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**RESEARCH ARTICLE**

# Detection of CD55- and/or CD59-deficient red cell populations in chronic lymphocytic leukemia Egyptian patients

**Ebtesam I. Ahmad [1] Nashwa M.A. Alazizi [1] and Rham Z. Ahmed [2]**

**1.**Clinical Pathology Departments, Zagazig University, Egypt
**2.**Medical Oncology Departments, Zagazig University, Egypt

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***Corresponding Author**

**Nashwa M.A. Alazizi**

## *Abstract*

**Background:** Paroxysmal nocturnal hemoglobinuria (PNH)-phenotype has been described in various hematological disorders, mainly aplastic anemia and myelodysplastic syndromes, while it has been reported that complete deficiency of CD55 and CD59 has also been found in patients with lymphoproliferative syndromes. We aimed to detect frequency and to assess the clinical significancd of the Paroxysmal Nocturnal Hemoglobinuria clones in chronic lymphocytic leukemia patients.

**Subjects and Methods:** The study comprised 70 subjects who were divided into thirty five patients with B-chronic lymphocytic leukemia and thirty five apparently healthy volunteers; anti-CD55 (DAF) and anti- CD59 (MIRL) were conducted using flow cytometry analysis for detection of PNH clone. Univariate analysis and correlations were performed to correlate between the presence of PNH clone and clinical and laboratory parameters.

**Results:** No concomitant negative CD55 and CD59 (CD55- CD59- phenotype) was found among the control group, while it was found in 8 /35 (22%) among the patient group. Isolated CD55 negative (CD55- CD59 + phenotype) was detected in 2/35 (5.7%) and 7/35 (20%) in control group and patient group, respectively. Isolated CD59 negativity (CD55+ CD59 - phenotype) was detected in 2/35 (5.7%) of the control group and 2/35 (5.7%) of patient group also. The PNH clone phenotype was significantly expressed among patient group compared to control group, ($p<0.05$). According to Binet staging, the median of CD55 negative phenotype in patients group was (11.17%) in stage A, (12.2%) in stage B and (13.8%) in stage C. There was no significant statistical difference between different stages, ($P>0.05$). While The mean ± SD of CD59 negative phenotype in patient group was (5.40 ±1.30%) in stage A, (9.32 ±3.66%) in stage B and (3.15 ±1.79%) in stage C, There was a highly significant statistical difference between different stages, ($P<0.001$) . Least significant difference of CD59 negative phenotype among the three stages showed significant elevation in CD59- phenotype in stages A & B compared to stage C, ($p<0.05$) & ($p<0.001$) respectively, while there was significant elevation in stage B compared to stage A, ($p<0.05$). There was a highly significant positive correlation between absolute lymphocytic count with both CD55 and CD59 negative expression, ($P < 0.001$).

**Conclusion:** From the present study we can conclude that flow cytometry is an important tool which can be used as a screening test for the detection of granulocyte populations deficient for CD55 & CD59 "PNH phenotype" in

CLL patient. The correlation between the lymphocytic count with PNH phenotype in our study may point to a role for PNH phenotype in the disease progression

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## INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a type of slow-growing leukemia affects developing lymphocytes which are under normal conditions produce immunoglobulin's that protect our bodies against infections and diseases **(Valent et al, 2007**)(1).

CLL remain stable for many months and years and around thirty - fifty percent of patients diagnosed with CLL never require any treatment For others, the leukemia cells multiply in an uncontrolled way, live longer than they are supposed to and accumulate in the bone marrow, blood stream, lymph nodes, spleen and other parts of the body making some people with CLL more susceptible to anemia, recurrent infections and to bruising and bleeding **(Elter et al., 2011**)(2). Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematological disorder characterized by increased number of cells with deficiency of glycosylphoshatidyl-inositol (GPI) - anchored membrane protein such as (CD55-CD59). Loss of these surface protein due to lack of GPI-anchor makes RBCs sensitive to complement Lysis as CD55, and CD59 are the most important membrane complement regulatory proteins (MCRPs) and they inhibit the complement cascade. The premature destruction lead to wide range of symptoms and complications **(Brodskyet al., 2008)*(3).***

PNH is divided into three subtypes: classic PNH, PNH accompanied with another specified bone marrow disorder (PNH-ad), and subclinical PNH (PNH-sc). In chronic lymphocytic leukemia it has recently been shown that the clonal expansion of granular lymphocytes occurs in patients with paroxysmal nocturnal hemoglobinuria (PNH)-phenotype in a subclinical fashion. Symptoms of hemolysis may be abscent in PNH- sc **(Kaiafa et al.,** 2008)(4).

The importance of detection of PNH clone in patients is that it could provide us with a useful prognostic tool depending on that the more we move from groups of better prognosis to the ones of the worse prognosis, the PNH clone is increasingly expressed **(Savage et al., 2009)(5).**

Patients are often overlooked for PNH testing due to the perceived rarity of PNH cells. Studies have suggested that, the presence of over a small number of PNH cells detected by flow cytometry may predict that patient’s has a higher a clinically important response to immunosuppressive therapy **(Pu et al., 2011)** (6).

The aim of this study is to detect the presence of the Paroxysmal Nocturnal Hemoglobinuria clones in chronic lymphocytic leukemia patients by assessment of CD55 and CD59 expression using flow cytometry and their correlation to clinical and laboratory parameters.

## Subjects and Methods

The study comprised 70 subjects who were divided into 2 groups:

1- **CLL group:** Comprised thirty five adult patients with CLL. They were 15 males and 20 females; with ages ranged from 50-70 years with mean±SD of 57±5 years. All patients had no history of previous malignancy and they gave their consent to participate.

2- **Control group:** Comprised thirty five apparently healthy individuals. They were 16 males and 19 females; with ages ranged from 45-56 years with mean±SD of 55±4.5.

All members of two groups were subjected to the following: Full history taking and clinical examination, - Routine investigations, including: Complete blood count (CBC), Liver and Kidney function tests, Bone marrow aspiration with examination of leishman stained smears for patients group when indicated, Direct coomb's test in all anemic patients and prior to starting treatment to identify autoimmune related hemolytic anemia and Immunophenotyping by using Flow cytometry (FACScan, Becton Dickinson, San Jose, California, USA). Acquisition and analysis were performed with CellQuest software (BD Biosciences) for establishment of the diagnosis of CLL in the patients group (Wood et al., 2007) (7). Radiological studies including: Chest X-Ray, CT scan and pelvi-abdominal US.

**Immunophenotyping**: Establishment of CLL diagnosis in our patients was done according to international scoring system. Five immunophenotyping markers are assessed in CLL: SIg, CD5, CD23, FMC7, and CD79b (or CD22). Cases with scores of 4 or 5 are considered typical for CLL. Scores of 3 or less are considered atypicalfor CLL (Moreau et al., 1997(8), Oscier et al., 2004)(9).

Detection of CD55 (DAF) and CD59 (MIRL) by flow cytometry for detection of PNH phenotype in patient and control groups. Ham test was done for all patients with PNH phenotype (CD55- CD59-).

PNH clone phenotype positivity threshold was defined as more than 5% of cells deficient for markers (CD55 and CD59) (Luzzatto and Notaro., 2012).(10).

***Statistical Analysis***

The data were tabulated and statistically analyzed using Microsoft Office Excel 2010, and Statistical Package for Social Sciences version 20 (SPSS).

Data were summarized using the arithmetic Mean standard deviation (SD), median and range for numerical variables. The frequency, distribution and percentage were calculated for categorized variables.

For Comparative Statistics; the following methods were used Chi-squared test ($\chi^2$) independent "t" test, and ANOVA "f" test and for non-parametric data Mann Whitney test and Kruskal–Wallis were used. For all above mentioned statistical tests done, the threshold of significance was fixed at 5% level (p-value).

## RESULTS

Seventy subjects participated in this study were composed of 31 male and 39 female the male to female ratio was (1:1.3) while it was (1:1.1) in the control group, with no significant statistical difference between both groups, ($p>0.05$) .The mean of age ±SD was (55±4.5) and (57±5) among the control and patient groups respectively with no significant statistical difference between both groups, ($p>0.05$), **table (1).**

As regard to hematological data, there were high significant elevation difference in mean±SD of TLC ($P<0.001$) and mean±SD of lymphocyte ($P<0.001$) in patient group compared to control group. Non significant difference in both mean value of R.C percentage ($P>0.05$) and median value of PLT ($P>0.05$), with high significant decrease in mean value of RBCs±SD ($P<0.001$) and mean value of Hb±SD ($P<0.001$), table(2).

Also the patient group showed high significant elevation in mean value of LDH±SD($P<0.001$)**,** significant elevation in both the mean value of urea±SD and mean value of creatinine ±SD ($P<0.05$) compared to control group, table(3)

According to Binet staging Out of 35 CLL patients, there were 8 (22.85%) in Stage A, 4 (11.42%) in stage B and 23 (65.71%) in stage C**, table (4)**. According to immunophenotyping score of CLL patients group there were 3/35 (8.57%) with score 3, 13/35 (37.14%) with score 4 and 19 /35 (54.28%) with score 5, table (4).

Among the 35 normal control no one had cells with concomitant negative both CD55 and CD59 (CD55- CD59 - phenotype), while this double negativity was found in 8 /35 (22.85%) among the patient group. Isolated CD55

negativity (CD55- CD59 + phenotype) was detected in 2/35 (5.7%) of the control group, while it was detected in 7/35 (20%) of the pati±ent group. Isolated CD59 negativity (CD55+ CD59 - phenotype) was detected in 2/35 (5.7%) of the control group and 2/35 (5.7%) of patient group also.

The PNH clone phenotype was significantly expressed among patients group compared to control group, (p<0.05).

There was no significant statistical difference between 3 phenotypes in control group, (p>0.05), while in patient group there was significant statistical difference between 3 phenotypes, (p<0.05) with significant elevation in double negativity (CD55- CD59- phenotype) compared to isolated CD55 negativity (CD55- CD59+phenotype) and isolated CD59 negativity (CD55+ CD59 – phenotype), **Table (5)**

Median of CD55 negative cells in control group was (1.1), while in patient group it was (12.8) with means ± SD (1.3±0.8 &14.3±7.2) respectively. There was a highly significant elevation in patient group compared to control group, (p<0.001), table (6).

The mean±SD of CD59 negative cells in control group was (1.8±0.7), while in patient group it was (4.2±2). There was a highly significant elevation in patient group compared to control group, (p <0.001), **table(6).** Out of 8 patients with double negative CD55 and CD59 (CD55- CD59 –) phenotype 1patient (12.5%) was in stage A, 2 patients (25.0%) were in stage B and 5 patients (62.5%) were in stage C. These differences was statistically significant, (p<0.001), **Table (7), figure(1).**

Median of CD55 negative phenotype in patients group was (11.17) in stage A, (12.2) in stage B and (13.8) in stage C. There was no significant statistical difference between different stages, (P>0.05).The mean ± SD of CD59 negative phenotype in patient group was (5.40 ±1.30) in stage A, (9.32 ±3.66) in stage B and (3.15 ±1.79) in stage C., There was a highly significant statistical difference between different stages, (P<0.001**), table( 8)**.

Least significant difference of CD59 negative phenotype among the three stages showed significant elevation in CD59- phenotype in stages A & B compared to stage C, (p<0.05) & (p<0.001) respectively, while there was significant elevation in stage B compared to stage A, (p<0.05).

Median of CD55 negative phenotype in patient group was (11.65) with score 3, (11.12) with score 4 and (14.97) with score 5. There was no significant difference between different scores, (P >0.05). Median of CD59 negative phenotype in patient group was (5.87) with score 3, (5.44) with score 4 and (2.89) with score 5. There was no significant difference between different scores, (P>0.05), **table(9)**

There was no significant correlation between Hb concentration versus CD55 and CD59 negative expression, (P >0.05). There was a highly significant positive correlation between absolute lymphocytic count and CD55 CD59 negative expression, (P < 0.001). There was no significant correlation between platelet count versus CD55 and CD59 negative expression, (p > 0.05)**, table( 10)** ,Figure(2).All patients with PNH (CD55- CD59-) phenotype had negative Ham test. We found that cases with PNH phenotype were coomb's test negative and had normal reticulocyte percentage except for one case presented with normocytic normochromic anemia, positive coomb's test and elevated reticulocyte percentage (9.5%) and was diagnosed as a case of CLL complicated with AIHA.

**Table (1): Demographic data of the patients and control groups**:

| | Control (N =35) | Patient (N =35) | Test of significance | P-value |
|---|---|---|---|---|
| **Sex N (%)** | | | | |
| Male | 16(45.7%) | 15(42.9%) | $\chi^2$ | > 0.05 (NS) |
| Female | 19(54.3%) | 20 (57.1%) | | |
| Male: Female ratio | 1 : 1.1 | 1 : 1.3 | | |

| **Age (years)** | | | | |
|---|---|---|---|---|
| Mean±SD | 55±4.5 | 57±5 | t-test | > 0.05 (NS) |
| Range (years) | 45-65 | 50-70 | | |

*NS= not significant*

**Table (2): Comparison of the hematological data between control and patient groups.**

| | Control group | Patient group | T-test | p-value |
|---|---|---|---|---|
| **TLC(×$10^3$ /$mm^3$ )**<br>mean±SD<br>Range | 7±1.4<br>(6.5-11) | 123.2±20.17<br>(30-150) | 3.98 | <0.001** |
| **Lymphocytes %**<br>mean±SD<br>Range | 27±6<br>(20-35) | 74±11<br>(42-90) | 18.6 | <0.001** |
| **RBCs (x$10^6$/µL)**<br>mean±SD<br>Range | 5.77±1.6<br>(4-6) | 4.07±0.85<br>(2-5) | -7.6 | <0.001** |
| **Hb( gm/dl )**<br>mean±SD<br>Range | 13±1.7<br>(10-14) | 10.5±1.8<br>(7-12.8) | 9.24 | <0.001** |
| **Reticulocytes %**<br>mean±SD<br>Range | 0.9±0.42<br>(0.5-1.8) | 1.39±1.49<br>(0.5-9.5) | -1.612 | >0.05<br>**(NS)** |
| **Platelet(×$10^3$/$mm^3$)**<br>Median<br>Range | 239.5<br>(150-400) | 100<br>(42-374) | **Mann Whitney**<br>**Z**=1.116 | >0 .05<br>**(NS)** |

**Table (3): Comparison between patient and control groups as regard laboratory data.**

| | Control group | Patient group | Test of Significance t-test (t) | p-value |
|---|---|---|---|---|
| **LDH(U/L)**<br>Mean±SD<br>Range | 256±58<br>(200-340) | 679.9±300<br>(300-2977) | 7.5 | <0.001** |
| **Total bilirubin(mg/dl)**<br>Mean±SD<br>Range | 0.9±0.1<br>(0.3-1.1) | 2±0.3<br>(0.1-3) | 1.7 | >0.05<br>**(NS)** |
| **Direct bilirubin(mg/dl)**<br>Mean±SD<br>Range | 0.2±0.07<br>(0.1-0.3) | 1.2±0.4<br>(0.1-2) | 0.5 | >0.05<br>**(NS)** |
| **Total protein(gm/dl)**<br>Mean±SD<br>Range | 7.4±0.4<br>(7-8) | 6.9±1<br>(5-8) | 1.6 | >0.05<br>**(NS)** |
| **Albumin(gm/dl)**<br>Mean±SD<br>Range | 3.8±0.3<br>(3.5-4.5) | 3.7±0.7<br>(3-4) | 2.5 | >0.05<br>**(NS)** |
| **ALT(U/L)**<br>Mean±SD | 22.3±6 | 63.5±13 | 1.8 | >0.05<br>**(NS)** |

| Range | (10-30) | (30-120) | | |
|---|---|---|---|---|
| **AST(U/L)**<br>Mean±SD<br>Range | 30.5±8<br>(13-40) | 57±10<br>(50-70) | 2.5 | >0.05<br>**(NS)** |
| **Urea(mg/dl)**<br>Mean±SD<br>Range | 22±6<br>(20-28) | 46.9±14<br>(40-60) | 2.9 | <0.05* |
| **Creatinine(mg/dl)**<br>Mean±SD<br>Range | 0.7±0.2<br>(0.4-1.2) | 1.2±0.4<br>(0.3-2) | 2.8 | <0.05* |

**Table (4): Binet staging and Immunophenotyping score of patients group.**

| **Binet Staging** | **n (35)** | **%** | **Immunophenotyping score** | **n (35)** | **%** |
|---|---|---|---|---|---|
| **A** | 8 | 22.85 | **3** | 3 | 8.57 |
| **B** | 4 | 11.42 | **4** | 13 | 37.14 |
| **C** | 23 | 65.71 | **5** | 19 | 54.28 |

**Table (5): CD55 and CD59 negative expression (PNH phenotype) among patient and control groups.**

| **Group / Phenotype** | **Control** (No =35) | **%** | **Patient** (No =35) | **%** | **P** |
|---|---|---|---|---|---|
| $CD_{55}^{-}$ $CD_{59}^{-}$ | (0) | 0.0 | (8) | 22.85 | < 0.05* |
| $CD_{55}^{-}$ $CD_{59}^{+}$ | (2) | 5.7 | (7) | 20 | |
| $CD_{55}^{+}$ $CD_{59}^{-}$ | (2) | 5.7 | (2) | 5.7 | |
| $X^2$ | 2.1 | | 11.3 | | |
| | > 0.05 | | <0.05* | | |

**Table (6): The mean value of CD55 and CD59 negative expression among patient and control groups:**

| | **Control group** | **Patient group** | **Test of significance** | **P** |
|---|---|---|---|---|
| **CD55 ⁻**<br>Median<br>Mean±SD<br>Range | <br>1.1<br>1.3± 0.8<br>(0.1-3.88) | <br>12.8<br>14.3±7.2<br>(0.7-39.4) | **Mann Whitney**<br>**Z**=7.4 | <0.001** |
| **CD59 ⁻**<br>Mean±SD<br>Range | <br>1.8±0.7<br>(0.16-5.06) | <br>4.2±2<br>(0.61-11.9) | **t**=5.2 | <0.001** |

**Table (7): The relation between Binet staging of patients and expression of double negative CD55 and CD59 ($CD55^-$ $CD59^-$) phenotype.**

| **-ve Markers expression / Stage** | **double negative phenotype ($CD55^-$ $CD59^-$)** (No = 8) | **%** | **P** |
|---|---|---|---|
| **A** | 1 | 12.5% | 0.001** |
| **B** | 2 | 25.0% | |
| **C** | 5 | 62.5% | |

**Table (8): Expression of CD55 and CD59 negative phenotypes among patients group according to Binet staging.**

| Binet stage<br>% of deficiency | Binet stages | | | Test of significance | P |
|---|---|---|---|---|---|
| | **A (No= 8)** | **B (No=4)** | **C (No=23)** | | |
| **$CD_{55}^-$**<br>median<br>Mean ±SD<br>Range | <br>11.17<br>12.88±8.66<br>(0.70-32.76) | <br>12.2<br>10.45±5.81<br>(1.89-17.20) | <br>13.8<br>15.23±9.18<br>(1.76-39.47) | **Kruskal – Wallis**<br>**K**=0.3 | >0.05<br>**(NS)** |
| **$CD_{59}^-$**<br>Mean ±SD<br>Range | <br>5.40±1.30<br>(3.27-7.23) | <br>9.32±3.66<br>(6.73-11.90) | <br>3.15±1.79<br>(0.61-6.11) | **F**=12.5 | <0.001** |

**Table (9): Expression of CD55 and CD59 negative phenotypes according to immunophenotyping score in all patients.**

| Score<br>phenotype | Score | | | Kruskal - Wallis | P |
|---|---|---|---|---|---|
| | **3 (No=3)** | **4 (No=13)** | **5 (No=19)** | | |
| **CD55-**<br>Median<br>Mean ±SD<br>Range | <br>11.65<br>16.19±11.3<br>(7.86-29.06) | <br>11.12<br>12.28±9.43<br>(0.70-35.76) | <br>14.97<br>15.20±9.38<br>(1.76-39.47) | **K**=0.3 | >0.05<br>**(NS)** |
| **CD59-**<br>Median<br>Mean ±SD<br>Range | <br>5.87<br>4.56±2.48<br>(1.70-6.11) | <br>5.44<br>5.16±1.27<br>(3.12-7.23) | <br>2.89<br>3.48± 2.80<br>(0.61-11.90) | **K**=1.3 | >0.05<br>**( NS)** |

**Table (10): Pearson correlation between expression of $CD55^-$ and $CD59^-$ versus (Hb, absolute lymphocytic and platelet counts) in patients group.**

| Phenotype<br>Laboratory Data | $CD55^-$ | | $CD59^-$ | |
|---|---|---|---|---|
| | **r** | **P** | **r** | P |
| **Hb( gm/dl )** | -0.17 | > 0.05(**NS**) | 0.13 | > 0.05 (**NS**) |
| **Lymphocytes $10^3$/µL (absolute count)** | 0.64 | <0.001** | 0.43 | <0.001** |
| **platelet(×$10^3$/$mm^3$)** | -0.12 | > 0.05(**NS**) | 0.10 | > 0.05 (**NS**) |

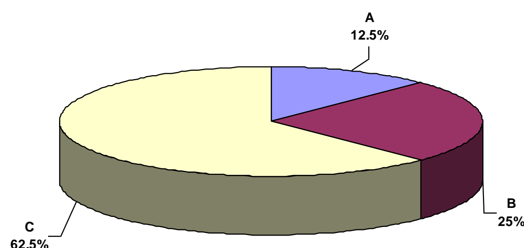

**Figure (1): The relation between Binet staging of patients and double negative CD55 &CD59 ($CD55^-$ $CD59^-$) phenotype**

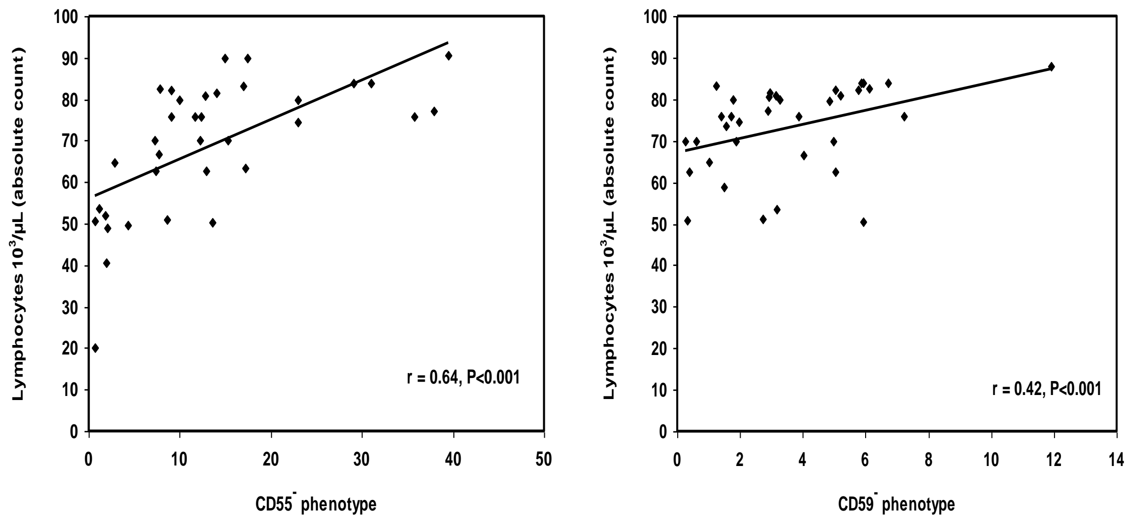


**Figure (2): Pearson correlation between expressions of $CD55^-$ and CD59- phenotype versus absolute lymphocytic count in patients group.**

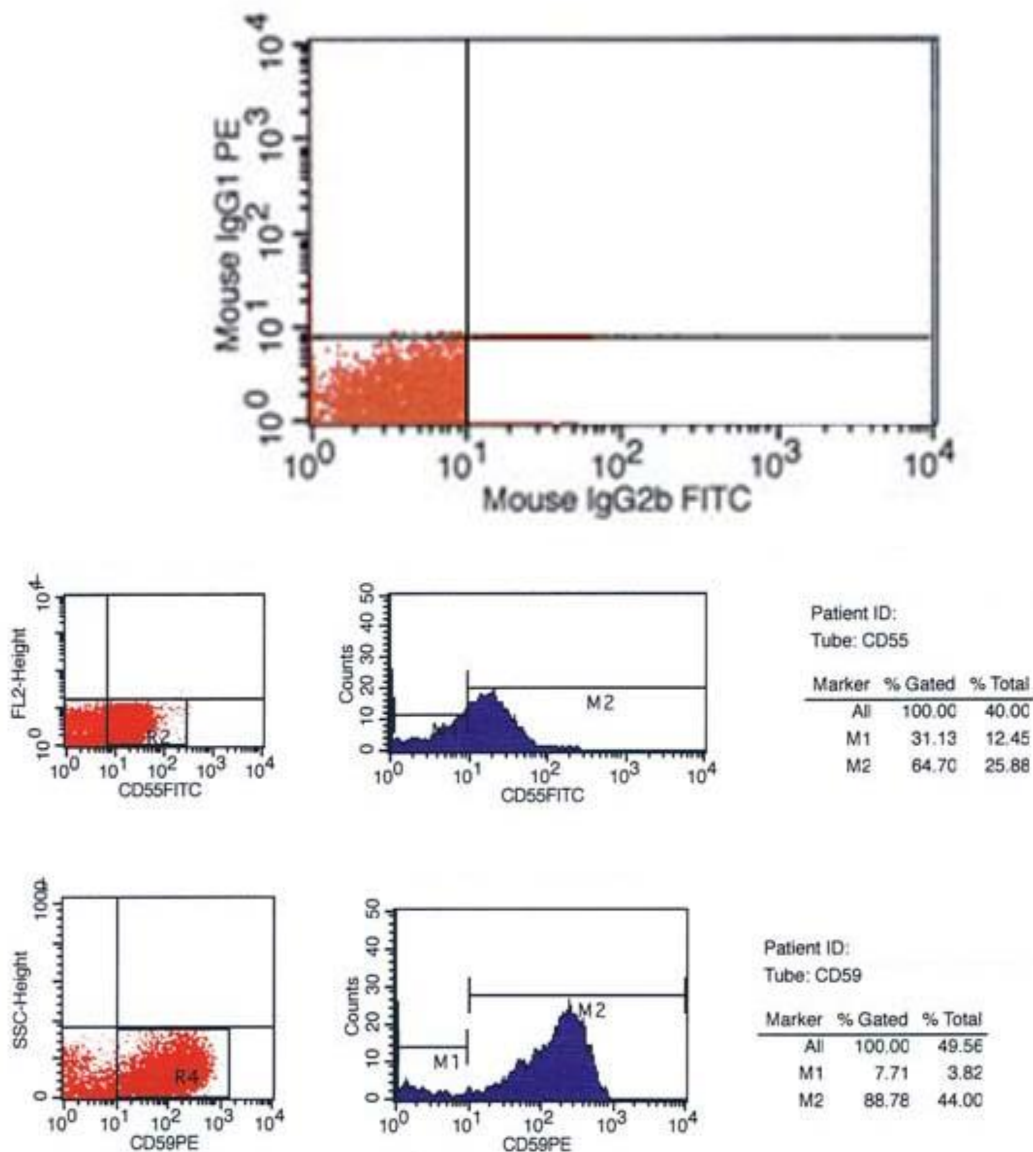


Figure (3) immunophenotyping of peripheral blood sample from CLL patient revealed double negativity of CD55 and CD59 ($CD55^{-}$ $CD59^{-)-}$ phenotype, so the patient is considered $PNH^{+}$.

## Discussion

The PNH-like defect has been studied in a series of patients with any form of lymphoproliferative syndromes and the results have been variable (Varma et al., 2012)(11) and Karadimitris et al., 2012) (12)

In the PNH haematopoietic cells, the first step in biosynthesis of the GPI-anchor is defective because of a somatic mutation in the gene encoding the α1-6-N-acetyl-glucosaminyl-transferase necessary for GPI-anchor formation and binding (the X-linked PIG-A gene), Rosti,( 2000) (13)

In the current study, we have screened the PNH-phenotype in CLL patients by flow cytometry to assess its frequency. Flow cytometry is useful in detecting PNH-sc as it is sufficiently sensitive to detect accurately 3% GPI-AP-deficient cells (Wang et al., 2012) (14)

In this study, double negative red cell populations (CD55- CD59– ) phenotype couldn't be detected among the control, while Isolated CD55- and CD59- negative expression (CD55- CD59+ & CD55+ CD59-) phenotypes both was detected in 2/35 (5.7%) of the control group. This result was in accordance with Sa Wang et al., 2009(15). The presence of extremely low levels of GPI-deficient neutrophils in eight out of eight normal individuals had been described, and PIG-A mutations had been identified in the majority of these cases in study of Araten et al., 1999 (16)

Also it has been reported that 5 of 121 healthy subjects had red cell populations deficient in CD55 and/or CD59 (Meletis et al., 2001)(17). These results suggest that the development of PNH may require not only a PIG-A mutation, but also a selection in favor of the PNH clone to allow it to proliferate preferentially compared with the residual hematopoiesis (Tremml et al., 2002) (18)

The “dual pathogenesis” model for the development of PNH was described according to Rotoli and Luzzatto. They suggested the presence of 2 components of PNH pathogenesis: (1) the presence, in very small numbers, of stem cells with the characteristic defect and proliferative disadvantage in the marrow of many individuals and (2) an aplastogenic influence suppressing the normal stem cells but not those bearing the mutated PIG-A gene, allowing the overgrowth of the PIG-A mutated cells However, it is crucial to distinguish PNH as a disease entity from the PNH-like defect that presents as the phenotypic abnormalities seen in various clonal disorders (Rotoli and Luzzatto., 1989) (19)

In our study, concomitant deficiency of CD55 and CD59 was found in 8/35 (22%) among our patients group, while isolated CD55- and CD 59- negativity (CD55- CD59 + & CD55+ CD59 -) phenotypes was detected in 7/35 (20%) & 2/35 (5.7%) respectively. There was significant elevation in numbers of patients expressing PNH phenotype compared with the control group.

This was in accordance with Hill et al. (2004(20)) who reported in his study a patient with CLL expressing a PNH phenotype. They concluded that somatic mutations in CLL could lead to another clonal entity and was in contrast with another study by Varma et al., (2012)(11) who could not detect that any of his patients had PNH (CD55- CD59–) phenotype which could be explained by in vivo lysis of cells showing deficit expression of GPI complex.

We found that the median of CD55 negative cells was significantly elevated in patient group compared to control group. Also, the mean ± SD of CD59 negative cells showed a significant elevation in patient group compared to control group.

Different proportions of PNH cells have been observed in a series of patients with different forms of LPS, before any kind of therapy (Fukuda et al., 1994(21) & Seya et al., 1994)(22) or after specific treatment, like anti-CD52 antibody administration (Hertenstein et al., 1995(23) & Taylor et al., 1997).(24)

Several hypotheses could be proposed trying to explain the presence of PNH phenotype in LPS and other clonal disorders. The stem cell of LPS may be prone to somatic mutations, and this could lead to another clonal entity (Seya et al., 1994)(22). As PNH phenotype is present at extremely low levels in a proportion of normal individuals, even if not usually detectable, we could suppose that LPS might offer a survival advantage to PNH cells, possibly by an immune-mediated mechanism, that should make this population detectable by different techniques (Dunn et al., 1999) (25)

The PNH cells are deficient in GPI-anchored proteins; it seems that a PNH clone reaches detectable proportions only if there is selection in its favor (Luzzatto & Bessler, 1996(26) and Hillmen & Richards, 2000) (27)GPI-deficient T lymphocytes have been detected in patients with chronic lymphocytic leukemia (CLL) after being treated with anti-CD52 antibodies (Campath-1H), supporting the hypothesis that an attack against the CD52 (GPI-anchored) protein may lead to the expansion of a GPI-anchor–deficient cell populations with the phenotype of “PNH” cells (Rawstron

et al., 2005)(28). Taylor and his colleagues demonstrated that T cells with a PNH phenotype can arise after Campath-1H therapy and that the GPI-deficient phenotype is stable in culture (Taylor et al., 1997)(24)

However it has been proven that PIG-A mutations were detectable in such patients prior to (Campath-1H) administration (Rawstron et al., 2005)(28), indicating that PNH-like clones are present in a very small proportion of cells prior to selection in their favor by (Campath-1H).

There was no significant statistical difference between the three PNH phenotypes in control group. while in patient group there was significant statistical difference between the three PNH phenotypes, with significant elevation in double negativity (CD55- CD59- phenotype) and isolated CD55 negativity (CD55- CD59+phenotype) compared to isolated CD59 negativity (CD55+ CD59 – phenotype).

This result was in accordance with Meletis et al. (2001)(17) who suggested that the incidence of CD55 deficient red cells was higher compared with CD59 deficient populations, may be due to a preferential preservation of CD59 in the red cells of PNH patients. As reported by other investigators (Hall and Rosse, 2000)(29), anti-CD55 did not delineate the abnormal erythrocyte population as well as did anti-CD59 due to the fact that CD55 antigen is weakly expressed on human erythrocytes.

It is of interest that patients with CLL and low grade B-cell NHL present a greater proportion of deficient CD55 and CD59 red cells compared to other types of NHL. The same phenomenon was observed in Hodgkin's disease where patients with nodular sclerosis had increased frequency of deficient CD55 and CD59 erythrocytic populations compared to other types of the disease (Meletis et al 2001) (17)

Among our patient group the double negative phenotype (CD55- CD59-) was significantly elevated in stage C compared to stage A and stage B. Our result can be explained by that 23/35 (65.7%) of our patients was in the C stage. In their study Meletis et al. (2001)(17) found that there was no correlation between the stages of CLL with the PNH phenotype. They also stated that clinical presentation of the disease or the hematological parameters do not seem to have any relationship with the development of PNH clone.

In our study, the median of CD55 negative phenotype showed no significant statistical difference between the three different stages of the disease. On the other hand, the mean ± SD of CD59- negative phenotype in patients group showed a highly statistical significant difference between the three disease stages, showing significantly high level in stage B compared to stage A & C. While it was significantly higher in stage A compared to stage C.

There was no significant correlation between Hb concentration and platelet count versus CD55- and CD59- negative expression, this was in accordance with Varma et al. (2012)(11). On the other hand Pramoonjago et al. (1999)(30) recorded some negative correlation between hemoglobin concentration and the percentage of CD59-negative granulocytes in 34 patients with PNH; this suggested that the degree of anemia depends partly on the size of the PNH clone.

There was a significant positive correlation between absolute lymphocytic count and CD55 or CD59 negative expression, this was in accordance with Kwong et al. (2001)(31) who hypothesed that LPS might offer a survival advantage to PNH cells and both were due to somatic mutations in LPS stem cells.

In this study all patients with PNH (CD55- CD59-) phenotype had negative Ham test. A result that is totally comparable with the data presented in the literature (Gupta et al., 2009(32) & Varma et al., 2012)(11) .This is possibly due to the small population of erythrocytes with reduced expression of GPI-AP, while in Ham test the result is positive if 10% to 50% cell lysis occurs in the sample (*Barbara* et al*., 2011).(33)* On the other hand it has been shown that CD55 or CD59 deficiency alone is not sufficient to produce homologous hemolysis (Sun et al., 1999)(34) and this phenomenon may also explain the absence of hemolysis in patients with isolated reduction of these antigens from cell membrane

Gupta et al. (2009)(32) stated that Ham test has limitations: firstly, this test is not specific for PNH; secondly, they can only screen for the protein defect on erythrocytes, and finally, these procedures cannot be applied after blood transfusion.

On the other hand Hill et al. (2004)(20) reported a case of CLL with positive Ham test that initially had no evidence of PNH, after treatment with alkylating agents and irradiation the Ham test became positive with some but not all of the sera tested. No evidence of intravascular hemolysis was noted. They postulated the development of an abnormal clone of erythroid cells either from the leukemia itself or marrow injury after treatment. Nevertheless, findings in that case were not typical of PNH.

From the present study we can conclude that flow cytometry is an important tool which can be used as a screening test for the detection of granulocyte populations deficient for CD55 & CD59 "PNH phenotype" in CLL patients.

We found that the PNH clone is present in CLL patients either in concomitant double negative phenotype or in single marker deficiency phenotype. The correlation between the lymphocytic count with PNH phenotype in our study may point to a role for PNH phenotype in the disease progression.

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