorable clinical outcome in solid tumors: a systematic review and meta-analysis. *Frontiers in physiology* 9**,** 452