

## Monocytes Activation and Some Haemostatic Parameters in Cases of $\beta$ -Thalassemia

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### ABSTRACT

**Background and objectives:** Thalassemia is a congenital hemolytic disorder caused by a partial or complete deficiency of alpha or beta globin chain synthesis. The aim of this matched case control study was to clarify the role of monocytes, platelets and endothelial activation in the pathogenesis of the chronic hypercoagulable state in  $\beta$ -thalassemia; both major ( $\beta$ -TM) and Intermediate ( $\beta$ -TI).

**Methods:** Whole blood of non-splenectomized and splenectomized  $\beta$ -thalassaemic patients, and healthy normal subjects were assayed for the expression of the cluster of differentiation molecule 14 as a marker for monocytes, the cluster of differentiation molecule 11b, as an indicator for monocytes activation; and Plasma levels of Von Willebrand factor Antigen and platelet aggregation ratio were assessed. The investigations were performed by the Beckman Coulter Flow cytometric analysis. Furthermore, complete blood count, hemoglobin electrophoresis, prothrombin time, partial thromboplastin time, liver functions test, and C - reactive protein were assessed.

**Results:** Both activated monocytes and the monocytes activation markers mean values show statistical significant differences between the studied groups and within the groups as well, where the F values were 6.798 and 3.781, while the P value for each group was  $\leq 0.0001$  and  $\leq 0.014$  respectively.

**Conclusions:** The monocytes activation is important determinant of the thrombotic risk observed in thalassaemic patients who had been splenectomized. These results might be important useful markers for the follow-up, diagnostic and therapeutic implications

**KEY WORDS:** thalassemia, CD14, platelet aggregation ratio, CD11b. Von Willebrand factor Antigen.

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### INTRODUCTION

Thalassaemias are a group of heritable anaemias with varying degrees of severity occurring early in life and caused by a partial or complete deficiency of  $\alpha$ -or  $\beta$ -globin chain synthesis resulting in chronic haemolysis<sup>[1]</sup>. The  $\beta$ -thalassaemias are also common in Saudi Arabia along the coastal strip of the Red Sea and in the Eastern province, although  $\beta$ -thalassaemia disease has been known for many years in these areas and many of its manifestations are recognized, the details of actual incidence, the natural history or clinical course of the disease from early childhood to death are unknown<sup>[2]</sup>. This is largely because of inadequate facilities for Thromboembolic phenomena (Hypercoagulability), both venous and arterial, are not uncommon in thalassaemic patients, particularly in patients who have undergone splenectomy and/or who undergo transfusion infrequently<sup>[3]</sup>. Abnormalities in the levels of coagulation factors and their inhibitors have been reported, resulting in a chronic hypercoagulable state<sup>[4]</sup>.

Thrombosis is typically an episodic complication associated with a temporary secondary activation of homeostasis. In contrast, markers of platelet and coagulation activation are persistently and consistently elevated in most thalassaemic patients (adults and children alike), even in the absence of overt thromboembolic events<sup>[5]</sup>.

The presence of a persistent hypercoagulable state combined with the infrequent occurrence of significant thrombotic events suggests that thrombosis is largely a subclinical process in thalassaemia that has been associated with autopsy findings of platelet and fibrin thrombi in the microvasculature of the lungs and brain<sup>[6]</sup>. These thrombi contribute to the development of pulmonary hypertension, low lung capacity, hypoxemia, and diffusion defects associated with right heart failure (cor-pulmonale)<sup>[7]</sup>, and the high frequency of ischemic brain lesions associated with asymptomatic brain damage (seen on MRI) in thalassaemic patients<sup>[8]</sup>.

Several etiologic factors may play a role in the pathogenesis of the hypercoagulable state in thalassaemia. These include low levels of protein S and C, increased platelet consumption and ongoing platelet, monocytes, granulocyte and

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protein activation and possible, continuous thrombin generation and enhanced fibrinolysis <sup>[9]</sup>. There is evidence suggesting that patients with  $\beta$ -thalassemia have activated platelets. Moreover, flow cytometric studies have also confirmed the chronic platelet activation status. In  $\beta$ -thalassemia, there is evidence of increased platelet aggregation <sup>[10]</sup>. Monocytes activation may also play an important role in increasing endothelial activation or injury in thalassemic patients. High levels of monocytes colony-stimulating factor were found in patients with  $\beta$ -TM <sup>[11]</sup>

It has been suggested that the absence of spleen in a variety of hematological diseases can contribute to an increased susceptibility to thrombosis <sup>[12]</sup>. In a series of  $\beta$ -TI patients followed for 10 years, 24 patients (29%) developed either DVT, pulmonary embolism, or portal vein thrombosis <sup>[11]</sup>. All patients except one had undergone splenectomy. The development of these complications has been ascribed to the presence of high platelet counts following splenectomy and/or to increased number of abnormal RBC <sup>[4]</sup>

We report the role of monocytes activation as indicated by monocytes markers including the cluster of differentiation molecule 14 (CD14) and monocytes activation marker as the cluster of differentiation molecule 11b (CD11b) in the pathogenesis of the chronic hypercoagulable state in  $\beta$ -thalassemia (Major and Intermediate). We also report on the role of platelet and endothelial activation or injury as indicated by the levels of Von Willebrand factor Antigen (vWF- Ag) % in plasma and the in-vivo or circulating platelet aggregates respectively.

## MATERIALS AND METHODS

The study was conducted in pediatric hematology unit in Ohoud hospital in Al-Madinah, Kingdom of Saudi Arabia between September 2009 and August 2010. Age matched 70 children were involved ( $13.4 \pm 5.15$ ) years. They were divided into 20 healthy normal children (group I), 20 non-splenectomized ( $\beta$ -TM) patients receiving blood transfusion (group II), 20 splenectomized  $\beta$ -TM patients (group III), 10 non-splenectomized ( $\beta$ -TI) patients (group IV). Informed consent was taken from all patients and patients guardians before including them in the study.

A specially designed interviewing format and examination checklist was fulfilled by the investigators included detailed medical history (with special emphasis on age at diagnosis, frequency of blood transfusion per year, requirement of chelation therapy, and splenectomy were taken from the patients or their parents), detailed history regarding treatment, and follow-up. The examination included the pre-transfusion hemoglobin levels, facial deformities, degree of splenomegaly, (if not splenectomized.)

### **Blood Sampling and Analyses:-**

4 ml of Peripheral venous blood was collected and divided into 2 tubes; the first 2 ml was collected on a tube Ethylene diamine tetra-acetate (EDTA) (2.2 mg/ml) for flowcytometric analysis and the second 2 ml of blood was collected with 3.8% sodium citrate as an anticoagulant, incubated for 20 minutes, then Plasma was obtained by a 10 minutes centrifugation at 1600 g for vWF Ag assay. More 2 ml blood was collected by 2 separate polyethylene syringes, 1 of them containing 4 ml fixative solutions and the other one containing 4 ml non fixative solution for measuring PAR to detect circulating platelet aggregates.

### **All cases were subjected to the following investigations:**

- CBC on Sysmex K-1000 cell counter.
- Examination of Lishman and Brilliant Cresyl Blue stained peripheral blood (PB) films for any abnormal cells as normoblasts and for reticulocytes respectively.
- Hemoglobin (Hb) electrophoresis to detect the percentages of Hb F, Hb A2 and HbA.
- Liver function tests on ADVIA 1650 Chemistry System and serum ferritin on Elecsys 2010 System.
- Prothrombin time (PT) and Partial thromboplastin time (PTT) on Sysmex CA 1500.
- C-reactive protein (CRP) by latex agglutination as acute phase reactant <sup>[13]</sup>
- Flow cytometric analysis for CD14 <sup>[14]</sup> as a marker for monocytes and CD11b <sup>[15]</sup> as an indicator for monocytes activation using Beckman Coulter flowcytometry (Beckman Coulter, Brea, CA) in the clinical biochemistry of College of Medicine, Taibah University.
- Plasma levels of vWF Ag by ELISA technique <sup>[11]</sup>
- Estimation the platelet aggregation ratio (PAR) by Sysmex K-1000 cell counter, was measured according to the method of Wu and Hoak <sup>[16]</sup> with minor modifications <sup>[17 & 18]</sup>

### **Statistical analysis**

The data were collated and analyzed using the Statistical Package for Social Studies version 13.0 (SPSS, Chicago, IL, USA). Complete confidentiality was maintained while the data were being processed. Descriptive statistics including frequencies, percentages and arithmetic mean were calculated. Comparison of factors was performed using Student's t

test or analysis of variance (ANOVA) as appropriate. Bivariate correlations were done using the corresponding Pearson correlation coefficient (r) and r squared (r<sup>2</sup>) values. Statistical significance was defined as p value equal or less than 0.05.

**RESULTS AND DISCUSSION**

Increased activated monocytes was indicated by the number of patients who had activated monocytes % more than the upper limit of the control group (48.9%) and by the cases with increased monocytes CD11b more than the upper limit of the control group (294.6). Platelet activation was indicated by decreased PAR (increased in-vivo or circulating platelet aggregates) below the lower limit of the control group (0.7).

Endothelial cell activation was indicated by increased vWF Ag % more than the upper limit of the control group (85%). Coagulation Defects were indicted by increased PT and/or PTT more than the upper limit of the control group (14 seconds) and (40 seconds) respectively. Affected liver functions were indicated by high liver enzymes (ALT and AST) more than the upper limit of the control group (40 U/L) and (37 U/L) respectively. In the current work, table (1) shows that there was highly significant decrease in the Hb concentration and RBCs counts in group II, III and IV in comparison with group I.

There was a significant increase of Hb concentration in group III and group IV (x ± SD 7.50 ± 1.48, 7.94 ± 1.58) respectively in comparison with group II (x ± SD 6.47 ± 1.40).

There was a highly significant increase of red blood cells count (RBCs) in group IV (x ± SD 3.65 ± 0.41) in comparison with group II (x ± SD 2.69 ± 0.72) and group III (x ± SD 3.06 ± 0.50).

As regard the platelet count (PC), there was highly significant increase in (PC) in group II, III and IV (x ± SD 294.45 ± 142.56; 594.40 ± 217.39 and 563.82 ± 172.83, respectively) in comparison with the control group (x ± SD 275.55 ± 62.63), there was highly significant increase in group IV compared with group III. (P ≤ 0.0001). There was insignificant slight decrease of (PC) in group II (P ≤ 0.01).

Table 1 Laboratory finding of all groups. (Data expressed as Range & mean ± SE ) and Statistical Variation. Compared by one way ANOVA test in

Variables	group I n=20	group II n=20	group III n=20	group IV n=10	One way ANOVA
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F & P Values
Hb	12.96 ± 0.96	6.47 ± 1.40	7.50 ± 1.48	7.94 ± 1.58	90.937***
RBCs	4.71 ± 0.78	2.69 ± 0.72	3.06 ± 0.50	3.65 ± 0.41	36.920***
Platelets Count (PC)	275.55 ± 62.63	294.45 ± 142.56	594.40 ± 217.39	563.82 ± 172.83	40.946***

- Significant (P ≤ 0.01), \*\* Highly Significant (P ≤ 0.001), \*\*\* Extremely highly significant (P ≤ 0.0001)

There was significant negative correlation between Hb concentration and platelets count where (r<sup>2</sup> = 0.882 and P ≤ 0.0001) showing an inverse relationship between the severity of anemia and the maximum platelet counts (Figure 1).

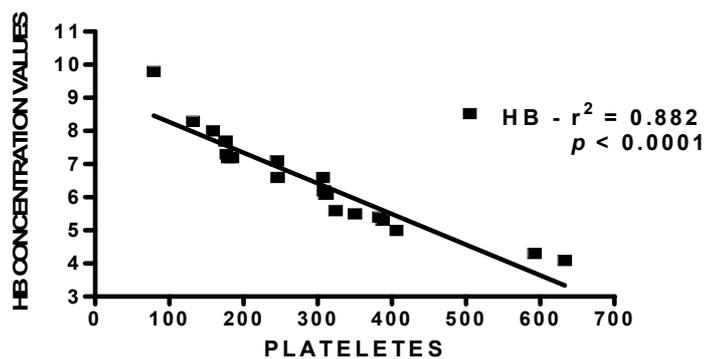


Figure1: shows the correlation between hemoglobin concentrations values and the platelets count values in group II of patients.

As regard PAR for platelet aggregation there was no significant difference between the studied group or within the groups using one way ANOVAs (F = 0.631, P ≤ 0.598) meanwhile vWF Ag % showed significant increase in groups II, III and IV compared to control group (x ± SD 91.75 ± 38.41; 103.80 ± 57.76 and 113.10 ± 39.97 and 60.49 ± 17.95, respectively), where F = 5.224 and P ≤ 0.003

There was significant differences of mean values of both activated monocytes and monocytes activation markers between and within the group where the (F = 6.798, and 3.781 where P ≤ 0.0001 and ≤ 0.014 respectively) (Figure 2).

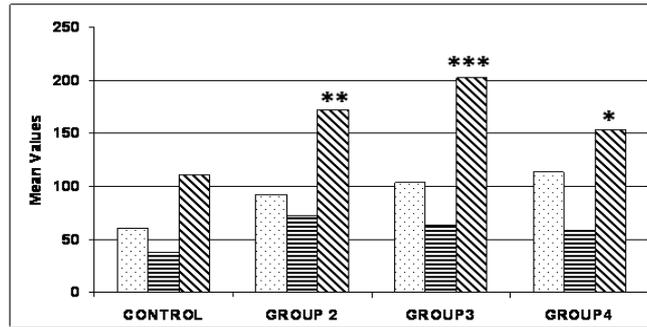


Figure2: shows the bars representing all groups' comparison in the vWF Ag %, Active monocytes and monocytes activation markers mean values. The light dotted bar representing vWF Ag %, dark horizontal lined bar representing active monocytes, and the wide dark downward diagonal lined bars representing the monocytes activation markers CD11b. \* Significant ( $P \leq 0.01$ ), \*\* Highly Significant ( $P \leq 0.001$ ), \*\*\* Extremely Highly significant ( $P \leq 0.0001$ )

The incidence of in-vivo platelet aggregation and platelet activation in our study were 40% in splenectomized patients and 26.6% in non-splenectomized patients (table 2).

Table 2: Incidence of some studied parameters related to hypercoagulability in thalassemia patients

Parameter	Increased activated monocytes		Platelet Activation		Endothelial activation		Coagulation defects		Affected liver activity		High P.C.	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Group II n = 20	18	90	6	30	12	60	12	60	19	95	3	15
Group III n = 20	14	70	8	40	9	45	12	60	20	100	16	80
Group IV n = 10	6	60	2	20	7	70	7	70	8	80	8	80

There were statistical significant positive linear correlations between the liver enzymes (AST and ALT) values and values of active monocytes ( $r^2 = 0.833$  and  $p \leq 0.0001$  and  $r^2 = 0.543$  and  $p \leq 0.0002$  respectively) (figure 3).

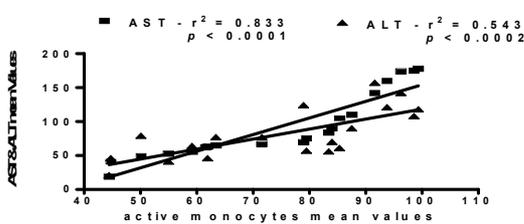


Figure3: shows the correlation between activated monocytes values and the liver function markers values (AST & ALT) in group II of patients.

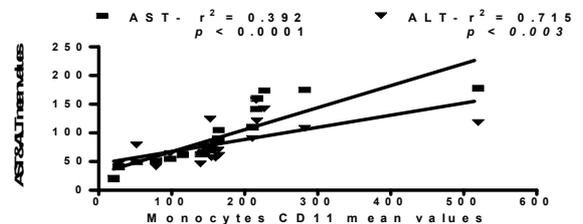


Figure4: shows the correlation between the monocytes activation markers values and the liver function markers values (AST & ALT) in group II of patients.

The same was found between values of the monocytes activations markers and liver enzymes ( $r^2 = 0.392$  and  $p \leq 0.0001$  and  $r^2 = 0.715$  and  $p \leq 0.003$ ) (figure 4).

## DISCUSSION

In 1978, Eldor<sup>19</sup> and a year later Houssain *et al*<sup>20</sup> found defective platelet aggregation in response to adenosine diphosphate, epinephrine, or collagen in  $\beta$ -TM patients. Most of the patients had undergone splenectomy and had high platelet counts. At that time, these anomalies were interpreted as signs of a mild bleeding disorder because many thalassemic patients experience frequent epistaxis as well as easy bruising<sup>19</sup>.

Venous and arterial Thromboembolic phenomena are common in patients with thalassemia, particularly in patients who have undergone splenectomy and transfusion infrequently<sup>13</sup>. Abnormalities in the levels of coagulation factors and their inhibitors have been reported, resulting in what can be defined as a chronic hyper-coagulable state<sup>14</sup>.

We studied the role of monocytes activation and platelets and endothelial activation as indicated by the in-vivo platelet aggregates and the levels of vWF antigen % in plasma to clarify their role in the pathogenesis of the chronic hypercoagulable state in  $\beta$ -TM and TI.

We found highly significant decrease in the Hb concentration and RBCs counts in group II, III and IV in comparison with the control group. These results confirm previous report by Premawardhena *et al* <sup>[11]</sup> and Goswami *et al* <sup>[12]</sup>, who have postulated that it is due to the nature of thalassaemia as being a form of severe anemia. These findings were explained by Erslev <sup>[21]</sup> who found that patients with  $\beta$ -TI have in general a milder clinical phenotype than those with  $\beta$ -TM. The marked anemia and the low RBCs count in splenectomized group are primarily caused by the removal of RBCs from the circulation and by increased destruction of RBCs <sup>[21]</sup>.

There was highly significant increase in PC in group II, III and IV in comparison with the control group and highly significant increase of PC in group IV in comparison with group III. The platelet count showed a slight (statistically insignificant) tendency to decrease in group II (non-splenectomized  $\beta$ -TM patients). This could be attributed to hypersplenic activity and to increased sequestration of platelets in enlarged spleens. Vento *et al* <sup>[22]</sup> found that in the splenectomized thalassaemic patients, there was a highly significant incidence of thrombocytosis. Increased platelet counts in these patients were a consequence of continuing anaemia with a hypercellular or hyperplastic BM and splenectomy. This may explain the significant negative correlation between Hb concentration and platelet count ( $r = -0.326$  where  $P < 0.05$ ) showing an inverse relationship between the severity of anemia and the height of platelet counts.

Platelets activation in our study was 40% in splenectomized patients and 26.6% in non-splenectomized patients and was not in agreement and lower than that observed by Wehmeier *et al*, <sup>[23]</sup> who reported that examination for circulating platelet aggregates according to Wu and Hoak <sup>[16]</sup> revealed increased circulating platelet aggregates in 71% of splenectomized and 35% of non-splenectomized thalassaemic patients. This may be causally related to the newly observed high incidences of pulmonary artery thrombosis and hypoxemia in splenectomized thalassaemic patients. So, increased platelet aggregation is an observation compatible with in vivo platelets activation and the existence of a hypercoagulable state <sup>[4]</sup>.

On the contrary, in our study, no significant difference was found between the studied groups as regard to the platelet aggregation done by the PAR test. This may be explained as the PAR test for platelet activation (aggregation) is non-specific and is affected by several factors, explaining the differences between our study and that of Winichagon *et al* <sup>[24]</sup> and Yamaga *et al* <sup>[25]</sup> noted that although additional evidence of abnormal platelet membrane phosphatidylserine (Ps) exposure was seen, the absence of any correlation between platelet (Ps) and haemostatic markers implies that platelet activation is not the predominant mechanism responsible for thrombin generation.

As regard to vWF Ag % it shows significant increase in groups II, III and IV in comparison with the control group. This is more or less consistent with results of Butthep *et al* <sup>[26]</sup> where significant increase in vWF was shown in  $\beta$ -TM patients with splenectomy as compared to normal. The detection of vWF in serum and plasma of thalassaemic patients suggests that endothelial activation or injury may be a feature of the hypercoagulable state in thalassaemia. Increase of activated monocyte % in thalassaemia patients can be explained by the excess of M-CSF in thalassaemia that is likely to originate from mononuclear phagocytes originating in response to their clearance for thalassaemic cells which is more rapid in  $\beta$ -thalassaemia than in  $\alpha$ -thalassaemia due to the more severe membrane damage in the former than in the later <sup>[27 & 28]</sup>.

In the current study the highest levels of activated monocytes were found in splenectomized patients in comparison to all other groups. These results could be explained by the previous cited study <sup>[29]</sup> which reported that aged or abnormal red blood cells with exposed phosphatidylserine (PSRBCs) are cleared from the circulation by splenic macrophages. In asplenic patients, other mononuclear phagocytic cells in tissues and in circulation may function in this capacity suggesting that splenectomy in E/ $\beta$ -Thal patients led to an increased amount of PSRBCs and activation in the mononuclear phagocytic system.

There were statistically significant positive linear correlations between the liver enzymes (AST and ALT) values and values of active monocytes and the monocytes activations markers. This finding could be explained by the previously shown overproduction of multiple cytokines [especially tumor necrosis factor (TNF) and interleukin (IL)-8] by monocytes in Thalassaemia. Moreover it was postulated that activation of monocytes and macrophages with subsequent proinflammatory cytokine production plays an important role in certain metabolic complications of  $\beta$ -TM patients and is a component of the liver injury of  $\beta$ -TM patients <sup>[29]</sup>.

## CONCLUSION

The study findings point to monocytes activation as an important determinant of the thrombotic risk observed in thalassaemic patients who had been splenectomized. These results might have important diagnostic and therapeutic implications

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