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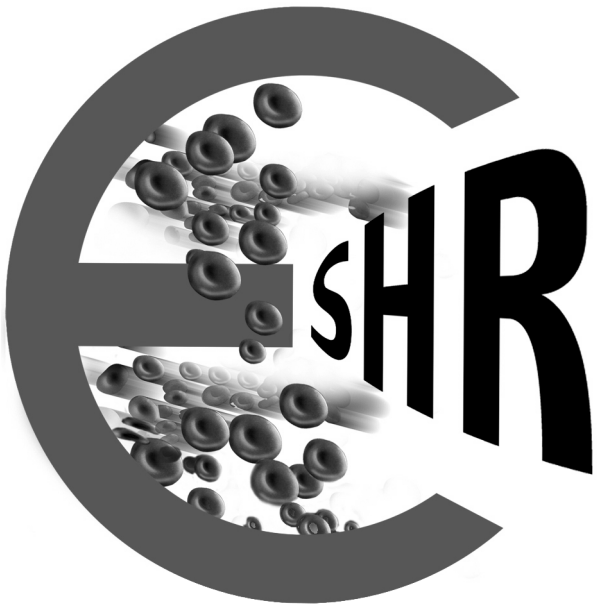

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Evaluation of Interleukin-17 and Gamma Interferon Levels in Primary Immune and Borderline Thrombocytopenia

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ABSTRACT

Background: Few studies have evaluated the Th17 cell associated cytokine in primary autoimmune thrombocytopenia (ITP); all of them included only Chinese populations with little agreement between their results. Monoclonal antibodies against interleukin 17 (IL-17) or its receptor have been developed for clinical application, so, further studies should determine if these inhibitors are clinically useful in the treatment of ITP.

Aims: The aim of this study was to explore the clinical significance of change in the level of IL-17 and gamma interferon (IFNγ) in peripheral blood mononuclear cells of newly diagnosed adult ITP patients before and one month following steroid treatment. We studied also IL-17 and IFNγ levels in borderline thrombocytopenia (healthy individuals with incidentally discovered platelet count between 100 and $150x10^9$/L).

Patients and Methods: Thirty three adult patients with thrombocytopenia and 11 healthy controls were enrolled. Patients were divided into two groups based on their platelet count at the time of the study: (i) Twenty newly diagnosed ITP patients (group 1) and (ii) Thirteen-borderline thrombocytopenia patients (group 2). Patients with newly diagnosed thrombocytopenia (n=20) were subdivided into 2 groups according to their response to steroid therapy for one month. Level of T-helper 1 (IFNγ), and T-helper 17 (IL-17) cytokines in peripheral blood mononuclear cells were investigated by enzyme-linked immunosorbentassay.

Results: The level of IL-17 and IFNγ was increased in patients with untreated ITP and borderline thrombocytopenia (p=0.0001 for IL-17 and p=0.0001 for IFNγ) when compared withcontrols. Furthermore, no statistically significant differencewas present in IL-17 and IFNγ level when comparing untreated ITP and borderline thrombocytopenia group. There was a significant positive correlationbetween IL-17 and IFNγ levels in ITP patients (r=0.621, p=0.003). There was statistically significant reduction in the level of IL-17 in responder patients (p=0.0001) while IL-17 level was insignificantly changed in non-responder patients (p=0.394).

Conclusion: Elevation of the level of IL-17 and IFNγ may be an important dysregulation factor of cellular immunity in ITP patients. Follow-up of persons with borderline thrombocytopenia is mandatory for early detection of future autoimmune abnormality in this group of persons.

Key Words: *ITP – IL-17 – IFN-gamma – ELISA – Autoimmunity.*

INTRODUCTION

Adult idiopathic thrombocytopenic purpura (ITP) is a chronic acquired organ-specific autoimmune hemorrhagic disease characterized by the production of antibodies against antigens on the membrane of platelets, resulting in enhanced Fc-mediated destruction of the platelets by macrophages in the reticuloendothelial system **[1]**.

Some patients have either no symptoms or minimal bruising, while others are at a risk of serious bleeding, which may include fatal intracranial hemorrhage, gastrointestinal hemorrhage or extensive skin and mucosal hemorrhage. The severity of thrombocytopenia correlates, to some extent, with the bleeding risk. Concepts surrounding the mechanisms of thrombocytopenia in ITP have shifted from the traditional view of increased platelet destruction mediated by autoantibodies to mechanisms in which both impaired platelet production and T cell-mediated effects play a role **[2]**.

T helper (Th)1/Th2 balance is essential in regulating immune system under normal conditions and is known to be dysregulated in many autoimmune diseases. The polarization of the

immune system towards either Th1 or Th2 immunity is dependent on the level of cytokines **[3]**.

Adult chronic primary ITP patients have high Th1/Th2 ("helper" CD4+ cells) ratio and high Tc1/Tc2 ("cytotoxic" CD8+ cells) ratio. Furthermore, the Th1/Th2 ratio imbalance is inversely correlated with disease severity, meaning the higher the Th1/Th2 ratio, the lower the platelet count. ITP patients also exhibit decreased numbers of CD4+CD25+ T-regulatory (T-regs) cells, which function to down-regulate T-cell responses. Not surprisingly, the degree of decrease in numbers of T-regs is associated with more severe disease in ITP. In addition to these changes, the total CD4:CD8 ratio is also observed to be diminished in ITP and improves with disease remission **[4]**.

Recently, a novel subset of CD4+ T cells, distinct fromTh1 and Th2, was identified. It is characterized by the production of interleukin 17 (IL-17) and, therefore, designated as Th17 cells. Th17 has been shown to play a crucial role in the induction of autoimmune diseases-including rheumatoid arthritis and experimental autoimmune encephalomyelitis which previously were considered to be mainly associated with dysregulated Th1 cell and IFNγ. It has been demonstrated that Th17 cells are more potent than Th1 cells in inducing autoimmune diseases **[3]**.

There are no data to date about Th17 levels in patients with ITP **[3]**. Few studies have evaluated the Th17 cell associated cytokines in ITP, all of them included only Chinese populations and there is little agreement between results **[5]**.

With advancements in the genetic manipulation of specific antigenic epitopes associated with the pathogenesis of ITP, the scope for a clearer understanding of the mechanisms contributing towards the pathophysiology of ITP will undoubtedly create a greater means by which potential therapies to manage ITP can be developed **[6]**. Because monoclonal antibodies against IL-17 or its receptor and a soluble IL-17 receptor have been developed for clinical application, further studies should determine whether these inhibitors are clinically useful in the treatment of chronic ITP **[7]**. The aim of this study was to explore the clinical significance of change in the level of IL-17 and IFNγ in peripheral blood mononuclear cells of newly diagnosed adult patients with primary immune thrombocytopenia before treatment and one month following steroid treatment.We studied also IL-17 and IFNγ levels in patients with borderline thrombocytopenia.

PATIENTS AND METHODS

Twenty adult patients with newly diagnosed ITP (group 1) according to the ITP diagnosis criteria proposed by an international working group (IWG) **[8]** were enrolled in this study. Six patients were females and fourteen were males. Their age ranged from 21-33 with a median of 25 years. They were randomly selected from outpatient clinics from Alexandria and Cairo Universities. All patients were symptomatic and their platelet counts were less than $30x10^9$/L. These patients were given treatment in the form of first-line corticosteroids according to international consensus **[8]** due to clinically significant bleeding and/or extremely low platelet count. One month after the initial treatment, response to therapy was validated. Eleven patients responded while nine patients did not respond to steroid treatment.

Thirteen patients with borderline thrombocytopenia (healthy individuals with incidentally discovered platelet count between 100 and $150x10^9$/L) **[9]** were considered group 2. Eleven healthy volunteers of matched age and sex were taken as normal controls (group 3).

Secondary ITP, pregnant patients, patients contraindicated to steroid therapy, patients who had received previous treatment for ITP, including immunosuppressive agents, or who had undergone splenectomy,were excluded[8].Newly diagnosed primary ITP was defined by the IWG as a platelet count less than $100x10^9$/L up to 3 months from diagnosis in the absence of other causes or disorders that may be associated with thrombocytopenia **[8]**.

Patients with ITP received treatment in the form of prednisone 1mg/kg orally for 21 days then tapered off. Response is defined as a platelet count ≥30 but $<100x10^9$/L and a doubling from baseline. Complete response is consistent with the new diagnostic threshold of $>100x10^9$/L **[8]**.

All participants in this study were subjected to:

- Thorough history-taking and clinical examination with special stress on evidence of bleeding, duration of ITP, previous treatment received for ITP and excluding other causes of thrombocytopenia including hepatitis C virus infection and helicobacter pylori infection (secondary ITP).
- Complete blood picture **[10]**.
- Bone marrow aspiration (for patients only) to exclude secondary causes of thrombocytopenia **[10]**.
- Hepatic and renal function tests **[11]**.
- Platelet specific antibody by modified antigen-capture ELISA.
- *Immunological tests:*

i- Quantitative estimation of IL-17 and IFNγ after mitogen; phytohemagglutinin (PHA) stimulated whole peripheral venous heparinized blood using commercial enzyme-linked immunosorbent assay (ELISA) kits. "RayBio® Human IL-17 ELISA USA" **[12]**.

ii- Estimation of IFN-γ levels were done by isolation of peripheral blood mononuclear cells from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Sigma Aldrich) and cultured at $2x10^5$ cells per 500µL in Roswell Park Memorial Institute medium (RPMI) 1640(Sigma Aldrich) supplemented with antibiotics and 5% fetal calf serum (Sigma Aldrich). For stimulation, 5µg/µl PHA mitogen (Wellcome Diagnostics) was used. Incubation of cultures was performed at 37ºC in a humidified atmosphere of 5% CO_2. After 48 hours incubation, culture supernatants were collected from each tube and stored at –20ºC to be assayed using commercial ELISA kits (Ray Biohuman IFNγ). We used a standard curve to detect IFNγ at the sub-nanogram level (≥100pg) **[13,14]**.

Statistical analysis:

Data were analyzed using SPSS program version 10. Data were expressed as mean±standard deviation (SD). Qualitative variables were compared using chi square (X^2) while one-way ANOVA test was done for normally distributed quantitative data. Between groups further analysis of ANOVA was done using least significant difference (LSD). Paired *t*-test was used to compare data before and following steroid treatment in group 1. Correlation between variables was assessed by Pearson's correlation (*r*). The level of significance was chosenas $p \leq 0.05$.

RESULTS

Table (1) shows clinical and laboratory criteria of the three studied groups. Patients' mean (±SD) platelet count was 22.15 (±5.87) $x10^9$/L. The duration of the disease was less than 3 months in all the studied patients in group 1.

Table (1): Clinical and laboratory parameters of the studied groups.

Parameter	Group 1 (n=20)	Group 2 (n=13)	Group 3 (n=11)	*p* value	LSD
Age (years)	21-33 25.3±3.06	14-34 25.54±6.996	19-30 22.91±3.33	0.311	
Gender:					
Male	14	9	5	0.35	
Female	6	4	6		
Platelets: ($x10^9$/L)	13-29 22.15±5.87	103-143 124±11.45	170-244 192.27±19.91	0.0001	1.2 1.3 2.3
Presence of platelet antibody	13	–	–		
IL-17 (pg/mL)	54-118 91.55±19.59	72-93 83.44±8.40	50-94 59.91±12.79	0.0001	1.3 2.3
IFNγ (pg/ml)	453-660 513.2±52.82	460-660 531.85±65.27	300-485 381.64±71.12	0.0001	1.3 2.3

Group 1: Immune thrombocytopenia.
Group 2: Border line thrombocytopenia.
Group 3: Normal Controls.
Values are expressed as range, mean ±SD.
LSD : Least significant difference, denote significant difference between groups
p : Is significant at the 0.05 level.

Table (2) shows comparison between age, platelet count and level of IL17 and IFNγ before and after steroid therapy in ITP patients responding (n=11) and non-responding (n=9) to steroid treatment.

Table (3) shows correlation between age, platelet count and level of IL17 and IFNγ in ITP patients (group 1). No correlation was detected between the level of IL17 and IFNγ and either age or platelets count in the three studied groups. Statistically significant direct correlation was present between IL17 level and IFNγ level in ITP group (r=0.621, p=0.003) (Fig. 1), borderline thrombocytopenia group (r=0.745, p=0.003) and controls (r=0.765, p=0.006).

Table (2): Comparison between responders and non-responders immune thrombocytopenic group as regards platelet count and cytokines level pre and poststeroid treatment.

Parameter	Responders (n=11)		*p* value	Non responders (n=9)		*p* value
	Before	After		Before	After	
Platelets:	13-29	200-375	0.0001	14-29	20-29	0.287
($x10^9$/L)	22.09±6.25	266.09±47.23		22.22±5.74	24.44±3.43	
IL-17 (pg/mL)	54-118	55-70	0.0001	67-116	61.4-107.8	0.394
	100.64±19.15	61.0±5.35		80.44±14.17	76.01±15.78	
IFNγ (pg/ml)	460-660	288-481	0.0001	453-512	430-485	0.121
	542.27±53.84	366.82±73.31		477.67±20.48	462.44±17.27	

Values are expressed as range, mean ±SD. *p* : Is significant at the 0.05 level.

Table (3): Correlation between age, platelet count and cytokines' level in twenty immune thrombocytopenic patients (group 1)

Parameter	Age		Platelets		IL-17		IFN-γ	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	–	–	–0.003	0.991	–0.252	0.284	–0.251	0.287
Platelets	–0.003	0.991	–	–	0.067	0.779	–0.094	0.692
IL-17	–0.251	0.287	0.067	0.779	–	–	0.621	0.003
IFN-γ	–0.251	0.287	–0.094	0.692	0.621	0.003	–	–

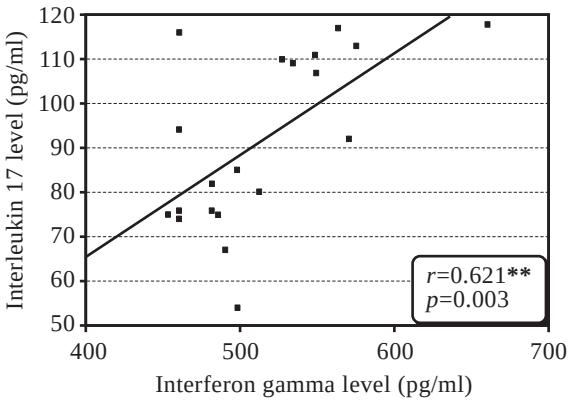

Fig. (1): Correlation between level of interleukin 17 and gamma interferon in immune thrombocytopenic patients (group 1).

DISCUSSION

Several studies have suggested that Th17 T cells may be the major cell type involved in orchestrating tissue inflammation and autoimmunity. Specifically, Th17 cells have been shown to play a crucial role in the induction of rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus (SLE) and psoriasis **[15]**. Semple et al., **[16]** first reported that T cells had reactivity against platelets, which initiated the investigation on T cell disorders in ITP.

Whether Th17 cells play a role in the pathogenesis of ITP remains undetermined because

several investigations on Th17 in ITP indicated contradictory conclusions **[17]**. While some authors demonstrated increased percentages of Th17 cells in the peripheral blood of ITP, Guo et al., **[18]** found comparable frequency of circulating Th17 cells (flow cytometry analysis) and comparable expression of IL-17 transcripts (RT-PCR evaluation) in patients and controls. In these studies Th17 cells were enumerated after stimulation of mononuclear cells with various molecules (phorbolmyristate acetate and ionomycin) and, therefore, not under physiological conditions.

IL-17 has potent immunogenic properties. It induces the release of colony stimulating factors, chemokines, metalloproteinases, tumor necrosis factor alpha, and IL-6. Moreover, IL-17 mobilizes and activates neutrophils **[19]**.

In our study,we detected statistically significant high mean value of IL-17 level in newly diagnosed immune thrombocytopenic group before treatment and in borderline thrombocytopenia group as compared to controls. Also, there was statistically insignificant difference between IL-17 levels when immune thrombocytopenia group was compared with borderline thrombocytopenia group. However, no correlation was detected between the level of IL17 and either age or platelet count in the three studied groups.

One possible link has been proposed by-Doreau et al., **[20]** who demonstrated that IL-17 alone or in combination with B-cell activating factor (BAFF) protects B cells from apoptosis, promotes B-cell proliferation and drives plasma cell differentiation, probably playing a role in the pathogenesis of SLE. Similar mechanisms might be involved in the pathogenesis of ITP, a disease also marked by a loss of B-cell tolerance, abnormal production of auto-antibody and high serum levels of BAFF **[21]**.

In 2009, Zhang and colleagues **[22]** first described up-regulation of Th17 cells along with Th1 in patients with ITP and suggested that Th17 cytokines promoted an imbalance favoring a more Th1-type immune response in ITP.Furthermore, in the natural course of experimental autoimmune encephalitis, an antigen-specific effector T cell secreting both IL-17 and IFNγ has consistently been found in vivo in the inflamed central nervous system **[7]**. Since ITP is also an autoimmune disorder, a search for a similar mechanism might be considered.

In our patients groups, we detected statistically significant high IFNγ level in both untreated newly diagnosed immune thrombocytopenia group and borderline thrombocytopenia group as compared to controls with statistically insignificant difference between IFNγ level in immune thrombocytopenia and borderline thrombocytopenia. There was also a significant positive correlation between IFNγ and IL-17 levels in the three studied groups. Thus, the most likely scenario is that both Th1 and Th17 cell types are involved.

In our study, statistically significant difference was detected in IL-17 level in immune thrombocytopenic patients responding to steroid therapy before and after treatment while there was no significant difference in IL-17 level between non responder immune thrombocytopenic patients before and after steroid treatment. This may reflect a role for IL17 in steroid responsiveness as reported by Hamid and colleagues **[23]** using both bronchial biopsies and primary bronchial epithelial cells in asthmatic patients. They concluded that IL-17, the signature cytokine of Th17 cells, can influence glucocorticoid receptor (GR) signaling capacity in asthmatic patients by reducing GRα levels and enhancing the expression of GRß which leads to further modulation of GRα function **[24]**. Reduced expression of GRα is also recently reported to be associated with glucocorticoid resistance in adult ITP patients **[25]**.

Based on the results of Stasi et al., **[9]**, healthy individuals with incidentally discovered platelet count between 100 and $150x10^9$/L have a 10-year probability of developing persistent thrombocytopenia of only 6.9% and of developing autoimmune disorders other than ITP of 12%. However, this criterion has not been formally validated yet. In our study, non-statistically significant difference was detected between the level of both IL17 and IFNγ in ITP patients in comparison with patients with borderline thrombocytopenia which confirm this observation and makes follow-up of these persons mandatory for early detection of future autoimmune abnormality.

In a previous study, patients with borderline thrombocytopenia have enhanced levels of pro-inflammatory cytokines linked to Th1 and Th17 cell response. They are also more frequently carriers of polymorphisms in genes that encode cytokines involved in the commitment of Th1 and Th17 immune response. This is similar to that observed in patients with chronic ITP, which points to the need of a search for pathogenic mechanisms associated with this condition **[26]**.

In conclusion, elevation of IL-17 and IFNγ may be an important dysregulation factor of cellular immunity in ITP patients. Follow-up of persons with borderline thrombocytopenia is mandatory for early detection of future autoimmune abnormality in this group of persons.

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Diastolic Dysfunction in Patients with Myeloproliferative Neoplasms

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ABSTRACT

Background: Cardiac diastolic dysfunction is largely unclear in patients with myeloproliferative neoblasms (MPNs).

Obgective: To evaluate the left ventricular diastolic function in MPNs patients by two-dimensional and Doppler echocardiographic studies.

Patients and Methods: The study included 44 MPNs patients, 20 of them have Chronic Myeloid Leukemia (CML), 7 with Essential Thrombocytosis (ET), 6 with Primary Myelofibrosis (PMF) and 11 have Polycythemia Vera.

Results: Echocardiographic studies showed that valvular lesions are common in MPNs patients with predominant tricuspid and mitral regurgitation. The Pulmonary Artery Systolic Pressure (PASP), Early Transmitral Deceleration Time (EDT) and IsoVolumic Relaxation Time of the left ventricle (IVRT) in MPNs patients are increased indicating Leftventricular diastolic dysfunction while, the ejection fraction (EF) representing systolic function is still within normal. Significant positive correlations between PASP and both EDT and IVRT confirming Left ventricular diastolic dysfunction with impaired diastolic filling.

Conclusion: The MPNs have deleterious effects on cardiac valves, pulmonary artery pressure and diastolic functions of the heart that can be early discovered by use of echocardiography in the initial clinical evaluation of subjects with MPNs.

Key Words: *MPNs – CML – PV – ET – PMF – PASP – Echo.*

INTRODUCTION

The myeloproliferative neoplasms (MPNs), previously termed the myeloproliferative disorders, are characterized by the clonal proliferation of one or more hematopoietic cell lineages, predominantly in the bone marrow, but sometimes in the liver and spleen **[1]**. The 2008 revision of the World Health Organization (WHO) classification of MPNs include: Chronic myelogenous leukemia (CML), chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia, mastocytosis, and unclassifiable MPNs **[2-4]**.

CML is the only MPN that is characterized by the chromosomal translocation t(9;22), BCR-ABL fusion gene. The most commonly recognized mutation in the remainder of the MPNs is Janus kinase 2 (JAK2) V617F. It is present in greater than 90% of patients with PV and approximately half of those with PMF or ET **[5,6]**.

The cardiovascular system is involved in 4% to 21% of cases of MPNs. Acute ischemic coronary artery disease is the presenting symptom in these cases **[7]**. Other cardiovascular complications in patients with MPNs are valvular heart involvement, pericardial involvement, aortitis, and thrombosis of major vessels and pulmonary embolism **[8-11]**. The development of an effective and safe strategy for preventing these cardiovascular complications is the main challenge in the treatment and management of patients with MPNs **[12-14]**.

Diastolic dysfunction refers to a condition in which abnormalities in mechanical function are present during diastole that occur in the presence or absence of a clinical syndrome of heart failure and with normal or abnormal systolic function **[15]**. Doppler echocardiography is

widely used for the noninvasive assessment of diastolic filling of the left ventricle **[16]**. The assessment of left ventricular diastolic function should be an essential part of routine examination, particularly in patients presenting with dyspnea or heart failure. About half patients with new diagnoses of heart failure have normal or near normal global ejection fractions. These patients are diagnosed with "diastolic heart failure" or "heart failure with preserved EF" **[17]**. The assessment of LV diastolic function and filling pressures is of paramount clinical importance to distinguish this syndrome from other diseases such as pulmonary disease resulting in dyspnea, to assess prognosis, and to identify underlying cardiac disease and its best treatment **[18]**.

Echocardiography has played a central role in the evaluation of LV diastolic function over the past two decades. The use of Doppler Echocardiography in evaluating diastolic function in MPNs patient is still lacking, so the purpose of this study is to evaluate the diastolic function in patients with myeloproliferative neoplasms.

PATIENTS AND METHODS

The study was conducted on 44 patients with Myeloproliferative Neoplasms (MPNs) who were attending the Clinical Hematology Unit of Assiut University Hospitals and South Egypt Cancer Institute including 20 males and 24 females. The age of the studied patients was ranged from 21 to 72 years with median age 50 years and mean±SD was 47.12±15.46 years. Twenty patients had Chronic Myeloid Leukemia (CML), seven had Essential Thrombocytosis (ET), six had Primary Myelofibrosis (PMF) and eleven had Polycythemia Vera.

Each patient was subjected to thorough history and clinical examination. Complete Blood Counts, Kidney and Liver functions, Bone Marrow Biopsy, Philadelphia Chromosome and JAK2 mutations were done to confirm diagnosis. M-mode, 2-Dimensional and Doppler (Pulsed wave, Continuous wave and Color Doppler) Echocardiography was performed to all patients.

Statistical analysis:

Statistical analysis was performed using the SPSS 16.0 statistical software package. Continuous variables were expressed as Mean±SD while Categorial variables were expressed as numbers and percentages. Paired-samples and independent-samples student *t*-tests were used to compare variables. Bivariate-Pearson correlation was used to investigate potential relationships between variables.

Ethical considerations:

An informed consent was obtained from every patient included in the study and the study was approved by the Ethical Committee in our Faculty.

RESULTS

The study included 44 patients 20 males and 24 females. The disease duration was 2.97±1.85 with median duation 3 years. As regarding treatment, 16 patients (36.3%) were on Imatinib therapy, 9 patients (20.5%) were on Hydroxyurea while 19 patients (43.2%) were not on specific treatment. The mean treatment duration is ranging from 1 to 5 years (2.75±1.65). The recorded valvular lesions in all studied MPNs patients were present in 26 patients (59%) of patients while 18 (41%) showed normal valvular morphology and function. Mitral regurgitation was present in 13 (29.5%) of patients, tricuspid regurgitation in 20 patients (45.5%), mitral stenosis in only one patient (2.2%) and aortic regurgitation in two patients (4.5%). The patterns of valvular affections in individual diseases are represented in Table (1); some patients had multiple valvular lesions. Pulmonary hypertension was observed in 19 patients (43%). The mean values of estimated echocardigraphic parameters in the different disease patterns are illustrated in Table (2). There is no correlation between Pulmonary Artery Systolic Pressure, (PASP) and Ejection Fraction (EF) r=0.064 and p=0.696 but positive correlations are present between PASP and both Early Transmitral Deceleration Time (EDT) and IsoVolumic Relaxation Time of the left ventricle (IVRT) (Fig. 1-A, B). No correlations were found between duration of the disease and duration of treatment on one side and PASP, EF, EDT or IVRT on the other side (Table 3). Table (4) represents the correlation between hematological parameters and these estimated echo parameters in MPNs patients; positive correlation is only found between white blood cells (WBCs) and EDT (r= 0.334 and p=0.035).

Table (1): Estimated valvular lesions in all MPNs patients.

Valve lesions	CML n=20	ET n=7	PMF n=6	PV n=11
Normal	5 (25%)	3 (43%)	3 (50%)	7 (64%)
MR	9 (45%)	1 (14%)	0 (0%)	3 (27%)
TR	11 (55%)	4 (57%)	3 (50%)	2 (18%)
MS	1 (5%)	0 (0%)	0 (0%)	0 (0%)
AR	0 (0%)	1 (14%)	0 (0%)	1 (9%)

CML : Chronic Myeloid Leukaemia.
ET : Essential Thrombocytosis.
PMF : Primary Myelofibrosis.
PV : Polycythaemia Vera.
AR : Aortic Valve Regurge.
MR : Mitral Valve Regurge.
MS : Mitral Valve Stenosis.
TR : Tricuspid Valve Regurge.

Table (2): Echocardiographic Parameters in MPNs Patients.

Echocardiographic parameter		Disease category			
		CML n=20	ET n=7	PMF n=6	PV n=11
PASP/mmHg	Mean±SE	30.95±2.18	29.80±5.82	26.75±3.94	24.27±2.39
	Median	32.0	22.0	26.0	20.0
	Range	18.0-59.0	18.0-48.0	20.0-35.0	18.0-45.0
	p-value		0.274		
EF%	Mean±SE	63.60±2.27	70.40±2.91	65.50±3.75	61.91±3.79
	Median	65.0	74.0	65.5	64.0
	Range	41.0-79.0	60.0-75.0	59.0-72.0	30.0-75.0
	p-value		0.286		
EDT/ms	Mean±SE	260.55±24.09	224.60±24.85	236.25±9.23	229.55±17.03
	Median	250.0	230.0	243.0	236.0
	Range	169.0-684.0	150.0-277.0	209.0-250.0	120.0-335.0
	p-value		0.875		
IVRT/ms	Mean±SE	142.15±20.83	91.40±19.26	135.00±13.67	114.36±14.83
	Median	115.0	107.0	148.0	101.0
	Range	54.0-493.0	15.0-120.0	94.0-150.0	12.0-192.0
	p-value		0.551		

CML : Chronic Myeloid Leukaemia.
ET : Essential Thrombocytosis.
PMF : Primary Myelofibrosis.
PV : Polycythaemia Vera.
AR : Aortic Valve Regurge.
MR : Mitral Valve Regurge.
MS : Mitral Valve Stenosis.
TR : Tricuspid Valve Regurge.
EF : Ejection Fraction.
EDT : Early Transmitral Deceleration Time.
IVRT : IsoVolumic Relaxation Time of the left ventricle.
PASP : Pulmonary Artery Systolic Pressure.
ms : Milliseconds.
mmHg : Milimeter Murcury.

Table (3): Correlation between duration of the disease and duration of treatment with PASP, EF, EDT and IVRT.

Echocardiographic parameter	Duration of disease		Duration of treatment	
	r-value	*p*-value	*r*-value	*p*-value
PASP mmHg	0.114	0.484	0.038	0.856
EF%	–0.111	0.494	–0.029	0.891
EDT ms	–0.017	0.918	0.094	0.655
IVRT ms	–0.075	0.647	–0.041	0.845

PASP : Pulmonary Artery Systolic Pressure.
EF : Ejection Fraction of the left ventricle.
EDT : Early Deceleration Time of the left ventricle.
IVRT : IsoVolumic Relaxation Time of the left ventricle.
ms : Milliseconds.
mmHg : Milimeter Murcury.

Table (4): Correlation between hematological parameters and PASP, EF, EDT and IVRT.

Echocardiographic parameter		WBCs	RBCs	Hb	Hct	Plt
EF%	*r*-value	0.218	–0.085	0.007	–0.070	0.132
	p-value	0.176	0.601	0.964	0.668	0.416
EDT ms	*r*-value	0.334	0.044	0.071	0.122	–0.118
	p-value	0.035*	0.789	0.663	0.452	0.470
IVRT ms	*r*-value	0.002	0.013	–0.008	0.108	–0.236
	p-value	0.988	0.938	0.962	0.509	0.142
PASP mmHg	*r*-value	0.125	–0.113	–0.111	–0.059	0.150
	p-value	0.443	0.488	0.497	0.719	0.356

PASP : Pulmonary Artery Systolic Pressure.
EF : Ejection Fraction of the left ventricle.
EDT : Early Deceleration Time of the left ventricle.
IVRT : IsoVolumic Relaxation Time of the left ventricle.
ms : Milliseconds.
mmHg : Milimeter Murcury.

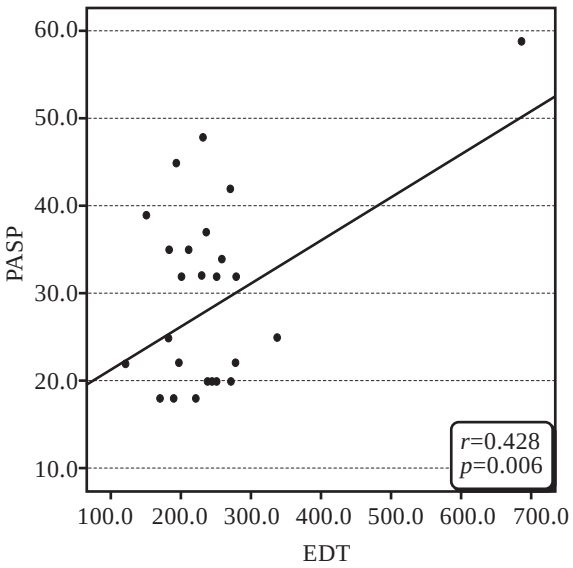

Fig. (1-A): Correlation between PASP and EDT.

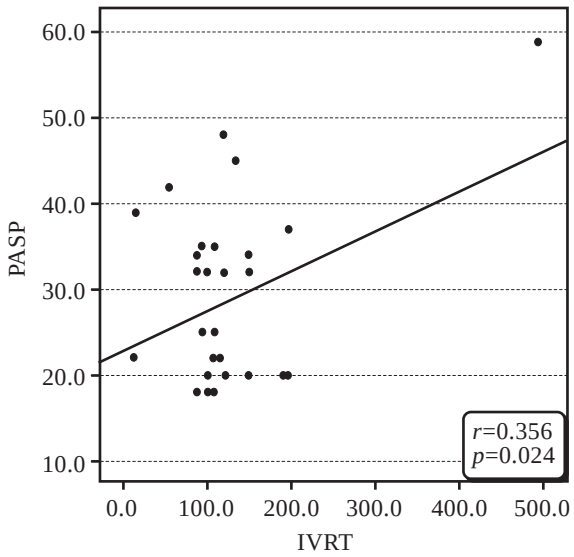

Fig. (2-B): Correlation between PASP and IVRT.

PASP : Pulmonary Artery Systolic Pressure.
EDT : Early Transmitral Deceleration Time.
IVRT : IsoVolumic Relaxation Time of the left ventricle.

DISCUSSION

Cardiac involvement in MPNs including coronary artery thrombosis, myocardial infarction, pulmonary hypertension, asymptomatic pericardial effusion, cardiac tamponade, intractable cardiac failure due to intra-ventricular thrombosis and stenosis of the Aortic and Mitral valves, even requiring surgical treatment, had been reported **[19]**.

The current study showed that cardiac valve lesions were encountered in 59% of MPNs patients. Tricuspid and mitral valves were the most commonly involved valves, a finding that is coinciding with previous reports **[8,20]**.

Pulmonary hypertension is well known in patients with MPNs but most of the literature consists of case reports or small studies as previously reported **[21-24,10]**. In the current study the pulmonary hypertension was present in 47.5% of the patients: 65% in CML, 40% in ET, 50% in PMF and 18.2% in PV. These results are higher than one previous report **[20]** but coinciding with others **[25-27]**. All patients in

the current study were asymptomatic with mild Pulmonary Hypertension, so none of them needed treatment. It is well known that symptoms of mild pulmonary hypertension are often subtle **[28]**. Since pulmonary hypertension is usually diagnosed after symptoms develop, it is possible that most cases of mild asymptomatic pulmonary hypertension remain clinically undiagnosed.

Trans-esophageal echocardiogram (TEE) is a good non-invasive method of diagnosing pulmonary hypertension **[29]** and has the advantage of excluding cardiac causes of pulmonary hypertension. The most important question is whether this high incidence of pulmonary hypertension in the current study is truly secondary to MPNs. These findings are coinciding with previous reports **[10,26,27]**. We believe that pulmonary hypertension in these cases is secondary to MPNs as the incidence of primary pulmonary hypertension in general population is very low and usually occurs in the third or fourth decade **[28]**. So the high incidence of Pulmonary Hypertension is not by chance.

Pathogenesis of pulmonary hypertension in MPNs is multi-factorial. It has been correlated to platelets in many studies **[20,21,24,30]**. Marvin and Spellberg **[22]** found obstruction of pulmonary capillaries by megakaryocytes leading to stasis and secondary micro-thrombosis in one patient with PMF and pulmonary hypertension; the right sided heart failure resolved with the correction of thrombocytosis in this patient. Furthermore, autopsy studies by **[31]** had demonstrated the presence of atypical megakaryocytes and thrombotic material in the lung capillaries of patients with pulmonary hypertension and MPNs. Other evidence implicating platelets in the pathogenesis of pulmonary hypertension is the presence of increased level of thrombopoietin in pulmonary arteries of patients with pulmonary hypertension **[30]**.

In the current study, left ventricular ejection fraction (LVEF), as an indicator of LV systolic function, was within normal expected values in different categories of MPNs patients. These results are similar to a previous report **[20]**. We did not find significant correlations between LVEF and duration of the disease, duration of treatment, WBCs, RBCs, Hb, Hct or Platelets that means the systolic function of the left ventricle represented by EF is not influenced by the disease process in our MPNs patients. Also, the used chemotherapeutic regemins was not containg direct cardiotoxic agents in treated patients.

In the current study, left ventricular diastolic function wass evaluated by measuring Early Transmitral Deceleration Time (EDT) and left ventricular Iso Volumic Relaxation Time (IVRT). LV diastolic dysfunction is diagnosed if EDT >220ms and IVRT >110ms. Diastolic dysfunction was found in 40% of all studied MPNs patients. These results are concordant with a previous report **[20]**.

The current study showed positive correlation between LV EDT and PASP and between LV IVRT and PASP. This can be explained by that prolonged LV EDT and LV IVRT indicate LV diastolic dysfunction. This, in turn, causes impaired diastolic filling of the LV with subsequent stagnation of blood in the LA causing pulmonary venous congestion. The end result is increased pulmonary vascular resistance and occurrence of pulmonary hypertension.

Conclusion:

Left ventricular diastolic dysfunction (LVDD) is a marker of evolving heart disease. Therefore, the high prevalence of LVDD in MPNs patients suggested by this study supports the use of echocardiography in the initial clinical evaluation of subjects with MPNs.

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Regulation of Iron Metabolism Through GDF-15 and Hepcidin in ß-Thalassemia Major Patients

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ABSTRACT

Background: Transfusion iron overload is the most important complication of ß-thalassemia and is a major focus in its management. Extensive iron deposition is associated with cardiac hypertrophy, dilatation and degenerative disorders of the myocardial fibers.

Aim of the Work: This work was done to investigate the role of hepcidin and growth differentiation factor15 (GDF-15) and their interaction in the progressive iron overload in ß-thalassemia major thus helping in prevention of iron overload in those patients.

Patients and Methods: The study was done on 60 patients with ß-thalassemia major, their age ranged from two to thirteen years with a mean of 7.50±3.21 and a median of 8 years, as well as 20 apparently healthy controls, their age ranged from two to twelve years with mean of 6.04±3.390 and a median of 6 years . The hepcidin hormone, GDF-15, ferritin and routine investigations were done for all patients and controls. The hepcidin hormone and GDF-15 were done using ELISA.

Results: There was high significant difference in serum ferritin between patients and controls (2180.75± 1438.858 VS 91.55±60.42ng/ml; *p*=<0.001). Significant difference was also found in serum GDF-15 between patients and controls (33880.33±7208.83 VS 451.05± 47.56pg/ml; *p*=<0.001). Furthermore, a high significant difference in serum hepcidin was found between patients and controls (22.81±14.353 vs. 92.87±53.373ng/ml; *p*=<0.001). Serum GDF15 level showed significant positive correlation with ferritin and significant negative correlation with hepcidin in patients with ß-thalassemia (*r*: 0.545 and –0.609 respectively, *p*<0001). Splenectomized patients showed significantly higher levels of serum ferritin and serum GDF-15 and significantly lower levels of hepcidin as compared to non-splenectomized patients (1763.43± 1327.97 vs. 1621.08 ±1297.27ng/ml,; *p*=0.008; 33255.71± 7753.62 vs. 22770.69±17081.887pg/ml, *p*<0.001 and 23.19±15.118 VS 46.42±46.792ng/ml, *p*=0.001 respectively).

Conclusion: GDF-15 hyper-expression occurs in unison with ineffective erythropoiesis and positively correlates with ferritin levels in patients with ß-thalassemia. In patients with ß-thalassemia, Hepcidin level decreases as a result of GDF-15 hyper-expression.

Key Words: *Hepcidin – GDF-15 – Iron overload – ß-Thalassemia major.*

INTRODUCTION

The thalassemia are heterogonous genetic disorders of hemoglobin synthesis, occurring more frequently in the Mediterranean region, the Indians, south east Asia and west Africa, divided according to their severity into major which is severe and transfusion dependent, intermediate and minor forms of illness **[1]**. In patients with ß-thalassemia, deficient ß-globin-chain production and accumulation of α-chains causes apoptosis of red blood cell precursors, which results in ineffective erythropoiesis and anemia of variable severity that is aggravated by reduced red blood cell survival secondary to hemolysis **[2,3]**. Transfusion iron overload is the most important complication of ß-thalassemia and is a major focus in its management, which can be prevented by adequate iron chelation therapy. Extensive iron deposition is associated with cardiac hypertrophy, dilatation and degenerative disorders of the myocardial fibers **[4]**. Hepcidin, a 25 amino acid iron peptide hormone, inhibits iron influx into plasma from duodenal enterocytes and macrophages, which recycles iron from erythrocytes and hepatocytes and stores it by inactivation of iron export pump ferroprotien **[5,6]**. This protective mechanism is suspended and iron uptake is enhanced despite normal or even increased body iron in patients with genetic ferroprotien defects and iron loading anemia **[7]**. Growth differentiation factor 15 (GDF-15) has been identified as a bone marrow

derived factor that abrogates hepcidin-mediated protection from iron overload under conditions of increased erythropoiesis **[8]**. In this work, we investigated the recently identified regulators of iron metabolism, hepcidin and GDF-15 in patients with ß-thalassemia major in order to understand their role and interaction in the progressive iron overload in this type of chronic anemia thus helping in the prevention of iron overload in those patients.

PATIENTS AND METHODS

Patients:

The present study included sixty patients with ß-thalassemia major (thirty six males and twenty four females, twenty one of them had been splenectomized). Their age ranged from two to thirteen years with mean of 7.50±3.21 and a median of 8 years. All patients were recruited from Pediatrics Department, Sohag University Hospital. Also, the study included twenty age and sex matched apparently healthy children who attend the pediatric outpatient clinic for different complaints (e.g. cough, diarrhea, etc.) as a control group. The study was approved by the Ethical committee and a written informed consent was obtained from parents of all cases in accordance with Sohag University Hospital Ethical Committee Guide Lines.

Methods:

Blood was drawn into standard EDTA vacutainers for the assay of CBC and Hb electrophoresis and plain tubes for the assay of ferritin, hepcidin and GDF-15. Separated serum was divided in aliquots, one aliquot was used for ferritin estimation, and the rest of aliquots were stored at –70ºC to be used for hepcidin and GDF-15 estimation.

Laboratory investigations:

Complete blood count (CBC) was done by the use of cell dyne-3700 (Abbott Diagnostics, Dallas, USA). Hemoglobin electrophoresis was done by the use of Genio Electrophoresis (Interlab S.R.l. Company, Roma, Italy). Serum ferritin was done by the use of Architect 2000 system (Abbott Diagnostic, Dallas, USA). Hepcidin was measured using hepcidin ELISA kit (Wuhan EIA-ab Science co; Ltd. Wuhan, China). Growth differentiation factor-15 (GDF-15) was measured using BioVendor GDF-15/MIC-1 ELISA kit (BioVendor Research and Diagnostic Products co., Candler, North Carolina, USA).

Statistical analysis:

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS-version 17). All data was expressed as mean±SD. For statistical evaluation, Student *t*-test was used. Correlation Coefficient (*r*) was used for showing positive and negative correlation between variables. *p*-value was considered significant if less than 0.05.

RESULTS

The study was carried out on sixty children with ß-thalassemia major (thirty-six males and twenty-four females, twenty-one of them had been splenectomized), as well as twenty apparently healthy controls. Hematological data of patients and controls are represented in Table (1).

Serum ferritin and GDF-15 levels were significantly higher while serum hepcidin was significantly lower in patients than controls (Table 1).

Serum GDF-15 level in patients showed significant positive correlation with ferritin and significant negative correlation with hepcidin (Figs. 1,2) and Hb level; no correlation was encountered with age. In the control group, it showed significant negative correlation with hepcidin and age, insignificant negative correlation with Hb level and no correlation with ferritin (Table 2).

In patients group, hepcidin showed significant positive correlation with Hb level, significant negative correlation with ferritin and no correlation with age. In the control group, it showed significant positive correlation with age, significant negative correlation with ferritin and no correlation with Hb (Table 3).

Splenectomized patients showed significantly higher levels of serum ferritin and serum GDF-15 and significantly lower levels of hepcidin as compared to non-splenectomized patients (Table 4).

Table (1): Hematological data of 60 ß-thalassemia major and 20 healthy children.

Parameter	Patients Mean±SD (range)	Controls	*p*
WBCs x10^9/L	10.69±5.24 (1.5-21.6)	7.96±2.80 (4-13.2)	0.001
RBCs x10^{12}/L	3.23±0.56 (2-4)	4.27±0.58 (3-5)	<0.001
Hb (g/dl)	7.03±1.75 (2.9-9.3)	11.68±0.81 (9.2-12.7)	<0.001
Plat. x10^9/L	364.95±183.32 (70-856)	241.05±72.54 (148-463)	<0.001
Hb A1%	33.16±22.05 (0-79)	97.18±0.39 (96-98)	<0.001
Hb A2%	3.91±1.60 (1-8)	2.52±0.29 (2-3)	<0.001
Hb F%	62.59±22.41 (16-99)	0.24±0.264 (0-1)	<0.001
Ferritin: ng/ml	2180.75±1438.86 (110-5800)	91.55±60.42 (8-210)	<0.001
Hepcidin: ng/ml	22.81±14.353 (2-63)	92.87±53.37 (41-230)	<0.001
GDF-15: pg/ml	33880.33±7208.84 (20100-44700)	451.05±47.56 (385-520)	<0.001

Table (2): Correlation between GDF15 and other variables in 60 ß-thalassemia patients and 20 controls.

Group	GDF15 X Hepcidin		GDF15 X Age		GDF15 X Hb		GDF15 X Ferritin	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Patients	–0.609	<0.001	–0.185	<0.157	–0.33	0.010	0.545	<0.001
Control	–0.718	<0.001	–0.451	0.046	–0.356	0.124	0.525	0.018

Table (3): Correlation between hepcidin and other variables in 60 ß-thalassemia patients and 20 controls.

Group	Hepcidin X Age		Hepcidin X Hb		Hepcidin X Ferritin	
	r	*p*	*r*	*p*	*r*	*p*
Patients	0.096	0.466	0.261	0.041	–0.288	0.025
Control	0.459	0.042	0.111	0.643	–0.529	0.016

Table (4): Comparison between splenectomized and non splenectomized ß-thalassemic patients as regards factors affecting iron homeostasis.

Parameter	Splene-ctomy	Mean±SD (range)	*p*
S. Ferritin (ng/ml)	Yes	1763.43±1327.97 (270-4567)	0.008
	No	1621.08±1297.27 (110-5800)	
GDF15 (pg/ml)	Yes	33255.71±7753.62 (21000-44700)	<0.001
	No	22770.69±17081.887 (20100-44500)	
Hepcidin (ng/ml)	Yes	23.19±15.118 (2-63)	0.001
	No	46.42±46.792 (5-62)	

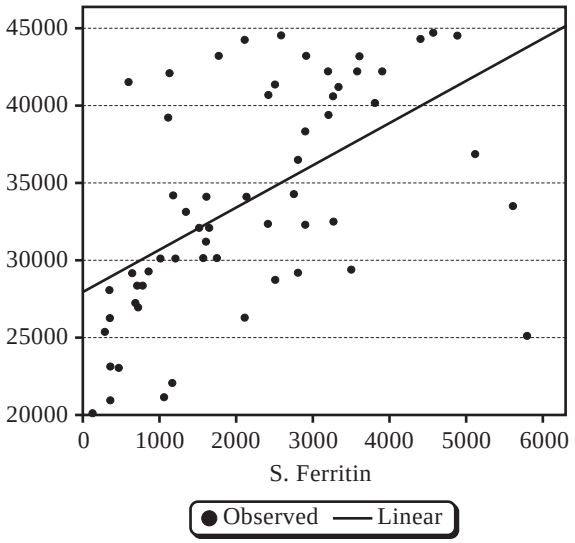

Fig. (1): Correlation between serum GDF15 and ferritin levels in patients group.

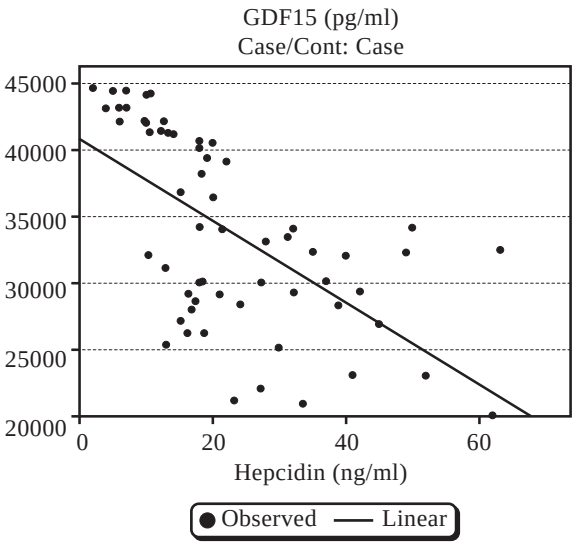

Fig. (2): Correlation between serum GDF15 and hepcidin levels in patients group.

DISCUSSION

ß-thalassemia is the most common chronic hemolytic anemia in Egypt (85.1%). A carrier rate of 9-10.2% has been estimated in 1000 normal random subjects from different geographical areas of Egypt **[9]**. ß-thalassemia is common in Mediterranean countries constituting a major public health problem **[9]**.

Iron absorption is increased in patients with congenital anaemias characterized by ineffective erythropoiesis. Clinically, increased intestinal iron absorption compounds the effects of transfusion iron overload in patients with thalassemia syndromes **[10]**.

Our results revealed highly significant elevation of serum ferritin in ß-thalassemia patients. These results agree with others **[11-13]**, who reported that even non-transfusion-dependent thalassemia patients often develop lethal iron overload.

The results of the present study revealed reduction of serum hepcidin level in ß-thalassemia patients. This observation was compatible with others **[14-16]**, who clearly demonstrated that even in ß-thalassemia major patients, who are highly iron overloaded, serum hepcidin levels are lower than would be expected because of the exuberant erythropoiesis. The main effect of hepcidin is negative regulation of cellular iron export from macrophages, duodenal enterocytes, and hepatocytes by promoting degradation of ferroportein, a transmembrane iron exporter.

In this work, there was marked elevation of serum GDF-15 in ß-thalassemia patients. These data agree with Zhao & Chang **[17]**, who reported that ineffective erythropoiesis is recognized as the principal reason of non-transfusion iron overload. In the process of expanded erythropoiesis, the apoptosis of erythroblasts induces the up-regulation of GDF15.

GDF15 suppresses hepcidin production by hepatocytes, subsequently low hepcidin level increases iron absorption from the intestine resulting in iron overload. Physiological dose of GDF15 can promote the growth and differentiation of erythroid progenitors, but a high dose of GDF15 can suppress the secretion of hepcidin. The regulation of GDF15 may also be related to iron level, epigenetic regulation and hypoxia **[18-21]**.

Erythropoietin-stimulated erythroblasts produce secreted mediators that act on the liver to suppress hepcidin production. Dying erythroblasts or erythroblasts that fail to mature appropriately may further contribute to secretion of hepcidin suppressors, perhaps explaining the paradoxical lack of iron overload in patients with expanded erythroblasts but normal maturation, such as in un-transfused chronic hemolytic anemia; GDF15 is one of these suppressors **[18-21]**.

In the present work, there was increase in serum ferritin level in splenectomized thalassemic patients than non splenectomized; this agree with Pootrakul **[22]**.

Our study showed increased serum GDF-15 in the splenectomized thalassemic patients than non splenectomized, consequently decreasing the serum hepcidin level in the splenectomized group.

In conclusion, GDF-15 hyper-expression occurs in unison with ineffective erythropoiesis and positively correlates with ferritin levels in patients with ß-thalassemia. In patients with ß-thalassemia, hepcidin level decreases as a result of GDF-15 hyper-expression. GDF-15 level decreases in splenectomized thalassemic patients than non-splenectomized, consequently decreasing the serum hepcidin level in the splenectomized patients. In the future, therapeutic use of hepcidin and hepcidin agonists may help to restore normal iron homeostasis in patients with ß-thalassemia who develop secondary iron overload.

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Platelet Count, Mean Platelet Volume and Aggregation as Markers of Disease Activity in Rheumatoid Arthritis

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ABSTRACT

Background: Rheumatoid arthritis (RA) is a chronic inflammatory disorder that affects small joints of hands and feet. Different forms of hematological disorders are associated with RA as anemia, neutropenia, thrombocytosis, thrombocytopenia, eosinophilia, and hematological malignancies. Thrombocytosis usually occurs during the active clinical stages of RA. Reactive megakaryocytopoiesis increases circulating platelets count and triggers hyperactivity. Hyperactive platelets target synovial membranes with subsequent local rheumatoid inflammation.

Objectives: To correlate the value of mean platelet volume (MPV), platelet count and platelet hyperactivity with Disease Activity Score (DAS 28 score) and their reflection on cardiovascular system in RA.

Patients and Methods: Fifty newly diagnosed RA patients attending the Clinical Rheumatology Unit during the year 2012 with fifteen age- and sex-matched control subjects were randomly selected. For all, CBC including platelet cont, MPV, platelet aggregation using ADP, echocardiography in addition to ECG, ESR and CRP were done.

Results: MPV, Platelet count, CRP and ESR were significantly higher in RA patients than controls, while Platelet aggregation, hemoglobin level, mean diastolic function and ejection fraction (EF) were significantly lower in RA patients than control group. Significant positive correlations were detected between DAS28 score and both MPV and platelet count, while significant negative correlations were found with both hemoglobin and EF. Significant increase of the platelet aggregation in sero-negative compared to sero-positive rheumatoid arthritis patients was detected.

Conclusion: Platelet count and MPV are inexpensive tests, may be useful for a rapid assessment of disease activity in patients with RA.

Key Words: *Platelet aggregation – MPV – Rheumatoid arthritis.*

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that affects many tissues and organs, but principally attacks synovial joints. Although the cause of rheumatoid arthritis is unknown, autoimmunity plays a pivotal role in both chronicity and progression, so RA is considered a systemic autoimmune disease **[1]**. Hematological manifestations in RA can be broadly categorized into anemia, thrombocytosis, neutropenia, thrombocytopenia, particularly autoimmune and drug induced thrombocytopenia; and hematological malignancies **[2]**.

Anemia associated with RA, known as rheumatoid anemia, is a typical example of anemia of chronic disease **[3]**. In the absence of effective treatment, anemia is highly prevalent among RA patients. The mechanisms involved in rheumatoid anemia include shortening of the erythrocyte lifespan, inadequate bone marrow erythropoiesis in response to anemia, and iron metabolism abnormalities **[4]**. Rheumatoid anemia is usually mild to moderate, normocytic normochromic or, less often, microcytic **[5]**.

Thrombocytosis in rheumatoid arthritis is common and correlates with disease activity. The exact pathogenetic mechanisms remain undetermined. Persistent overproduction of certain thrombocytopoietic factors can induce megakaryocytopoiesis and thrombocytopoiesis **[6]**. The megakaryocytopoiesis and inflammatory cascade of RA share hematopoietic cytokines and respond to a number of colony-stimulating factors. Progenitors of osteoclasts, important

during the development of erosion, are of hematopoietic lineage **[7]**. Moreover, the bone marrow could also be diseased in this inflammatory cascade. Therefore, increased platelet mass could be a reflection of the affected bone marrow. Thrombocytosis usually occurs during the active clinical stages of RA **[8]**. Reactive megakaryocytopoiesis increases circulating platelets count and triggers hyperactivity. Hyperactive platelets target synovial membranes with subsequent local rheumatoid inflammation. Hyperactive platelets interact with other cells, and target the vascular wall. Considerable evidence indicates that patients with RA are prone to premature ischemic heart disease (IHD), myocardial infarction (MI) and heart failure. Disease modifying anti-rheumatic drugs (DMARD) decrease platelet activity **[9]**.

Availability of automated blood cell analyzers has made the measurement of platelet count and morphology common practice. Mean platelet volume (MPV) is emerging as an indicator of platelet reactivity, which could estimate cardiovascular risk **[10]**. Increased MPV is related to acute vascular events such as destabilization of atherosclerotic plaque, unstable angina, MI and paroxysmal atrial fibrillation **[11]**. MPV is an independent risk factor and predictor of MI in predisposed subjects **[12]**.

PATIENTS AND METHODS

Fifty newly diagnosed RA patients, 38 males and 12 females were recruited from Rheumatology Unit of Internal Medicine department of Assuit University hospital. Their ages ranged from 18 to 42 with a mean of 29.2±6.2 and a median of 24 years. Thirty six patients (72%) of them were diagnosed as sero-positive, while the remaining 14 patients (28%) were diagnosed as sero-negative rheumatoid arthritis. Twenty five controls, 8 males and 17 females were included; their ages ranged from 19 to 40 with a mean of 28.5±4.5 and a median of 27 years. Patients with history of bleeding tendency, cardiovascular diseases, anemia, liver diseases or receiving NSAIDs were excluded. All patients were subjected to complete medical history and examination, Disease Activity Score (DAS28) was measured for all patients. Written Informed consents were taken from all subjects; this study was approved by the ethical committee of Assiut University. To all patients and controls the following investigations were performed: CBC including MPV, ESR, CRP, RF, blood urea, serum creatinine, random blood glucose, platelet function tests for platelet aggregation using Platelet Aggregation Profiler (PAP-4, USA); Reagent: ADP. Also, Complete M-mode, 2-dimensional, and Doppler echocardiography was performed at rest.

Statistical methods: The data obtained were calculated and statistically analyzed by using SPSS data analysis program. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Patients with RA showed significantly increased ($p<0.001$) MPV (fL) and platelet count ($x10^9$/L) than controls, while platelet aggregation tests using ADP, hemoglobin level (gm/dl), and Ejection fraction (%) were significantly lower ($p<0.001$) in patients than control group (Table 1). The mean levels of ESR (in the 1st and 2nd hours), CRP in mg/L and RF in u/mL of the studied patients were significantly higher ($p<0.001$) when compared with controls (Table 2). Also 21 patients (42%) showed grade I diastolic dysfunction and 14 (28%) showed grade II diastolic dysfunction, while all controls showed normal diastolic function ($p<0.001$).

According to the Disease Activity Score (DAS28) 36 patients (72%) showed moderately active RA >3.2 ≤5.1, and 14 (28%) showed very active RA >5.1, while all control group showed RA ≤3.2. There was a significant positive correlation between DAS28 score and MPV and platelet count ($r=0.58$, $p=0.0001$ and $r=0.408$, $p=0.003$ respectively), while a significant negative correlation was detected between DAS28 score and hemoglobin level ($p=0.000$ and $r=–0.798$) and between DAS28 score and EF ($p=0.011$ and $r=–0.358$) (Fig. 1). No correlation was encountered between DAS28 on one side and ESR 1st and 2nd hour, CRP or platelet aggregation on the other side ($r=0.117$, 0.082, 0.064 and 0.096) respectively.

From the total 50 RA patients, 36 patients (72%) were diagnosed as sero-positive, while the remaining 14 patients (28%) were diagnosed as sero-negative rheumatoid arthritis. A significant increase ($p<0.05$) of the platelet aggregation

in sero-negative rheumatoid arthritis was detected when compared to sero-positive rheumatoid arthritis patients. The DAS28 was significantly higher (p<0.05) in sero-positive when compared to sero-negative rheumatoid arthritis patients, while no significant differences were detected between sero-positive and sero-negative patients as regards MPV, platelet count, ESR, CRP, WBC count, EF or diastolic function (Table 3).

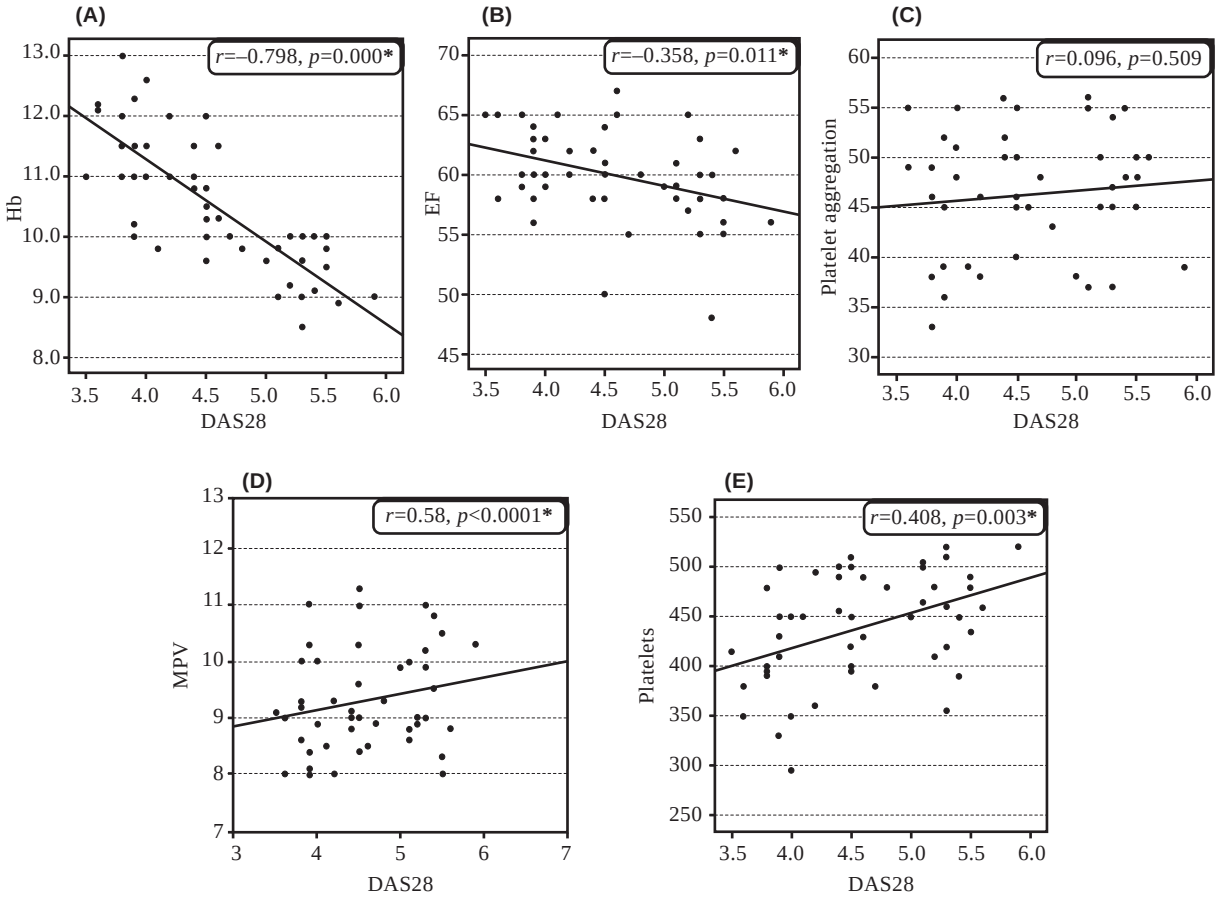

Fig. (1): Correlation between DAS28 score and (A) Hemoglobin (Hb), (B) Ejection Fraction (EF), (C) Platelet aggregation, (D) Mean Platelet Volume (MPV) and (E) Platelet count.

Table (1): Comparison of Hb level, Platelet Count, MPV, platelet aggregation and Ejection Fraction between Rheumatoid Arthritis patients and controls.

Variables	Patients (n=50)	Control (n=25)	p-value
MPV (fl)	8-11 (9.3±0.9)*	8-9 (8.2±0.3)	<0.001
Platelet aggregation using ADP (%)	33-56 (46.2±6.0)	55-70 (61.6±5.1)	<0.001
Platelet x10^9/L	295-520 (438.8±54.8)	160-400 (269.2±59.9)	<0.001
Hb (gm/dl)	8.5-13 (10.5±1.1)	12-14.8 (13.2±0.7)	<0.001
EF %	48-67 (59.9±3.7)	58-66 (63.8±2.6)	<0.001

*Range (mean±SD) ADP: Adenosine Diphosphate.

Hb = Hemoglobin level.
MPV = Mean platelet volume.
fl = Femtolite.
EF = Ejection Fraction.

Table (2): ESR, CRP and RF levels in patients and controls.

	Patients (n=50)	Control (n=25)	*p*-value
ESR 1st hour	17.7±35.6*	7.5±1.4	***$p<0.001$
ESR 2nd hour	60.6±19.4	14.7±2.9	***$p<0.001$
CRP	39.8±28.5	3.1±4.3	***$p<0.001$
RF	26.2±25.5	1.2±2.4	***$p<0.001$

ESR : Erythrocytic Sedimentation Rate.
CRP : C-reactive protein.
RF : Rheumatoid factor.
$p<0.001$: Highly significant.
* : Mean±SD.

Table (3): Comparison between sero-positive and sero-negative Rheumatoid Arthritis patients.

Variables	Rheumatoid factor		*p* value
	Negative (n=14)	Positive (n=36)	
ESR1 (mm/h)	34.64±18.76*	36.03±17.58	NS
ESR2 (mm/h)	60.57±19.73	60.64±19.61	NS
CRP	46.14+39.27	37.28+23.23	NS
WBCs $x10^9$/L	6.92±1.79	6.71±1.19	NS
Platelet $x10^9$/L	440±95.5	438.31±89.56	NS
MPV (Fl)	9.06±1.24	9.39±0.79	NS
Platelet aggregation using ADP (%)	43.29±6.38	47.39±5.49	<0.05
DAS28	4.20±0.55	4.72±0.64	<0.05

* : Mean±SD.
ESR : Erythrocyte Sedimentation Rate.
CRP : C-reactive protein.
WBCs : White Blood Cells
MPV : Mean platelet volume.
fl : Femtoliter.
ADP : Adenosine Diphosphate.
NS : Not significant.

DISCUSSION

Hematological manifestations in RA can be broadly categorized into areas of anemia, thrombocytosis, leukocytosis, thrombocytopenia, particularly autoimmune and drug induced thrombocytopenia even hematological malignancies **[2]**.

In the current study patients with RA showed significantly increased mean platelet count than controls with positive correlation between DAS28 score and platelet count, a finding which is similar to the results of Kisacik et al. **[13]**. The pathogenesis of increased platelet count in those patients is mostly due to enhancement of cytokines release with increased disease activity **[14,15]**.

Rheumatoid patients in the current study are mostly demonstrating a pattern of normocytic normochrmoic anemia, their mean hemoglobin level is lower than that documented by Yazici et al. **[16]**, but similar to Kisacik et al. **[13]** study. Moreover, there was strong positive correlation between DAS28 score and the degree of anemia. Severity of anemia and thrombocytosis is associated with the disease activity in RA **[14]**. These hematopoietic presentations particularly thrombocytosis are presumably mediated by cytokines and growth factors, including Il-1, IL-3, IL-4, IL-6, IL-11 and TNF-α. Amongst these mediators, IL-6 is one of the major cytokines responsible for inflammation in RA, which has also a regulatory function on acute phase response **[15]**. Moreover, some authors suggested that IL-6 is the primarily responsible cytokine in secondary thrombocytosis; therefore, it is reasonable to find an association between disease activity, acute phase markers and platelet characteristics in inflammatory disorders. Some studies suggested participation of platelets in the inflammation of RA **[14]**. There is evidence of platelet activation in RA **[16]** and MPV reflects platelet activation **[17]**. Mean platelet volume is an important platelet histogram index reported by hospital laboratories in daily clinical practice. Several previous reports showed the utility of MPV as a marker of platelet activation based mainly on the fact that in this activation process the platelets change their shape and the volume. Otherwise, MPV as a marker of platelets activation has been demonstrated to have prognostic importance in patients with cardiovascular disease and a large volume may be regarded as a marker of platelets activation **[18,19]**. Mean platelet volume (MPV) in the current study was significantly higher in RA patients; this finding is similar to the study of Gasparyan et al. **[9]**.

Yazici et al. **[16]** demonstrated a significant decrease in platelet count and MPV after treatment by tocilizumab for 6 months. Another study done by Kisacik et al. **[13]** revealed that MPV is significantly decreased in active rheumatoid

arthritis patients than control (osteoarthritis patients) with mild degree of increase after treatment. They suggested that small MPV may reflect accelerated maturation and short life span of platelets in active RA. The difference between the 2 previous studies may be attributed to technical reasons, age of patients, sample size and/or inclusion of patients with co-morbidity.

In the current study, MPV was correlated with DAS28 score in RA patients; furthermore, other disease activity markers demonstrated significant associations. Otherwise, certain studies previously revealed discordance between ESR and CRP levels and disease activity in RA **[20]**. Therefore, MPV is an inexpensive test that may be useful for a rapid, at a glance assessment of disease activity in patients with RA.

In the current study we reported a positive correlation between platelet aggregation test using ADP and DAS28 score. This is coinciding with Mac Mullan et al. **[21]** study. However it is worth mentioning that the cause of weak platelet aggregation may be attributed to that most of our patients might have received empirical treatment including non steroidal anti inflammatory drugs (NSAIDs) before attending to the outpatient clinic.

By using Doppler Echocardiography, 68% of patients were suffering from diastolic dysfunction. Early diastole depends on active relaxation of the ventricle, as well as passive properties of the ventricle that include wall thickness, chamber geometry, and myocardial stiffness **[22]**. Relaxation is an energy dependent process and influenced by load **[23]**. The cause of diastolic dysfunction in our patients is not well recognized. It may be explained by ischemia due to premature atherosclerosis of the coronary arteries which may be attributed to increase in MPV and platelet hyperactivity. Early detection of ischemia for these patients may need stress ECG which is practically difficult as many of RA patients have knees arthritis. Stress echocardiography can be an alternative with sensitivity reaching 80% in many patients but should be done under careful monitoring to avoid fatal arrhythmia. Coronary angiography is the gold standard for detection of coronary arterial atherosclerosis, but it is invasive and expensive. Multi Slice Computed Tomography (MSCT) coronary angiography can be a substitution and it has got good negative results to exclude ischemia, but the major disadvantage for using it is that it may give false positive results especially in patients having calcification in their coronaries.

Moreover, diastolic dysfunction in these patients may be explained by myocardial fibrosis causing restrictive filling of the heart and reducing diastolic volume of either or both ventricles. Constrictive pericarditis could be an explanation in some patients and should not be missed. Constriction is due to chronic inflammation and fibrosis of the pericardium often superimposed by calcification resulting in decreased ventricular compliance. There is increased end-diastolic pressure for any given end diastolic volume. The increased pressure affecting both ventricles equally and effectively decreases diastolic filling and thus end-diastolic volume of both ventricles. The increased pressure is transmitted backward and results in elevated pulmonary and systemic pressures. Equalization of end diastolic pressures in all four cardiac chambers is the hallmark of constrictive pericarditis. Other causes of diastolic dysfunction were excluded before collecting data from patients as morbid obesity, elderly persons, and uncontrolled diabetes mellitus to avoid misinterpretation.

In the current study, only 4% of patients had ejection fraction (EF) <55% (below normal range) with systolic wall motion abnormalities. As in patients with high output state due to anemia, the heart's systolic function index and EF are expected to be higher than in normal subjects. So, it has been recommended that a normal LVEF should be above 60% in anemic patients. Results of the current study are similar to that of Rudominer et al. **[24]** in which the vast majority of their patients had preserved EF. Also, diastolic dysfunction, lower LV mass, higher pulmonary arterial pressure and higher left atrial volume index.

In the current study, there was no difference between sero-positive and sero-negative patients as regards ESR, CRP, WBC count, platelet count and MPV. However, the mean DAS 28 of sero-positive patients was significantly higher than that of sero-negative patients. The same applies for platelet aggregation. ESR and CRP correlates closely with clinical disease activity in patients

with RA. However, certain studies revealed discordance between ESR or CRP levels and the disease activity in RA **[20]**.

Conclusion: Platelet count and MPV are inexpensive tests, may be useful for a rapid assessment of disease activity in patients with RA.

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The Relation between Multidrug Resistance Gene (MDR1) Polymorphism and Response to Nilotinib in Egyptian Patients with Chronic Myeloid Leukemia (CML)

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ABSTRACT

Background: For the past 10 years, the tyrosine kinase inhibitors (TKI), first generation Imatinib and second-generation Dasatinib and Nilotinib have been the standard treatment of chronic phase (CP) CML. However, a subset of patients does not respond even to the 2nd generation TKIs. One of the possible mechanisms of resistance to TKIs is the inappropriate expression of the multidrug resistance (*MDR1*) gene encoding the P glycoprotein (Pgp).

Patients and Methods: Thirty-one upfront Ph+ve CML patients, planned to receive Nilotinib, were included in this study. Detection of *MDR1* gene polymorphism C3435T, using PCR Restriction Fragment Length Polymorphisms (PCR-RFLP) was done initially for every patient. We prospectively followed up the patients between February 2012 and February 2014 with PCR for *BCR-ABL1* transcripts every 3 months. The molecular response to Nilotinib, according to the level of *BCR-ABL1* by PCR, was correlated to the different MDR1 3435 genotypes.

Results: Fifteen/31 patients (48.4%) carried the CC genotype, 9 (29.1%) carried TT genotype, while 7 (22.5%) carried CT genotype. Molecular response was optimal in 56%, 60% and 80% of the patients at month 3, 6 and 12 months respectively. Patients carrying *MDR1* 3434CT genotype showed a higher, yet insignificant, molecular response to Nilotinib.

Conclusion: In our study, the *MDR1*-C3435T genotype did not significantly affect the molecular response to Nilotinib. Further studies in larger series of patients are needed to define the genetic polymorphisms with therapeutic relevance in patients on Nilotinib among Egyptians.

Key Words: *Chronic myeloid leukemia (CML) – Multidrug resistance (MDR1) gene polymorphism – Molecular response – Nilotinib.*

INTRODUCTION

Chronic Myeloid Leukemia is one of the first neoplasms that are linked to genetic aberration namely the Philadelphia (Ph) chromosome. Ph chromosome results from a translocation between chromosome 9 and 22 with fusion of *RBC* gene on chromosome 22 to the *ABL1* gene on chromosome 9, with formation of the *BCR-ABL1* chimeric oncogene. This oncogene codes for a constitutively active cytoplasmic tyrosine kinase, which is implicated in the development of CML and has become a primary target for the treatment of this disorder **[1]**.

CML is the first human Cancer that responds to molecular target therapy. Imatinib, a member of TKIs works through competitive inhibition at the adenosine triphosphate (ATP) binding site of the BCR-ABL1 protein, which results in the inhibition of phosphorylation of proteins involved in BCR-ABL1 signal transduction. The BCR-ABL1 inhibition results in apoptosis of the malignant cells that express BCR-ABL1 without affecting the normal cells **[2]**.

However, some patients develop Imatinib-resistant disease or intolerance to Imatinib because of toxicities **[3]**.

Nilotinib (AMN107), a second generation TKI, represents viable alternative to Imatinib with approximately 30 folds more potency **[4]**.

These products were initially launched for use as second line therapies and were approved for first line use by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2010 on the basis of the results from two ongoing multinational RCTs **[5]**.

In newly diagnosed CP-CML patients, Nilotinib 600mg and 800mg daily were compared to Imatinib 400mg daily in ENESTnd clinical trial. Nilotinib was statistically superior at one year on the same end points for all comparisons **[5]**.

However, despite their excellent efficacy, a subset of patients does not respond to TKIs, and are deemed to have resistance to the drug **[6]**.

Currently, resistance to TKIs is believed to be a consequence of the interaction of multiple factors such as treatment compliance, bioavailability, pharmacodynamics, genetic changes, *BCR-ABL1* kinase domain mutations, or combinations of these **[7]**.

Multi drug resistance gene (*MDR1*) [*ABCB1* (ATP-binding cassette, sub-family B (*MDR* /ATP), member 1)] product is an ATP-driven efflux pump contributing to the pharmacokinetics of drugs that are P-glycoprotein (P-gp) substrates. The generally accepted action of MDR1 is to reduce intracellular drug accumulation through Pgp-mediated efflux, thus hampering the achievement of effective drug levels at the target site **[8]**.

The inappropriate expression of the *MDR1* gene has been frequently implicated in resistance to different chemotherapeutic drugs as *MDR1* single nucleotide polymorphisms (SNPs) are associated with drug clearance **[9]**.

Nilotinib had been identified as a substrate of P-gp in Nilotinib-resistant cell lines **[10]**.

More than 50 single nucleotide polymorphisms (SNPs) have been identified concerning the *MDR1* gene, and SNP polymorphisms may affect the expression and function of the P-gp. The SNPs T1236C, G2677T/A, and C3435T are the most common variants in the coding region of *ABCB1* SNPs in *MDR1* gene and have the potential to alter protein function and could also influence the efficiency of absorption or elimination **[8]**.

PATIENTS AND METHODS

Between February 2012 and March 2013, 31 chronic phase CML patients who were consecutively admitted to clinical hematology unit, kasr Alainy Hospital or visited its outpatient clinic were enrolled in this study. The study was approved by the Ethical Committee of Kasr Alain and all patients signed an informed consent before enrollment. The patients were prospectively followed for 12 months to evaluate their response to TKI Therapy.

A total of 31 patients, aged 18 years or older, were eligible. They were 15 males (48.3%) and 16 females (51.7%) with an age range of 18 to 78 with a median of 38 years. All had Ph-positive chronic-phase CML. The diagnosis of CML was based on standard clinical data and confirmed by cytogenetics and molecular analysis.

Chronic-phase (CP) CML was defined according to WHO criteria, by the presence of less than 10% blasts, less than 20% basophils, and a platelet count of $\geq 100 \times 10^9$/L with no extramedullary involvement **[11]**.

Patients with the following criteria were excluded: Patients under the age of 18 years, pregnant females, Philadelphia negative CML and Patients treated before with interferon or underwent autologous bone marrow transplantation.

Initial assessment included: Detailed Medical history recording, complete Physical examination, complete blood count and examination of peripheral blood film, estimation of Sokal and Hasford risk scores, quantitative measurement of *BCR-ABL1* transcripts using RQ-PCR at diagnosis and every 3 month after staring TKIs and the detection of *MDR1* gene polymorphism C3435T, using PCR Restriction Fragment Length Polymorphisms (PCR-RFLP).

Treatment:

All patients were planned to receive nilotinib 400 mg od.

Follow-up:

We prospectively followed-up the patients between February 2012 and February 2014. Patients were regularly monitored on an outpatient basis; biweekly Physical examinations, Blood counts, and biochemistry were obtained during the first month of TKI Therapy and then monthly until a complete hematological response was achieved, and then every 3 months with RQ-PCR for *BCR-ABL1* there after.

Response to treatment:

It was evaluated according to ELN criteria 2013 (Table 1) using CBC at month 3 to assess hematological response and *BCR-ABL1* transcripts % at month 3, 6 and 12 to assess molecular response **[12].**

Table (1): Molecular Response According to ELN criteria 2013.

Time point	Optimal	Warning (Suboptimal)	Failure
3 months	*BCR-ABL1* ≤10%	*BCR-ABL1* >10%	Non-CHR
6 months	*BCR-ABL1* <1%	*BCR-ABL1* 1-10%	*BCR-ABL1* >10%
12 months	*BCR-ABL1* ≤0.1%	*BCR-ABL1* 0.1-1%	*BCR-ABL1* >1%

Drug toxicity:

It was evaluated according to the common Toxicity Criteria for Adverse events (CTCAE) version 4.3.

RNA extraction and cDNA synthesis:

Total RNA extraction was carried out from fresh peripheral blood leucocytes according to the initial silica extraction method described by Boom et al., **[13]**, using QIAmp RNA Blood minikit (Qiayen Hilslen, Germany). For cDNA synthesis, 1 µg total RNA was used to synthesize first-strand cDNA according to the manufacturer protocol (Fermentas).

BCR-ABL1 transcripts measurement:

Real-time quantitative polymerase chain reaction (RQ PCR) was used for detection of *BCR-ABL1* transcripts level.

MDR1 C3435T genotyping:

MDR1 C3435T polymorphism was detected using a PCR-RFLP assay. The following primers were used: 5'-GCTGG TCCTGAAGTTG ATCTGTGAAC-3' as forward and 5'-AC ATT-AGGCAGTGACTCGATG AAGGCA-3' as reverse primer. The PCR mixture included: 1µM primer, 200µM of each dNTP (Sigma), Taq DNA polymerase, 1X buffer with 1.5mM MgCl2, and 2.5 units Taq polymerase (5U/µL, Sigma). The PCR product (248bp in size) was digested for 3h at 37°C with 2U *MboL* restriction enzyme. The expected fragment sizes are: a 238-bp fragment for TT genotype, 172-and 60-bp fragments for the CC genotype, and 238, 170 and 60bp for the CT genotype. DNA fragments generated were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed with an ultraviolet transilluminator.

Statistical analysis:

Pre-coded data was entered on the computer using "Microsoft Office Excel Software" program (2010) for windows. Data was transferred to the Statistical Package of Social Science Software program, version 21 (SPSS). Comparison between groups was performed using independent sample *t*-test or one way ANOVA with Tukey's post hoc test for quantitative variables and Chi square or Fisher's exact test for qualitative ones. Repeated measures were tested using Friedman test and pairwise Wilcoxon test with Bonferroni adjustment of P values. Spearman correlation coefficients were calculated to get the association between different quantitative variables. *p*-values less than 0.05 were considered statistically significant, and less than 0.01 were considered highly significant.

RESULTS

The present study included 31Ph positive CML patients in chronic phase of the disease. Patient's age ranged from 18 to 78 with a median of 38 years. Male patients were 24 (48.3%) and female patients were 19 (51.7%).

Drug toxicities leading to discontinuation of treatment:

Three patients (10%) stopped Nilotinib treatment after 6 months because of drug toxicity (1 had persistent Grade 2 hepatotoxicity; 2 had Grade 4 hematological toxicity).

Response to treatment:

At month 3, molecular response was optimal (*BCR-ABL1%* ≤10%) in 56%, warning zone was identified (*BCR-ABL1%* >10%) in 41% and 3% failed to achieve hematological response.

At month six, 60% achieved optimal molecular response, warning zone was identified (*BCR-ABL1%* >10%) in 20% and molecular failure occurred in 20% of patients.

At month 12, 80% achieved major molecular response, warning zone was identified (*BCR-ABL1%* >10%) in 15% and molecular failure occurred in 5% of patients.

Progression of the disease:

According to WHO definition of blastic transformation of CML **[11]**, no patient on Nilotinib arm transformed into acute leukemia.

Progression of BCR-ABL1 transcripts % by time after treatment with Nilotinib:

BCR-ABL1 transcripts % significantly decreased at month 3 and 12 in comparison with the previous *BCR-ABL* transcripts % (p=<0.001, and 0.004 respectively). There was statistically significant reduction in *BCR-ABL1* transcripts % in relation to the baseline *BCR-ABL1* transcripts (p=<0.001, <0.001, <0.001 and <0.001 at month 3, 6, 9 and 12 respectively, Table 2).

Distributions of genotypes among study group:

The overall frequency of different *MDR-1* 3435 genotypes among CML patients enrolled in our study showed that the majority, 15 patient (48.4%) carried the CC genotype, 9 patients (29.1%) carried TT genotype, while 7 patients (22.5 %) carried CT genotype.

The Relation between the MDR C3435T Genotypes and Response to TKIs:

Although patients carrying *MDR1* 3434CT genotype showed a higher molecular response to Nilotinib, there was no statistically significant difference between *MDR*- C3435T genotypes and the molecular response to treatment with Nilotinib according to ELN 2013 criteria of response (Table 3).

Table (2): Kinetics of *BCR-ABL1* by time after Nilotinib therapy in 31 chronic phase CML patients.

Time point	*BCR-ABL1*	Transcript level		*p*-value	
	Mean±SD	Median	Range	Baseline	Stepwise
At Diagnosis	138.6±126.2	97.0	0.0–465.0	–	–
Month 3	38.8±78.7	8.0	0.0–382.0	< 0.001	<0.001
Month 6	34.6±71.5	1.0	0.0–225.0	<0.001	0.26
Month 9	6.4±16.4	0.3	0.0–55.0	<0.001	0.255
Month 12	1.2±4.0	0.1	0.0–18.0	<0.001	0.004

Table (3): The Relation between the *MDR* C3435T Genotypes and Response to Nilotinib.

Response		*MDR* Genotype			*p*-value
		CC	CT	TT	
% Response at 3 months (n=30):	Failure	7.1	0.0	0.0	0.8
	Suboptimal	42.9	28.6	44.4	
	Response	50.0	71.4	55.6	
% Response at 6 months (n=26):	Failure	23.1	16.7	28.6	0.6
	Suboptimal	23.1	0.0	28.6	
	Response	53.8	83.3	42.9	
% Response at 12 months (n=20):	Failure	10.0	0.0	0.0	0.7
	Suboptimal	20.0	0.0	16.7	
	Response	70.0	100.0	83.3	

DISCUSSION

Despite the excellent efficacy of TKIs in treatment of chronic myeloid leukemia, a subset of patients does not respond to TKIs, and are deemed to have resistance to the drug. Resistance to BCR-ABL1 TKIs has become a pressing challenge in the treatment of CML. Thus, studies on the mechanisms of resistance to TKI have been driven by the need to improve response and prevent or overcome drug resistance **[6]**.

Although point mutations in the *BCR-ABL1* kinase domain is the most common mechanism, several mechanisms can play a role in the resistance to TKIs but the possible importance of drug-transporter proteins has been only recently appreciated with the demonstration that TKIs is a substrate of P-glycoprotein (Pgp), the product of *MDR1* gene. The generally accepted action of MDR1 is to reduce intracellular drug accumulation through Pgp-mediated efflux, thus hampering the achievement of effective drug levels at the target site **[14]**.

P-glycoprotein is encoded by the multidrug resistance *ABCB1* gene, and the functional variation in this gene could explain, at least in part, variable responses to this drug **[6]**.

(SNPs) in *ABCB1* genes have the potential to alter protein function and could also influence the efficiency of absorption or elimination. The up-regulation of drug transporters (ABCB1-ABCG2) is one of specific causes of resistance to Imatinib **[15]**.

In our study, genotype distribution revealed elevation of CC genotype frequency in CML patients (48.4%), followed by TT genotype (29.1%) and CT genotype (22.5%).

It has been found that the distribution of *MDR1*-C3435T polymorphism is significantly influenced by ethnicity. It is clear that people of African origin carry predominantly the wild-type (CC) allele and not the homozygous allele (TT). Ameyaw et al., reported high frequency of CC allele in Ghanian, Kenyan, African American and Sudanese populations (83%, 83%, 84% and 73% respectively) compared with British Caucasian, Portuguese, south-west-Asian, Chinese, Filipino and Saudi populations who showed lower frequencies of the C allele (48%, 43%, 34%, 53%, 59%, and 55%, respectively) **[16]**.

In Caucasian people, the frequency of CC and TT alleles is approximately the same. However, the TT is the predominant genootype among Asian and Indian populations **[17]**; in the Indian population, the frequency of the homozygous TT variant was 43%, CC 18% and C/T 39% **[18]**.

In our study, patients carrying *MDR1*-3434 CT genotype showed a higher response at month 3, 6 and 12 compared to CC and TT genotypes with no statistical significance.

To the best of our knowledge, no study addressed the impact of *MDR* C3435T polymorphism on the molecular response to Nilotinib. However some studies revealed a significant relation between molecular response to Imatinib and *MDR* C3435T polymorphism.

Dulucq et al., reported overall frequency of the *MDR1* 3435 CC, CT, and TT genotypes of 18.9%, 51.1%, and 30%, respectively in 90 French CML patients treated with imatinib; the haplotype (1236C-2677G-3435C) was statistically linked to less frequent major molecular response (70% vs 44.6%; p<0 .021) **[19]**.

Deenik et al., studied *ABCB1* gene single nucleotide polymorphisms (SNPs), C1236T, G2677T/A, and C3435T, with respect to molecular response in a cohort of 46 early chronic phase CML patients, in Nederland, receiving high-dose imatinib (800mg); patients homozygous for 3435T and 2677T showed lower probabilities to obtain a major molecular response (MMR) and complete molecular response CMR **[20]**.

Vivona et al., investigated the relation between *ABCB1* polymorphisms c.1236C>T, c.3435C>T and c.2677G>T/A with markers of response to Imatinib in patients with CML in 118 Brazilian patients initially treated with a standard dose of Imatinib for 18 months. In the responder group, the frequency of *ABCB1* 1236CT/2677GT/3435CT haplotype was higher in patients with MMR than in patients without MMR (51.7% vs. 8.3%, p=0.010). Furthermore, carriers of this haplotype had increased probability of reaching the MMR compared with the non-carriers (OR: 11.8; 95% CI: 1.43-97.3, p=0.022) **[21]**.

Conclusion:

In our study, although patients carrying *MDR1*- 3434CT genotype showed a higher response to Nilotinib at month 3, 6 and 12 compared to CC and TT genotypes, statistical significance was not achieved.

In view of the significant influence of *MDR1*-C3435T genotypes on the response to Imatinib in CML patients previously reported, we would expect a similar impact on response to Nilotinib. This discrepancy may be due to racial differences or to the small number of patients in our cohort. Further studies on larger series of patients with a longer follow-up period (2 years) are needed to verify. Other mechanisms of resistance such as point mutation of *BCR-ABL1* gene, OCT level, etc should also be incorporated in such studies.

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