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RESEARCH ARTICLE

Detection Of Paroxysmal Nocturnal Hemoglobinuria Clones In Patients With Pancytopenia.

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Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) clones lack complement regulatory molecules (CD55,CD59) and therefore probably "weakened", have no malignant potential, occur at low levels in normal individuals. The presence of PNH clones in the setting of aplastic anemia (AA) or myelodysplastic syndrome (MDS) have been shown to have prognostic and therapeutic implications. The current study aimed to detect expression of PNH clones (CD55 and CD59) in patients with pancytopenia by flowcytometric analysis. The study included 48 newly diagnosed pancytopenic patients. Twenty four apparently healthy volunteers' age and sex matched with patients were chosen as control group. Peripheral blood samples were be analyzed by flowcytometry for detection of CD55&CD59. 33.3% of patients having ($CD55^{-}$ and $CD59^{-}$) were detected, also $CD55^{-}$ and $CD59^{-}$ were significantly increased in pancytopenic patients with PNH clone subgroup compared to the other two patients subgroups (aplastic anemia and MDS). PNH clone was higher in patients with A.A (40%). than in patients of MDS (22.2%). In conclusion**,** flowcytometry is a sensitive and reliable method of PNH clone assessment in bone marrow failure syndrome. Our findings confirmed the presence of $CD55^{-}$ and $CD59^{-}$cell clones in granulocytes of pancytopenic patients who diagnosed as A.A and MDS. This suggests that these disorders may be part of bone marrow failure syndrome. The assessment of PNH clone may be important for monitoring therapy. This needs to be confirmed with further studies on larger cohorts of patients

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INTRODUCTION

Pancytopenia is an important clinico-haematological entity encountered in our day to day clinical practice. It is a disorder in which three major formed elements of blood (red blood cell, white blood cell and platelets) decrease in number [(1)]. It is not a disease entity but a triad of findings that may result from number of diseases; primary or secondary; involving the bone marrow such as paroxysmal nocturnal haemoglobinuria(PNH), aplastic anemia, myelodysplastic syndrome (MDS) and bone marrow replacement by leukemia or metastasis or may be secondary to hypersplenism[(2)]**.** PNH is a rare acquired hematological disorder characterized by anemia, intravascular hemolysis, bone marrow hypoplasia, as well as tendency to thrombosis and infection. Increased sensitivity of erythrocytes to complement-mediated cell lysis is due to deficiency of membrane-bound GPI (glycosylphosphatidyl inositol)-anchored proteins which normally function as the inhibitors of reactive hemolysis. Diagnosis requires flowcytometry. Treatment is supportive and with eculizumab, a terminal complement inhibitor [(3)]. Aplastic anemia is a hematopoietic stem cell disorder characterized by pancytopenia associated with hypocellular bone morrow which

is replaced by fat cells (a sponge-like tissue) and results in deficient production of all bloods cells. Although most cases of aplastic anemia are idiopathic, the condition may occur in patients exposed to high dose of radiation, chemotherapeutic agents, drugs such as chloramphenicol and phenylbutazone and infections such as hepatitis and Epstein – barr virus[(4)]. MDS is a heterogeneous group of acquired clonal stem cell disorders characterized by dysplasia of one or more haematopoietic cell lines with increased risk of transformation to acute myeloid leukemia. In a patient with MDS, the blood stem cells (immature cells) do not become healthy red blood cells (R.B.Cs), white blood cells (W.B.Cs), or platelets.This leaves less room for healthy W.B.Cs, R.B.Cs, and platelets to form in the bone marrow, so ineffective haematopoiesis should be occured[(5)]. **.**PNH and MDS commonly arise in patients with aplastic anemia showing pathophysiological link between these disorders that are included in bone marrow failure syndrome[(6)]**.** Moreover it has been demonstrated that the lack of GPI anchored membrane proteins such as CD55 and CD59 has diagnostic value in PNH. Because PNH often develops in patients with aplastic anemia and pancytopenia, we attempted to detect a PNH clone (CD55 and CD59) in patients with pancytopenia using flowcytometry with monoclonal antibodies against CD55 and CD59[(7)].

The objective of this work is to detect expression of PNH clones (CD55 and CD59) in patients with pancytopenia by flowcytometric analysis and its importance in prediction of disorder such as PNH, A.A and MDS.

PATIENTS AND METHODS

1) This case-control study was carried out at Medical Oncology and Clinical Pathology Departments, Zagazig University Hospitals; it comprised 48 newly diagnosed pancytopenic patients ,They were 30 males (62.5%) and 18 females (37.5%), with male to female ratio of 1.6:1. Their ages ranged from 28-69 years with mean±SD of 48.9±11.59 years. Twenty four apparently healthy volunteers' age and sex matched with patients were chosen as control group. They were 11 males (45.83%) and 13 females (54.2%) with male to female ratio 1:1.1. Their ages ranged from 30-62 years with mean±SD of 43.29±9.9 years.

2) **Selection criteria of the patients:** The patients were selected for the study on the basis of standard clinical and hematological criteria for pancytopenia without evidence of hemolysis and all patients provided informed consent.

3) **Exclusion criteria:**-Patients who known to suffer pancytopenia secondary to hypersplenism or liver disease, patients with known hematological or non-hematological malignancy receiving chemotherapy or radiotherapy, or patients with cytopenia proved to be secondary to vitamin B12 or folate deficiency.

4) All the members of the study were subjected to the following:-Complete history taking and thorough clinical examination. Complete blood count (CBC) and reticulocytic count. Leishman- stained peripheral blood (PB) smears examination. Routine investigations {Liver function tests, Kidney function tests, LDH and Serum ferritin} Bone marrow aspiration with Prussian blue staining of the smears and bone marrow biopsy with silver staining whenever needed (for patients only). Ham's test for acid hemolysis: it is based on RBCs deficient of CD55 and CD59 are sensitive to lyses by activated complement fixation.

Flowcytometric analysis of CD55 and CD59:-

***Reagents:-**Monoclonal antibodies: Anti-Human CD55 Fluorescein isothiocyanate conjugate (FITC) also known as antibody of decay accelerating factor (DAF) and Anti-Human CD59 Phycoerythrin conjugate (PE) also known as antibody of membrane inhibitor of reactive lysis (MIRL) purchased from eBioscience, United States. Negative isotypic control for determining the non-specific binding of the monoclonal antibodies (MoAbs) against mouse IgG_2 and kappa light chain. Phosphate buffered saline (PBS) (8.5g Nacl, 1.07g NaH_2PO_4\ anhydrous and 0.39g NaH_{24}-$2H_2O$) was added to 1 liter of distilled water (DW), pH 7.4 (sigma, chemical co.st. Louis Mo.). BD FACS Lysing solution diluted 1:10 with deionized water.

***Staining:-** Blood samples were collected on EDTA vaccutainer tubes. 10µL monoclonal antibody (anti-human CD59 or CD55) was added to100 µL blood in respective tubes of samples and isotypic control. The tubes were vortexed and incubated in dark for 30 minutes at room temperature. 500 µL FACS Lysing solution (diluted 1:10) was added that lyses erythrocytes under gentle hypotonic conditions while preserving leucocytes. The tubes were vortexed and incubated 10 minutes at room temperature. The tubes were centrifuged at 1500 rpm for 5 minutes, and then the supernatant was discarded. Two wash steps were started by adding 500 µL PBS, vortexed then the tubes were centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. Finally, the sample was mixed by gently swirling the sample using a vortex, and 500µl PBS was added to the sample that was ready for reading. Aquisition was done by flowcytometer (FACS Calibur, BD, USA) using CellQuest software (BD, USA).

5) ***CD55 and CD59 expression analysis and interpretation [(8)]:-**For cytometric analysis, granulocytes were analyzed using light-scatter parameter which in terms of FSC/SSC (size/ cell granularity). The presence of negative

cell population for CD55% and CD59 above 5% of cells was adopted as a criterion for recognition of the PNH cell clone.

6) ***According to the foredone laboratory investigations,** the patients was categorized into 3 subgroups: **(A)-** Eighteen patients with aplastic anemia (14 males and 4 females and their ages ranged from 28-56 years with mean±SD of 45.7±10.7 years). (**B**)-Fourteen patients with MDS (8 males and 6 females, their ages ranged from 47-69 years with mean±SD of 56.4±8.04 years). Among MDS group, there were 10 patients with RA, 2 patients with RA with ringed sideroblast (RARS) and 2 patients with RA with excess blast type 1. **(C)-**Sixteen patients having pancytopenia with PNH clone (6 males and 10 females, their ages ranged from 26-62 years with mean±SD of 46±13.2 years), 12 with aplastic anemia and 4 having MDS (RA). All patients were Ham's test negative.

7) **STATISTICAL ANALYSIS:**-The data were collected, presented and analyzed using SPSS-PC (version 10) software. Comparisons between measures (Mean+SD) of two groups were done using student t-test. While, comparisons between measures (Mean+SD) between multiple groups were done by one way **ANOVA** test. The test results were considered significant when P. value < 0.05 and considered non significant when P. value > 0.05. Kruskall-Wallis test: Used instead of ANOVA in non-parametric data (SD>50% mean).Correlation co-efficient rank test used to rank different variables against each other in linear correlation which was positive or negative**.**

RESULTS

LDH and Ferritin were significantly increased in the patients' subgroups than the control but lower count of TLC, Hb and Platelets were reported in patients' subgroups than in control. This was accompanied by increase of $CD55^{-}$ and $CD59^{-}$ among patients groups than control group, (**tabs;1&2** respectively).

$CD55^{-}$ and $CD59^{-}$ were significantly increased in pancytopenic patients with PNH clone subgroup compared to two other patients' subgroups (aplastic anemia and MDS) and the control group. On the other hand PNH clone was higher in patients with A.A. (40%) than in patients of MDS (22.2%), (**tabs; 3&4 -fig;3).**

There was a significant negative correlation between $CD59^{-}$ and TLC in pancytopenia with PNH clone subgroup, but no significant correlation as regard Hb and platelets, ,(**tab;5-fig;4)**. Patients with A.A and MDS had showed no significant correlation between $CD55^{-}$& $CD59^{-}$ and (TLC, Hb and Platelets),**(tabs;6&7)**.

1)

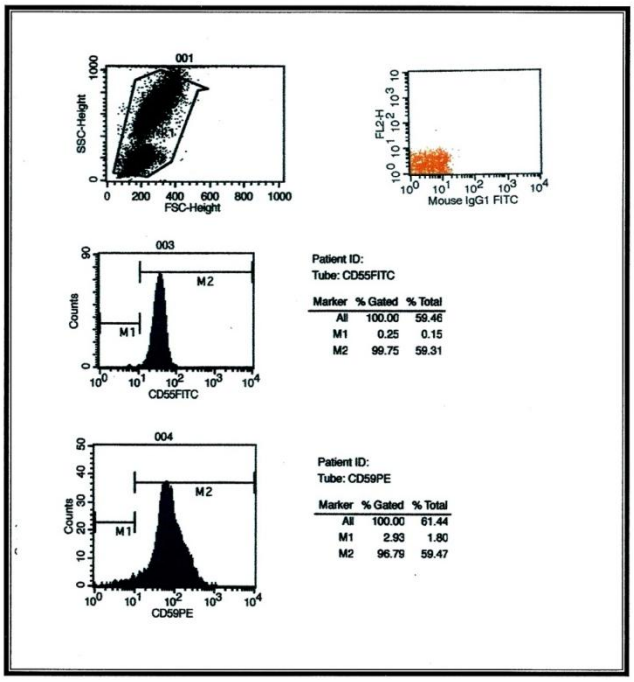

Figure (1):-Immunophenotyping of peripheral blood sample of healthy control revealing normal expression of PNH clone ($CD55^-$ and $CD59^-$).

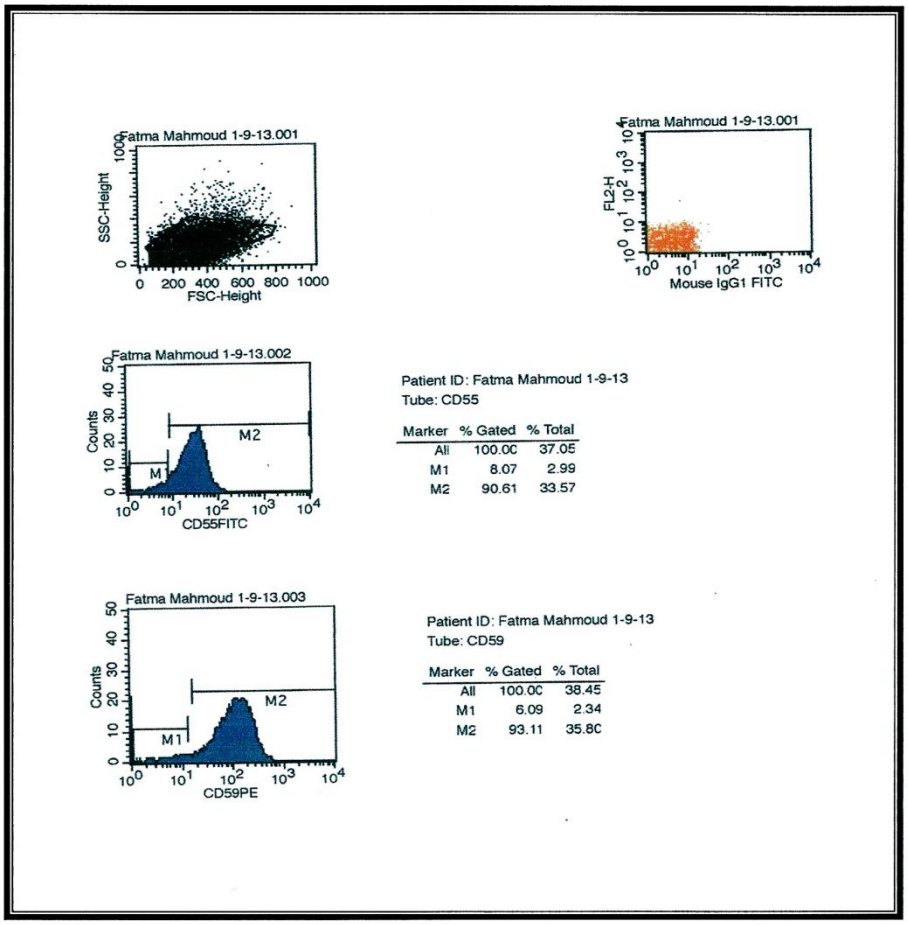

Figure (2): Immunophenotyping of peripheral blood sample of pancytopenic patient revealing positive expression of PNH clone ($CD55^-$ and $CD59^-$)

Table (1):-Comparison of the mean values of different parameters between patients subgroups and control group using ANOVA test

Parameters	Patients			Control N=24	F	P. value
	Aplastic anaemia N=18	MDS N=14	Pancytopenia with PNH clone N=16			
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD		
TLC ($x10^3/mm^3$)	2.6±0.8	2.7±0.9	2.8±0.8	7.7±1.6*	62.3	<0.001**
Hb (gm/dl)	9.2±1.1	9.4±0.8	8.5±0.9	14.4±1.1*	92.31	<0.001**
Platelets ($x10^3/mm^3$)	30.5±26.4	54.4±50.8	48.7±37.7	257.17±62.7*	Z=71.23	<0.001**
LDH (U/l)	554.5±115	611.6±141.2	537.3±32.6	319.9±61.7*	32.56	<0.001**
Ferritin (ng/ml)	826.4±259.5	704.7±321.4	848.7±321.6	82.5±30.3*	45.09	<0.001**

*****group which significantly different from other groups.

Table (2):-Comparison of $CD55^{-}$ &$CD59^{-}$ between patients and control

Parameters	Patients (Mean ±SD)	Control (Mean ±SD)	MW Test	P. value
$CD55^{-}$ (%)	7.08±5.3 **Median**=6.4	2.7±1.8 **Median**=2.09	**Z**= -2.9	<0.01*
$CD59^{-}$ (%)	12.7±10.8 **Median**=7.6	1.6±1.5 **Median**=1.08	**Z**= -4.5	<0.001**

Z= MW=Mann-Whitney U test (non parametric test).

Table (3): Comparison of the mean values of $CD55^{-}$ and $CD59^{-}$ between patients subgroups and control using Kruskal Wallis test.

Parameters	Patients			Control	KW	P. value
	Aplastic anaemia N=18	MDS N=14	Pancytopenia with PNH clone N=16	N=24		
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD		
$CD55^{-}$ (%)	4.9±2.4 Median= 5.43	4.6±2.3 Median= 1.46	11.5±5 * Median= 9.8	2.7±1.8 Median= 2.09	Z 16.6	0.001**
$CD59^{-}$ (%)	6.9±3.3+ Median= 6.4	4.3±3.01 Median= 3.39	26.7±9.2* Median= 27.1	1.6±1.5+ Median= 1.08	Z 28.01	0.001**

(*) group which was a highly significantly increase from other groups, p.values < 0.001
(+) group which was a significantly decrease compared to aplastic anaemia, $p < 0.05$

Table (4): Distribution of PNH clone among patients with aplastic anemia and MDS.

	With PNH clone	Without PNH Clone	Total	X^2	P. value
Aplastic anaemia	12(40%)	18(60%)	30	0.8	0.3
MDS	4(22.2%)	14(77.8%)	18		
Total	16(33.3%)	32(66.7%)	48		

X^2= Chi-Square test.

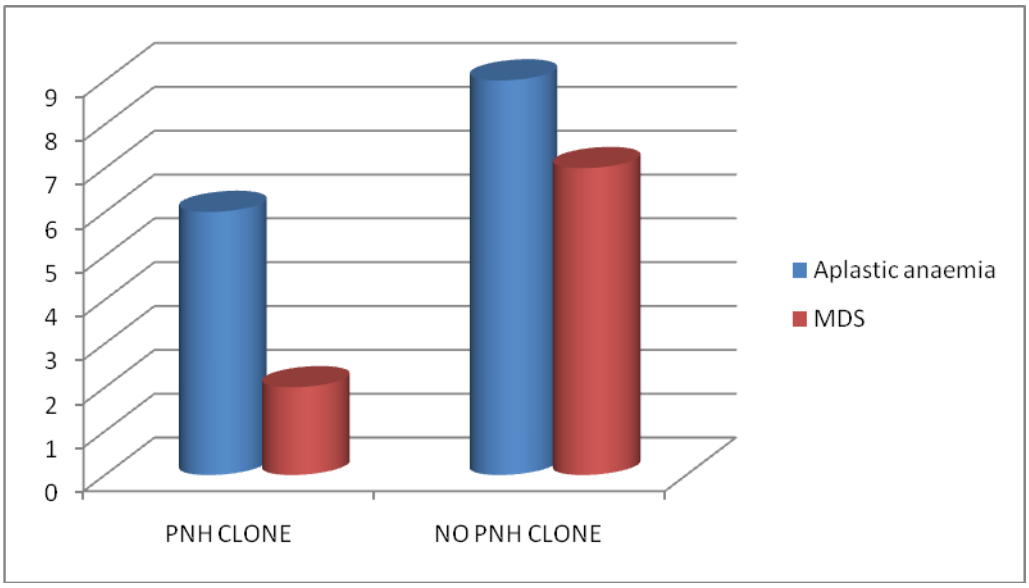

Figure (3): Distribution of PNH clone among cases of aplastic anemia and MDS.

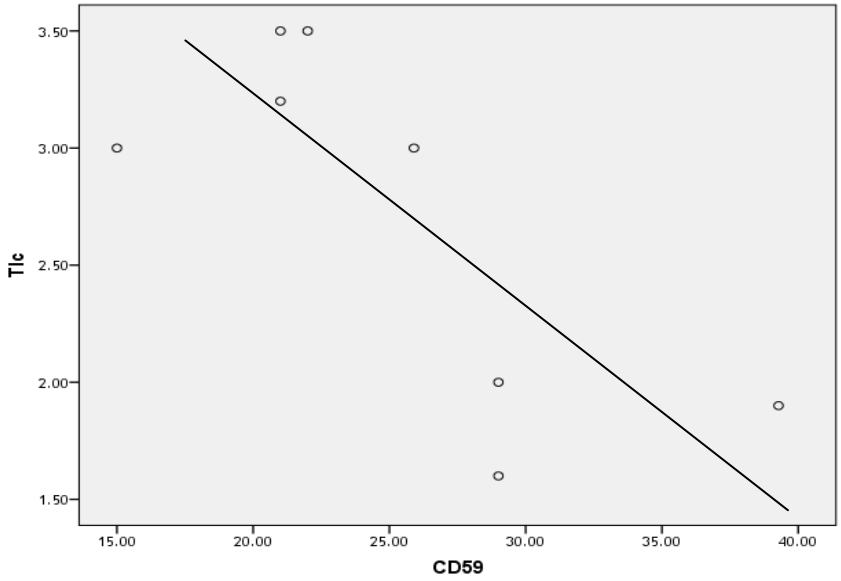

Figure (4): Negative correlation between $CD59^{-}$ and TLC in pancytopenia with PNH clone subgroup.

Table (5): Correlation between $CD55^{-}$ & $CD59^{-}$ and other parameters in patients with (pancytopenia with PNH clone).

Pancytopenia with PNH clone	$CD55^{-}$		$CD59^{-}$	
	R	**p. value**	**R**	**P. value**
TLC($x10^3/mm^3$)	0.45	0.25	-0.69	0.05*
Hb(gm/dl)	0.01	0.93	0.24	0.62
Platelets($x10^3/mm^3$)	-0.14	0.7	-0.292	0.5

Table (6): Correlation between $CD55^-$ & $CD59^-$ and other parameters in patients with aplastic anemia.

Aplastic anemia	$CD55^-$		$CD59^-$	
	R	P. value	R	P. value
TLC($x10^3/mm^3$)	-0.06	0.86	-0.13	0.61
Hb(gm/dl)	-0.13	0.73	-0.08	0.81
Platelet($x10^3/mm^3$)	-0.48	0.12	-0.53	0.11

Table (7): Correlation between $CD55^-$ & $CD59^-$ and other parameters in patients with MDS.

MDS	$CD55^-$		$CD59^-$	
	R	P. value	R	P. value
TLC($x10^3/mm^3$)	-0.21	0.61	-0.17	0.71
Hb(gm/dl)	-0.11	0.81	-0.48	0.24
Platelet($x10^3/mm^3$)	-0.39	0.38	-0.42	0.33

DISCUSSION

PNH is a clonal disorder of hematopoietic stem cells and all blood cell lineages are involved. Disturbances of GPI-anchored proteins are commonly due to somatic mutation of phosphatidylinositol glycan complementation class A (PIG-A) gene [(9)]. Various tests are used for the determination of PNH. Routine diagnostics involves: the Ham's test (acidified serum lysis test) but it determines only the sensitivity of red blood cells to complement-mediated lysis. For the evaluation of reduced expression of GPI anchored proteins on all blood cells, flowcytometric analysis is probably the best method to date [(10)].

In the present study, by using monoclonal antibodies against CD55 and CD59 and flowcytometry, we searched for PNH-type clone in the peripheral blood of patients without clinical symptoms of haemolysis.

In this study, PNH clone ($CD55^-$ and $CD59^-$) was significantly increased in patients with pancytopenia compared to normal control. This result in agreement with finding of **Kiafa et al.** [(11)] who reported that in patients with myelodysplastic syndrome (MDS) granulocytic populations were mainly deficient of CD59 rather than CD55. **Dingli et al.,** [(12)] hypothesized mechanisms by which PNH clones arises in immune-mediated BM failure syndrome: they proposed that GPI-deficient cells have a conditional growth advantage by evading an immune attack directed against normal hematopoietic progenitor cells. Another assumption is that GPI-deficient cells, which can be detected at very low numbers in healthy individuals, can expand without a conditional advantage, especially in conditions with reduced stem cell numbers.

The most common test for monitoring hemolysis in PNH patients is evaluation of serum lactate dehydrogenase (LDH) enzymatic activity. In our study, the level of LDH was significantly higher in patients with pancytopenia compared to normal control however, in all of them no clinical or laboratory findings supporting hemolysis were elicited. **Scheinberg et al.,** [(13)] reported that the levels of LDH were 250-300 U/L (normal range 113-226) in 55% and greater than 300 U/L in 37% of patients with severe aplastic anemia. They observed that despite elevated LDH levels could accompany an expanded clone; hemolysis was mild and subclinical in most patients. Also, serum ferritin was significantly increased in patients with pancytopenia in this study.

Jong et al., [14] reported that many patients with AA require blood transfusions as supportive therapy. Regular transfusions lead to the development of iron overload, which can cause significant damage to the heart, liver, and endocrine glands.

In this study, patients having pancytopenia with PNH clone had higher mean values of $CD55^{-}$ and $CD59^{-}$ than the control group and the two other patients subgroups (MDS and aplastic anemia). **Gupta et al., [15]** stated that fewer than 10% GPI-AP-deficient granulocytes are detected in AA patients at diagnosis, but occasional patients may have larger clones. Our results in this respect are in accord with previous study in which mean values of $CD55^{-}$ and $CD59^{-}$ granulocytes were 4.9% and 6.9% respectively. In the meantime, **Mikhalva et al., [16]** reported that the PNH clone has been detected in more than 60% of de novo sever aplastic anemia patients. The disease was characterized by pancytopenia and aplasia of the bone marrow without clinical signs of intravascular hemolysis.

Among patients with aplastic anemia and MDS in this study, the incidence of PNH clones were 40% in aplastic anemia and 22.2% in MDS patients. This is in agreement with findings of **Wang et al., [17]** who reported that with the sensitive flowcytometry, increased PNH-type cells were detected among RA patients at a lower prevalence (17.6%) level than AA patients (52.0%). The prevalence of PNH^{+} clone among our MDS patients was 22.2%. This lower prevalence, compared with AA patients, was in sharp contrast to the results of a previous report by **Dunn et al., [18]** that showed similar prevalence in AA and MDS patients.

Parker[19] reported that PNH clones in AA and MDS suggests a pathogenetic link to immunologically mediated forms of bone marrow failure. Six out of 23 (26.1%) of his PNH patients had prior history of aplastic anemia, and the duration between diagnosis of AA and PNH was 1 to 5 years. Most (five out of 6; 83.3%) of these patients developed hemolytic PNH.

One of the possible causes of expansion of PNH clone in AA patients may be that the PNH stem cells acquire survival advantages due to a deficiency of GPI-anchored proteins required for being attacked by cytotoxic T cells [20] .

A negative correlation was demonstrated between $CD59^{-}$ and TLC in pancytopenia with PNH clone subgroup but no significant correlation as regards Hb and platelets were elicited. This was in accord with **Varma et al. [21] . Gupta et al., [15]** showed that PNH clone size on granulocytes negatively correlated with the hemoglobin of PNH patients. Earlier, **Pramoonjago et al., [22]** recorded negative correlation between Hb and $CD59^{-}$granulocytes in PNH patients. They stated that the severity of anemia in PNH depends partly on the size of the PNH clone.

A quick response to immunosuppressive therapy was observed by **Mikhalva et al., [16]** at 3 months in sever aplastic anemia patients with PNH clone (38.9%) but in patients without PNH clone at time of diagnosis, achievement of partial remission at 6 months occurred and followed by PNH clone appearance and persistence.

Immunosuppressive treatment is reported to improve cytopenia in some patients with MDS. As regard the response to induction therapy with cyclosporine, it was noted that response rate was significantly higher in PNH +ve (88.9%) group than that in PNH -ve ones (21.4%), indicating that presence of PNH cells in MDS patients confer a good outcome[23]. Similar findings were reported by other researchers **(Wang et al., [24]** ; **Sugimori et al., [25] 2006 and Ishikawa et al., [26] 2007),** which further supported the hypothesis that the minor population of PNH –ve cells in MDS patients might be predictor for response to immunosuppressive therapy (IST) or to survival advantage.

The response to immunosuppression in MDS requires explanation, and even though the pathophysiology is not fully elucidated. Good outcome observed in PNH clone +ve group and other studies pointed to the importance of an immune-mediated component of MDS haematopoiesis being driven to apoptosis and the potential to remove this drive by immunosuppressive treatment[23] .

Clinical studies of subclinical PNH in association with MDS have been conducted predominantly on patients diagnosed as having refractory anemia (RA) according to the French-British-American (FAB) classification of MDS. The presence of PNH clones in different categories of MDS has been reported in a number of studies, and the conclusions have been contradictory. **Iwanaga et al., [27]** and **Wang et al., [24]** found that cells with a PNH –ve phenotype (PNH+) were only present in patients with RA (according to the FAB definition), but not in patients with

other subtypes of MDS. **Wang et al., [24]** also found that the RA patients with a detectable PNH +ve clone had a more indolent clinical course as compared with PNH –ve RA patients.

On the other hand, **Kaiafa et al., [11]** reported that PNH +ve cells were significantly more pronounced in high-grade MDS such as refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEB-t) and chronic myelomonocytic leukemia (CMML). They concluded that the presence of a higher level of PNH +ve cells in MDS predicted a poor clinical outcome.

In conclusion, flowcytometry is a sensitive and reliable method of PNH clone assessment in bone marrow failure syndrome. Our findings confirmed the presence of $CD55^{-}$ and $CD59^{-}$cell clones in granulocytes of pancytopenic patients who diagnosed as A.A and MDS. This suggests that these disorders may be part of bone marrow failure syndrome. The assessment of PNH clone may be important for monitoring therapy. This needs to be confirmed with further studies on larger cohorts of patients.

Conflict of interest: the authors indicated no potential conflict of interest

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