

## Expression of ZAP 70 in Chronic Lymphocytic Leukemia (CLL) and its Correlation with Clinical Stages

Zahra Tasleem, Hamid Saeed Malik, Hassan Shaukat, Halima Babar, Kehkashan Hasan, Saima Bashir

### ABSTRACT

**Objective:** To ascertain the levels of ZAP-70 expression within the context of Chronic Lymphocytic Leukemia (CLL) patients and to subsequently analyze the potential correlations between ZAP-70 expression and the clinical staging of the disease.

**Study Design & Setting:** Cross-sectional, descriptive study. Department of Hematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi from July 2021 to December 2021.

**Methodology:** We included 73 CLL patients of various ages and both genders. and patients having lymphoproliferative disorders other than CLL were excluded. Sysmex XN-3000 was used to do complete blood counts. Diagnosis of CLL was confirmed immunophenotypically by flow cytometry. Samples were processed by standard methods for ZAP70 analysis. Descriptive statistics were expressed in terms of mean  $\pm$  standard deviation (SD). A Chi-square test was conducted, with a significance level of p-value = 0.05 being considered significant.

**Results:** Among 73 patients, 24 were females (32.9%) and 49 were males (67.1%). Females had a mean age of  $69.00 \pm 9.47$  years, whereas males had a mean age of  $65.73 \pm 11.12$  years. ZAP-70 expression was positive in 8 (11%) and negative in 65 (89.0%) cases. The expression of ZAP-70 in CLL and its correlation with Binet Stages was significant ( $p=0.002$ ). ZAP-70 expression in males and females showed no significant difference ( $p=0.059$ ). ZAP-70 analysis showed no significant difference in patients with age more than 60 years from = 60 years of age ( $p=0.275$ ).

**Conclusion:** ZAP-70 gene expression should be used in routine laboratory settings and can be correlated with the clinical stages of CLL.

**KEYWORDS:** ZAP-70, Chronic Lymphocytic Leukemia (CLL), Binet Stages, Multiparameter flow cytometry(MFC).

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### INTRODUCTION:

In the Western world, Chronic Lymphocytic Leukemia (CLL), is the most prevalent hematologic cancer, and it constitutes approximately 25% of all leukemias in the United States. It is characterized by the gradual buildup of immature monoclonal CD5+ B-cells in various body tissues.<sup>1</sup> CLL cells rely heavily on signals from the microenvironment. CLL is related to a characteristic immune phenotype in lymphoid tissue, peripheral blood, and bone marrow.<sup>2</sup>

There is a great deal of heterogeneity in the disease, whether it is in terms of the stage of the disease at diagnosis, or terms of the cytogenetic abnormalities, as well as the clinical outcomes among patients. CLL prognosis relies on specific markers, e.g. immunoglobulin heavy chain gene (IgVH) mutational status and ZAP-70 expression. The disease can progress quickly in some patients and result in early death, but in others, it remains stable without the need for treatment and lasts for several years.<sup>3,4</sup> To identify stable or progressive forms of CLL, other prognostic factors should be considered in the early stages of the disease.<sup>5</sup>

Somatic mutations in the immunoglobulin heavy-chain variable region (IgVH) are among the most influential

prognostic factors for distinguishing two B-CLL subsets.<sup>5</sup> Patients are classified into two categories based on the presence (50 to 60%) or absence of somatic hypermutation in the immunoglobulin heavy chain variable region (IGHV) genes of the clonotypic B cell receptor (BCR). Those with low IGHV mutation levels (<2% change from germline) are termed unmutated (um-CLL) and typically experience more aggressive disease than mutated (m-CLL) patients.<sup>1</sup> These mutations lead to anergic B-cell receptors, which hinder the proliferation of leukemic cells. Patients with mutated IgVH genes tend to have a more favorable prognosis compared to those with unmutated IgVH genes who tend to have reduced survival and are lower response to chemotherapy.<sup>1,5</sup>

While IGHV mutation status is a strong prognostic marker, it does not fully capture CLL's heterogeneity. In contrast to other prognostic factors, it is not suitable for routine practice due to the complexity of the method of analysis, unlike other prognostic factors.<sup>6</sup> While noting IgVH mutations are costly and not routinely available, ZAP-70, a protein found to increase in CLL, offers a more accessible prognostic marker. It has been observed that protein tyrosine kinase ZAP-70 expression in CLL cells, measured through flow cytometry, is closely linked to IGHV mutational status, survival, and disease progression. ZAP-70's abnormal expression affects CLL cell biology by enhancing B-cell receptor (BCR) signaling (reducing threshold), proliferation, and migration toward the tumor microenvironment. Assessing ZAP-70 expression is crucial, with multiparameter flow cytometry (MFC) being a valuable method. MFC measures the fluorescence intensity of anti-ZAP-70 labeling in different cell subtypes, improving our ability to assess B cell clonality. This understanding is valuable for patient care and the development of interventions to manage the risk of additional B cell malignancies in CLL patients.

A genome-scale gene expression analysis has shown that Ig-mutated and Ig-unmutated CLL can be distinguished by the expression of a few hundred genes, and ZAP-70 expression is the most useful discriminator.<sup>7</sup> Additionally, its expression would remain stable throughout B-CLL, making it a prognostic factor. ZAP-70 is a protein tyrosine kinase that belongs to the Syk-ZAP-70 family. ZAP-70 was found to be expressed on B-CLL cells but not on normal B lymphocytes.<sup>8,9</sup> In routine laboratory settings, ZAP-70 gene expression has emerged as a promising surrogate marker for IGHV mutation status, aiding in the identification of patients with a more aggressive disease course.<sup>4,10</sup> This study was designed to investigate ZAP-70 expression patterns in B-CLL patients to gain insights into the prevalence of low-expression cases and their correlation with the clinical stages.

#### **METHODOLOGY:**

Our investigation constituted a six-month cross-sectional descriptive study conducted from July 2021 to December 2021, within the Department of Haematology at the Armed

Forces Institute of Pathology (AFIP) Rawalpindi. Ethical approval for the study was granted by the institutional review board (IRB) under the reference FC-HEM20-5/READ-IRB/21/1297. For sample size determination, we meticulously reviewed existing literature and employed the WHO calculator. This calculation yielded a sample size of 45, factoring in a 5% margin of error, a 95% confidence level (CI), and a previously reported ZAP-70 expression incidence of 2.97%.<sup>11</sup> Our sampling methodology adhered to nonprobability consecutive sampling principles, and a total of 73 eligible participants were included in the study during the designated research period, encompassing the maximum sample size available for our investigation.

**Inclusion criteria:** This research encompassed individuals newly diagnosed with Chronic Lymphocytic Leukaemia (CLL), spanning all age groups and both genders.

**Exclusion Criteria:** This study excluded individuals who had previously received a CLL diagnosis and were actively undergoing treatment.

Before enrolling patients, written consent was obtained diligently, rigorously adhering to stringent confidentiality protocols throughout all research phases. Thorough patient history collection and comprehensive physical examinations were meticulously conducted. To assess their condition, complete blood counts were carried out using the Sysmex XN-3000 system. The confirmation of Chronic Lymphocytic Leukemia (B-CLL) diagnoses was achieved through immunophenotypic analysis, utilizing flow cytometry and immunohistochemistry (IHC). Standard techniques were applied to process samples for ZAP70 analysis. The antibodies employed in this procedure consisted of CD22FITC/HLADRPE, ZAP70FITC/CD38PE, and CD45FITC/14PE. As a negative control, an isotype control (antimouse IgG1FITC/IgG2aPE) was meticulously utilized. In the context of the ZAP-70 assessment, a positivity threshold was set at an antibody population exceeding 20%, while antibody populations below this threshold were considered negative.

#### **Method for ZAP70 analysis**

3mL of whole blood samples obtained in an EDTA tube were used to measure total leucocyte count and differential leucocyte count. Fluorochrome labelled monoclonal antibodies were used including Anti CD22 FITC, Anti HLA-DR-PE, Anti ZAP-70 FITC, Anti CD38 PE, Anti CD45 FITC and Anti CD14 PE. Tubes were labelled according to the defined panel and 10µL of antibody was added in each tube and 50µL of whole blood sample. It was then mixed thoroughly followed by incubation in the dark for 30 minutes at room temperature. 1:10 dilution of FACSlyse solution was prepared in distilled water 2mL of this was added in each tube and incubation was done in the dark for 10 minutes. Tubes were centrifuged for 5 minutes at 300g and the supernatant was discarded. The remaining almost

50µL fluid was shaken to resuspend the cells. 2mL RPMI 1640/PBS was dissolved in distilled water in each tube. Then 2mL of this was added in each tube and incubated for 10 minutes in the dark. Centrifugation of the tubes was done again for 5 minutes at 300g, the supernatant was discarded and the remaining solution was shaken. 0.5mL of 3.3% Formalin solution was added to each

Data was inputted into Microsoft Excel, and then it was subjected to analysis using the Statistical Package for Social Sciences (SPSS) 21.0. Descriptive statistics were presented in terms of mean ± standard deviation (SD). Additionally, a Chi-square test was conducted, with a significance level of p-value =0.05 being considered significant. tube and kept at 4°C till analysis on a flow cytometer.

**RESULTS:**

Among 73 patients, males were 49 (67.1%) and females were 24 (32.9%) with male to female ratio of 2:1. Females had a mean age of 69.0±9.47 years, whereas males had a

Table 1: Gender distribution in different age groups

Age Range	Females (%)	Males (%)	Total (%)
<60	5 (20.8)	19 (38.8)	24 (32.9)
>60	19 (79.2)	30 (61.2)	49 (67.1)
<b>Total</b>	<b>24 (32.9)</b>	<b>49 (67.1)</b>	<b>73 (100)</b>

Table 2: Correlation of ZAP-70 with Binet Stages using the Chi-Square test

ZAP-70	Binet Stages			Total (%)	p-value
	A	B	C		
Positive	0 (0.00%)	4 (12.9%)	4 (40.0%)	8 (11.0%)	0.002
Negative	32 (32.0%)	27 (87.1%)	6 (60.0%)	65 (89.0%)	
<b>Total</b>	<b>32 (100%)</b>	<b>31 (100%)</b>	<b>10 (100%)</b>	<b>73 (100%)</b>	

Figure 1: Distribution of ZAP-70 within Gender

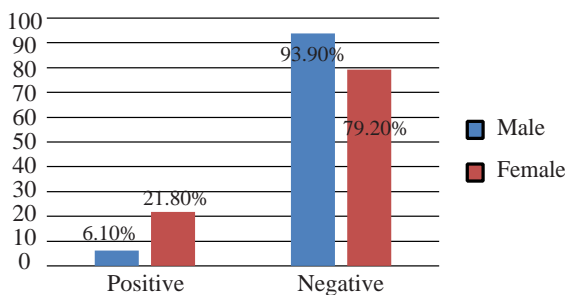
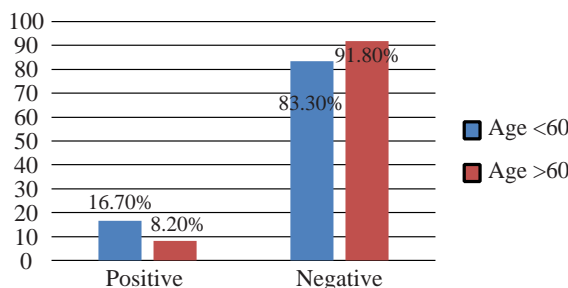


Figure 2: Distribution of ZAP-70 within age group



mean age of 65.7±11.12 years. Both males and females showed the highest frequency of cases in the age group > 60 years. (Table 1). ZAP-70 expression was positive in 8 (11%) and negative in 65 (89.0%) cases. We assessed the correlation between ZAP-70 expression and Binet Stages in Chronic Lymphocytic Leukemia (CLL) using the Chi-Square test, yielding a significant result (p-value=0.002). (Table 2). Distribution of ZAP-70 within gender is shown in Figure 1. Forty nine 49 (67.1%) patients were above 60 years of age. Distribution of ZAP-70 within age group as shown in Figure 2.

**DISCUSSION:**

CLL is a heterogeneous disease and has widely variable outcomes i.e in a study by Strati P et al, the analysis of causes of death revealed the multifaceted nature of mortality in CLL patients. Few patients have sluggish disease and they have a survival of up to 20 years and usually, in such cases, death is not directly related to CLL. Infections, other malignancies, and comorbid health conditions emerge as pivotal determinants of patient outcomes, exerting significant influence. This underscores the importance of adopting a comprehensive approach to caring for CLL patients, to enhance the care and prognosis of individuals with CLL.<sup>12</sup>

However, in some patients, a benign course is followed by an aggressive disease. The findings from a study by Greipp PT et al, provided significant insights into a rare CCL subgroup, having male predominance corresponding to our research and diagnosis at an advanced stage of the disease, associated with the aggressive nature of this particular subtype of CLL. These patients experience a significantly shorter overall survival (OS) i.e. death occurs within three to five years of diagnosis, underscoring the severe consequences. These deaths are usually because of complications that are directly related to CLL.<sup>13</sup>

In our study, the majority of cases were males( 67.1% n 49/73) while females accounted for about one-third. Similar to our study, Diehl F et al.<sup>14</sup> also observed that males consistently represented the majority of CLL cases. This study was conducted on a much larger scale providing a comprehensive overview of CLL demographics and outcomes. They noted a mean age of 69.6 years. We also observed the same mean age in females 69±9.47SD years while 65.7±11.12 years was the mean age in males. It revealed that CLL primarily affects older individuals, with a substantial proportion diagnosed at the age of 60 or older. This could be due to the fact that older patients were more likely to opt for no treatment, as compared to younger patients. This study reported 5-year and 10-year overall survival rates which got worse for older patients, particularly those aged 80 or older.

Similarly in alignment with our study, Mjaliet et al,<sup>15</sup> also reported that CLL primarily affects patients over the age of 50, with no reported cases in individuals below 30. This



study specifically emphasized different types of leukemia across various age groups. This is likely due to differences in sample sizes and the inclusion of diverse leukemia types in the later study, showing the multifaceted nature of leukemia and the need to consider diverse factors, including age and gender, in both leukemia research and clinical practice. However, Strati P *et al.*,<sup>12</sup> noted a slightly higher mean age of 72 years.

In our study, ZAP-70 expression was positive in 8 (11%) and negative in 65 (89.0%) cases however, no significant differences were observed in ZAP-70 expression between genders or age groups. A previous study done by Shaikh M *et al.*,<sup>16</sup> in the year 2020 in our country gave a greater sample size for analysis, showing male predominance having an M: F ratio of 2:1 in resonance with our study. Similarly, the median age for the entire study population was 62 years with minimal age difference between males and females. The analysis of ZAP-70 expression revealed that 15% exhibited high expression, 43.3% had low expression, and 41.7% were negative for ZAP-70, giving similar positive results, delving further into categorizing expression levels. However, ZAP-70 expression showed no significant distinction between males and females echoing the findings of our research.

In a study done in India by A Gogia *et al.*,<sup>17</sup> ZAP -70 positivity was noted in 25% of cases of CLL which is roughly double of noted by us. These variations may arise from differences in patient demographics and testing Techniques. The median age of CLL patients was 57 years, indicating slightly younger average ages for both genders with 64 male and 16 female consistently showing male predominance, emphasizing this gender pattern within the disease. These discrepancies in their findings are likely due to differences in patient populations and assay methods. For defining positivity of ZAP 70 expression Shaikh M *et al.*,<sup>16</sup> and A. Gogia *et al.*,<sup>17</sup> both used a cut off of 20%. The same was used by us.

The expression of ZAP-70 and its correlation with Binet Stages was significant in our study (p-value =0.002). Out of total 73(100%), all 32/73 (43%) analysed cases show negative results for Binet Stage A, while out of 31/73 (42.4%) patients which were analysed for Binet Stage B, 4 (12.9%) showed positive results while 27 (87.1%) showed negative results. Out of total 10/73 (13.4%) patients 4 (40%) showed positive results and 6 (60%) negative results. Attia HR *et al.*,<sup>18</sup> documented in their study that in the Binet staging system, there was a noteworthy increase in the expression of ZAP-70 in group C patients compared to groups A and B (p-value 0.008, 0.034 and 0.017 respectively ) which were linked to higher CD38 expression, hepatomegaly, splenomegaly, and specific genetic markers. This study showed a more comprehensive analysis of prognostic markers with males being more prevalent as in our study.

R. Zeeshan *et al.*,<sup>19</sup> in a local study in Pakistan indicated a

similar male predominance with a slightly lower mean age of 57.5 years. In our study, females tended to be older than males. Conversely, the second study did not reveal a significant age difference between genders. However, both studies concurred that CLL predominantly affects individuals in their middle to later years. This study also correlated age and gender with ZAP-70 expression in CLL and noted that no correlation existed between ZAP-70 with age and gender (p <0.05). ZAP-70 positivity was identified in 13.5% of the cases (slightly higher rate) associated with stage III disease and a high lymphocytic count. These associations underscore the potential prognostic value of ZAP-70. Similar results were noted in our study and by Rossi D *et al.*<sup>20</sup>

## CONCLUSIONS:

ZAP-70 expression was shown to be substantially associated with advanced binet stages, extensive intrathoracic/abdominal lymphadenopathies, splenomegaly, and cytopenias, all of which are signs of active and aggressive illness. Increased ZAP-70 expression is an important predictor of disease progression and a helpful indicator for risk assessment in newly diagnosed CLL patients. It predicts overall survival in CLL patients

### Authors Contribution:

**Zahra Tasleem:** Conception, Data Collection, Drafting, Statistical Analysis

**Hamid Saeed Malik:** Design of study, Proofreading, Drafting

**Hassan Shaukat:** Data collection, Literature review

**Halima Babar:** Literature Review, Statistical Analysis

**Kehkashan Hasan:** Literature Review, Data Collection

**Saima Bashir:** Data collection, Drafting

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