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Expression of CD41 (GPIIb) and CD61 (GPIIIa) in Patients with Glanzmann Thrombasthenia Using Flow Cytometry

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Abstract

Background Many genetic mutations causing severe reduction or defect in GPIIb (CD41) and/or GPIIIa (CD61) receptors in Glanzmann Thrombasthenia (G.T.). Flow cytometry can be used for quantification of these two receptors for diagnosis and further identification of G.T. types which are type I, II and III.

Objective To detect the occurrence of CD41 (GPIIb) and CD61 (GPIIIa) in patients diagnosed with Glanzmann thrombasthenia and to classify them accordingly by flow cytometry.

Methods A descriptive cross sectional study was conducted on 28 patients with G.T. collected from the National Center of Hematology and Children Welfare Teaching Hospital over 5 months from Dec. 2014 to Apr. 2015. Those patients were subjected to all hematological investigations required for the diagnosis of G.T. Glanzmann Thrombasthnia Italian Team Protocol (GLATIT) was used to assess the severity of bleeding in those cases at time of collecting samples after taking clinical information either directly with their full agreement or from their files.

Results Majority of cases were below 20 years of age with male to female ratio 1:1. According to the results obtained by flow cytometry. Majority of cases (86%) were classified as type I. A single case (3%) was found to be of type II whereas type III constituted 11% of the total no. of cases. Family history and consanguinity were found in 79% and 93% of the affected families respectively.

Conclusion Type I is the most common followed by type III then type II. Most cases were mild bleeders followed by moderate then severe bleeders. Platelet function test is essential to diagnose the variant form of G.T. (type III) which reveal a normal or near normal expression of both receptors as detected by flow cytometry. More than one type of G.T. had been found in one family with variable bleeding severity suggesting that more than one mutation can occur in the same family.

Keywords CD41, CD61, Glanzmann thrombosthenia.

List of abbreviation: PT = prothrombin time, PTT = partial thrombopastin time, TT= thrombin time G.T. = Glanzmann thrombasthenia, PRP = Platelet rich plasma, CD = Cluster of differentiation, GP = Glycoprotein, GIT = Gastrointestinal tract

Introduction

Glanzmann thrombasthenia (G.T.) is a rare genetic autosomal recessive hemorrhagic disease caused by a defect in platelet function leading to the development of bleeding manifestations as epistaxis, gum bleeding, menorrhagia, petechiae, bruises. It is found mainly in regions where consanguineous marriage is common as in Iraq, Iran, India and among Jews. It affects males and females equally [(1)]. Historically, the disease was first discovered in 1918 by Edward Glanzmann, a Swiss pediatrician, who noticed that one of his cases presented with spontaneous bleeding symptoms with normal platelet count and morphology, prolonged

bleeding time, normal prothrombin time (PT) and partial thrombopastin time (PTT), normal level of coagulation factors [2]. At the molecular level, two important platelet surface glycoprotein are implicated in the disease, GPIIb (CD41) and GPIIIa (CD61). These two glycoproteins form a complex that functions as a receptor for fibrinogen and other molecules included in platelet functions, leading eventually to platelet aggregation and formation of platelet plug [3-5]. Genetically, two genes responsible for the biosynthesis of GPIIb, IIIa complex, both are located on chromosome no. 17, which are ITGB3, ITGA2B. Various types of mutation can affect them leading to the development of G.T. [2,3]. In this disease, where either one or both glycoproteins are absent or have defective function, this will render the other glycoprotein also either absent or defective in its function causing no binding between platelet and fibrinogen, no platelet aggregation, leading to a defect in primary hemostasis and hemorrhagic manifestations will appear [4,6]. Patients with Glanzmann thrombosthenia presents with normal platelet count and morphology, normal PT, PTT, TT, normal level of clotting factors and VWF. Platelet function test by aggregometry is used for diagnosis of those patients where it reveals absent aggregation of platelets in response to all physiological agonists as ADP, epinephrine, collagen except for high dose of ristocetin, which is the characteristic pattern of G.T. [5]. However, flow cytometry is another technique that has an increasing application in this field, the corresponding antibodies (anti CD41 and CD61) is directed by flow cytometry against CD41 (GPIIb) and CD61 (GPIIIa) respectively, so the percentage of these receptors expression can be determined and hence the types and sub types of G.T. can be identified [7,8]. Severe reduction (< 5% for both receptors) in the expression was consistent with type I, moderate reduction (5-20%) goes with type II whereas > 20% expression is regarded as type III [7].

Glnazmann thrombosthenia is divided into three types:[8]

Type I: severe reduction in expression of both receptors.

Type II: weak expression of both receptors.

Type III: normal or near normal expression of both receptors (a qualitative defect).

The objectives of this study was to detect the occurrence of CD41 (GPIIb) and CD61 (GPIIIa) in patients diagnosed with Glanzmann thrombasthenia and to classify them into types accordingly by flow cytometry.

Methods

This descriptive cross sectional study was conducted on 28 patients who attended the National Center of Hematology and Children Welfare Teaching Hospital as highly suspected cases of Glanzmann thrombasthenia; collected over a period of time from December 2014 till April 2015.

Those patients were selected randomly regardless their age and gender (there was no specific age or gender to be included neither excluded from the study; suspected cases of all age groups and both sexes were taken). They presented with clinical and laboratory features of G.T. as mucocutaneous bleeding as epistaxis, bruises, gum bleeding, petechia, gastrointestinal bleeding; either spontaneously or after trauma, family history, normal PT, PTT, TT and platelet count and morphology.

Ethical aspects was taken in consideration while carrying out this research. Assuring that no physical and psychological impacts would be gained to patients who subjected to the tests, no mention of their names while announcing the results. As the majority of cases were in the pediatric age group, the full agreement of their parents was taken prior to sampling. The same agreement was taken from the adult ones. However, the leftover of the blood sample was taken for the tests of this research for the majority of cases who were already referred from other centers to evaluate their platelet by platelet function test and flow cytometric analysis. The blood samples were collected

with EDTA anticoagulated tube for flow cytometric analysis. Trisodium citrate was used for samples to be analyzed by light transmission aggregometry. The samples centrifuged to obtain.

Those patients were subjected to platelet function test to diagnose G.T. at the National Center of Haematology. Various platelet agonists were used in platelet function test, which include ADP, collagen, epinephrine and ristocetin; all with the light transmission aggregometer were manufactured by BIODATA CORP. USA.

The bleeding severity of those patients were assessed according to Glanzmann thrombasthenia Italian Team protocol (GLATIT) [(9)] as follows:

- Mild: minor bleeding symptoms or patients who bleed only after trauma or surgery.
- Moderate: spontaneous and life threatening bleeding as GIT bleeding.
- Severe: repeated bleeding episodes requiring blood or platelet transfusion.

The leftover of the blood samples of some patients were used in the tests of the study whereas other patients were referred from other hospitals and specialists specifically for performing the tests, which are included in the study.

Flow cytometric analysis was done at private lab. Anti CD41 manufactured by Abcam. Cambrige and anti CD61 manufactured by Partec. USA; both are anti human primary conjugated antibodies of mouse origin, were used for immunophenotyping of CD41 and CD61 markers respectively by flow cytometry.

A sample of 2 ml of peripheral venous blood were collected in EDTA containing tube from patients; who already submitted to platelet function test, for immunophenotyping. Whereas additional 5 ml of blood were collected in tube containing trisodium citrate from newly diagnosed cases for performing platelet function test.

The blood samples were centrifuged either by PDQ platelet function centrifuge manufactured by BIODATA, USA, double centrifugation protocol or by single centrifugation at 1000 rpm for 10 min. the platelet rich plasma which were transferred to glass tubes and transported to the lab at room temperature by hand to be analyzed within 8 hrs in maximum for flow cytometry and 4 hrs for aggregometry.

100 µl of PRP was taken for flow cytometry. 0.45 ml for each agonist was added in platelet function test. The result of flow cytometry is represented in a dot blot while the result platelet function test is recorded as waves or traces for each agonist.

Statistical analysis

The statistical analysis was performed with the statistical package for social sciences (SPSS) 21.0 and Microsoft Excel 2013.

Results

The three types of G.T. has been identified in the studied sample when flow cytometry is applied as shown in table 1. Males and females were equally affected 1:1. 86% of patients were of type I with severe reduction in receptor expression; 92% of them were classical, in which, there was < 5% expression of both receptors and 8% were heterogeneous, in which, one receptor has < 5% expression while the other one has > 5% expression. Type II constituted only 3% (1 out of 28 patients) and the remaining 11% were of type III.

More than one type of G.T. had been identified in members of the same family. The bleeding severity was assessed in the studied cases and revealed that 43% of the patients were mild bleeders, 39% were moderate and 18% were severe bleeders as shown in figure 1. There was no correlation between the severity of bleeding and the level of receptor expression as well as with the types of G.T. as illustrated in table 2. Majority of cases 82% were below 20 years of age. Mean age was 12 year ± 11.78 SD, median was 11 year and the range was (3-58 year). A single case was at the sixth decade of life.no cases were found in the 4^{th} and 5^{th} decade of life and age had no impact on

bleeding severity as shown in table 3. Family history was found in 79% and consanguinity reached 93% of the cases. Flow cytometry results had shown reduction of expression of CD 41 and CD 61 receptors in comparison with normal levels as in figures (3, 4). Platelet function test showed some variation in the trace of high dose of ristocetin as shown in figure 5 and figure 6. In some patients, there was persistent agglutination while other case showed no response at all; with no statistical significant correlation between it and the percentage of receptor expression. Epistaxis was the most common bleeding manifestation 75% followed by petechiae and bruises 64%, gum bleeding 46% and the least frequent was GIT bleeding that occurred in 18% of the patients as illustrated in figure 2.

Moreover, age and gender had no effect on type of G.T. neither severity of bleeding as in table 4.

Table 1. Types of Glanzmann thrombasthenia

Type of Glanzmann thrombasthenia	No. of patients	Percentage of types	Median of CD41 expression	25-75 Percentile of CD41 expression	Median of CD61 expression	25-75 Percentile of expression
Type I	**24**	**86%**	**2.18**	**1.2- 2.47**	**3.22**	**1.72-3.99**
Type II	**1**	**3%**	**5.93**	**5.93**	**5.66**	**5.66**
Type III	**3**	**11%**	**92.85**	**45.89-95.82**	**92.76**	**44.25-94.66**
Total	**28**	**100%**	---	---	---	---

Table 2. Correlation between severity of bleeding and percentage of receptor expression showing no statistically significant correlation between them

Receptor expression	Median & percentile	Severity of bleeding Mild (n=12)	Moderate (n=11)	Severe (n=5)	P value
CD41	Median 25-75 Percentile	2.58% (1.46-3.98)%	2.28% (1.22-2.64)%	2.13% (1.56-4.03)%	0.737 NS
CD61	Median 25-75 Percentile	3.85% (2.15-5.19)%	3.35% (1.64-4)%	3.58% (2.85-4.98)%	0.840 NS

Chi-square test was used to describe the association between data, α =0.95

Table 3: Correlation between age and bleeding severity

Age groups	Mild	Moderate	Severe	Total	P value
≤ 10 years	**6**	**3**	**4**	**13**	**0.745**
11-20 years	**4**	**6**	**0**	**10**	
21-30 years	**1**	**2**	**1**	**4**	
31-40 years	**0**	**0**	**0**	**0**	
41-50 years	**0**	**0**	**0**	**0**	
> 50 years	**1**	**0**	**0**	**1**	
Total	**12**	**11**	**5**	**28**	

Chi-square test was used to describe the association between data, α =0.95

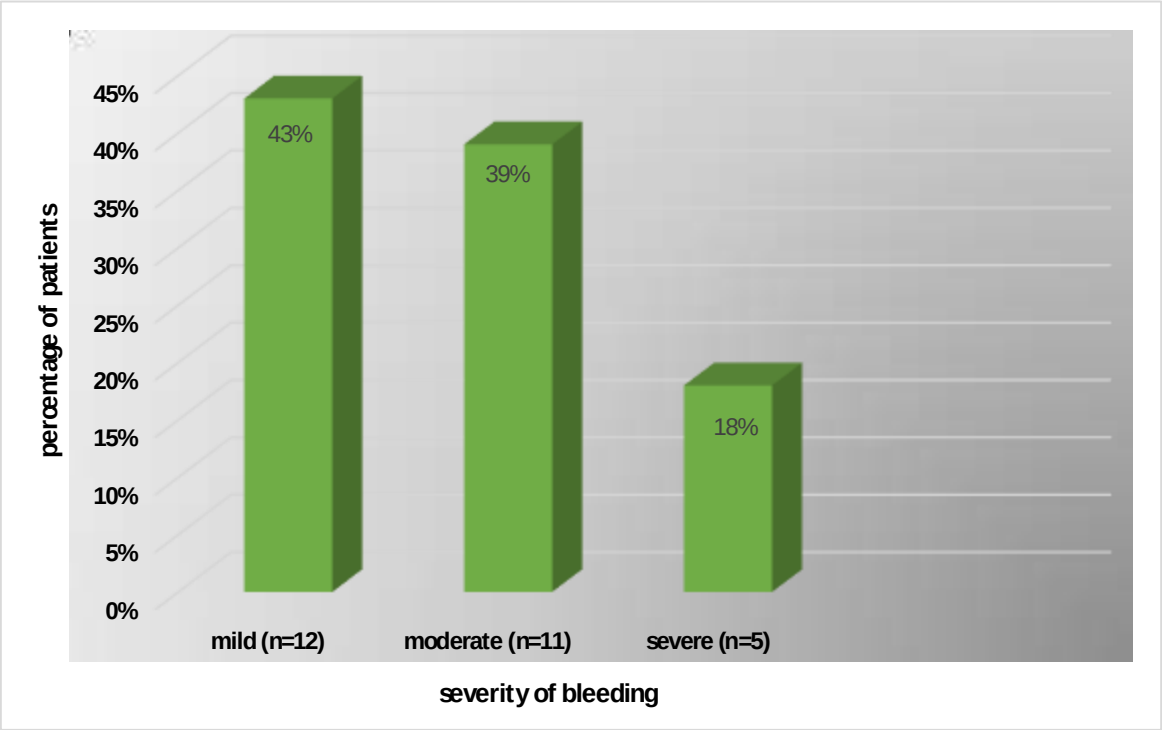

Fig. 1. Distribution of patients according to severity of bleeding

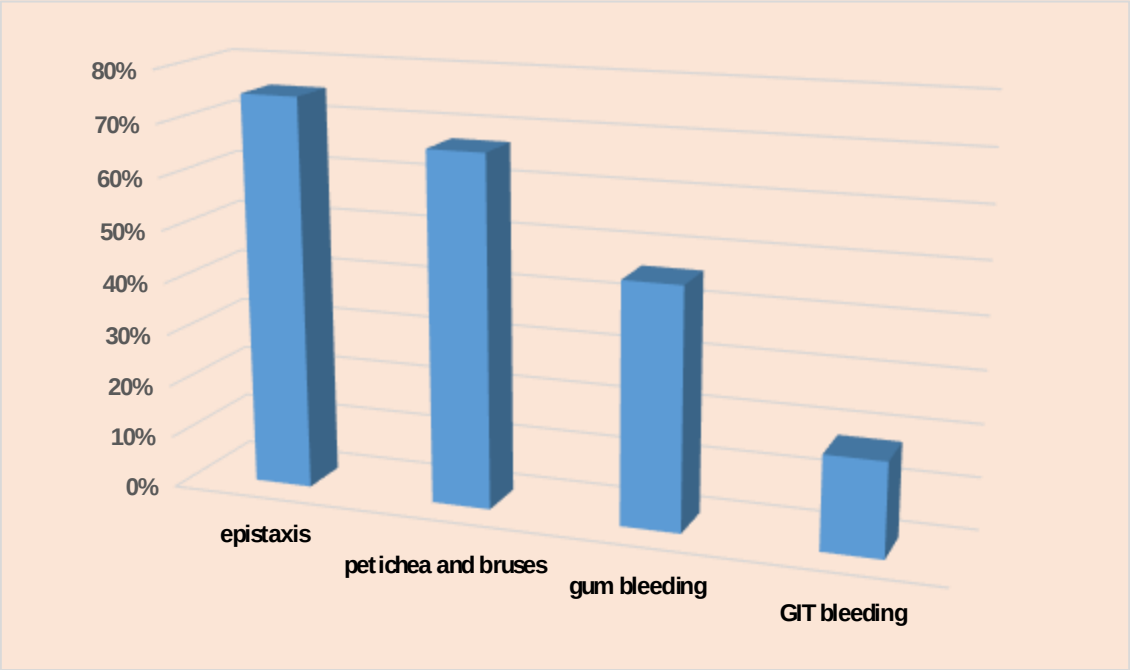

Fig. 2. Percentage of bleeding manifestations

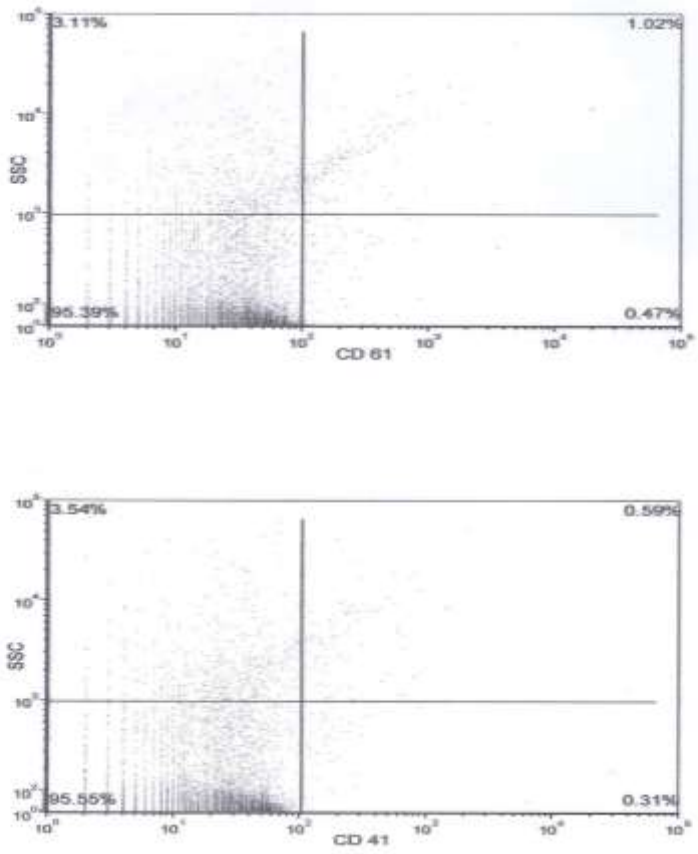

Fig. 3. Dot blot showing reduced expression of both CD41 and CD61 receptors

Discussion

When flow cytometry was applied on platelet rich plasma samples of 28 patients with G.T, it revealed that type I G.T. was the most common type (86%), followed by type III (11%), then type II (3%). This result was in agreement with the result of Kannan et al study (India 2003) that revealed highest frequency in type I (64%) and higher frequency in type III (24%) rather in type II (12%) (10). However, other studies as Alireza Farsinejad (Iran 2010) (11) and Layla Beshwari (kingdom of Saudi Arabia 2005) (12) showed similar result regarding type I but type III was more common than type II. More than one type of G.T. was found in the same family (two sisters, one of them was of type I while the other was of type II). Such finding suggests than more than one type of mutations can affect the genes leading to the occurrence of G.T. In the current study, most cases were below 20 years with absence of cases within the 4^{th} and 5^{th} decade of life. This can be related to chance or the small studied sample or racial factors that may affect the type of the causative mutations. Consanguinity was found in 93% of the patients and it goes with the fact that G.T. as other autosomal recessive disorders can be a common finding in areas where consanguineous marriage is common as in Iraq.

Seventy nine percent of the cases had a positive family history of the disease while the remaining 21% had no such history. This suggest that despite that G.T. is an inherited disease; de novo cases can occur in some individuals due to an acquired mutation

affecting the genes. These results approached the results of other studies. When Glanzmann thrombasthenia Italian Team Protocol was used to assess the severity of bleeding in the studied sample; majority of cases were mild and moderate bleeders and severe ones were of least frequency. However, they gave a history of severe bleeding manifestations when they were at a younger age, but such finding was not proved statistically when the correlation between the severity of bleeding with age and expression was tested and that goes well with the fact that the type of genetic mutation and other unidentified factors can affect the bleeding severity rather than the level of expressed receptors.

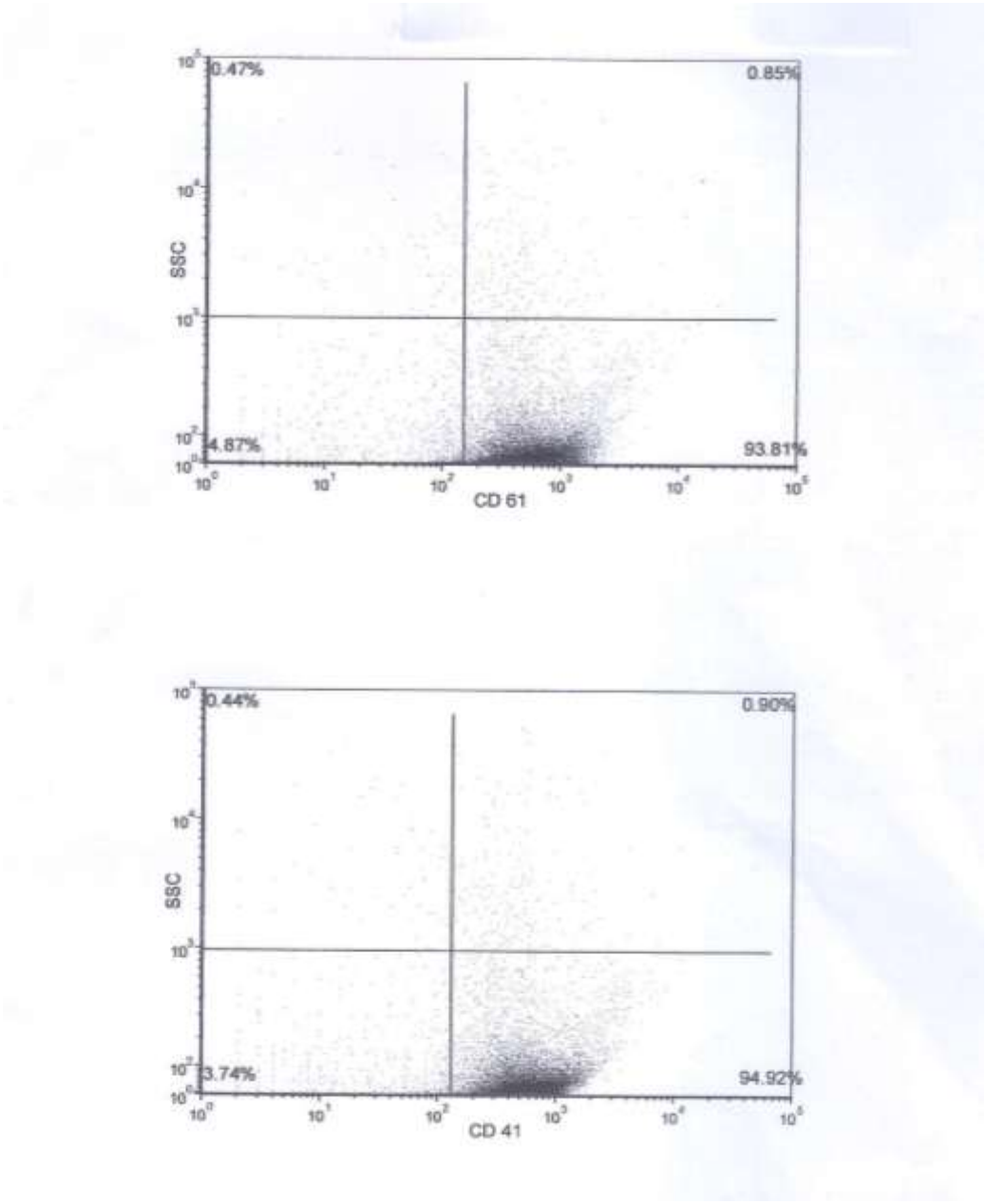

Fig. 4. Dot blot showing normal expression of both CD41 and CD61

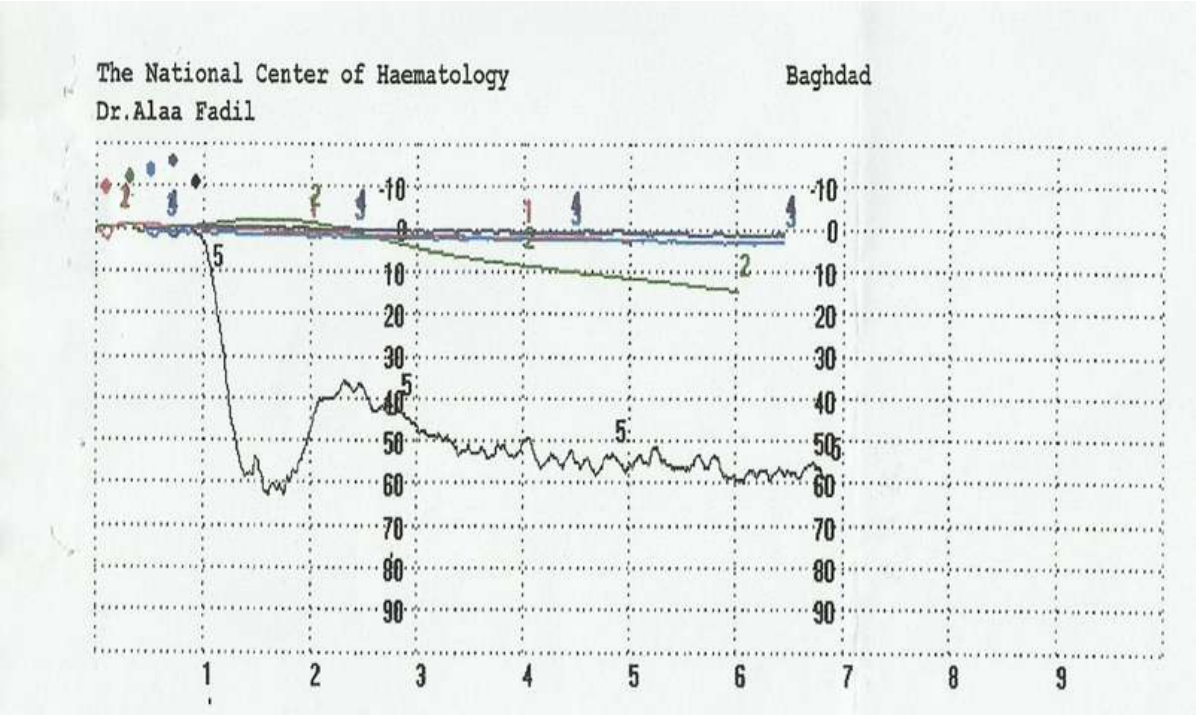

Fig. 5. Platelet function trace with persistent aggregation in response to high dose of ristocetin 1.2 mg/ml. 1- Red: ADP. 2- Green: collagen. 3- Blue: epinephrine. 4- Black: 0.5 mg/ml ristocetin. 5- Purple: 1.2 mg/ml ristocetin. X-axis represents time in minutes. Y-axis represents transmission of light.

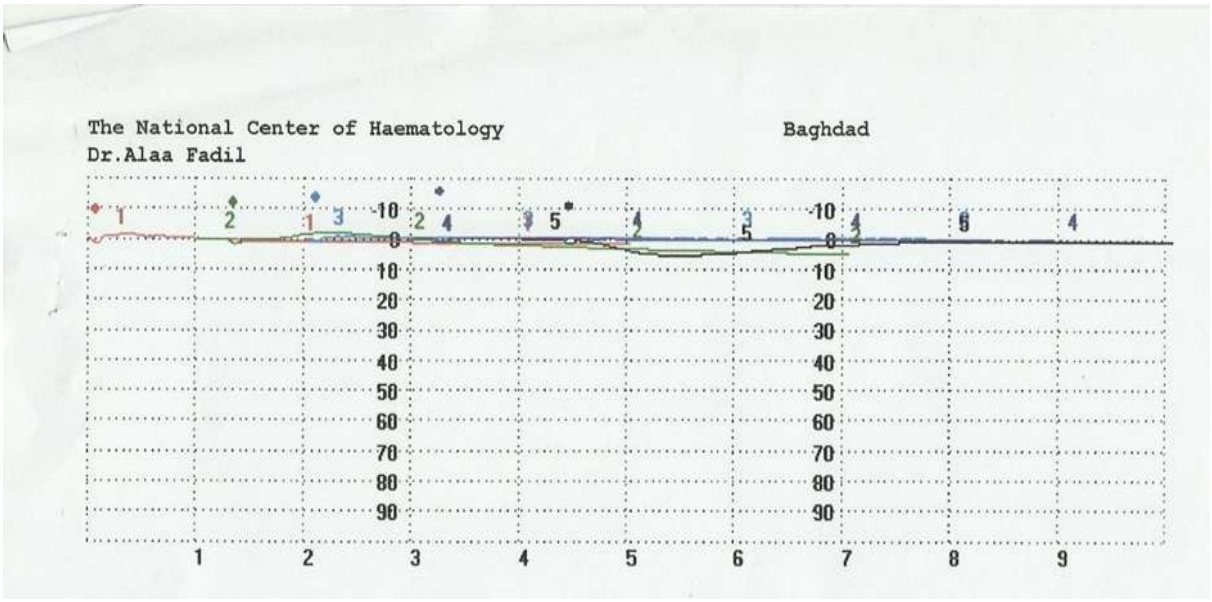

Fig. 6. Platelet aggregation trace with no aggregation in response to high dose of ristocetin 1.2 mg/ml. 1- Red: ADP. 2- Green: collagen. 3- Blue: epinephrine. 4- Black: 0.5 mg/ml ristocetin. 5- Purple: 1.2 mg/ml ristocetin, X-axis represents time in minutes. Y-axis represents transmission of light.

Table 4. Correlation between age, gender and types of Glanzmann thrombasthenia

		Scoring system						p value
		Type I	%	Type II	%	Type III	%	
Age groups	≤ 10 years	11	45.8%	0	0.0%	2	66.7%	0.186 NS
	11-20 years	8	33.3%	1	100.0%	1	33.3%	
	21-30 years	4	16.7%	0	0.0%	0	0.0%	
	58 years	1	4.2%	0	0.0%	0	0.0%	
	Total	24	100.0%	1	100.0%	3	100.0%	
Gender	Female	13	54.2%	1	100.0%	0	0.0%	0.502 NS
	Male	11	45.8%	0	0.0%	3	100.0%	
	Total	24	100.0%	1	100.0%	3	100.0%	

This study concluded that all types of Glanzmann thrombasthenia had been found in Iraq. Majority of them were of type I. More than one type had been identified in one family. The expression of both receptors had no impact on the severity of bleeding. Consanguinity can be considered as risk factor for G.T.

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Author Contribution
Hala made the contact with patients, gathered information regarding their clinical history and results of the hematological investigations, performed the centrifugation of majority of blood samples taken for flow cytometry as well as assisted in performing the immunophenotyping by flow cytometry, attending the platelet function test procedure, sorting and organization of data and results, organization of some figures and tables, writing thesis. Dr. Subh was the supervisor and guidance throughout the whole work. Yusra provided data of some cases, performed platelet function test for the vast majority of the patients. Dr Nidal provided data of some patients.

Conflict of interest
None.

Funding
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