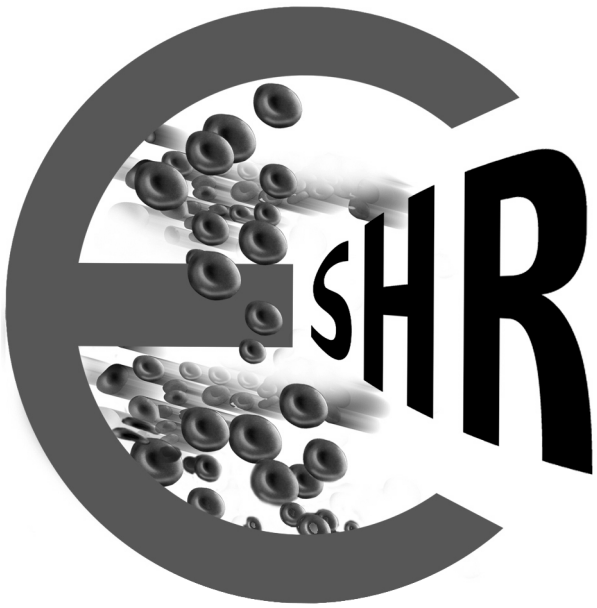
The Journal of the Egyptian Society of Haematology & Research



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T Cell Immunoglobulin Mucin (TIM)-3 Expressions on Peripheral Blood Lymphocytes from Patients with Chronic Hepatitis Virus C Infection: A Possible Role in the Pathogenesis of the Disease

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ABSTRACT

Background: T cell immunoglobulin mucin (TIM)-3 acts as a negative regulator of Th1/Tc1 cell function by triggering cell death upon interaction with its ligand, galectin-9. This negative regulatory function of TIM-3 is involved in establishing and/or maintaining a state of T cell dysfunction or "exhaustion" observed in chronic viral diseases. 25-hydroxyvitamin D (vitamin D) plays a role in decreasing the risk of many chronic illnesses, including common cancers, autoimmune diseases, infectious diseases, and cardio-vascular diseases.

Objectives: To evaluate the level of expression of TIM-3 on Peripheral Blood Mononuclear cells (PBMs) in cases of chronic hepatitis C (HCV), and to evaluate its association with vitamin D level.

Patients and Methods: In this study flow cytometric detection of expression TIM-3 was performed on PBMs of 70 chronic HCV cases. Patients were divided into compensated and decompensated groups (35 each) and 20 apparently healthy persons were included in the study as a control group. Quantitative detection of HCV RNA was performed by real time PCR and assay of serum 25-hydroxyvitamin D for all cases and controls was performed using fully automated ARCHITECT.

Results: The absolute number and percentage of lymphocytes were highest in control group followed by decompensated group and minimum in the compensated group (p<0.001). CD4% cells was higher in the compensated and control groups, than in the decompensated group (p=0.221) while CD8% was significantly higher in decompensated group compared to compensated and control groups (p=0.004), CD14%, showed significant elevation in decompensated compared to compensated and control groups (p=0.008). CD56 showed insignificant differences between the three groups (p=0.792). Increase in the percentage of TIM +ve CD4, CD8, CD14 and CD56 cells showed maximum percentage expression in the decompensated group, and least in the control group. The differences were significant regarding CD8 and CD56 (p=0.043 and 0.007 respectively) and highly significant regarding CD4 and CD14 +ve cells (p=<0.001). The mean level of 25-(OH) vitamin D was significantly lower in decompensated group compared to compensated and control group (p<0.001). There was no correlation between vitamin D and TIM-3 percentage expression in compensated and decompensated HCV patients. In all chronic HCV patients, negative correlation was encountered between vitamin D and CD4 TIM +ve% (r=–0.328, p=0.013) and strong negative correlation was obtained between vitamin D level and CD14 TIM +ve% (r=–0.518, p=0.000). No correlation was encountered between TIM-3 expression and ALT except for negative correlation between CD56 Tim-3 +ve% and ALT in decompensated group (r=–0.505, p=0.046). No correlation was found between TIM-3 expression and INR except for positive correlation with CD56 in chronic HCV patients which was good in the compensated (r= 0.560, p=0.005) and strong in the decompensated (r=0.74, p=0.001) group. In multivariate regression analysis, low vitamin D level was a significant risk factor for chronic HCV (p=0.006, OR: 1.81, 95% CI=1.18-2.78).

Conclusions: Our findings demonstrated that expression of TIM-3 + T cells on lymphocytes of chronic HCV patients is associated with sever HCV disease as levels were higher in decompensated patients than the compensated patients. Low vitamin D level may be an independent risk factor for chronic HCV infection.

Key Words: *Flow cytometry – Vitamin D – TIM-3 – Lymphocytes – Hepatitis C.*

INTRODUCTION

Hepatitis C Virus (HCV) is major causative agent of chronic hepatitis, affecting approximately 200 million people throughout the world; the majority of individuals exposed to HCV become persistently infected. A broad array of functional impairments of virus-specific T cells from early to chronic stages of infection, including exhaustion (decreased antiviral cytokine production, cytotoxicity, and proliferative capacity and arrested stages of differentiation **[1,2]**.

T cell Immunoglobulin Mucin-(TIM-3) was first identified as a molecule specifically expressed on IFN-gamma-secreting CD4 (+) T helper 1 (Th1) and CD8 (+) T cytotoxic (Tc1) cells in both mice and human. TIM-3 acts as a negative regulator of Th1/Tc1 cell function by triggering cell death upon interaction with its ligand, galectin-9. This negative regulatory function of TIM-3 has now been expanded to include its involvement in establishing and/or maintaining a state of T cell dysfunction or "exhaustion" observed in chronic viral diseases. In addition, it is now appreciated that TIM-3 has other ligands and is expressed on other cell types, where it may function differently. This supports an important role for TIM-3 in both autoimmune and chronic inflammatory diseases in human **[3]**. TIM-3 expression may play an important pathogenic role in patients with long-standing chronic **[1]**.

HCV infection vitamin D is metabolized by the liver and converted to 1, 25-dihydroxyvitamin D3, which is the active form of the vitamin. Individuals with chronic liver disease may have poor conversion from vitamin D3 or any of its other biologically active metabolites **[4]**.

In this study we aimed to determine the level of expression of TIM-3 on Peripheral Blood Mononuclear cells (PBMs) in cases of HCV during different disease stages and to clarify its possible role in the pathogenesis of the disease. We also aimed to determine the level of vitamin D concentration in different HCV disease status and to correlate it with the level of TIM-3 expression.

PATIENTS AND METHODS

The study included 70 (40 males and 30 females) chronic HCV patients, who were consecutively selected from the Inpatient Unit of the Internal Medicine Department, Sohag University Hospital, from March 2014 to July 2014. Their ages ranged from 40-70 years with a mean of 41.4±9.7 and a median of 40 years. All patients had established HCV infection, previously proved by PCR of HCV-RNA. Twenty age and gender matched apparently healthy subjects were included as a control group (12 males and 8 females); their age ranged from 43-68 with a mean of 49±12 and a median of 46 years. They had no clinical signs of liver disease as assessed by medical history and clinical examination and they were HCV antibody negative. Patients were divided into two groups: Group 1 (35 patients) included HCV antibody positive/HCV RT-PCR positive patients with normal liver functions (compensated), Group 2 (35 patients) comprised HCV antibody positive/HCV RT-PCR positive patients with abnormal liver functions (decompensated). Exclusion criteria included pregnancy and taking interferon therapy and other causes of chronic liver diseases. The study was approved by the Ethics Committee of the Faculty of Medicine, Sohag University and informed consent was obtained from all the participants.

Methods:

All subjects underwent a complete screening panel, including history taking and physical examination. Blood samples were withdrawn for routine laboratory investigations (complete blood picture, AST, ALT, Alkaline Phosphatase (ALP) albumin, Total Bilirubin (TBIL), Direct Bilirubin (DBIL), Total Protein (TP), albumin, prothrombin time and concentration and INR and abdominal ultrasound was performed. The degree of liver decompensation was evaluated using the Model of the End-stage Liver Disease (MELD) score **[5]** and Child-Turcotte-Pugh score (CPTS) **[6]**.

Anti-HCV was tested using commercially available micro particle enzyme immunoassay kits (AXSYM, Abbott Laboratories).

- Detection of HCV RNA was performed on 7500 fast real time PCR system (Applied Biosynthesis) using ready to use PCR kit supplied by artus® HCV RG RTPCR Kit 24, Version 1, catalog no. 52963, QIAGEN GmbH, Germany) **[7]**.
- Serum 25-hydroxyvitamin D test was performed using fully automated ARCHITECT instrument (Abbott Diagnostics Division, Chicago) based on Chemiluminescent Microparticle Immunoassay (CMIA). The level was measured by Abbott Architect i1000 Chemiflex device (kits manufactured by Abbott#3L52-46) **[8]**.

TIM-3 expression on various cell populations: Detection of expression of TIM-3 on different cell populations was performed using

monoclonal antibodies and analysed by flow cytometry. TIM-3 PE, CD4 FITC, CD 14 Per-CP, CD8 FITC and APC CD56 were obtained from BD Biosciences (BD Pharmingen, USA) and from BioLegend, USA. Dual staining was performed to detect TIM-3 expression on each cell population. PBMCs were stained in the dark for 15 minutes at room temperature (20-25ºC). The cells were washed twice with the washing solution and re-suspended in 0.5ml PBS. Stained cells were analysed by flow cytometry within 3 hours of staining using FACS Calibre FCM (Becton Dickinson) flow cytometry and Cell Quest software. Lymphocytes were gated on a dot plot was created for CD4 versus TIM-3, CD8 versus TIM-3, CD56 versus TIM-3 and CD14 versus TIM-3 **[9]**. The percentage of cells of the lineage+/TIM-3 was used for statistical analysis of the data.

Statistical analysis:

SPSS version 19.0 was used for statistical analysis. Data were summarized as mean ± SD, range or median. Student *t*-test was used to compare the means between two groups, and one-way analysis of variance (ANOVA) test was used to compare means of more than two groups. Multivariate and multivariate regression analysis was used to examine the role of TIM-3 positive cells and vitamin D as risk factors. *p*-value <0.05 was considered significant.

RESULTS

Laboratory variables of the studied groups are demonstrated in (Table 1).

The mean level of HCV-RNA in compensated group was insignificantly lower than in the decompensated group (144.906±479.509 vs. 162.59±308.7926, p=0.89) TIM-3 expression on different lymphocytes subsets in chronic HCV patients is presented in (Table 2). The absolute numbers and the percentage of lymphocytes are percentage and absolute lymphocyte count were significantly higher in control group followed by the decompensated group and lowest in the compensated group (p<0.001). CD4% cells was higher in the compensated and control groups, than in the decompensated group, with in significant difference (p=0.221) while CD8% was significantly higher in decompensated group compared to compensated and control groups (p=0.004). CD14% showed significant elevation in decompensated compared to compensated and control groups (p=0.008). CD56 showed insignificant differences between the three groups (p=0.792). TIM-3 CD4, CD8, CD14 and CD 56% were highest among the decompensated group, and least among the control group. The differences were significant regarding CD8 and CD56 (p=0.043 and 0.007 respectively) and highly significant regarding CD4 and CD14 cells (p=<0.001).

The mean level of 25-(OH) Vitamin D was significantly lower in decompensated compared to compensated and control groups (p<0.001), (Table 1).

No correlation was encountered between TIM-3 expression and ALT except for negative correlation with CD56 TIM-3 +ve% in the decompensated group (r=–0.505, p=0.046). Also, no correlation was encountered between TIM-3 expression on all lymphocyte subsets and serum albumin or bilirubin (Table 3).

No correlation was found between TIM-3 expression and INR except for positive correlation with CD56 TIM-3 +ve% in chronic HCV patients group which was good in the compensated (r=0.560, p=0.005) and strong in the decompensated group (r=0.74, p=0.001; Figs. (1,2).

There was no correlation between vitamin D and TIM-3 percentage expression in compensated and decompensated HCV patients (Table 4). In all chronic HCV patients (n=70), negative correlation was encountered between vitamin D level and CD4 TIM-3 +ve% (r=–0.328, p= 0.013) and good negative correlation between Vitamin D and CD14 TIM-3 +ve% (r=–0.518, p=0.000).

In multivariate regression analysis, low vitamin D level in HCV cases was found to be a significant risk factor for development of chronic HCV (p=0.006, OR: 1.81, 95% CI=1.18-2.78).

Univariate regression analysis showed that low vitamin D level is a highly significant risk factor in chronic HCV cases (p<0.001 OR=0.58, 95% CI: 0.425-0.793), followed by CD14 TIM-3 positive cells (p=0.003, OR=1.04, 95% CI: 1.016-1.079), then CD4 TIM-3 positive cells (p=0.022, OR=1.411, 95% CI: 1.051-1.895).

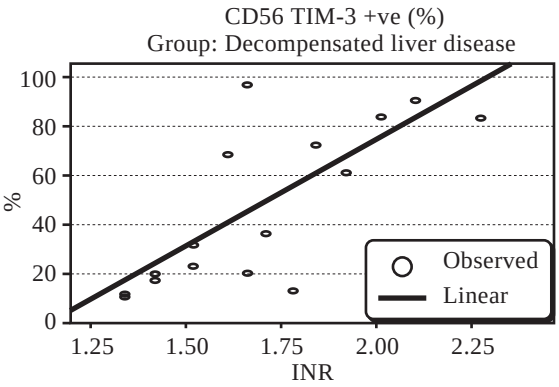

Fig. (1): Correlation between INR and CD56 TIM-3 positive cells in decompensated chronic HCV patients.

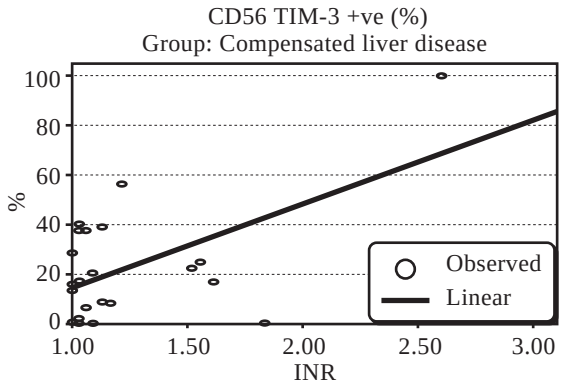

Fig. (2): Correlation between CD56 TIM-3 positive cells and INR in compensated chronic HCV patients.

Table (1): Laboratory variables of the studied groups.

Parameter	Chronic HCV n=70		Control n=20 (Group III)	*p*-value		
	Decompensated n=35 (Group I)	Compensated n=35 (Group II)		I vs. III	II vs. III	I vs. II
PLT: 10^9/L	69.28±17.53	173.9±78.01	276±82.4	<0.001	<0.001	<0.001
HB: gm/dL	8.47±0.96	16.28±5.13	13.33±1.25	<0.001	0.030	<0.001
WBC: 10^9/L	4.94±1.46	7.026±2.43	8.13±1.72	<0.001	0.126	<0.001
INR	1.70±0.27	1.28±0.38	1.02±0.03	<0.001	0.034	<0.001
PC: %	44.28±9.35	79.78±22.95	97.5±5.77	<0.001	0.005	<0.001
PT: Seconds	19.58±2.97	14.30±4.46	11.86±0.33	<0.001	0.036	0.003
IBIL: mg/dL	1.68±0.79	0.85±0.97	0.36±0.12	<0.001	0.037	0.002
DBIL: mg/dL	1.69±0.83	0.70±1.09	0.19±0.08	<0.001	0.068	0.003
TBIL: mg/dL	3.37±1.54	1.54±1.94	0.54±0.17	<0.001	0.049	<0.001
TP: g/Dl	6.56±0.789	7.421±0.915	7.74±0.26	<0.001	0.189	<0.001
ALB: g/dL	2.35±0.504	3.726±0.931	4.86±0.41	<0.001	<0.001	<0.001
SGPT: U/L	70.56±38.89	33.087±27.09	21.06±5.62	<0.001	0.090	<0.001
SGOT: U/L	93.5±40.93	33.347±26.72	17.31±7.47	<0.001	0.025	<0.001
Vit D: ng/mL	10.83±3.79	14.43±3.41	22.84±6.07	<0.001	<0.001	0.003

PLT : Platelets.
HB : Hemoglobin.
WBCs : White Blood Cells.
INR : International Normalized ratio.
PC : Prothrombin Concentration.
PT : Prothrombin Time.
IBIL : Indirect Bilirubin.
DBIL : Direct Bilirubin.
TBIL : Total Bilirubin.
TP : Total Protein.
ALB : Albumin.
SGPT : Serum Glutamic-Pyruvic Transaminase.
SGOT : Serum Glutamic Oxaloacetic Transaminase and Vit D:25 (OH) hydroxyl vitamin D.

Table (2): Lymphocyte subsets in chronic HCV patients and control.

Parameter	Group I n=35	Group II n=35	Group III n=20	*p*-value	I vs. III	II vs. III	I vs. II
TLC X 10^9/L	26.72±93.87	19.19±84.14	29.1±10.65	0.004	0.512	0.001	0.001
Lymphocyte	28.87±8.95*	19.19±8.41	31.49±9.57	<0.001	0.416	<0.001	0.001
CD4%	32.13±14.32	38.56±12.02	37.17±8.21	0.221	0.225	0.691	0.126
CD4/TIM-3 +	16.61±16.82	5.78±5.3	3.12±2.13	<0.001	0.003	0.066	0.016
CD8%	36.67±29.12	17.95±9.07	20.82±5.79	0.004	0.042	0.272	0.02
CD8/TIM-3 +	27.52±30.86	17.82±10.16	11.18±4.18	0.043	0.046	0.008	0.228
CD14%	7.76±3.27	9.51±3.99	5.99±2.17	0.008	0.077	0.001	0.149
CD14/TIM-3 +	79.09±17.26	55.55±16.19	42.09±26.3	<0.001	<0.001	0.08	<0.001
CD56%	0.5±0.34	0.45±0.5	0.54±0.33	0.792	0.732	0.5	0.724
CD56/TIM-3 +	46.26±31.92	22.18±22.94	21.36±18.3	0.007	0.013	0.93	0.016

Group I : Decompensated.
Group II : Compensated.
Group III : Control.
* : Percent.
CD : Cluster of Differentiation.
TIM-3 : T-cell immunoglobulin domain and mucin domain 3.
TLC : Total Lymphocyte Count.
$p<0.05$ is significant.
$p<0.001$ is highly significant.

Table (3): Correlation between TIM-3 +ve expression on lymphocytes subsets and ALT in HCV patients and control.

Parameter	Controls	Chronic HCV patients	
		Com-pensated	Decom-pensated
CD4 TIM3 +ve (%):			
r	0.071	–0.175	–0.245
p	0.794	0.425	0.328
CD8 TIM3 +ve (%):			
r	0.169	–0.065	–0.457
p	0.532	0.767	0.065
CD14 TIM3 +ve (%):			
r	0.203	0.117	–0.281
p	0.451	0.594	0.274
CD56 TIM3 +ve (%):			
r	–0.215	–0.113	–0.505
p	0.442	0.607	0.046

Table (4): Correlation between vitamin D and TIM-3 +ve expression on lymphocytes subsets in chronic HCV patients and control.

Parameter	All subjects	Control	Chronic HCV patients	
			Com-pensated	Decom-pensated
CD4 TIM-3 +ve (%):				
r	–0.328	0.070	–0.259	–0.018
p	0.013	0.797	0.233	0.945
CD8 TIM-3 +ve (%):				
r	–0.168	0.072	–0.094	0.243
p	0.217	0.791	0.668	0.347
CD14 TIM-3 +ve (%):				
r	–0.518	–0.364	–0.027	–0.095
p	0.000	0.165	0.904	0.717
CD56 TIM-3 +ve (%):				
r	–0.212	0.233	–0.101	–0.085
p	0.123	0.403	0.647	0.755

CD : Cluster of Differentiation.
TIM-3 : T-cell immunoglobulin domain and mucin domain 3.
r : Pearson correlation.
p : Probability value.

DISCUSSION

T-cell Immunoglobulin and Mucin domain-containing molecule 3 (TIM-3) plays an important role in regulating T cells in chronic hepatitis C virus infection and Hepatocellular Carcinoma (HCC).

Our study included 90 subjects (70 HCV +ve patients and 20 normal controls) and the HCV infected patients were subdivided into two groups; patients with compensated and patients with decompensated liver functions, and each subgroup involved 35 patients.

TIM-3 is expressed on only a very small percentage of CD4+ or CD8+ T cells, and its over-expression may indicate T cell exhaustion and represent a pathological immune state. However, innate immune cells including monocytes, macrophages and DCs show constitutive and high-level TIM-3 expression that can be further elevated in some diseases. Gerlach et al., **[11]** found that in patients with acute HCV infection if the T-helper (CD4+) immune response was weak, less efficient or not maintained for a sufficient length of time, patients would proceed to persistent infection and chronic hepatitis. Furthermore, Hoffman et al., **[12]** found that HCV-RNA positive individuals without clinical or histopathologic evidences of liver disease had a statistically significantly higher CD4+ proliferative response to the HCV core protein than patients with chronic hepatitis, suggesting a protective role of CD4+ cells against hepatocellular damage. Bowen and Walker **[13]** evidently showed the role of both CD4+ and CD8+ T cells in HCV viral clearance. Strong and sustained CD4+ and CD8+ T cell responses together lead to the resolution of HCV. Also, DCs play a major role by processing and presenting the antigens on MHC-I and MHC-II molecules and providing optimum stimulation to CD8+ and CD4+ T cells, respectively **[14]**.

Tim molecules are expressed on T cells, monocytes, and antigen-presenting cells, including macrophages and dendritic cells **[15]**. TIM-3 has been shown to be involved in the suppression of T-cell effector function in chronic HIV infection, defining a population of exhausted T cells that is distinct from the PD-1-expressing population **[16]**.

Mason et al., **[1]** stated that TIM-3 expression may play an important pathogenic role in patients with long standing chronic HCV infection.

In our study, CD8-TIM-3 positive cells were significantly higher in decompensated group compared to compensated and control groups (p=0.004). Similar results were found by McMahan et al., **[2]** who reported that TIM-3 expression by both CD4 and CD8 increases significantly in HCV infected patients, compared to control (p=0.0047 and (p=0.0002 respectively), regardless of the liver functions. They, also, demonstrated that early accumulation of PD-1+ TIM-3+ T cells is associated with functional impairment, and consequently with development of persistent HCV.

Our results are consistent with Mason et al., **[1]** who examined the expression of Tim-3 by flow cytometry on PBMCs from 42 patients with persistent hepatitis C viremia and 10 normal controls. They found that, chronic HCV infection is associated with elevated frequencies of TIM-3-expressing CD4+ and CD8+ T cells relative to those uninfected with the highest expression on HCV-specific cytotoxic T lymphocytes (CTLs) (p=0.02 and 0.008 respectively).

In the current study, CD14 cells were maximum in the compensated group, followed by decompensated and minimum in the control group, again with a significant difference (p= .0.008).

CD56 showed insignificant differences between the three groups. There was steady increase in the percentage of TIM-3 +ve CD4, CD8, CD14 and CD56 cells, with maximum percentages among the decompensated liver disease group, and least percentage among the control group. This is consistent with a study reported by Vali et al., **[17]** which revealed that HCV-specific T cells in HCV/HIV co-infection show elevated frequencies of dual TIM-3/PD-1 expression that correlate with liver disease progression.

In the current study, no correlation was encountered between TIM-3 expression and ALT except for negative correlation with CD56/ TIM-3 +ve% in decompensated group (r= –0.505, p=0.046). Also, no correlation was encountered between TIM-3 expression on all lymphocyte subsets and serum albumin or bilirubin. No correlation was found between TIM-3 expression and INR except for good positive correlation with CD56 TIM-3+ve% chronic HCV group which was good in the compensated (r=0.560, p=0.005) and strong in the decompensated (r=0.74, p=.0.001) group. This is contrary to Wu et al., **[18]** findings who studied TIM-3 in regulating the antiviral CD8+ T-cell response in Chronic Hepatitis B (CHB) patients. TIM-3 expression on peripheral virus-specific CD8+ T cells from 20 CHB patients and 20 healthy controls was determined by flow cytometry. They reported that TIM-3 expression may also indicate the severity of liver injury because its expression was markedly and positively correlated with ALT, AST, INR and TB. TIM-3 expression correlated with ALT levels (a surrogate marker for hepatic injury) suggesting that TIM-3 expression is more likely a feedback response to prevent over-activation of the host immune system than a viral strategy to counteract immune attack. This hypothesis was also supported by their previous study which showed that TIM-3 expression levels returned to normal after attenuation of liver inflammation **[19]**. In a study done by Rong et al., **[20]** TIM-3 3+CD14+ cells and TIM-3 3+CD3+CD16/CD56+ cells were analyzed by flow cytometry. Results showed that expression of TIM-3 was significantly increased on both the monocytes and NKT-like cells in CHB patients than in controls (p=0.002 and p<0.001, respectively). TIM-3 levels on monocytes and NKT-like cells were further upregulated in patients with acute liver failure. TIM-3 expression on both monocytes and NKT-like cells was positively correlated with level of ALT (r=0.59, p<0.001, and r=0.60, p<0.001). Mason et al., **[21]** analysed TIM-3 expression on NKs in chronic HCV-infected subjects, demonstrating not only elevated expression of TIM-3 on these cells but also a positive correlation between TIM-3 and NK activity; this suggests that TIM-3 on NKs is associated with activation of this innate lymphocyte population that is polarized towards cytotoxicity in chronic HCV. These findings reveal roles for TIM-3 in the regulation of NKs that might represent targets for treatment of chronic viral infections.

Vitamin D is metabolized by the liver and converted to 1,25-dihydroxyvitamin D3, which is the active form of the vitamin. Individuals with chronic liver disease may have poor con-

version from vitamin D3 or any of its other biologically active metabolites **[22]**.

In the present work, vitamin D was significantly lower in decompensated group ($p<0.001$). Severe liver disease may increase the risk of vitamin D deficiency and/or there might be a relationship between vitamin D deficiency and fibrosis. DeLuca **[23]** and Arteh et al., **[24]** also showed that low levels of 25 (OH) D are associated with fibrosis and suggested that low 25 (OH) D levels may predict hepatic decompensation and mortality in patients with chronic liver failure.

In the current study, there was no correlation between vitamin D and TIM-3 percentage expression in compensated and decompensated HCV patients. In all chronic HCV patients (n=70), negative correlation was encountered between vitamin D and CD4 TIM3 +ve% (r= –0.168, p=0.013) and strong negative correlation between vitamin D and CD14 TIM3 +ve% (r= –0.518 and p=0.000). In this work, low vitamin D level in HCV cases was found to be a significant risk factor for chronic HCV. Vitamin D has been shown to be a key regulatory element of the immune system, and its serum concentrations correlate with the severity of liver damage and the development of liver fibrosis/ cirrhosis. Furthermore, supplementation with vitamin D could be beneficial in increasing the response rate to Peg-INF-α based therapy in CHC patients **[25]**.

During chronic HCV infection, TIM-3 expression on CD4+ and CD8+ T cells is elevated, and these TIM-3+ T cells exhibit a CD 127 low CD 57 high and CD45RACC R high phenotypes, indicating the impaired function of these effector cells. Accordingly, blockade of TIM-3 expression enhances cell proliferation and promotes cytokine production **[1]**.

In conclusion, our study showed that accumulation of TIM-3+ T cells is associated with development of persistent HCV. TIM-3 expression may also indicate the severity of liver injury because its expression has been found to be higher in decompensated than compensated HCV patients. Adequate vitamin D level is a protective factor for development of CHC. The correlation between TIM-3 expression and vitamin D concentration will be critical for exploiting the therapeutic potential of TIM-3 and vitamin D for the treatment and follow-up of the disease.

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The Incidence of Expression of ZAP-70 and CD38 in Chronic Lymphocytic Leukemia Patients

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ABSTRACT

Background: Chronic Lymphocytic Leukemia (CLL) is the most common chronic lymphoproliferative disorder. ZAP-70 and CD38 are among the prognostic parameters in CLL.

Aim of Study: This study was undertaken to know the prevalence of ZAP-70 and CD38 in the treatment naive patients of CLL seen at the Dammam University Hospital, Saudi Arabia.

Material and Methods: ZAP-70 and CD38 were tested by flow cytometry on peripheral blood samples. ZAP-70 and CD38 positivity was defined as expression on 20% and 30% of CLL cells, respectively. Clinico-hematological profile and its correlation with ZAP-70 and CD38 expression was assessed in consecutive 80 CLL patients.

Results: There were 64 males and 16 females with an age range of 52-75 with a median of 58 years. Sixteen patients (20%) were asymptomatic and diagnosed incidentally. Median Total Lymphocyte Count (TLC) at presentation was 62 X 10^9/L. Rai stage distribution was: Stage 0-6, stage I-20, stage II-36, stage III-5, and stage IV-13. ZAP-70 and CD38 positivity were detected in 20 patients (25%) and 29 patients (36%), respectively. Eleven patients were positive and 34 were negative for both ZAP-70 and CD38 yielding a concordance rate of 56%. There was no statistically significant difference between ZAP-70 and CD38 positivity and negativity with regard to age, sex, Lymphocyte count, lymphadenopathy, organomegaly, and Rai staging.

Conclusion: ZAP-70 and CD38 positivity were detected in 25% and 36%, respectively, with concordance rate of 56%, which is higher than Western literature. There was no correlation of ZAP-70 or CD38 positivity with age, sex, lymphadenopathy, organomegaly, or Rai staging.

Key Words: *CD38 – Chronic lymphocytic leukemia – ZAP-70.*

INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is a common leukemic disorder in the West, with an estimated incidence in the United States of 5.17 per 100,000, representing 20% of all mature B-cell neoplasm **[1]**.

CLL patients with advanced stage disease and those who progress from early stage disease are treated with chemotherapy **[2]**. Overall about 70% of all patients will require therapy during the course of the disease. Although Rai staging system predicts overall survival, it does not predict which patients in the early stages (0-I) will progress and require therapy **[3]**. The traditional prognostic parameters based on routine and well-established tests involving the blood or bone marrow (clinical stage, pattern of bone marrow infiltration, lymphocyte doubling time, beta-2 microglobulin levels, and lactate dehydrogenase level) are useful but they may not accurately predict progression for a given patient **[4,5]**. In the past few years the focus of research in prognostic factors in CLL has changed from clinical to biological factors. The IgVH mutational status, CD 38 and ZAP-70 expression are few of such markers. Till date the strongest independent prognostic factor for survival in CLL is the presence of somatic mutations of the variable region of the immunoglobulin heavy chain gene **[6]**. Patients with unmutated IgVH gene have aggressive disease, requiring early treatment and often show poor response to chemotherapy. However, IgVH mutation studies are cost and labor intensive and are not widely available at most centers. Therefore, there was a need for simpler, reliable, and easily standardized surrogate marker, which can substitute IgVH mutation testing. The best studied of these new prognostic parameters have been proteins residing on the cell surface (CD-38) or in the

cytoplasm (ZAP-70), recurring genetic defects in the CLL cells (detected by Fluorescence In-Situ Hybridisation (FISH)), and the mutation status of Ig VH gene. Zeta-associated protein 70 (ZAP-70) is a tyrosine kinase protein normally expressed in T cells and natural killer cells. ZAP-70 has been reported to be expressed preferentially in CLL un-mutated IgVH **[7]**. The ligation of B-cell receptor on CLL cells that express ZAP-70 is associated with excessive tyrosine phosphorylation **[8]**. There are several methods used to study the expression of ZAP-70 in patients with CLL; flow cytometric detection of ZAP-70 is relatively reliable and could be placed into more routine use **[9]**. CD 38 is a 45-kDa, non-lineage restricted, type II transmembrane glycoprotein that has many protein functions. It can serve as an ectoenzyme that catalyzes the synthesis and hydrolysis of cyclic ADP-ribose, a Ca2+ mobilizing agent that acts independently of inositol triphosphate **[10]**. CD 38 also functions as receptor that induces proliferation and increases survival of CLL cells **[11]**. CD 38 positivity (defined as at least 30% positive cells) is an independent prognostic marker for an unfavorable clinical course in CLL **[12]**. CD 38 positive patients may progress faster to advanced stage. These patients not only have more aggressive disease but also do not respond to chemotherapy as others do **[13]**. However, it is not a surrogate marker for IgVH mutational status. It has been found that there is significant correlation between CD 38 positivity and intermediate/high modified Rai stages, multiple bulky lymphadenopathy, and splenomegaly **[14,15]**. There are no much published data available in developing countries about prevalence of ZAP-70 and CD 38 positivity in CLL patients, so this study was undertaken to know the prevalence of CD 38 and ZAP-70 in CLL patients in a developing country and to correlate this with baseline parameters. With longer follow-up we will be able to know the prognostic value of ZAP-70 and CD 38, which will be reported later on.

MATERIAL AND METHODS

Between January 2012 and December 2013, in Dammam University Hospital, consecutive 80 cases of treatment naive CLL patient were selected for this analysis. All cases fulfilled the National Cancer Institute working group criteria for diagnosis of CLL **[16]**. The protocol was approved by the ethical committee of the hospital. Two milliliters of peripheral venous blood were collected in ethylenediaminetetraacetic acid from each patient after taking informed consent as per guidelines of the ethics committee. A standard whole blood lysis method was used for sample preparation. Briefly, 1 X 10^6 cells were incubated with preconjugated monoclonal antibodies: CD 38 conjugated to Alexa Fluor 488, CD 19 to phycoerythrin cyanine 5.5 (PE-Cy 5.5), and CD 5 to allophycocyanin (APC), (BD Pharmingen, San Diego, CA, USA) at Room Temperature (RT) in the dark for 20 minutes. Two milliliters of Fluorescence Activated Cell Sorter (FACS) Lyse (BD Pharmingen, San Diego, CA, USA) were added and incubated for 2 hours in the dark at RT followed by incubation for ZAP-70 Phycoerythrin (PE) for 30 minutes (BD Biosciences, San Jose, CA, USA). The cells were then washed and re-suspended in phosphate buffer saline and kept at 4ºC till acquisition. A total of 10.000 events were acquired on a flowcytometer (FACS Canto, BD Biosciences, San Jose, CA, USA) equipped with facility for at least 4-color immunophenotyping and analysis was done using FCS express software version 3.0 (Denovo software, Los Angeles, CA, USA). CLL cells were identified as CD 5+ CD 19+ events and expression of CD 38 and ZAP-70 was evaluated on these gated CLL cells. A cutoff value of 30% to define expression of CD38 and 20% to define expression of ZAP-70 was used Fig. (1).

Statistical analysis:

Quantitative variables were summarized as median and qualitative variables as proportions. Baseline categorical variables were analyzed using Chi-Square test/Fisher's Exact *t*-test. Multivariate logistic regression for independent prognostic value of CD38 and ZAP-70 expression was performed using STATA software version 11.1 (StataCorp, Texas, USA) and $p< 0.05$ was defined as significant.

RESULTS

The median age was 57 years (range 28-90 years). There were 64 males and 16 female patients. Eighteen patients were asymptomatic. Out of 80 patients, 6 (7.5%) were in Rai stage 0, 20 (25%) in Rai stage I, 36 (45%) in Rai stage II, 5 (6.25%) in Rai stage III, and 13 (16.25%) were in Rai stage IV. The median

hemoglobin was 11g/dL with range of 4.7-16 g/dL. The median total leucocytic count was 62 X 10^9/L and absolute lymphocyte count was 51 X 10^9/L. The median platelet count was 150 X 10^9/L. Out of 80 patients, 26 (32.5%) were in Rai stage 0 and I, 36 (45%) were in Rai stage II, 18 (22.5%) were in Rai stage III and IV. CD 38 was positive in 29 (36.25%) of patients. ZAP-70 was positive in 20 (25%) patients. Stage wise distribution of ZAP-70 and CD 38 positive patients is given in (Table 1). Out of 80 patients in whom both ZAP-70 and CD 38 were tested, 11 were concordant ZAP-70+, CD38+ and 34 were ZAP-70-and CD 38-, yielding a concordant rate of 56%. After analyzing ZAP-70 and CD38 as continuous variables no definite correlation was found with age, sex, lymphadenopathy, organomegaly or Rai staging (Table 2). With a median follow-up of 17 months, 11 patients of early stage disease progressed; 3 were ZAP-70 positive, 4 were CD 38 positive, and 4 patients were negative for both. There was no difference between progression free period of ZAP-70 and CD38 positive group.

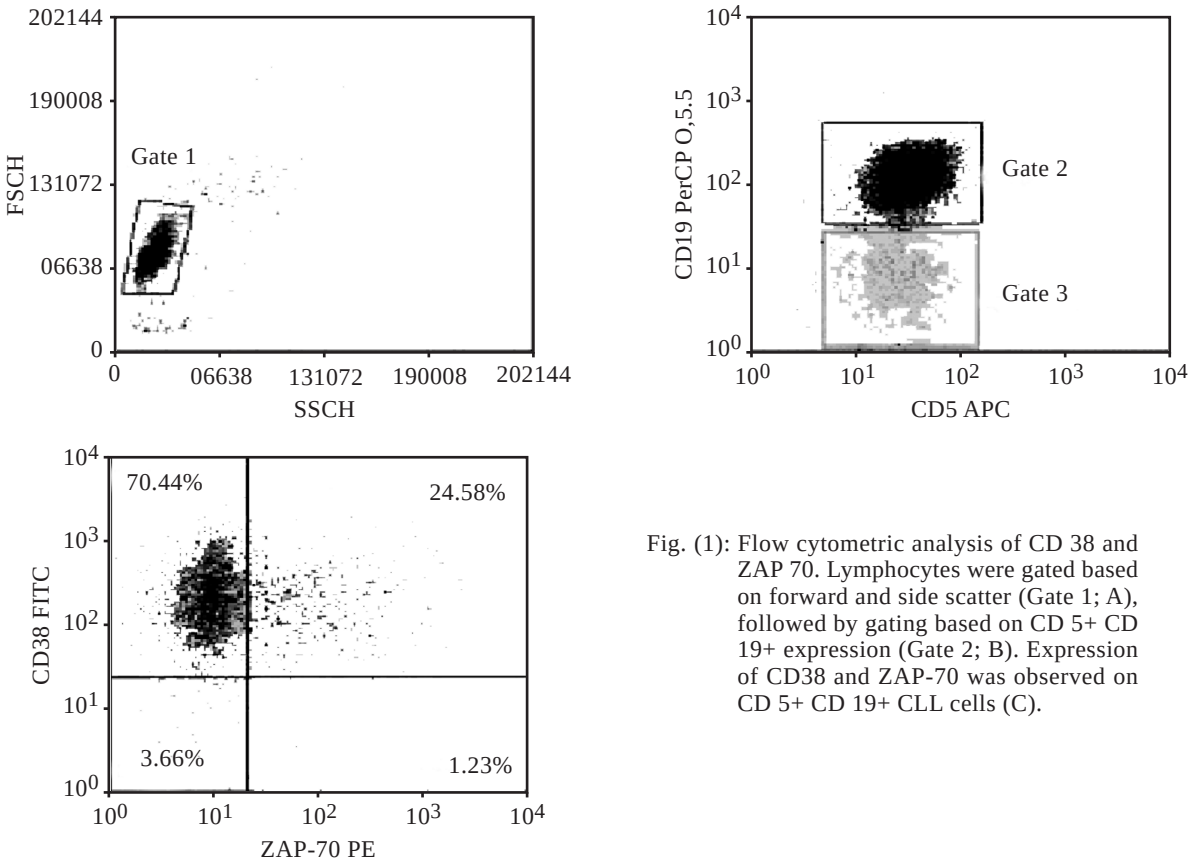

Fig. (1): Flow cytometric analysis of CD 38 and ZAP 70. Lymphocytes were gated based on forward and side scatter (Gate 1; A), followed by gating based on CD 5+ CD 19+ expression (Gate 2; B). Expression of CD38 and ZAP-70 was observed on CD 5+ CD 19+ CLL cells (C).

Table (1): Stage distribution as per ZAP-70 and CD38 in 80 chronic lymphocytic leukemia patients.

	Stade no (%)			
	Rai 0 and I (%)	Rai II (%)	Rai III and IV (%)	*p*-value
ZAP (n=80):				
Positive	9 (11.25)	7 (8.75)	4 (5)	0.249
Negative	17 (21.25)	29 (36.25)	14 (17.5)	
CD 38 (n=80):				
Positive	8 (10)	15 (18.75)	6 (7.5)	0.130
Negative	18 (22.5)	21 (26.25)	12 (15)	

Table (2): Co-relation of ZAP-70 and CD38 with age and base line clinical and laboratory parameters in 80 chronic lymphocytic leukemia patients.

Variables	ZAP-70		CD 38	
	Odds ratio	*p*-value (95% confidence interval)	Odds ratio	*p*-value (95% confidence interval)
Age	0.99	0.843 (0.93-1.06)	1.05	0.110 (0.99-1.11)
Gender (Female)	0.45	0.323 (0.09-2.19)	0.39	0.196 (0.09-1.62)
Hb	1.16	0.456 (0.78-1.72)	0.96	0.813 (0.67-1.36)
TLC	1.00	0.171 (1.0)	1.00	0.205 (1.00)
ALC	1.00	0.213 (1.0)	1.00	0.213 (1.00)
Platelets	0.56	0.396 (0.15-2.14)	1.58	0.451 (0.48-5.24)
Lympadenopathy (No)	0.43	0.212 (0.12-1.61)	1.00	0.997 (0.31-3.27)
Hepatomegaly (No)	1.13	0.875 (0.24-5.33)	1.56	0.486 (0.45-5.44)
Splenomegaly (No)	0.37	0.192 (0.08-1.66)	0.65	0.488 (0.19-2.20)
Stage (Stage 0):				
I	1.11	0.927 (0.11-11.21)	1.36	0.786 (0.15-12.48)
II	0.35	0.370 (0.03-3.52)	3.08	0.310 (0.35-27.02)
III	0.17	0.310 (0.01-5.25)	1.26	0.874 (0.07-22.16)
IV	0.33	0.490 (0.01-7.58)	1.50	0.785 (0.08-27.04)

Hb : Hemoglobin.
TLC : Total Leukocytic Count.
ALC : Absolute Lymphocyte Count.

DISCUSSION

Rai clinical stage is the most robust and established prognostic factor in CLL. The limitation with this staging system is that a significant percentage of patients with early stage disease will rapidly escalate to advanced disease that requires therapy. Unfortunately, the Rai system is unable to prospectively differentiate the rapidly evolving patient from more stable patients who may not progress for decades. It was found that patients positive for ZAP-70 in early stage disease had a shorter time to therapy, with a median time from diagnosis to initial therapy of 2.9 years, compared with 9.2 years for ZAP-70 negative patients for the same stages **[7]**. ZAP-70 holds significant promise as a prognostic marker; it was reported to be highly predictive of time to treatment in a large cohort of early stage (Rai 01) and untreated CLL **[7,8]**. Various studies have reported ZAP-70 positivity in CLL ranging from 36% to 57% (Table 3). The optimal cutoff for defining ZAP-70 positivity was reported as 20% by various authors **[9,14,15,17]**. Del Poeta et al., and Hus et al., found significant correlation between high ZAP-70 levels and advanced Rai stage and splenomegaly **[12,14]**. In this study among the 80 patients 25% (20 patients) were ZAP-70 positive. There was no correlation of ZAP-70 with age, sex, hemoglobin, lymphocyte count, organomegaly or clinical Rai stage. Zeeshan R. et al., **[18]**, said "The frequency of ZAP-70 positivity in B-CLL patients was found to be 13.5%. ZAP-70 positivity was significantly correlated with stage III disease and high absolute lymphocytic count ($p<0.05$). No correlation of ZAP-70 could be established with age and gender ($p>0.05$)".

Various studies have reported CD38 positivity in CLL ranging from 29% to 60% (Table 3). In this study 80 patients were tested and 29% were CD38 positive. In this study 80 patients were tested and 29% were CD38 positive.

Table (3): Comparison of ZAP-70 and CD38 with literature.

Study	No. of patients	ZAP 70%	CD 38%
Crespo et al., **[9]**	56	57	60
Hus et al., **[14]**	156	36	33
Schoroer et al., **[13]**	252	46	29
D'Arena et al., **[15]**	157	36	29
Present study	80	25	36

ZAP: Zeta-Associated Protein.

The Rai system is based upon the concept that in CLL there is a gradual and progressive increase in the body burden of leukemic lymphocytes, starting in the blood and bone marrow (lymphocytosis), progressively involving lymph nodes (lymphadenopathy), spleen and liver (organomegaly), with eventual compromise of bone marrow function (anemia and thrombocytopenia). In the original series describing the Rai system **[19]**, the stage at the time of initial diagnosis was approximately: Stage 0 (lymphocytosis) 25%, Stages I to II (lymphadenopathy, organomegaly) 50%, Stages III to IV (anemia, thrombocytopenia) 25%. Since this time, some reports have described a shift to earlier stages at initial presentation **[20]**, while others have not **[21]**. This may reflect changes in the use of "routine" laboratory testing with complete blood counts. In addition, the estimated median survival by stage has improved as new treatments have evolved. In our study, out of 80 patients, 8 (10%) were in Rai stage 0 and I, 15 (18.7%) were in Rai stage II, 6 (7.5%) were in Rai stage III and IV. This differs from early literature because of the low number and short follow-up period of time.

There was no correlation of CD38 with age, sex, hemoglobin, lymphocyte count, organomegaly, or clinical Rai stage. Because of short follow-up this data is not mature to do survival analysis.

Conclusion:

The present study was aimed to study the incidence of two prognostic markers namely ZAP-70 and CD38 positivity in CLL patients. ZAP-70 and CD38 positivity were detected in 20 patients (25%) and 29 patients (36%), respectively, with concordance rate of 56%. There was no correlation of ZAP-70 and CD38 positivity with age, sex, lymphadenopathy, organomegaly, and Rai staging. The low prevalence rate of ZAP-70 and high prevalence of CD38 was due to biology of disease in the Middle East population.

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Prevalence of XPO1 and NOTCH1 Gene Mutations in CLL and its Association with Trisomy 12

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ABSTRACT

Background: Recent studies reported whole genome sequencing of Chronic Lymphocytic Leukemia (CLL) samples and found repeated mutations in the XPO1 and NOTCH1 genes. XPO1 was found mutated in 2.4% of cases, while NOTCH1 was found mutated in 12.2% or 15.1% of CLL samples.

Aim: Our aim is to detect the prevalence of XPO1 and NOTCH1 gene mutation in CLL patients and its association with different subtypes of CLL and trisomy12 positive cases.

Methods: The coding XPO1 (exons 15 and 16), and the NOTCH1 (exon 34; RefSeq NM-017617.2) mutations hotspot were analyzed by direct sequencing of genomic DNA extracted from blood mononuclear cells. Purified amplicons were subjected to conventional DNA Sanger sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, and USA). We reported the results of sequencing of XPO1 and NOTCH1 in 186 CLL cases presenting to Dammam University Hospital in Saudi Arabia.

Results: Our results confirmed frequency of XPO1 mutations. However, we found only 5 NOTCH1 mutations in 127 IGVH unmutated/ZAP70^{+} CLL samples (4%), and one mutation was found in IGVH mutated/ZAP70^{-} CLL for a total percentage of 1.5%. Because 4 of 6 mutated samples also showed trisomy 12, we sequenced NOTCH1 in an additional 77 cases with trisomy 12 CLLs, including 47 IGVH unmutated/ZAP70^{+} cases. We found 41.9% NOTCH1 mutation frequency in aggressive trisomy 12 CLL cases.

Conclusions: Our data suggest that activation of NOTCH1 plays a critical role in IGVH unmutated/ZAP70^{+} trisomy 12 CLL.

Key Words: *CLL – XPO1 – NOTCH1 – Gene mutation – Trisomy 12 – IGVH mutation – ZAP70.*

INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in adults and is characterized by the monoclonal expansion of CD5+ B cells. CLL shows clinical heterogeneity, from patients with very stable to patients with a rapidly progressive disease that is refractory to therapy. In progressive disease, there is transformation to diffuse large B-cell lymphoma, a condition known as Richter's Syndrome (RS) **[1]**.

Although biological factors, such as mutational status of IGHV genes, TP53 disruptions, chromosomal aberrations and CD38 and Zap70 expression, have been associated with clinical outcome, they do not entirely explain the molecular pathogenesis and the clinical heterogeneity of the disease. The development of new powerful sequencing technologies has made it possible to perform unprecedented detailed genetic analyses which have led to the discovery of novel genetic alterations in CLL and shed light on the understanding of this complex disease. In this way, two unexpected pathways have been identified to be mutated in CLL, and indicate that activated NOTCH1 signaling and defects in the splicing machinery play a prominent role in the development of specific subsets of CLL **[1,2]**.

The observation of a high expression of IgM in the group harboring NOTCH1 mutations also suggests that those alterations occur preferentially in cells highly responsive to external stimuli and sustaining NOTCH1 signaling **[3]**. It remains to be determined whether NOTCH1 mutations represent a primary event occurring in the first stage of transformation or a secondary event driving disease progression.

B-cell Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in Western societies **[4]**. Genetic aberrations can be identified in the CLL samples of more than 80% of patients **[5]**. CLL cases can be subgrouped into 2 major types, aggressive or indolent, which are defined as cases that express high levels of ZAP70 and un-mutated IgH V region genes (IGHV), or low-to-negligible ZAP-70 and mutated IGHV. The most frequent recurrent genetic alterations include deletion/inactivation of 13q14 (>50%), deletion of 11q22-23 (18%), trisomy 12 (15%-18%), and deletion 17p (7%-10%) **[5]**. Two studies reported whole-genome sequencing of CLL samples and found 40 somatic mutations in 5 samples and 46 somatic mutations in 4 samples, respectively **[6,7]**. Subsequent sequencing of larger numbers of CLL samples revealed NOTCH1 mutations in 18%-20% of IGVH unmutated/ZAP70^{+} CLL samples, but only in 4%-7% of IGVH mutated/ ZAP70^{-} CLL samples **[6,7]**. One of these 2 reports also showed recurrent mutations in the XPO1 gene **[7]**. These mutations were found in 4 of 165 CLL samples or in 2.4% of cases. All these mutations were found in IGVH unmutated/ ZAP70^{+} CLL samples, and the percentage in this cohort was 4.6% **[7]**. The XPO1 gene encodes a member of the importin-β/karyopherin-β family of nuclear transport factors, namely Xpo1, which mediates nuclear export of proteins and ribonucleoprotein **[8]**. Xpo1 also is involved in the control of several cellular processes by controlling the localization of cyclin B and members of the MAPK pathway **[9]**. NOTCH1 encodes a class I trans-membrane protein functioning as a ligand-activated transcription factor **[10,11]**. On ligand binding, Notch1 undergoes several proteolytic cleavages resulting in translocation of the Notch1 intracellular domain (ICN) to the nucleus where it plays an important role in cell differentiation, proliferation, and apoptosis leading to transcriptional activation of multiple target genes, including c-Myc **[12]**. ICN contains PEST domain targeting ICN for ubiquitinylation and degradation **[10,11]**. Almost all NOTCH1 mutations in CLL are represented by the 2 base deletion frameshift resulting in a truncated constantly active protein, lacking the C-teminal PEST degradation domain Fig. (1) **[6,7]**. In addition, one frameshift insertion and 2 nonsense mutations were observed, each resulting in truncated Notch1. It has been reported that treatment with Y-secretase inhibitors induces cell growth arrest and apoptosis in different cell lines by decreasing NOTCH1 signal transduction **[13]**. Finally, antagonists that act by directly targeting the NOTCH trans-activation complex are under investigation **[14]**. These findings bring hope that these new molecular insights can be translated into new therapeutic approaches for the treatment of CLL.

In this work, we studied the prevalence of XPO1 and NOTCH1 gene mutation in CLL patients and its association with different subtypes of CLL and trisomy12.

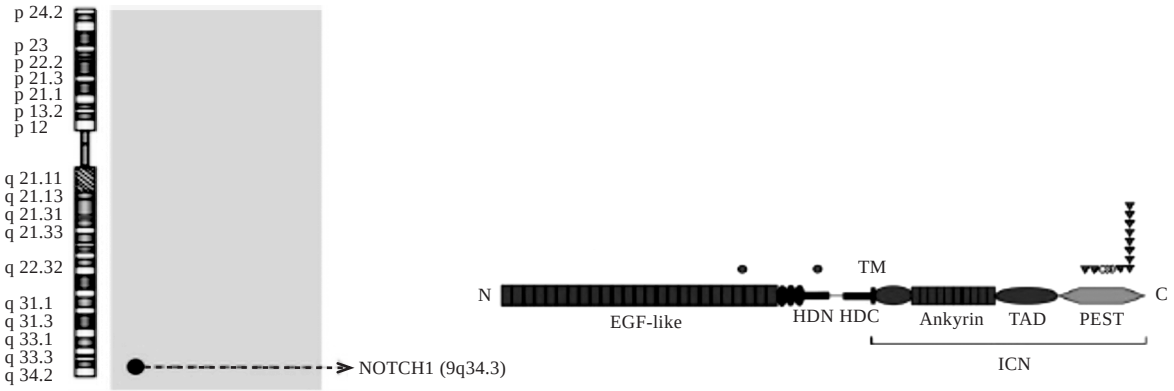


Fig. (1): Schematic representation of the Notch1 receptor on chromosome 9 and showing the PEST domain **[15,16]**.

PATIENTS AND METHODS

Patients:

The study was carried out in accordance with the institutional review board protocol approved by the Dammam University. Samples were obtained from 186 CLL patients enrolled in the CLL Research Consortium and presented to Dammam University Hospital; a written informed consent was obtained from all patients.

They included 155 males and 31 females with an age range of 50-75 and a median of 65 years. For 6 of these patients, 2 time points were provided, for a total of 192 samples analyzed. The 2 time points represent different stages of the disease: The first time point was provided in a clinically indolent stage while the last time point was provided during the aggressive stage. All cases satisfied the WCLL diagnostic criteria for CLL **[17]** and were selected on the basis of: I) Untreated disease; II) Availability of biological material. Lymphocyte morphology, immunophenotype, FISH analysis and IGHV sequencing were performed as previously described **[18]**, where there were 127IGVH unmutated/ ZAP-70$^+$ CLL and 65 IGVH mutated/ZAP-70$^-$ CLL samples.

Methods:

Progression was determined by clinical parameters such as increase in spleen size, white blood count, and overall Rai stage. Aggressive status was defined as unmutated IGVH (>98% of homology to the germline), and >20% of ZAP70-positive cells. Indolent status was defined as mutated IGVH, and <20% of ZAP70-positive cells **[19]**.

Sequencing:

DNA was extracted with the DNeasy Blood and Tissue Kit (QIAGEN). The coding XPO1 (exons 15 and 16), and the NOTCH1 (exon 34; RefSeq NM-017617.2) last coding exon, which encodes the portion of the PEST domain, mutations hotspots previously identified in CLL **[20]**, were analyzed by direct sequencing of genomic DNA extracted from blood mononuclear cells. Purified amplicons were subjected to conventional DNA Sanger sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, and USA). For amplification, we used high-fidelity advantage 2 polymerase master mix (Clontech). The primer sequences were: Xpo15-16dir2: Ttaggaaatgtacttgtagtttcta, xpo15-16rev2: Gggtctctaacaagacaaaaacat; notch33dir: Acccagcctcacctggtgcaga, notch-33rev: Tcggccctggcatccacagag. Purified amplicons were subjected to conventional DNA Sanger sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, and USA). If mutated peak (s) on chromatograms were as high as the Wild-Type (WT) peak, we concluded that mutations were in 100% of cells. Otherwise, mutations were found in 50% and 25% of cells accordingly.

RESULTS

Samples were obtained from 186 CLL patients, male to female ratio was 5 to 1, with an age range of 50-75 with a median of 65 years. We sequenced XPO1 coding region (exons 15 and 16) in our set of samples from 186 CLL patients. Six cases had 2 samples collected at 2 different time points, resulting in total of 192 CLL samples analyzed, 127 IGVH unmutated/ ZAP70$^+$, and 65 IGVH mutated/ZAP70. We found the E571K mutation in 4 of 192 samples (2.1%), Fig. (2). All the mutated samples were in the IGVH unmutated/ZAP70$^+$ cohort, with a frequency of 4/127 (3.1%). In addition, we found the V565I (ex16-61719490 G-A) mutation in the first and second samples collected from a patient who first had indolent disease (sample collection 1) that later became progressive (sample collection 2). The other IGVH unmutated/ZAP70$^+$ sample displayed a V520A mutation in exon 15 (ex15-61719700 [T-C], in ~ 25% of cells). In summary, we found XPO1 mutations in 6 of 127 IGVH unmutated/ZAP70$^+$ cases (4.7%), but in only 1 of 65 IGVHmutated/ZAP70$^-$ cases (1.5%).

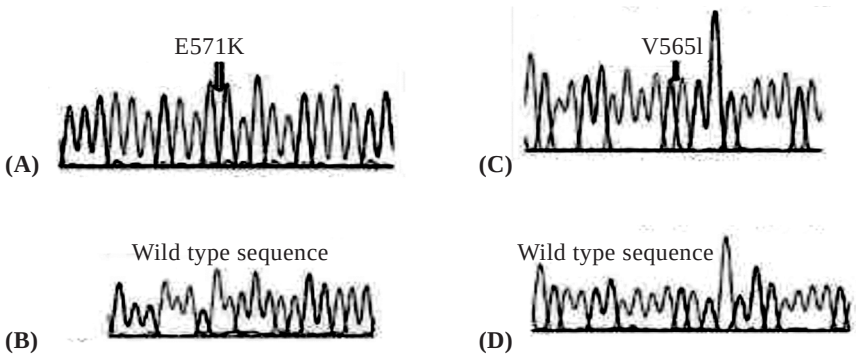


Fig. (2): XPO1 gene mutation. (A,B) Showing E571K mutation and its wild type, (C,D) Showing V565I mutation and its wild type.

We used the same set of samples to screen for NOTCH1 mutations (exon 34) Fig. (3). Interestingly, we found only 5 mutations among 127 IGVH unmutated/ZAP70$^+$ CLL samples (4%). One mutation was found in 6/192 IGVH-mutated/ZAP70$^-$ samples for a total percentage of 3.1% (Table 1).

Table (1): NOTCH1 mutation frequency in CLL.

Sample description	Total mutation	Aggressive	Indolent
• Total samples, first set	6/192 (3.1)*	5/127 (4.0)	1/65 (1.5)
• Trisomy 12 samples in the first set	4/19 (21.1)	4/15 (26.7)	0/4 (0)
• Trisomy 12 set	23/77 (29.9)	22/47 (46.8)	1/30 (3.3)
• Total trisomy 12 samples	27/96 (28.1)	26/62 (41.9)	1/34 (2.9)

* No. (%)

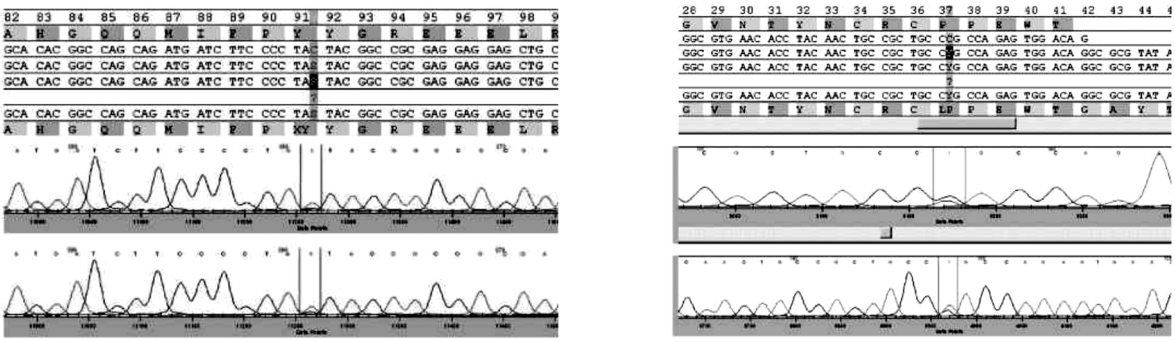
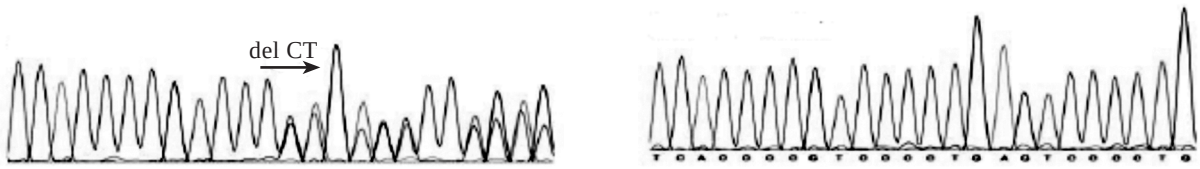


Fig. (3): DNA sequencing electropherograms demonstrating the NOTCH1 sequence mutations.

Because 4/6 samples with NOTCH1 mutations had trisomy 12 Fig. (4), we examined for NOTCH1 mutations in 77 additional cases that also had trisomy 12. This set of samples included 47 IGVH unmutated/ZAP70$^+$ aggressive cases, that were discordant for ZAP70 expression; however, they were characterized as aggressive because they were treated within 1 year of diagnosis), and 30 IGVH mutated/ZAP70$^-$ cases. Among these samples, we found NOTCH1 mutations in 22/47 (46.8%) IGVH unmutated/ZAP70$^+$ aggressive cases, but in only 1/30 (3.3%) IGVH-mutated/ZAP70$^-$ cases. Collectively, for all cases examined with trisomy 12, we found NOTCH1 mutations in 26/62 (41.9%) IGVH unmutated/ZAP70$^+$ aggressive cases, and in 1/34 (2.9%) IGVH-mutated/ZAP70$^-$ indolent cases. Twenty-five cases had mutations in NOTCH1 that were similar to those described **[6,7]** namely a heterozygous 2-bp frameshift deletion P2515fs. Two other cases had mutations resulting in Q2409stop or L2457V. All mutations were observed in 100% of cells in each sample, except in 2 cases in which the P2515fs mutation was observed in ~50% of the cells, and in one case, in ~25% of the cells.



(A) **(B)**

Fig. (4): NOTCHI1 mutation in CLL trisomy 12 patient: A) Chromatogram of a heterozygous CT coding sequence deletion at position chromosome 9:138510470-71. B) Wild type of NOTCHI1 gene.

DISCUSSION

In the current study, we found XPO1 mutations in 6 of 127 IGVH unmutated/ZAP70$^+$ cases (4.7%), but in only 1 of 65 IGVH mutated/ZAP70$^-$ cases (1.5%). These data confirmed previously reported results **[7]**.

Notch1 mutations were independent of gender, thus suggesting that Notch1 mutations might be an important marker of unfavorable prognosis in both male and female CLL patients. Interestingly, we found only 5 mutations among 127 IGVH unmutated/ZAP70$^+$ CLL samples (4%). All these changes were previously de-

scribed 2-bp frame-shift deletion P2515fs, resulting in truncated Notch1 protein **[6,7]**. One mutation was found in 6/192 IGVH-mutated/ ZAP70^{-} samples for a total percentage of 3.1% (Table 1). These results show 4- to 5-fold lower Notch1 mutation frequency in IGVH unmutated/ZAP70^{+} CLL compared with previous reports (4% vs 18%-20%) **[6,7]**, suggesting that Notch1 mutations may not be as prevalent as previously reported which may be due to ethnic variation.

In fact, the first studies reported a high frequency of Notch1 mutations in IGVH unmutated cases and in aggressive clinical phases of CLL as chemo-refractory with disease progression towards transformation into RS. A significant adverse impact on outcome has also been reported independently of other clinico-biological features, including TP53 alterations and unmutated IGHV genes, as Notch1 positive patients showed a significantly shorter overall survival, a shorter time to progression and a high risk of RS **[21]**.

Analyses on larger number of patients and on specific subgroups of patients have now documented a particularly high frequency of NOTCH1 mutation in CLL cases harboring trisomy 12 (+12), one of the cytogenetic alterations recurrently observed in CLL and classically associated with an intermediate prognosis **[22]**. Del Giudice and colleagues documented a high frequency of Notch1 mutations (30%) in CLL cases harboring trisomy 12 as the sole cytogenetic abnormality **[21]**. Importantly, this study also revealed a significant shortening of survival in the Notch1 mutation positive patients, refining the intermediate prognosis of CLL cases with trisomy 12. Moreover, the study highlighted that the presence of Notch1 mutations in +12 CLL cases is associated with a peculiar gene-expression profile characterized by an over representation of cell cycle related genes that are located on chromosome 12.

Although 2 previous studies reported high mutation frequency for NOTCH1 in IGVH unmutated/ZAP70^{+} CLL **[6,7]**, in our set of samples we only observed 4% frequency. On the other hand, our data suggest that almost half of IGVH unmutated/ZAP70^{+} trisomy 12 CLL patients (41.9%) harbor Notch1 mutations, indicating that Notch1 activation is strongly associated with trisomy 12. These differences could be explained, at least in part, by the fact that previous reports did not specifically study Notch1 mutations in trisomy 12 CLL, and did not specify how many trisomy 12 samples were present in their sample pools. All NOTCH1 mutations, except one, resulted in a truncated protein, lacking the C-terminal PEST degradation domain, rendering it constitutively active **[6,7]**. Functional significance of L2457V mutation remains to be elucidated.

In conclusion, Notch1 represents a new target of genetic lesions that could be involved in the pathogenesis of CLL and identifies a subgroup of patients with poor prognosis. Considering the high frequency of Notch1 mutations in a subgroup of patients harboring trisomy 12 and the prognostic implications of this, these mutations should be evaluated at diagnosis and progression. As Notch1 represents a new therapeutic target in CLL, future studies should evaluate the sensitivity of Notch1 mutation positive CLL cases to Notch1 inhibitors, as has been documented in T-ALL.

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Assessment of Red Cell Fragility by Flow Cytometry and Red Cells Antioxidant Enzymes (Glutathione Peroxidase and Reductase) in End Stage Renal Disease Patients Undergoing Hemodialysis

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ABSTRACT

Background: Anemia is common and multifactorial in hemodialysis (HD) patients. Oxidative damage is known to cause erythrocyte deformability.

Objectives: We aimed to assess the influence of the HD procedure on red cell antioxidant enzymes and red cell fragility using Flow Cytometry (FCM).

Patients and Methods: This is a case-control study involving 70 HD patient and 30 healthy controls; all patients received recombinant erythropoietin (rEPO) and iron. Two blood samples were taken pre-and post-HD for assessment of biochemical and hematological parameters, red cell fragility by flow cytometry and red cell antioxidant enzymes glutathione peroxidase (GSH-Px) and reductase (GSH-Rx).

Results: The mean age of HD patients was 48.42± 14.02 yrs; the causes of ESRD were hypertension 35 (50%), obstructive nephropathy 11 (15.7%), chronic glomerulo-nephritis 7 (10%), diabetes mellitus 6 (8.6%) and unknown etiology 11 (15.7%). GSH-Px and GSH-Rx decreased significantly in post-HD compared to pre-HD and control group (p=0.0001). Percentage of residual red cells in pre-HD and post-HD were significantly lower than healthy control (p=0.0001) with insignificant difference between pre-HD and post-HD group (p=0.6709). Patients were categorized into responder 27 (38.57%) and non-responder to rEPO therapy 43 (61.43%). Non-responders to rEPO therapy had significantly lower percentage of residual red cells and GSH-Px activity compared to responders.

Conclusions: HD aggravates oxidative damage; nevertheless the increased red cell fragility detected in pre-HD patient was not intensified immediately after dialysis (i.e. no acute hemolysis). However; gradual deterioration of RBCs due to mechanical stress cannot be excluded following extracorporeal blood circulation in the dialysis machine. We therefore suggest that antioxidant supplementation might be capable of recovering antioxidant defense in red cells and plasma and thus preventing oxidative damage induced by HD.

Key Words: *RBCs osmotic fragility – Hemodialysis – Flow cytometry – ESRD – Glutathione.*

INTRODUCTION

The pathogenesis of anemia in hemodialysis (HD) patients comprises different mechanisms **[1,2]**. Decreased RBCs lifespan was observed in nearly all HD patients; this may be due to extrinsic (extracorporeal circuit) or intrinsic (uremic milieu) factors that lead to modifications in RBCs membrane composition **[3,4]**.

Patients with End Stage Renal Disease (ESRD) have reduced capacity to handle oxidative stress as indicated by decreased levels of antioxidants (such as glutathione) and increased lipid peroxidation within the RBC membrane which may contribute to shortening of RBC lifespan **[5]**.

There is great evidence that HD patients are in a continuous state of oxidative stress, which may be aggravated by bio-incompatibility of the dialyzer membrane, that may induce formation of Reactive Oxygen Species (ROS) e.g.

H2O2 and superoxide which are neutralized by the extensive RBC antioxidant system involving both non-enzymatic and enzymatic antioxidants like glutathione peroxidase **[6]**. Furthermore, both dialyzable components of uremic plasma and dialysis procedure trigger premature suicidal erythrocytes **[7]**.

The RBC deformability is determined by cell geometry, internal viscosity, rheological properties of the membrane, osmotic pressure, calcium, nitric oxide, temperature, ageing and depletion of adenosine triphosphate. Hence, a slight decrease in RBC deformability causes a significant increase in micro-vascular flow resistance and blood viscosity **[8]**.

Osmotic deformability is frequently measured in RBCs and provides information on their viability, cellular water content, surface area and deformability, particularly in relation to several pathological conditions **[9]**. Therefore we tried to investigate red cell fragility by flow cytometry.

The aim of this study was to assess the influence of the HD procedure on both RBC fragility (using flow cytometry) and red cells antioxidant enzyme GSH-Px and GSH-Rx.

MATERIAL AND METHODS

Study design and subjects:

A cross-sectional case-control study was carried out at the dialysis unit in Qena-General Hospital, Qena-Upper Egypt-Egypt, seventy patients on maintenance HD, (34 males and 36 females) and thirty age and sex matched healthy volunteers (21 males and 9 females), who were free of kidney disease and with normal hematological and biochemical values, did not receive any medication known to interfere with the studied variables, served as control. All participants provided an informed consent. The study was approved by the ethical committee in Qena Faculty of Medicine.

Exclusion criteria:

Patients with autoimmune disease, pregnancy, malignancy, hematological disorders, infections, HBV or HCV and HIV positivity, severe hyperparathyroidism, blood loss or transfusion and known cause of anemia (e.g., haemoglobinopathies) were excluded.

All patients attended the dialysis unit 3 times per week; each dialysis session lasted for 4 hours, using bicarbonate dialysate solution with polysulphone F7/F9 membrane dialyzers (Bio-140; Dialife SA, Taverne, Switzerland).

The medication taken by patient's population includes: Anti-hypertensive drugs (calcium antagonists, angiotensin converting enzyme inhibitors), phosphate binders (calcium carbonate), folic acid, vitamin C and vitamin B6, recombinant erythropoietin (rEPO) Epoetin therapy by the subcutaneous route and iron by intramuscular injection by the end of HD.

Classification of the patients, as responders or non-responders to rEPO therapy, was performed in accordance with the European Best Practice Guidelines which defines resistance to rEPO as a failure to achieve target Hb levels of (11-12g/dL) with maintained doses of rEPO **[10]**.

The following characteristics were observed or calculated: Age, gender, duration of dialysis, weight, height, Body-Mass Index (BMI) and blood pressure measured using standard mercury sphygmomanometer. The average of two blood pressure readings, recorded at an interval of 5min., was used for analysis.

Blood samples:

Seven ml venous blood was taken twice from HD patients, pre-HD and post-HD on a midweek dialysis sessions. The pre-HD sample was drawn from arteriovenous fistulas at insertion of the arterial needle, before heparinization of the line and after an overnight fasting period and before the patient was being connected to the dialysis machine and the post-HD sample just prior to patient disconnection, and blood samples were taken once from the control subjects.

Blood samples were collected in EDTA, heparin and plain vacutainer tubes. EDTA tube was used for Complete Blood Count (CBC) using cell dyne-1800 (Abbott diagnostics, USA), and for flow cytometry RBCs osmotic fragility assessment. Plain tube blood was allowed to clot and then centrifuged at 3000rpm for 10

minute and serum separated for assessment of urea, creatinine using Cobas c311 automated chemistry analyzer (Roche diagnostics, Germany). The heparinized tube was centrifuged immediately at 2700 X g for 10min. at 4ºC, and the plasma was separated from the packed RBC. RBCs were washed 3 times with cold saline, and the buffy coat carefully aspirated from the surface of the pellet, then red cell lysate prepared, aliquoted in 1ml cryo-tubes and stored at –80ºC for later analyses of GSH-Px and GSH-Rx.

GSH-Px and GSH-Rx activity were measured using kits provided by Cayman Chemical Michigan-USA; cat no 703102 and 703202 respectively according to manufacturer instructions. The activity of GSH-Px was indirectly measured by a coupled reaction with GSH-Rx. Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GSH-Px, is recycled to its reduced state by GSH-Rx and NADPH and the oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340nm at 37ºC using the Humalyzer 2000 analyzer. A standard curve was prepared by using the standard provided in the kit, and the value for each sample was read from the curve. GSH-Px activity precision: The intra-assay coefficient of variation was 5.7%. GSH-Px assay range 50-344nmol/min/ml and the GSH-Rx activity intra-assay coefficient of variation was 3.7%; GSH-Rx assay range 20-255 nmol/min/ml.

Flow-Cytometry Osmotic Fragility Test (FCM OF): In this test a hemolysis inducing agent, deionized water, is spiked to a red cell suspension during acquisition, and the count of red cells is measured sequentially in real-time using FCM according to the method described by Won and Suh **[11]** with a modification in the acquisition time.

The red cell suspension was prepared by a two-step dilution with normal saline. In the first step, 20-30µL blood was diluted with 1.1mL of normal saline. The volume of blood taken was calculated based on the following formula so that the same number of red cells would be present in each tube:

$$\text{Blood volume (ML)} = 130 \div (\text{Number of red cells}/\mu L)/10^6$$

In the second step, 10µL from the first diluted blood was added to the flow cytometry tube that already contained 1.1mL of normal saline. This second diluted blood was the final red cell suspension ready for flow cytometry acquisition and analysis.

Acquisition: Forward Scatter Characteristics (FSCs) and Side Scatter Characteristics (SSCs) were acquired via linear amplification on the FACS caliber flow cytometer using cell Quest soft wear (Becton Dickinson, San Jose, CA).

A time/FCS acquisition plot, in which eight identical regions were demarcated, was created to acquire events based on time over a period of ~102.4sec. This dot plot showed dots moving from left to right, assuming time is the X parameter; one region took ~11s. The FACS tube containing the thoroughly mixed final red cell suspension was added at the sample injection port. After the first region passed (R1) during acquisition (the area through which the cells pass), the tube was removed without ending acquisition and 0.9ml Deionized Water (DW) was added and acquisition was again continued up to the eighth region. The acquisition was stopped either when a 200000 events were acquired or when 102.4sec passed. It took ~2min to run one tube.

Analysis: The event count per single region was chosen as a parameter reflecting the number of residual red cells in each region of the time/FSC plot prior to and after DW spiking. The average of the total number of events in the last two regions (R7) and (R8) were used for calculation of the percentage % of residual red cells Fig. (1).

The degree of osmotic hemolysis was expressed as "% residual red cells", i.e. the percentage of residual red cells divided by the number of red cells initially present, and was calculated based on the following formula:

$$\%\ \text{Residual red cells} = \frac{\text{Mean event count of last two regions (R7 and R8)}}{\text{Event count of first region (R1)}} \times 1.1/2 \times 100\%$$

1.1/2.0 is the multiplying factor for correction as it is assumed that the first region is

also in a diluted state for comparison with the remaining regions which are diluted by spiking with 0.9mL DW. Increased osmotic fragility is indicated by a low percentage of residual red cells. The FCM-OF was proofed to be valid for screening for red cell membrane disorders **[12]**.

Calculations were done for estimated glomerular filtration rate (eGFR) **[13]** and the percentage blood urea nitrogen reduction ratio (URR %), to assess the intensity of dialysis; inadequate dialysis was defined as a reduction in URR% of less than 65% after a dialysis session **[14]**.

Statistical data:

Data analysis was performed using SPSS version 22 software. Paired-sample and independent-sample two tailed student *t*-tests and Chi-Square were applied where appropriate. Data are expressed in mean, standard deviation and percentage and parametric or non-parametric tests were used for comparison of data. Pearson correlation coefficient was used to explore the relationship between quantitative variables. A *p*-value of less than 0.05 was considered significant.

RESULTS

Baseline demographic characteristics and clinical data of the studied HD patients are presented in (Table 1).

Laboratory data of the studied group are presented in (Table 2).

Correlations between % residual red cells and other clinical and laboratory parameters in pre-and post-HD patients are shown in (Table 3).

Comparison between responder and non-responder to rEPO therapy in pre-HD patients is presented in (Table 4).

No correlation between % of residual red cells and other clinical or laboratory parameters in pre-HD patient was encountered except for a mild significant positive correlation with eGFR (r=0.417, p=0.031) and mild negative correlation with patient age (r=–0.453, p=0.018) in responders as well as mild significant positive correlation with MCHC (r=0.347, p=0.023) in non-responders.

Table (1): Demographic and clinical data of 70 end stage renal disease patients under hemodialysis.

Parameter	ESRD patients
Gender:	
Male: Female	34:36
Age:	
Mean ± SD (yrs)	48.42±14.02
Median (Range)	50 (21-80)
Dialysis duration (month):	
Mean ± SD	31.086±26.49
Median (Range)	144 (6-24)
Pre-HD systolic blood pressure (mmHg):	
Mean ± SD	145.7±18.3
Median (Range)	150 (110-180)
Pre-HD diastolic blood pressure (mmHg):	
Mean ± SD	97.8±11.27
Median (Range)	100 (70-120)
BMI (kg/m^2):	
Mean ± SD	21.5±6.04
Median (Range)	21.1 (11.3-37.8)
Blood urea nitrogen reduction ratio (URR%):	
Mean ± SD	59.74±14.51
Median (Range)	61.33 (16.48-86.43)
<65% URR: No (%)	43 (61.43%)
>65% URR: No (%)	27 (38.57%)
Cause of ESRD:	*No (%)*
Hypertension	35 (50%)
Obstructive nephropathy	11 (15.7%)
Chronic glomerulonephritis	7 (10%)
Diabetic nephropathy	6 (8.6%)
Unknown	11 (15.7%)

BMI : Body Mass Index.
URR : Urea Nitrogen Reduction Ratio.

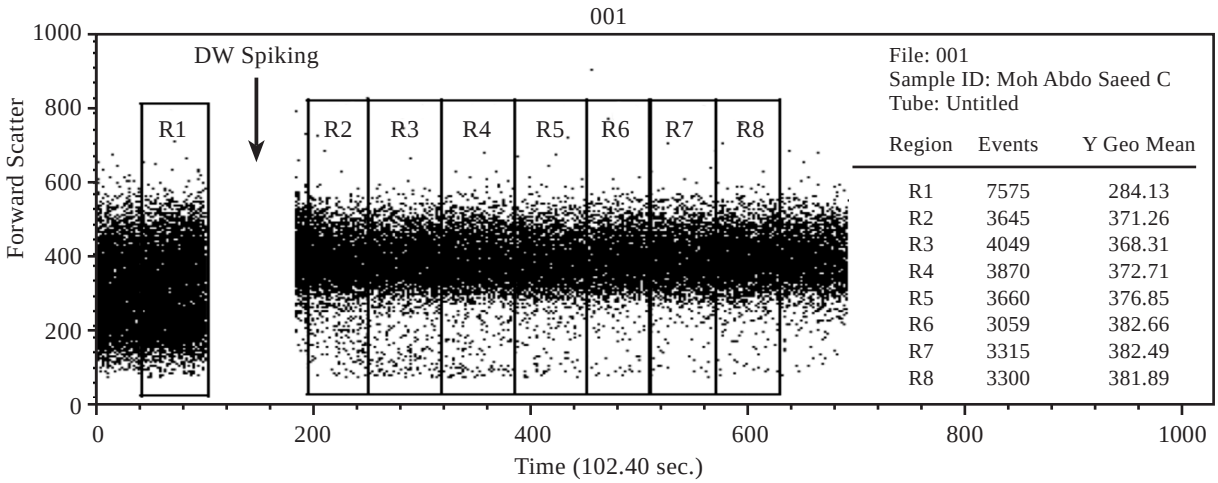

Fig. (1A): The FSC/time plot of red cells from a healthy [% residual red cells 98%].

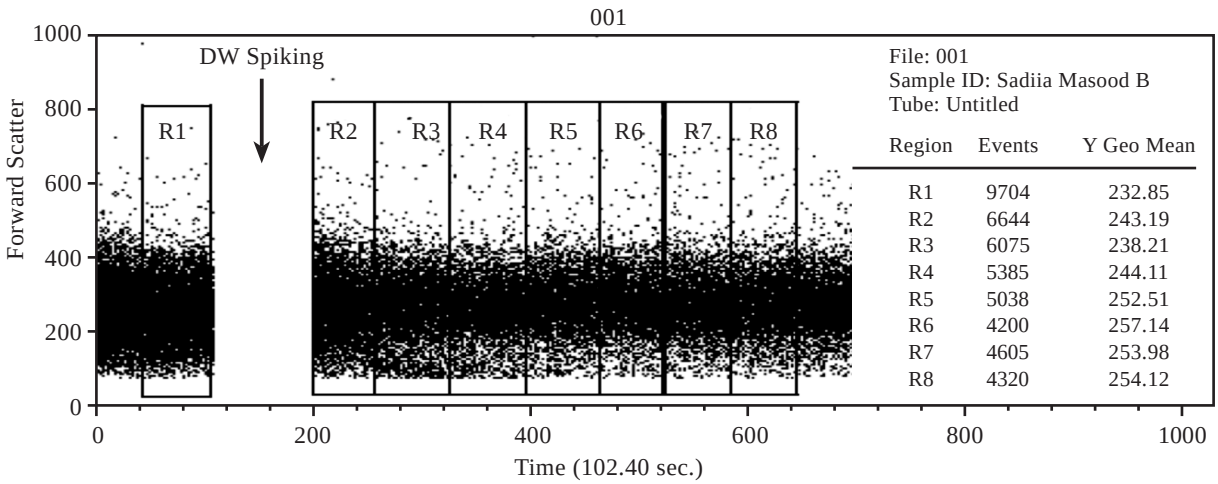

Fig. (1B): The FSC/time plot of red cells [% residual red cells was 83.6% before HD].

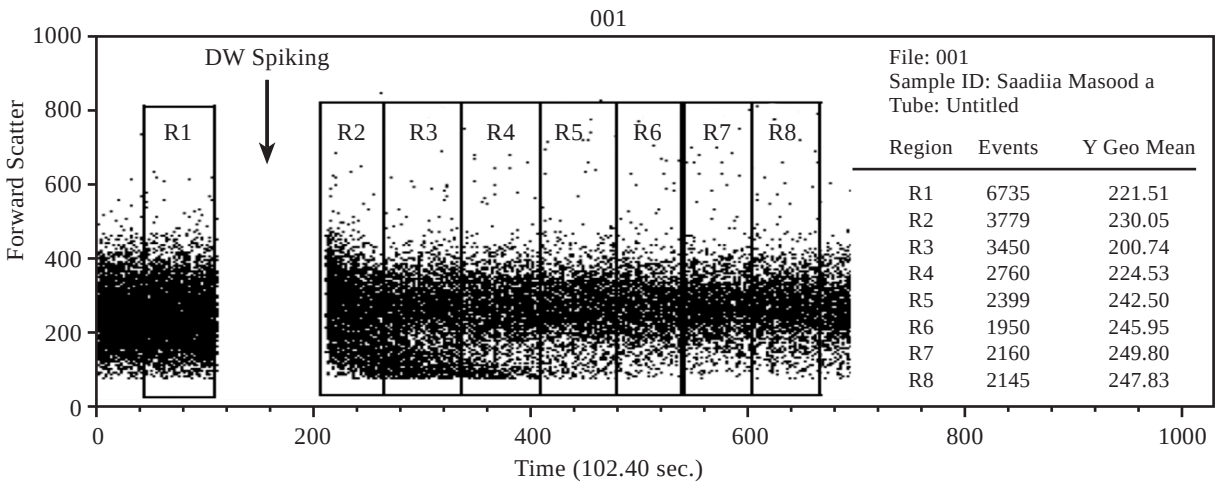

Fig. (1C): The FSC/time plot of red cells [% residual red cells was 58% after HD].

*: DW indicates deionized water.

Fig. (1): Flow cytometry FSC/time plot for % red cell acquisition and analysis form: A) Healthy control; B) A case before hemodialysis; and C) A case after hemodialysis.

Table (2): Comparison of laboratory data between end stage renal patients and controls.

Parameter	ESRD patients		Controls (No=30)	*p*-value
	Pre-HD (No=70)	Post-HD (No=70)		
Urea (mg/dl)	128.3±39.2** 54-254 123.5	51.3±26.3 16-141 48	27.4±7.9 15-46 26	0.0001[a]* 0.0001[b]* 0.0001[c]*
Creatinine (mg/dl)	11.07±3.14 4.74-21.28 10.54	5±2.17 1.88-12.38 4.66	1.06±1.3 0.56-1.8 0.8	0.0001[a]* 0.0001[b]* 0.0001[c]*
EGFR (mL/min/1.73m^2)	4.7±1.7 2-10 4	13.14±5.9 4-32 11	>60	0.0001[a]* 0.0001[b]* 0.0001[c]*
Hb (g/dl)	10.5±2.4 3.9-17.3 10.4	10.7±2.6 3.9-18.7 10.3	13.3±1.3 12-17.7 12.85	0.0001[a]* 0.0001[b]* 0.6831[c]
MCV (fl)	88.39±6.79 68.9-110 87.55	87.47±7.28 68-106 86.2	87.13±6.39 77-102.2 86.8	0.3908[a] 0.8245[b] 0.0371[c]*
MCH (pg)	27.78±2.2 21.3-33.7 27.55	27.88±2.44 21-35.4 27.7	28.2±2.14 27-32.8 28	0.3671[a] 0.5227[b] 0.5176[c]
MCHC (g/dl)	31.42±0.76 29.5-32.9 31.5	31.86±0.84 29.5-33.8 32	32.37±0.68 31.1-34.2 32.3	<.00001[a]* 0.0040[b]* 0.00001[c]*
RDW %	14.7±1.4 12-19.6 14.5	14.8±2.03 12.3-18.8 14.6	14.6±1.15 12.1-17.4 14.6	0.7513[a] 0.5995[b] 0.6853[c]
Platelets count X 10^9/L	219.4±66.7 113-420 203	213.9±71.6 122-493 200	304.6±78.9 165-467 288	0.0001[a]* 0.0001[b]* 0.7773[c]
WBCs X 10^9/L	6.76±2.5 3-15.6 5.85	7.2±3.1 2.4-19.9 6.1	6.0±1.8 3.7-9.4 6.0	0.1437[a] 0.0636[b] 0.3884[c]
Absolute neutrophil count X 10^9/L	4.0±2.082 1.170-1.2324 3.423	4.658±2.914 0.462-1.612 3.640	3.351.7±1.866 3.257-7.392 3.270	0.1447[a] 0.026[b]* 0.1265[c]
Absolute lymphocyte count X 10^9/L	1.790.7±0.453 1.020-2.880 1.761	1.587.7±0.566 0.574-2.700 1.616	1.981.8±0.568.6 0.616-2.960 1.932	0.0697[a] 0.0019[b]* 0.041[c]
Absolute Mid cells count X 10^9/L	0.926±0.488 0.225-2.604 0.792	0.860±0.474 0.270-2.587 0.728	0.557±0.095 0.370-0.801 0.553	0.000014*[a] 0.000662*[b] 0.391532*[c]
Red cell GSH-Px (nmol/min/ml)	37.47±7.59 22-48 37.5	26.833±6.15 15-36 36	270.3±75.79 124-432 233	0.0001[a]* 0.0001[b]* 0.0001[c]*
Red cell GSH-Rx (nmol/min/ml)	21±4.89 14-35 21	14.3±3.96 9-22 15	169.93±43.32 103-298 138	0.0001[a]* 0.0001[b]* 0.0001[c]*
% residual red cells	62.62±28.15 18-120 62.45	64.5±24.5 21-131 64.1	91±24.8 57-133 90	0.0001* 0.0001* 0.6709

ESRD: End Stage Renal Disease.
HD : Hemodialysis.

* : Significant.
**: Mean ± SD, range, median.

a: Pre-HD vs. controls.
b: Post-HD vs. controls.
c: Pre-HD vs. post-HD.

Table (3): Pearson correlation between % residual red cells and other clinical and laboratory parameters in end stage renal disease patients pre-and post-Hemodialysis (HD).

Variable	% residual red cells in ESRD patients			
	Pre-HD		Post-HD	
	r	*p*-value	*r*	*p*-value
Dialysis duration (month)	–0.002	0.986	–0.051	0.675
Systolic blood pressure (mmHg)	–0.167	0.167	–0.074	0.543
Diastolic blood pressure (mmHg)	–0.118	0.331	–0.055	0.651
Urea (mg/dl)	0.002	0.986	–0.157	0.194
S. Creatinine (mg/dl)	0.068	0.575	–0.153	0.663
eGFR (mL/min/1.73m^2)	0.023	0.850	0.139	0.251
Hb (g/dl)	0.216	0.072	0.261	0.261
Hct (%)	0.180	0.135	0.249	0.376
MCHC (g/dl)	0.295*	0.0131*	0.240*	0.045*
Mid cells absolute count X 10^9/L	–0.155	0.200	–0.259*	0.030*
Absolute lymphocyte count X 10^9/L	–0.276*	0.0207*	–0.063	0.604
Platelets count X 10^9/L	–0.152	0.209	–0.097	0.4243
Red cell GSH-Px (nmol/min/ml)	–0.0996	0.4149	–0.2384*	0.0468*
Red cell GSH-Rx (nmol/min/ml)	0.0841	0.4888	–0.1396	0.2511

*: Significant weak correlation.

Table (4): Comparison between responder and non-responder to rEPO therapy in end stage renal disease patients pre-hemodialysis.

Pre-hemodialysis parameter	Responder 27 (38.57%)	Non-responder 43 (61.43%)	*p*-value
Male: Female	16:11	18:25	0.1563
Dialysis duration (month)	32.41±21.35** 7.0-84 (24)	25.84±24.59 6-96 (24)	0.2598
Hb (g/dl)	12.97±1.54 11-17.3 (12.7)	9.06±1.52 3.9-10.9 (9.3)	<0.00001*
MCHC (g/dl)	31.67±0.55 30.7-32.9 (31.8)	31.26±0.82 29.5-32.9 (31.3)	0.0259*
RDW (%)	14.49±1.16 12-16.6 (14.4)	14.77±1.28 12.6-17.5 (14.5)	0.6297
% URR	61.62±12.98 36.2-86.43 (61.38)	58.52±15.45 16.48-77.05 (61.29)	0.3676
Platelets count X 10^9/L	188.42±45.18 138-282 (193)	230.14±75.87 113-420 (219)	0.0362*
WBCs X 10^9/L	5.976±1.387 3.6-10.9 (5.4)	7.26±2.77 3.0-15.6 (6.5)	0.0401*
Absolute neutrophil count X 10^9/L	3.352±1.279 1.200-6.867 (3.29)	4.407±2.373 1.170-12.324 (3.894)	0.0494*
Absolute lymphocyte count X 10^9/L	1.715±0.457 1.080-2.739 (1.744)	1.839±0.449 1.020-2.880 (1.776)	0.1230
Absolute Mid cells count X 10^9/L	0.812±0.407 0.225-2.289 (0.696)	1.015±0.473 0.576-2.604 (0.810)	0.0712
Red cell GSH-Px (nmol/min/ml)	36.70±7.48 22-48 (37)	37.23±7.76 21-47 (37)	0.3904
Red cell GSH-Rx (nmol/min/ml)	21.07±5.05 14-35 (21)	20.88±4.72 14-33 (21)	0.9064
% residual red cells	70.78±30.21 18-112.8 (74.6)	57.51±25.56 18.5-119.9 (49)	0.0337*

Urea nitrogen reduction ratio (URR %). *: Significant. **: Mean ± SD, range, (median).

DISCUSSION

HD is a common therapeutic strategy for patients with ESRD. This procedure promotes a complex biological response when the patient's blood interacts with the artificial HD membranes **[3]**. It is known that the mechanical properties of RBC change with age and mechanical stress in the extracorporeal circuit accelerates the aging process and thus might reduce the lifespan of RBC. This reduced lifespan is treated with erythropoietin which does not only increase red cell production but also increase RBC lifespan **[15]**.

RBCs of patients undergoing HD are more susceptible to oxidative stress. These factors may contribute to the shortened survival of RBC **[16]**. The increased oxidative stress levels in patients undergoing HD may promote the oxidation of surface phosphatidylserine, thereby further contributing to phagocytic removal of RBC **[17]**.

In the present study; patients under HD were more anemic than controls, and non-responder were more anemic than responder patients. Both pre-and post-HD the Hb concentration and hematocrit were significantly decreased compared to controls; this reflects the extent of reduction of the circulating red cell mass. On the other hand, RBCs count, Hb, MCH, MCHC and RDW showed insignificant increase while WBCs count, neutrophils significantly increased in post-HD compared to pre-HD. This is in agreement with a previous study **[18]**. In contrast another study **[19]** showed statistically significant increase in RBCs count, Hb, Hct, RBCs indices in ESRD patients in post-HD compared to pre-HD. This was attributed to the fact that pre-HD patients are usually hyper volemic and the values of RBCs count, Hb levels are lower and the increase in post-HD may be associated with a translocation of RBCs from the splanchnic circulation in order to compensate the hypovolemic stress during dialysis **[20,21]**.

The increase in MCHC determines cytoplasmic viscosity; loss of erythrocyte water in hypertonic media leads to a local increase in RBC cytoplasmic viscosity resulting in increased MCHC and loss of deformability **[8]**.

In the present study; there was no significant difference between pre-and post-HD group compared to control in WBCs count. This is in agreement with a previous study **[19]**.

In the present study; the absolute lymphocyte counts in the post-HD group were significantly lower than control group with further significant decrease in post-HD compared to pre-HD. This is in agreement with one study **[23]** and in contrast to another **[19]** which found significant increase in WBCs and lymphocyte counts in post-HD when compared to the pre-HD count.

In the present study; the absolute neutrophil counts and mid cell count (mainly monocytes) were significantly higher in post-HD patients than healthy control but with insignificant difference between pre-HD and post-HD. Furthermore, non responders showed significant higher WBC (mainly neutrophils) counts and platelets (as an inflammatory marker) compared to responders. This is in agreement with previous studies **[19,23]**. These data further strengthen previous findings which indicated that HD procedure activates neutrophils and monocytes; although we cannot exclude enhanced inflammatory process particularly in patients not responding to rEPO therapy **[10,24]**.

The present study revealed significant decrease in platelet counts, though still within the normal range, in the pre-and post-HD groups when compared to control group, with insignificant difference between pre-HD and post-HD.

In contrast, significant decrease in platelet count post-HD compared to pre-HD was reported by several studies **[19,25,26]**; this was attributed to platelet's activation and degranulation caused by the use of polysulfone membranes. This finding is in contrast to Yu et al., study **[18]** that reported mild increase in platelet counts after HD.

In post-HD; the mean concentrations of red cell GSH-Px and GSH-Rx were significantly decreased in post-HD compared to pre-HD and control group which could be related to the loss of antioxidant enzymes through the membranes during HD; this is in agreement with previous studies **[27,28]**.

In the present study RBCs FCM-OF analysis established that the percentage of residual red cells from pre-and post-HD group was significantly lower than healthy control but with no significant difference between pre-and post-HD

group. This means that red cell fragility was significantly increased in HD patients but not aggravated by the HD procedure (i.e. no acute hemolysis). However, gradual deterioration of RBCs due to mechanical stress following extracorporeal blood circulation in the dialysis machine cannot be excluded. This is in agreement with previous studies showing that the mechanical properties of RBC were markedly altered in HD patients compared to controls **[29-32]**. The effect of HD may be executed through reduction in spectrin, which is normally associated with a reduction in RBC deformability; reduction in spectrin, is higher in patient's not responding to Epo therapy **[4]**.

Candan et al., **[33]** reported increased red cell osmotic fragility in HD patients due to peroxidation of membrane lipid and increase in Malondialdehyde (MDA) and they found that supplementation with zinc and vitamin C decreases osmotic fragility and MDA in zinc-deficient HD patients. Furthermore, Vlassopoulos et al., **[34]** reported that low RBC osmotic resistance (RBCOR) in HD patients aggravates anemia and raises EPO needs. This is related to the type of dialysis membranes used and L-carnitine supplementation normalizes osmotic resistance in some patients.

In the present study, non-responders to rEPO therapy showed significantly lower Hb, red cell GSH-Px, GSH-Rx activity and % of residual red cells and significantly higher platelets and absolute neutrophil counts compared to responders. This may be attributed to the enhanced inflammatory process observed in non-responder patients and it was explained by pro-inflammatory cytokine's generation from activated T-cells which then promote apoptosis in erythroid progenitor cells in the bone marrow **[35]**.

Gallucci et al., **[36]** concluded that increased oxidative damage of RBC membrane detectable in HD patients is considered a factor of resistance to rEPO.

Mihaljević et al., **[33]** suggested that the administration of rEPO does not affect Red Blood Cell Osmotic Resistance (RBCOR) in HD patients, that RBCOR is not always reduced in those population and that it correlates with a small number of laboratory parameters (serum calcium and hydrogen ions characteristic for the uremic syndrome).

Finally, to our knowledge no previous study investigated red cell fragility in ESRD by flow cytometry; this study was the first to demonstrate a positive correlation between RBC oxidative damage and red cell fragility.

In conclusion, our results showed that HD aggravates oxidative damage. Results of fragility test showed that the red cell fragility is increased in ESRD as detected in pre-HD patients and it is not intensified immediately after dialysis procedure (i.e. no acute hemolysis). However, gradual deterioration of RBCs due to mechanical stress cannot be excluded following extracorporeal blood circulation in the dialysis machine.

We therefore suggest that antioxidant supplementation is capable of recovering antioxidant defense in red cells and plasma thus preventing oxidative damage induced by hemodialysis.

Conflict of interest statement: The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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