CELLULAR ORIGIN OF PERIPHERAL BLOOD MICROPARTICLES IN THALASSEMIC PATIENTS

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ABSTRACT

Microparticles (MPs) are membrane vesicles released from many different cell types during cell activation or apoptosis. MPs abnormally display phosphatidylserine (PS) on their outer leaflets which create negatively charged (PS) which bind to the site of the prothrombonase complex. There are many reports in which a low amount of MPs in healthy subjects were found, whereas their number are elevated in patients at risk of thrombosis, nevertheless it has never been reported in thalassemia patients.

Thalassemia is a hereditary hemolytic disease characterized by absent or decreased production of normal hemoglobin. Patients with thalassemia have thromboembolic complications and a tendency to increase MPs in circulation by abnormal red blood cells (RBCs) and activated cells in the circulation. This study used a flow cytometer to determine the absolute number and surface markers of MPs in whole blood from thalassemic patients and healthy subjects. The values obtained by the study were used to relate to hematological parameters and markers of coagulation activation including platelets factor 3-like activity.

The results indicated that in thalassemia patients especially with splenectomies, β-thalassemia/hemoglobin E has a significantly high percentage and the absolute number of annexin V-positive MPs when compared with healthy subjects ($p<0.05$). The percentage of annexin V-positive in MPs were correlated well with the percentage of annexin V-positive in platelet population and the percentage of annexin V-positive in RBCs population. Cellular origin of annexin V-positive MPs are mainly from platelets which expressed activation and adhesion molecule markers (CD41, CD36, CD62P). MPs were also found from RBCs, endothelial cells, monocytes, granulocytes and leukocytes. A low amount of tissue factor was also detected on MPs. The positve correlation between platelet factor 3 like activity and the number of annexin V-positive in MPs, platelets and RBCs population were found. While the highest correlation with MPs that expressed platelet activation markers were observed. This result suggests that MPs may be generated from activated platelets by the expression of PS on ineffective thalassemic RBCs since a correlation between them was found, and these MPs could be involved with coagulation state by PS and adhesion molecule expression which is consequent to clinical severity and risk of thrombosis in thalassemia patients.

KEY WORDS : MICROPARTICLES / THALASSEMIA / ORIGIN / PLATELET FACTOR 3-LIKE ACTIVITY

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การศึกษาเซลล์ต้นกำเนิดของไมโครพารติเคิ้ลในเลือดผู้ป่วยธาลัสซีเมีย (CELLULAR ORIGIN OF PERIPHERAL BLOOD MICROPARTICLES IN THALASSEMIC PATIENTS)

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บทคัดย่อ

ไมโครพารติเคิ้ล คือ ส่วนของผนังเซลล์ที่หลุดออกมาจากเซลล์ต่าง ๆ ในระบบหมุนเวียนเลือด อันเนื่องมาจากเซลล์นั้นถูกกระตุ้น หรือ เป็นผลมาจากกระบวนการสลายตัวของเซลล์ ผนังด้านนอกของไมโครพารติเคิ้ลจะมีโมเลกุลของฟอสฟาทิดิลเซอร์รินปรากฏอยู่ซึ่งฟอสฟาทิดิลเซอร์รินนี้มีประจุลบและเป็นตำแหน่งที่ทำให้เกิดการจับของโปรธอมบิเนส-คอมเพลคในกระบวนการแข็งของเลือดได้ ไมโครพารเคิ้ลนี้พบได้ทั้งในเลือดของคนปกติซึ่งจะมีปริมาณน้อย และพบปริมาณที่สูงขึ้นในผู้ป่วยที่มีอาการเกี่ยวข้องกับภาวะเสี่ยงต่อการแข็งตัวของเลือดสูง แต่ทั้งนี้ยังไม่เคยมีรายงานของไมโครพารติเคิ้ลในผู้ป่วยธาลัสซีเมียมาก่อน

ธาลัสซีเมียคือโรคทางพันธุกรรมที่เป็นผลมาจากการสร้างฮีโมโกลบินที่ผิดปกติ และมีรายงานถึงพยาธิสภาพแทรกซ้อนที่เกี่ยวข้องกับอุดตันของเลือด รวมถึงเคยมีรายงานถึงการปรากฏของฟอสฟาทิดิลเซอร์รินบนผนังเซลล์เม็ดเลือดแดงของผู้ป่วย และการหลุดของส่วนผนังเซลล์เกิดเป็นเวซิเคิ้ลของเม็ดเลือดแดงในกระแสเลือด อีกทั้งมีรายงานเกี่ยวกับภาวะของเซลล์ต่าง ๆ ถูกกระตุ้นในกระแสเลือด อันน่าจะเป็นสาเหตุให้เกิดไมโครพารติเคิ้ลได้ ดังนั้นการศึกษานี้จะใช้เทคนิคโฟลไซโทรมิเตอร์ศึกษาจำนวนของไมโครพารติเคิ้ล และเซลล์ต้นกำเนิดของไมโครพารติเคิ้ลเหล่านี้ จากนั้นนำไปหาความสัมพันธ์กับค่าการแข็งตัวของเลือด และค่าอื่น ๆ ทางโลหิตวิทยา

ผลการศึกษาพบว่าผู้ป่วยธาลัสซีเมีย โดยเฉพาะอย่างยิ่งผู้ป่วยชนิด เบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอี ที่ตัดม้ามแล้ว มีค่าร้อยละและค่าสัมบูรณ์ของไมโครพารติเคิ้ลในปริมาณสูงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับคนปกติ ($p<0.05$) และมีความสัมพันธ์กับค่าร้อยละของการปรากฏฟอสฟาทิดิลเซอร์รินบนผิวเม็ดเลือดแดง และบนผิวเกร็ดเลือดด้วย เซลล์ต้นกำเนิดของไมโครพารติเคิ้ลที่พบมากที่สุดคือ เกร็ดเลือด ซึ่งมีโมเลกุลที่แสดงการถูกกระตุ้นและใช้ในการยึดติด (adhesion moleclue) เช่น CD41, CD36 และ CD62P ปรากฏอยู่ นอกจากนี้ยังพบเซลล์ต้นกำเนิดมาจาก เซลล์เม็ดเลือดแดง, แอนโดทีเลียลเซลล์, โมโนไซท์ , แกรนูโลไซท์ และ เซลล์เม็ดเลือดขาวตามลำดับ อีกทั้งยังพบ ทิชชู แฟคเตอร์ปรากฏอยู่ในปริมาณเล็กน้อยด้วย จากการศึกษาพบความสัมพันธ์ของค่าการแข็งตัวของเลือดกับการปรากฏของฟอสฟาติดิลเซอร์รีนบนผิวของไมโครพารติเคิ้ล, เกร็ดเลือด และเซลล์เม็ดเลือดแดง นอกจากนี้ยังพบความสัมพันธ์ที่สูงของค่าการแข็งตัวของเลือดนี้กับปริมาณไมโครพารติเคิ้ลที่ปรากฏโมเลกุลของเกร็ดเลือดที่ถูกกระตุ้น ซึ่งสันนิษฐานว่าไมโครพารติเคิ้ลนี้อาจกำเนิดมาจากการเกิดภาวะเกร็ดเลือดถูกกระตุ้นจากฟอสฟาติดิลเซอร์รีนที่ปรากฏบนผิวของเม็ดเลือดแดง เนื่องจากการพบความ สัมพันธ์ของเปอร์เซ็นต์ฟอสฟาติดิลเซอร์รีนบนไมโครพารติเคิล เกร็ดเลือด และเซลล์เม็ดเลือดแดง จากการศึกษานี้ทำให้เราทราบข้อมูลเกี่ยวกับไมโครพารติเคิ้ลในผู้ป่วยธาลัสซีเมีย ทั้งด้านปริมาณร้อยละ, ค่าปริมาณสัมบูรณ์ และความสัมพันธ์กับค่าการแข็งตัวของเลือด ซึ่งอาจชี้ให้เห็นถึงสภาวะความเสี่ยงที่จะเกิดการอุดตันของเลือดในผู้ป่วยธาลัสซีเมียได้

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell cytotoxicity
ADP	adenosine diphosphate
ATP	adenosine triphosphate
α	alpha
BDB	Becton Dickinson Biosciences
β	beta
Ca	calcium
CBC	complete blood count
CD	cluster designation
Cl	chloride
DNA	deoxyribonucleic acid
δ	delta
EC	endothelial cell
ε	epselon
ELAM-1	E-selectin adhesion molecule-1
FPA	fibrinopeptide
FITC	fluorescein isothiocyanate
FL	fluorescence
FCS	forward scatter
γ	gramma
Hct	hematocrit
Hb	hemoglobin
HbCS	hemoglobin Constant Spring
HCII	heparin cofactor II
hr	hour
Ig	immunoglobulin
ICAM-1	intercellular adhesion molecule-1
kDa	kilo Dalton

LIST OF ABBREVIATIONS (cont.)

MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume micro
µl	microlite
µm	micrometer
MP	microparticle
nm	nanometer
βE	nonsplenectomized β-thalassemia
OD	optical density
PerCP	peridinin chlorophyll protein
φ	phi
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
PE	phycoerythrin
PAF	platelet activation factor
PF3	platelet factor 3
$F_{1,2}$	prothrombin fragment
ψ	psi
RBC	red blood cell
R	region
ROCK I	Rho-associated kinase I
SCD	sickle cell disease
SSC	side scatter
βES	splenectomized β-thalassemia
TAT	thrombin-anti trombin III
VCAM-1	vascular cell adhesion molecule-1
VWF	von Willebrand factor
WBC	white blood cell count
ζ	zeta

CHAPTER I

INTRODUCTION

The thalassemia syndromes are hereditary hemolytic diseases, which are found in many parts of the world, including Thailand (1, 2). The most prominent metabolic defect in thalassemia resulting from unbalance synthesis of globin chains of the hemoglobin molecule, leading to a relative excess of one of the globin chain: alpha chain in β- thalassemias and beta chain in the α-thalassemias (2, 3).

The pathophysiology relates primarily to the degree of anemia that is caused by both intramedullary hemolysis and red blood cell (RBC) destruction (2-4). Many clinical symptomes have been described in thalassemia patients, including infection, iron overload, pericarditis, abnormal endocrine function, hypoxemia, pulmonary artery occlusion, extravascular hemopoiesis and leg ulcer (2, 5-13). In addition, there have been numerous reports of thromboembolic complications associated with thalassemias (7, 14-19). Hemostatic changes have been observed in patients with β-thalassemia major and β-thalassemia intermedia and also in patients with α -thalassemia (hemoglobin H disease) (20, 21).

Both α and β thalassemic RBCs have an altered morphology and exhibit a decreased deformability besides its membranes which are more rigid than normal RBCs (22, 23). A comparison of normal RBCs with those isolated from thalassemia patients found that thalassemic RBCs can provide a source of negative charged phospholipid (expression of phosphatidylserine (PS) on outer leaflet) which is resulted from their loss of membrane phospholipid asymmetry (4, 14, 24, 25).

The presence of PS exposure of thalassemic RBCs is most likely physiologically important. They can provide a surface for enhancing hemostasis, activating of other blood cells including the platelets, monocytes and granulocytes alone or together and may induce activation of the vascular endothelium, which further contributes to the thrombotic process (4, 14). They also mediate the rapid removal of these RBCs from the circulation by macrophage, which are contributing to the anemia (14). Moreover, it has also been found that PS exposed on thalassemic

RBC vesicles. The studies have found that both percentage and absolute number of RBC vesicles in thalassemic patients are higher than in healthy subjects and relates to hematological parameters (26).

Microparticles (MPs) are membrane vesicles released by cells upon activation or during apoptosis (27-29). MPs can be easily released by the increase in cytosolic calcium concentration (29, 30). Since the process of MPs formation entails a loss of normal membrane lipid asymmetry in which aminophospholipids are confined to the inner leaflet of the cell membrane, MPs abnormally display PS on their outer leaflets (27). MPs of various cellular origins are presented in blood and typically beared cell membrane antigens that reflect their cellular origin (31). MPs have a potent pro-inflammatory effect, affect vascular function and promote coagulation (28, 32-34).

There are extensive studies regarding MPs in various diseases such as patients undergoing cardiac surgery or plasmapheresis and in patients suffering from diabetes, heparin-induced thrombocytopenia, myocardial infarction, uremia, idiopathic thrombocytopenic purpura, or thrombotic thrombocytopenic purpura (35-48). These patients have tendency to increase risk for thromboembiolic complication, and also have increased numbers of PS expressing MPs. MPs provide the catalytic surface necessary for the assembly of the procoagulant enzyme complexes, prothrombinase and tenase (14, 29). In the blood flow, the presence of high levels of procoagulant MPs, stemming from lysed RBCs, apoptotic cells, or activated platelets, therefore could be responsible for the dissemination of prothrombotic seats (49, 50). Although there have been studies about MPs in several diseases, but there is no specific study concerning thalassemias which also have reports of thromboembolic complications and have tendency to increase MPs in circulation by abnormal RBCs and activated cell in circulation (4, 15, 51, 52).

This study used flow cytometer to determine the absolute number and surface markers of MPs in whole blood from thalassemic patients. This study also relate MPs with hematological parameters and coaglation parameter, platelets factor 3 like activity. The result from this study provides a data on absolute number and cellular origin of MPs and their correlation with coagulable state in thalassemias and healthy subjects.

CHAPTER II

OBJECTIVES

1. To determine cellular origin of peripheral blood MPs in thalassemic patients.
2. To determine absolute number of peripheral blood MPs in thalassemic patients.
3. To determine the correlation between absolute number of peripheral blood MPs and hematological parameters in thalassemic patients.
4. To determine the correlation between the number of peripheral blood MPs and platelet factor 3-like activity in thalassemic patients.

CHAPTER III

LITERATURE REVIEW

1. Thalassemia

Thalassemia is a hereditary hemolytic disease characterized by absent or decreased production of normal hemoglobin (Hb), resulting in a microcytic anemia of varying degree (2, 3, 53). The thalassemia has a distribution concomitant with areas where *Plasmodium falciparum* malaria is common, probably because they provided partial protection against malaria (2). The thalassemia is found in many parts of the world. α thalassemia is more prevalent in Southeast Asia and Southern China. The β thalassemia is seen primarily in the areas surrounding Mediterranean Sea, Africa and Southeast Asia (2). In Thailand, there are prevalence of both α thalassemia, β thalassemia and two Hb variants including HbE and Hb Constant Spring (HbCS) (1, 2). Due to global migration patterns, there has been an increase in the incidence of thalassemia in North America in the last ten years, primarily due to immigration of thalassemia patients from Southeast Asia (2).

1.1 Hemoglobin

Hemoglobin is a soluble globular tetrameric protien of molecular mass of 64.5 kDa found within vertebrate RBCs, at a concentration of about 5 mM. The structure of Hb is similar, each consists of two separate pairs of globin chain. In the normal adult, HbA, which is composed of two α and two β globins (α2β2), comprising about 95% of all Hb. Two minor Hbs are HbA2 and HbF. HbA2 composed of two α and two delta (δ) globins (α2δ2) comprises 2-3.5% of Hb, while HbF, composed of two α and two gamma (γ) globins (α2γ2), comprises less than 2% of Hb (2).

During early development of fetus, there are several embryonic Hbs. However, the main Hb during intrauterine life is HbF or fetal Hb. HbF has a high oxygen-affinity in order to attract oxygen from maternal blood and deliver it to the fetus. After birth, the production of adult Hb rapidly increases and HbF production drops off (2).

In normal assembly of adult Hb (HbA - α2β2), α and β globin are synthesized by genes on different chromosome: chromosome 11 and chromosome 16, whereas heme is synthesized primarily on mitochondria. The β-like globin chains are controlled by gene cluster on chromosome 11 in which the different genes are arranged in the order 5'-ε-^{G}γ-^{A}γ-ψβ-δ-β-3'. The α-like gene cluster is on chromosome 16 and the genes are arranged in the order 5'-ζ-ψζ-φα-α2-α1-θ-3'. The α globin molecule concentration is rather stable in fetal and adult life, because it is needed for both fetal and adult Hb production. The β globin appears early in fetal life at low levels and begins to rapidly increase after 30 weeks gestational age, reaching a maximum about 30 weeks postnatally. The γ globin molecule reaches a high level early in fetal life at about 6 weeks and begins to decline about 30 weeks gestational age, reaching a lowest level about 48 weeks postgestational age. The δ globin appears at a low level at about 30 weeks gestational age and maintains a low profile throughout life (2, 53).

2. Clinical Severity and Pathophysiology of Thalassemia

A mutation or deletion of the genes that control globin production leads to a decreased production of the corresponding globin chains and (or) an abnormal Hb. This abnormal Hb synthesis leads to the expression of thalassemia (2, 53). The globin that is produced in normal amounts winds up in excess and forms RBC aggregates or inclusions. These aggregates become oxidized and damage the cell membrane, leading either to hemolysis, ineffective erythropoiesis, or both. The quantities and properties of these globin chain aggregates determine the characterstics and severity of the thalassemia. The result from the RBC defects cause many clinical symptoms in thalassemic patients including autoimmune hemolytic anemia, infection, iron overload, leg ulcer, gall stone, pericandritis, abnormal endocrine function, extravascular hemopoiesis, mineral and vitamin disturbances, hypersplenism, post-transfusion hypertension, hypoxemia and pulmonary artery occlusion (3, 7, 8, 13, 16, 18, 19, 53-61).

In clinically, thalassemia can be classified into 3 forms depend on the degree of severity, namely: thalassemia minor, thalassemia intermedia and thalassemia major. Thalassemia minor or thalassemia trait that occur in subjects who are heterozygous for a thalassemia gene on the chromosome, such as homozygous α-thalassemia 2 and α-

thalassemia/β-thalassemia double heterozygous, are clinically and hematologically completely silent. The second form is thalassemia intermedia which may involve in defective of many genes on chromosomes and describes conditions that are associated with a more severe degree of anemia than the trait, although they are not as severe as the major forms, thalassemia intermedia includes β-thalassemia/Hb E, HbH disease and homozygous HbCS. Finally, the thalassemia major which occurs in subjects who are homozygous for the thalassemia gene, such as Hb Bart's hydrops fetalis, homozygous β-thalassemia and β-thalassemia/Hb E. The thalassemia major is the most severe disorder of all thalassemia forms characterized by hypochronic anemia and have to receive constant medical care and supportive therapy involving principally blood transfusion (2, 3, 53).

2.1 α-Thalassemia

The α-thalassemia is caused by a decrease in production of α globin chains and results in an excess of β globins. The α-thalassemia can be generally categorized as: Silent carrier (-α/αα) which will experience no health problems in his/her lifetime, α-thalassemia trait [(-α/-α) or (--/αα)] which are identified by microcytosis, erythrocytosis, hypochromia, and mild anemia. The individual with a thalassemia trait will experience no significant health problems except a possible slight anemia which cannot be treated with iron. HbH disease (--/-α) is resulting from the formation of β globin tetramers (β4) called HbH. These tetramers are more stable and soluble, but under special circumstances can lead to hemolysis, generally shortening the life span of the RBC. Conditions of oxidative stress cause HbH to precipitate, interfering with membrane function and leading to RBC breakage. HbH-Constant Spring (HbH/CS) (--/α^{cs} α) is an elongated α globin due to a termination codon mutation and is a more severe form of this hemolytic disorder. Finally, α-thalassemia major (--/--) or hydrops fetalis is the most severe α-thalassemia, in which a fetus produces no β globins, which is generally incompatible with life (2, 3, 53).

2.2 β-Thalassemia

β-thalassemia results in an excess of α globins, which leads to the formation of α globin tetramers (α4) that accumulate in the erythroblast (immature RBC). These aggregates are very insoluble and precipitation interferes with erythropoiesis, cell maturation and cell membrane function, leading to ineffective erythropoiesis and

anemia. Unlike the deletions of globin gene that constitute most of the α-thalassemia syndromes, β-thalassemia is caused by over 150 diverse mutations on chromosome 11 that affect all aspects of β globin production: transcription, translation, and the stability of the β globin product, so the β-thalassemia syndromes are much more diverse than the α-thalassemia syndromes. There are three main clinical phenotypes of β-thalassemia. The first is β-thalassemia trait which due to a single β-thalassemia allele mutation and is usually asymptomatic. Second, β-thalassemia intermedia which have a homozygous or heterozygous β globin mutation. In this condition, the lack of β protein in the Hb is great enough to cause a moderately severe anemia and significant health problems, including bone deformities and enlargement of the spleen, but not to the degree that chronic transfusion therapy is required. Finally, β-thalassemia major resulted from the inheritance of two β-thalassemia alleles (homozygous or compound heterozygous) causes a life-threatening anemia that requires regular blood transfusions and extensive ongoing medical care. These extensive, lifelong blood transfusions lead to iron-overload which must be treated with chelation therapy to prevent early death from organ failure. In β-thalassemia, it is clear that important factors include the severity of the particular α-thalassemia allele, or alleles in compound heterozygotes, the co-inheritance of different forms of β-thalassemia, and varying ability to produce HbF (2, 3, 53, 62).

The interaction of excess α- and β-globin chains with membrane products cause different cellular changes that contribute to the difference in the pathophysiology of α- and β-thalassemia. This altered deformability is in part due to rigidity of their membranes and the state of hydration of these cells. Thalassemic RBCs present PS-exposing subpopulations of that are most likely physiologically important. They could provide a surface for enhancing hemostasis as hypercoagulable state found in many studies. Because such exposure of PS on RBCs may mediate the rapid removal of these RBCs from the circulation, thereby contributing to the anemia. Morover, potential consequences of RBC–PS exposure in these hematologic disorders include an exacerbation of the anemia owing to enhanced phagocytic recognition and removal of PS-positive RBCs, cell apoptosis, and activation of coagulation because surface PS provides a "docking site" for the involved hemostatic proteins (3, 4, 17, 24, 25, 27).

3. Thalassemia and Hypercoagulation

There have been many reports of thromboembolic complications associated with thalassemia (6, 7, 14, 18, 19, 54, 61, 63-65). The thromboembolic complications include recurrent and transient ischaemic cerebral attacks, strokes as well as deep venous thrombosis, pulmonary embolism, and recurrent arterial occlusion (18, 19, 54). In many studies, they observed hemostatic changes in patients with both β-thalassemia major, β-thalassemia intermedia and also in α-thalassemia (HbH disease). In many surveys, the prevalence of thrombosis was 1.1 to 29 % in thalassemic patients. The autopsy findings in patients with thalassemia have clearly demonstrated hypercoagulability as a pathologic feature. They also found atherosclerotic changes and obstructive lesions consisting of organized, recanalized thrombi in the pulmonary arteries and microvasculature (14, 15, 17).

Thalassemic patients, who have low levels of protein C and protein S, show enhanced platelet consumption, and ongoing platelet, monocyte, granulocyte, and endothelial activation. Increased plasma levels of activation peptides, thrombin-ATIII (TAT), prothrombin fragment ($F_{1.2}$), fibrinopeptide A (FPA), and D-dimer, are suggestive of continuous thrombin generation and enhanced fibrinolysis. This suggestion is supported by autopsy findings of platelet and fibrin thrombi in the microvasculature in the lungs and the brain. These thrombi could contribute to the pulmonary hypertension, low lung capacity, hypoxemia, and diffusion defects associated with right heart failure (cor pulmonale) and to the high frequency of ischemic brain lesions associated with asymptomatic brain damage (14, 16, 64, 66).

Thalassemia is associated with partial or complete deficiency of α or β globin chain synthesis, which lead to denaturation and degradation of the remaining globin chains. These processes are associated with loss of the normal asymmetrical distribution of the RBC membrane phospholipids and translocation of PS to the external membrane leaflet (flip-flop). The membrane damage may be related to lipid peroxidation mediated by free iron and increased amounts of membrane-bound hemichromes and immunoglobulins and modifications in the membrane band 3 protein and spectrin. The membrane changes may partly explain the enhanced aggregation of PS-exposing RBCs, their increased adherence to endothelial cells (ECs), and their capacity to enhance thrombin generation via the assembly of the prothrombinase

complex. The enhanced thrombin generation leads to activation of platelets, monocytes, granulocytes, and ECs and expression of tissue factor, which further enhances the thrombotic process. The low levels of the coagulation inhibitors, protein C and protein S, further facilitate the resultant hypercoagulable state (4, 14, 24, 25, 27, 66-69). (Figure 1)

3.1 Hemostatic Changes in Thalassemia

Hemostatic changes have been observed in patients with β-thalassemia major and β-thalassemia intermedia and also in patients with α-thalassemia (HbH disease) (14, 16, 20). Defective platelet aggregations were found in response to adenosine diphosphate, epinephrine, or collagen in β-thalassemia major (70). Most of the patients had undergone splenectomy and had high platelet counts. There are found increased circulating platelet aggregates in splenectomized and nonsplenectomized patients with β-thalassemia/HbE disease, an observation compatible with in vivo platelet activation and the existence of a hypercoagulable state (71). Thalassemia platelet life span was significant shorter than normal platelet. The mean platelet life span in patients who underwent splenectomy was 107 ± 36 hours compared to 248 ± 51 hours in healthy individuals who underwent splenectomy because of trauma (p<0.001). The mean platelet life span in nonsplenectomized patients was 102 ± 64 hours compared to 224 ± 23 hours in healthy individuals (p<0.01) (72). These data suggested that the shortened platelet life span was caused by enhanced platelet consumption, a feature usually associated with active thrombotic disease, severe atherosclerosis, diabetes mellitus, and other chronic hypercoagulable states (64, 72, 73).

The existence of chronic platelet activation in thalassemia was further confirmed by flow cytometric studies, which demonstrated the presence of an increased fraction of platelets carrying the activation markers CD62P (P selectin) (14, 69). In addition, morphologic changes in thalassemic platelets, elevated plasma platelet factor 3 (PF3), and increased spontaneous whole blood platelet aggregation were reported (71, 73, 74). Chronic platelet activation is also presented in β-thalassemia major and β-thalassemia intermedia. The presence of morphologic platelet abnormalities in splenectomized patients with β-thalassemia/HbE disease may also contribute to an enhanced risk of vascular complications (70, 75).

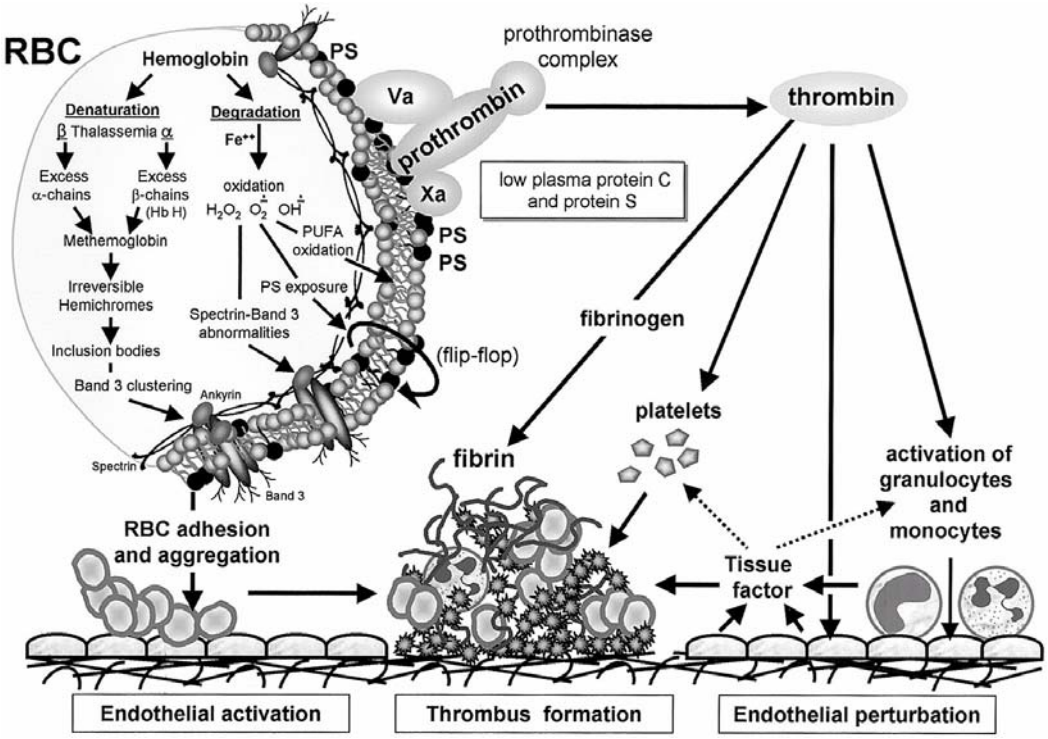


Figure 1 The hypercoagulable state in thalassemia. Thalassemia is associated with partial or complete deficiency of α- or β-globin chain synthesis, which leads to denaturation and degradation of the remaining globin chains. This process is associated with loss of the normal asymmetrical distribution of the RBC membrane phospholipids and translocation of PS to the external membrane leaflet (flip-flop). The membrane damage may be related to lipid peroxidation mediated by free iron and increased amounts of membrane-bound hemichromes and immunoglobulins and modifications in the membrane band 3 protein and spectrin. The membrane changes may partly explain the enhanced aggregation of PS-exposing RBCs, their increased adherence to Ecs, and their capacity to enhance thrombin generation leads to activation of platelets, monocytes, granulocytes, and ECs and expression of tissue factor, which further enhances the thrombotic process. The low levels of the coagulation inhibitors, protein C and protein S, further facilitate the resultant hypercoagulable state (15).

Detection of elevated levels of endothelial adhesion proteins (intercellular adhesion molecule-1 [ICAM-1], E-selectin adhesion molecule-1 [ELAM-1], vascular cell adhesion molecule-1 [VCAM-1], von Willebrand factor [VWF], and thrombomodulin) in serum and plasma of thalassemic patients suggested that endothelial activation or injury may be a feature of the disorder (51, 76). The adherence of RBCs to ECs correlates with microvascular occlusions in sickle cell disease (SCD) and malaria and is considered a major contributor to microcirculatory disorders (77). RBCs from patients with β-thalassemia major and β-thalassemia intermedia showed enhanced adhesion to cultured ECs (10-to 25-fold increase compared to normal RBCs) (78). Similar findings were described in SCD where the interaction of sickle RBCs with ECs induced a state of oxidative stress leading to enhanced transendothelial migration of blood monocytes (77-80).

Activated granulocytes could also contribute to the endothelial damage and the hypercoagulable state in thalassemia. Elevated granulocyte phagocytic function, as manifested by enhanced chemiluminescence, was observed in patients with β-thalassemia major, with greater prominence of the abnormality in patients (53). Removal of leukocytes from transfused blood with a Leukostop filter resulted in improved pulmonary function tests (forced expiratory volume in 1 second/forced vital capacity ratio) in patients with β-thalassemia major, 6 months after the procedure (81). This clinical observation illustrates the deleterious effect that activated granulocytes can induce in the lungs of patients with thalassemia.

Monocyte activation may also play a significant role in heightening endothelial activation or injury in thalassemia. In patients with HbH disease and β-thalassemia major, a high serum levels of monocyte colony-stimulating factor and increased monocyte phagocytic activities (antibody-dependent cell cytotoxicity [ADCC]) toward RBCs were found (81).

Several investigators have reported profound changes in the levels of coagulation factors, coagulation factor inhibitors, and components of the fibrinolytic system. Studies of the coagulation proteins provide strong evidence for the existence of a chronic hypercoagulable state in thalassemia (15, 17, 66, 82).

Plasma prothrombin levels were significantly lower in β-thalassemia major compared to healthy controls (17). Low levels of the coagulation inhibitors, protein C and protein S, have been observed in patients with β-thalassemia from a variety of ethnic backgrounds. The decreased levels of free protein S were also found in thalassemia patiens (15, 17, 66, 82). Low levels of heparin cofactor II (HCII), known to be associated with increased thrombotic risk, have been found in thalassemic patients. Frequent blood transfusions resulted in a slow normalization of HCII levels, suggesting that the low HCII levels could be related to increased RBC turnover that had been suppressed by hypertransfusion (83).

The possibility of a genetic basis for the hypercoagulable state in thalassemic patients seems unlikely because a study of congenital thrombophilic mutations were not found an increase prevalence in thalassemia patients (17).

The existence of a chronic and lifelong hypercoagulable state in thalassemia was further supported by the elevated levels of TAT complexes found in β-thalassemia major whom none or had any clinical signs of overt thrombosis. Significantly elevated levels of $F_{1.2}$ and fibrinopeptide A (FPA) were found in splenectomized patients with β-thalassemia intermedia, and these patients also had high plasma D-dimer levels, a manifestation of enhanced fibrinolysis. Elevated TAT levels were also observed in patients with α-thalassemia (15, 17).

3.2 Contribution of the Hypercoagulable State in Thalassemia.

In thalassemic patients, they may provide a source of negatively charged phospholipids from the RBC, platelet, RBC vesicle and may be MPs like a SCD which can increase thrombin generation, as measured by prothrombinase assay or flow cytrometry (17, 24-26, 68, 69, 84). The procoagulant effect of thalassemic RBCs seems to be due to an increased surface expression of anionic phospholipids such as phosphatidylethanolamine (PE) and PS. This was demonstrated by experiments that showed that annexin V, which binds anionic phospholipids, could block the procoagulant effect of isolated thalassemic RBCs (85). The loss of the normal asymmetrical distribution of the RBC membrane phospholipids and translocation of PS to the external membrane leaflet (flip-flop) may serve as a signal for their recognition and removal by the reticuloendothelial system, like an aged RBCs contain

higher amounts of PS on the outer leaflet of their membranes compared to young cells (4, 86).

The membrane damage of thalassemic RBC may be related to lipid peroxidation mediated by free iron and increased amounts of membrane-bound hemichromes and immunoglobulins and modifications in the membrane band 3 protein and spectrin (87-89). Exposure of PS on thalassemic RBCs increases their adherence to ECs and enhances thrombin generation via the assembly of the prothrombinase complex. The enhanced thrombin generation leads to activation of platelets, monocytes, granulocytes, ECs and expression of tissue factor, which further enhances the thrombotic process (77). The low levels of the coagulation inhibitors, protein C and protein S, further facilitate the resultant hypercoagulable state (66).

Moreover, in the thalassemic patients, a highly significant correlation was found between the number of RBC-bound annexin V molecules and the fraction of CD62P (P selectin) platelets. These results support the idea that the procoagulant surface of thalassemic RBCs promotes thrombin generation in vivo leading to platelet activation (69).

Thalassemic RBCs were demonstrated to enhanced cohesiveness, which may contribute to the hypercoagulable state. Using a novel image analysis system to measure RBC aggregation in a flow chamber, an increased cohesion of thalassemia RBCs was detected, demonstrated by the formation of large aggregates (91). It is noteworthy that RBC aggregate size was reduced to normal after patients received a blood transfusion and this observation was confirmed by in vitro experiments where the addition of normal RBCs to thalassemic RBCs resulted in reduced aggregation under flow (91). These in vitro findings could partly explain the recent clinical observation that patients with β-thalassemia intermedia who do not receive transfusions regularly had a much higher incidence of thrombotic events compared to the incidence of such events in those receiving regular transfusions (16).

The contribution of the abnormal RBCs to the thrombotic process has been also demonstrated in animal models of congenital hemolytic anemias (92-95). A lethal hypercoagulable state manifested by large thrombotic lesions in the heart and the liver and large venous thrombi was found in mice in which the expression of erythroid band 3 had been eliminated via targeted mutagenesis (92). The abnormal RBCs from these

mice significantly shortened the Russell viper venom clotting time of normal plasma in a dose-dependent fashion, whereas RBCs from normal mice had no effect. These experiments suggested that the membrane of band 3 null RBCs provides a suitable surface for activation of the prothrombinase complex and, indeed, PS exposure on the outer membrane leaflet of the affected RBCs was demonstrated by increased FITC-annexin V binding. A high incidence of thrombosis in the heart and brain was also found in α-spectrin and β-spectrin-deficient mice with hereditary spherocytosis (93). Thrombosis incidence in these animals was significantly reduced following the transfusion of normal RBCs or transplantation of normal bone marrow (94). The presence of normal RBCs in the peripheral circulation of these α-spectrin-deficient mice prolonged the survival of young animals and abrogated the development of thrombosis in adult animals (94).

4. Microparticles

Microparticles (MPs) are membrane vesicles released from many different cell types, vary in size (submicron elements from 0.1 to 1 μm), phospholipid and protein composition depends on their cellular origin and the cellular process triggering their formation (29, 32, 35, 41, 96). MPs are hallmark of cellular alterations that are shed from the plasma membrane of most eukaryotic cells undergoing activation and apoptosis. In vitro study shown that MPs were released from ECs, vascular smooth muscle cells, platelets, leukocytes, lymphocytes and RBCs (30, 32, 36, 96, 97). Some of these MP populations occur in the blood of healthy individuals and patients who have an increase risk for thromboembiolic composition in many disease such as patients undergoing cardiac surgery, patients undergoing plasmapheresis, patients suffering from diabetes, heparin-induce thrombocytopenia, myocardial infraction, uremia, Idiopathic thrombocytopenia purpura, meningococal sepsis, patients with lupus anticoagulant, multiple sclerosis, preeclampsia, acute coronary syndromes and severe hypertention patients. They have a potent pro-inflammatory effect, promote coagulation and affect vascular function (32, 35, 36, 38, 40, 41, 46, 50, 98-104).

4.1 Plasma Membrane Composition and Structure

The plasma membrane of eukaryotic cell is phospholipid asymmetry membrane, the outer leaflet is formed predominantly with the cholinephospholipids

(sphingomyelin and phosphatidylcholine [PC]), whereas the aminophospholipids (PS and phosphatidyleethanolamine [PE]) are formed in the inner leaflet. This selective localization dictates that asymmetric biomembranes are assembled and maintained by specific mechanisms that control transbilayer lipid sidedness. Normal membrane lipid asymmetry is regulated by the cooperative activities of three transporters. The ATP-dependent aminophospholipid-specific **translocase**, which rapidly transports PS and PE from the cell's outer-to-inner leaflet and they are targets for Ca^{2+} that directly regulates the transporter's activities.; the ATP-dependent nonspecific lipid **floppase**, which slowly transports lipids from the cell's inner-to-outer leaflet; and the Ca^{2+}-dependent nonspecific lipid **scramblase**, which allows lipids to move randomly between both leaflets (28).

4.2 Generation of Microparticles

There are two mechanisms which different pathways are involved in MP generation, cell activation and apoptosis (29, 30, 96) (Figure 2). The activation can be induced by many agonist such as thrombin, calcium ionophore A23187 ADP plus collagen, the terminal complement complex C5b-9, shear stress, bacterial lipopolysaccharides, cytokines such as tumor necrosis factor-α or interleukin-1, and hydroperoxide. When the cell is activated, there is an increase of cytosolic calcium concentration, especially at the site of vesiculation. Increased cytosolic calcium can activates kinases, inhibits phosphatases and activates calpain. MP formation requires the breakdown of the membrane skeleton, the subcellular system that provides the cell membrane with structural stability. This skeleton mainly consists of actin, vinculin and talin. Talin is degraded by calpain, which is one of the direct pathways through which the increased cytosolic calcium concentration. Chelation of extracellular calcium ions can blocks the increase in cytosolic calcium as well as the release of MP. Thus the increase in cytosolic calcium is essential for MP release (29, 30, 96).

Another pathway of MP generation is cell apoptosis. Apoptotic membrane blebbing depends on activation of the Rho-associated kinase, ROCK I, which is cleaved by activated caspase. ROCK I promotes increased actin–myosin force generation, couples actin–myosin filaments to the plasma membrane and disruption of the membrane skeleton structure, thought to drive the formation of membrane blebs.

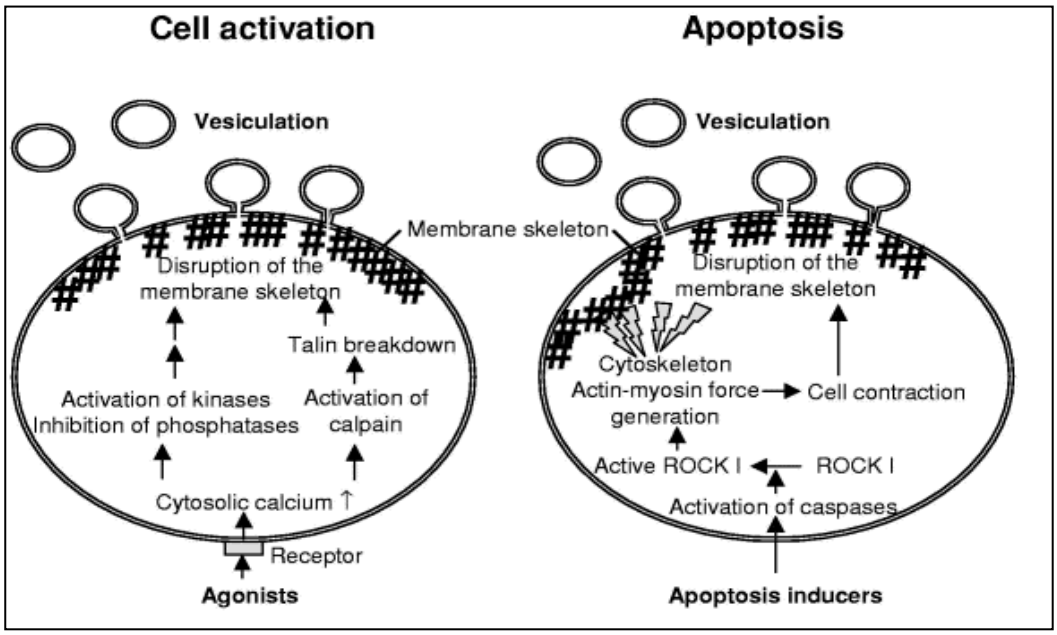


Figure 2 Schematic representation of general mechanisms involved in microparticle formation during cell activation (left panel) and apoptosis (right panel) (29).

ROCK I activity and, as a consequence, membrane blebbing are required for redistribution of fragmented DNA from the nuclear region into the membrane blebs and apoptotic bodies. Such MPs may contain fragmented DNA and may differ from MPs formed by cell activation in size, lipid and protein composition and (patho-) physiological effects (30, 97).

4.3 Origin of Microparticles

From the studies of numbers and cellular origin of MPs in the blood of healthy men and women show that circulating MPs were mainly derived from platelets, but also from RBCs, leukocytes and ECs. MPs have also been studied in various disease states, in which numbers, cellular source and composition are altered (29, 32, 96).

5. Clinical Significance of Microparticle

MPs may have various (patho-)physiological functions, namely transport of membrane components from the parent cell to other cells and (in-)direct activation of inflammation, coagulation or vascular function. (Figure 3)

5.1 Coagulation

Coagulation requires not only activated coagulation factors and calcium ions, but also membranes exposing negatively charged phospholipid, such as PS. Exposure of PS facilitates binding of activated coagulation factors to the membrane, thereby enabling the formation of tenase and prothrombinase-complexes. MPs have a negatively charged phosholipid surface, readily bind activated coagulation factors and expose tissue factor in various condition. Both in vitro and in vivo generated MPs initiate and support thrombin generation in vitro, and the numbers of MP in blood are correlate with the $F_{1,2}$ in vivo in many diseases. Furtermore, infusion of artificially prepared phospholipid vesicles triggers the development of severe disseminated intravascular coagulation in primates, and infusion of these vesicles in pregnant rats induces placental congestion and growth restriction in offspring. Moreover the negative charge on MPs, P-selectin which is often presents on platelet-derived MPs is likely to induce tissue factor expression by monocytes. Thus MPs may also indirectly promote coagulation (28, 49, 50, 105-109).

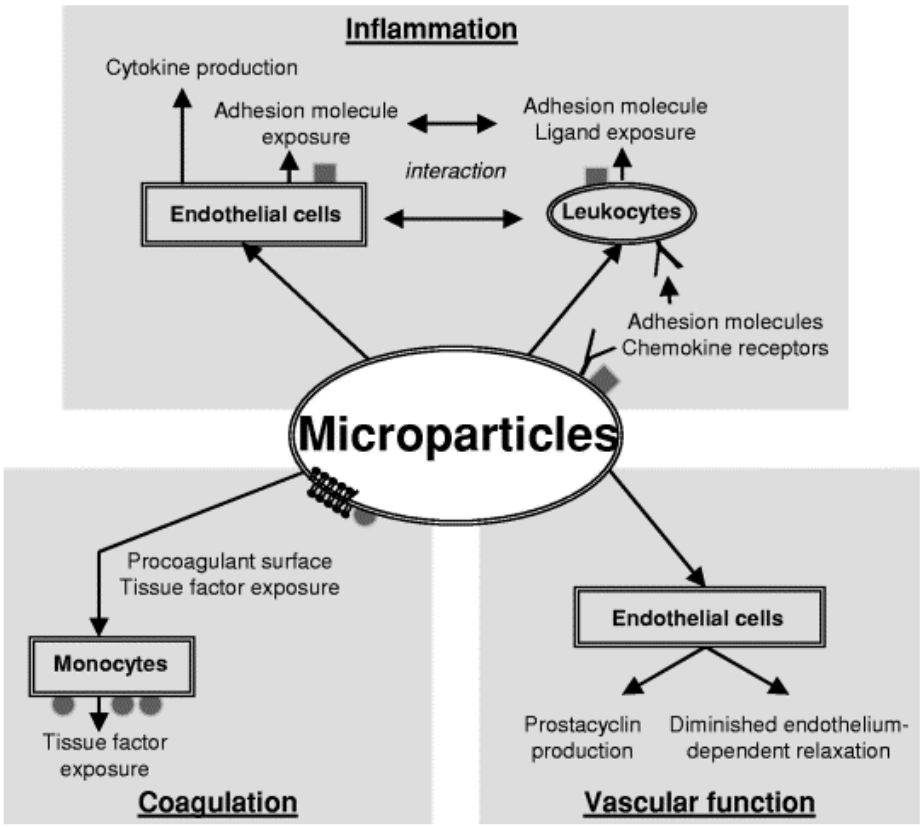


Figure 3 Schematic representation of functions attributed to microparticles.(29)

5.2 Inflammation

Adhesion of monocytes and neutrophils to the endothelium is an early event in inflammation and susequent transendothelial migration of the leukocytes. Specific adhesion receptors exposed on endothelial cells attract various classes of leukocytes to the vascular wall. The ligands for these receptors are present on leukocytes as well as present on the leukocyte-derived MPs, which of these ligands are involved in the adhesion of leukocyte-derived MPs to endothelial cells. In addition, endothelial-derived MPs are also promote monocyte-endothelial cell binding. Platelet-derived MPs can upregulate adhesion molecules on monocytes and endothelial cells resuting in an increase cellular interaction as well as an increase phagocytic activity and increase leukocyte-leukocyte binding (110-112).

Endothelial-derive MPs can induce neutrophils activation and chemotactic attraction of leukocytes. The MP derived from peripheral mononuclear cells can transfer chemokine receptor to another mononuclear cells, monocytes and endothelial cells (102, 103, 113). Platelet-derived MPs may initiate inflammation. They deliver arachidonic acid to endothelial cells, which resulted in regulation up of ICAM-1 and the subsequent adhesion of monocyte. Moreover, MPs trigger the release of cytokines by endothelium which is the subsequent events when leukocytes are bound to the vascular wall and migrate into intima. The secreted cytokines and growth factors promote the migration and proliferation of vascular smooth muscle cells and thus plaque formation (114, 115).

The components of MPs that are involved in inflammation may be oxidized phospholipids. Oxidized phospholipids may form (one of) the biologically active components of MPs that cause monocyte adherence to endothelial cells and neutrophils activation. Oxidized phospholipids exert their actions through platelet activation factor (PAF) receptor, which are exposed on both endothelial cells and leukocytes. The exact pathways of oxidized phospholipid are not yet clarified. MPs are also capable of delivering arachidonic acid to endothelial cells, monocytes and platelets. Thus, MPs are actively involved in inflammatory processes (109, 110, 116).

5.3 Vascular Dysfunction

There are many studies reported the effects of MPs on endothelial activation and function in vitro (29, 50). In in vivo studies, MP numbers are elevated or the

composition of the MP population is altered in cardiovascular diseases that are characterized by endothelial dysfunction, such as acute coronary syndromes, hypertension, artherosclerosis and pre-eclampsia. Also, high levels of presumably apoptotic MPs are presented in atherosclerotic plaques. These MPs are mainly derived from monocytes and lymphocytes. Almost all tissue factor activity in the plaque is located on these MPs (29).

5.4 Hypertension

The study investigated the effects of extreme blood pressure elevation by using endothelial- and platelet-derived MPs as a marker of endothelial and platelet activation. They found that the endothelial MPs values were elevated in the severe hypertension group at highest risk for acute vascular target organ injury compared with the mild hypertension and control groups. They also found a strong, positive correlation of endothelial MPs with the level of both systolic and diastolic blood pressure. Moreover, endothelial MPs also correlated with the presence of diabetes mellitus and smoking, factors also known to produce endothelial activation/injury. Platelet-derived MP values were also elevated in the high-risk, severe hypertension group but not the mild hypertension and control groups. They also found a strong correlation of platelet-derived MPs with the absolute level of blood pressure (117-119).

6. Platelet Factor 3

Thrombin generation is the culminating event of the coagulation cascade. It is initiated after the expression of tissue factor by endothelial cells and monocytes exposed to thrombogenic stimuli. Anionic phospholipids, chiefly PS, are necessary for the optimal activity of tissue factor and completion of the clotting process. They display a catalytic potential by allowing the formation of the characteristic enzyme complexes at the membrane surface (120).

After thrombin generation, thrombin autoamplification is exerted through feedback activation loops involving either coagulation factors or platelets. A new pharmacological approach of thrombosis is presented, based on the control of the exposure of procoagulant phospholipids and membrane MP shedding (120, 121).

Platelets are viewed as the main source of procoagulant phospholipid referred to as platelet factor 3. PF3 is a platelet membrane component that plays an important role in the activation of the coagulation mechanism. Whenever platelet activation occurred, PF3 is released and participates in thrombin formation (120-122).

The plasma membrane of resting cells presents an asymmetrical distribution of phospholipids, aminophospholipids being sequestered in the inner leaflet. Procoagulant phospholipids become available at the outer surface after cell stimulation. The collapse of the membrane asymmetry is thought to promote a phospholipid scrambling accompanied by the shedding of MPs. The plasma membranes of such vesicles bear irreversibly externalized procoagulant PS and contain glycoproteins that testify to their tissue origin. Hence, MPs could disseminate a dual procoagulant and adhesive potential including PF3 like activity. This is confirmed by the study in RBC which show that RBC membrane fraction has also some PF3 like activity, and in abnormal RBC membrane disorders, eg thalassemia, some of the membrane fraction accelerates platelet activation by increasing the PF3 activity (120-122).

Opartkiattikul et al. developed a method for determination of PF3 activity in whole blood, which could detect changes in PF3 activity with time. The principles of this method are the amount of thrombin generated in a fixed reaction time correlates with the amount of PF3 and to avoid inhibition of thrombin activity by antithrombin III. The intrinsic coagulation pathway is activated by elagic acid and $CaCl_2$. A synthetic thrombin inhibitor, MD 805, was added to the system for irreversible process and the activity of thrombin generated was measured by synthetic thrombin substrate S-2238 using A405 as an indicator of the availability of PF3 (74, 123).

CHAPTER IV

MATERIALS AND METHODS

1. Blood Sample Collection

Four milliters of venous blood samples from 35 healthy volunteers and 148 thalassemic patients were collected after informed consent and divided in 2 tubes; 2 ml of blood were added into sodium heparin containing tube (Sigma, MO, USA) and another 2 ml were added in 3.8% sodium citrate tube (Sigma, MO, USA). One hundred and twenty four thalassemic patients who have been diagnosed by using electrophoresis and/or molecular method consist of 38 β-thalassemia/HbE (splenectomy), 53 β-thalassemia/HbE (nonsplenectomy), 34 hemoglobin H disease (HbH) and 23 HbH constant spring (HbH/CS). Patients were free from blood transfusion at least 3 months.

2. Complete Blood Count (CBC)

The complete blood count was performed after blood sample collection by automate hematological analyzer SysmexK-800 (TOA Medical Electronic, Kobe, Japan) at the Division of Hematology, Faculty of Medicine Siriraj Hospital. The hematology parameters include RBCs, white blood cell count (WBCs), platelet count, Hb, hematocrit (Hct), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC).

3. Flow Cytometric Analysis

3.1 Percentage Calculation of PS-Positive Events of Microparticles, Platelets and RBCs in Peripheral Blood

Two microliters of heparinized blood samples were added into polystyrene tube containing 2 μl of fluorescein isothiocyanate (FITC)-conjugated annexinV (Becton Dickinson Biosciences (BDB), San Jose, CA, USA) or anti-Mouse IgG1-

FITC/IgG2-conjugated phycoerythin (PE) (BDB) for negative control. Samples were stained immediately, or at 4 hr and 24 hr after blood collection for time dependent test. Then, 94 µl of annexinV binding buffer was added and incubated in the dark at room temperature for 15 minutes. After that, 300 µl of annexinV binding buffer was added into the stained blood samples and analyzed by BDB FACSort flow cytometer. Acquisition and data analysis were performed using CellQuest Software (BDB). The light scatter and fluorescence channels were set at logarithmic gain. The MP population was defined by size in forward and side scatter dot plot (Figure 4, R1) followed by their morphology which was smaller than platelets and RBC population in whole blood sample (presented in region R1). The platelet population and RBCs were represented in region R2 and region R3 respectively. From the population of MPs in region R1, platelets in region R2 and RBCs in region R3, the annexinV-positive events of MPs, platelets and RBCs were analyzed by histogram plot as represented in positive area curve (M2) and the percent of annexinV-positive was obtained by histogram statistics.

3.2 **Absolute Number of AnnexinV-Positive Peripheral Blood Microparticle Calculation**

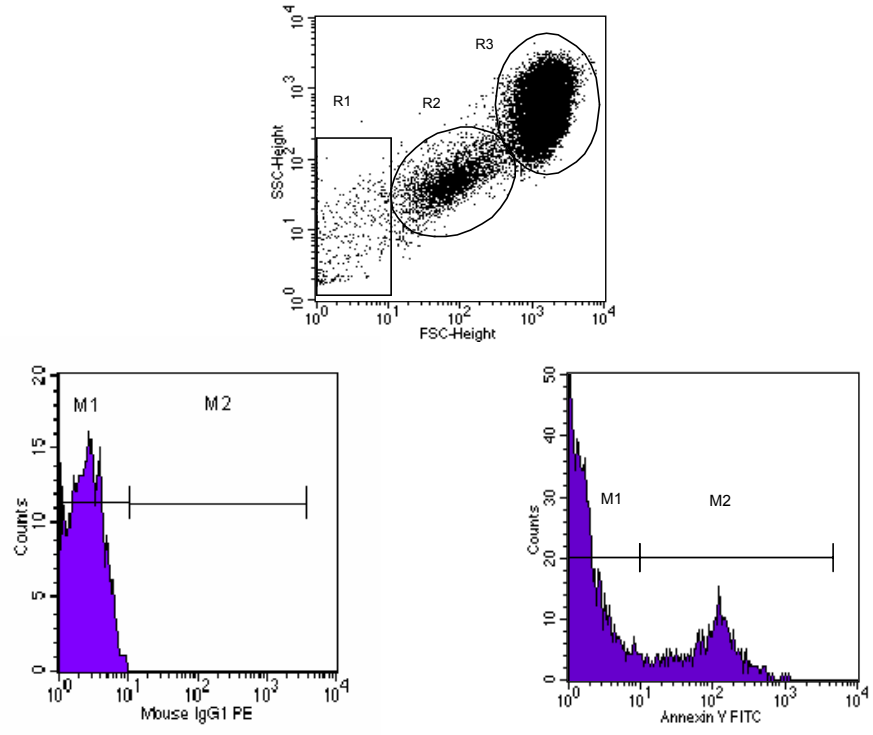
TruCount tube (Lot No. 57004, Bead Count 51440, BDB) which contains a lyophilized pellet of 4.2 µm fluorescent dye beads with known density was used for calculation of absolute annexinV-positive MPs.

Two microliters of heparinized blood sample (diluted 1:4 by annexin V binding buffer) were stained with 2 µl of annexinV-PE (BDB) to distinguish MPs from events due to nonspecific noise. For internal quality control of the method, two microliters of monoclonal antibody directed against platelet specific antigen, CD41, (PharMingen, BDB) conjugated with FITC were added for calculating absolute platelet number, and compared with platelet count from the complete blood count (CBC) using automate hematological analyzer. After adding 94 µl of annexin binding buffer and incubation at room temperature for 15 minutes in the dark, 50 µl of stained blood was added into the TruCount tube (BDB). Samples were analyzed by BDB FACSort flow cytometer. Acquisition and data analysis were performed using

CellQuest Software (BDB). The light scatter and fluorescence channels were set at logarithmic gain. The MP population was presented in region R1 (Figure 5) and the annexinV-possitive MP population was represented in region R2. The fluorescent bead events were gated by using FL1 and FL2 dot plot as region R3. The bead were acquired at least 1,500 of bead events for absolute annexinV-positive MP calculation. In addition, absolute platelet was also calculated by using CD41-positive events which were gated on platelet population as region 4 (R4) for comparison with platelet count from hematological analyzer. The absolute annexinV-positive MPs and CD41-positive platelets were calculated by using the following formula:

$$\text{Absolute annexinV positive microparticles} = \frac{\text{Events in annexinV-positive microparticles population (R2)}}{\text{Events in absolute count bead region (R3)}} \times \frac{\text{Total bead per test}}{\text{Test volume}} \times \text{Dilution factor}$$

$$\text{Absolute Platelet} = \frac{\text{Events in CD41-positive in platelet population (R4)}}{\text{Events in absolute count bead region (R3)}} \times \frac{\text{Total bead per test}}{\text{Test volume}} \times \text{Dilution factor}$$



Histogram Statistics

File: 1BE_S_.002
Acquisition Date: 04-Sep-03
Gate: G1
Gated Events: 51433
Total Events: 514919

Marker	Events	% Gated	% Total	Mean	Geo Mean	Median
All	51433	100.00	9.99	3.60	1.33	1.00
M1	48447	94.19	9.41	1.19	1.14	1.00
M2	3000	5.83	0.58	42.63	14.97	9.65

Figure 4 Calculation of percent annexinV-positive microparticles, platelets and red blood cells. The MP population was defined by size in forward and side scatter dot plot followed by their morphology which was smaller than platelets and RBC population in whole blood sample (R1). The platelet population and RBCs were represented in region R2 and region R3 respectively. From the population of MPs in region R1, platelets in region R2 and RBCs in region R3, the annexinV-positive events of MPs, platelets and RBCs were analyzed by histogram plot as represented in positive area curve (M2) and the percent of annexinV-positive was obtained by histogram statistics.

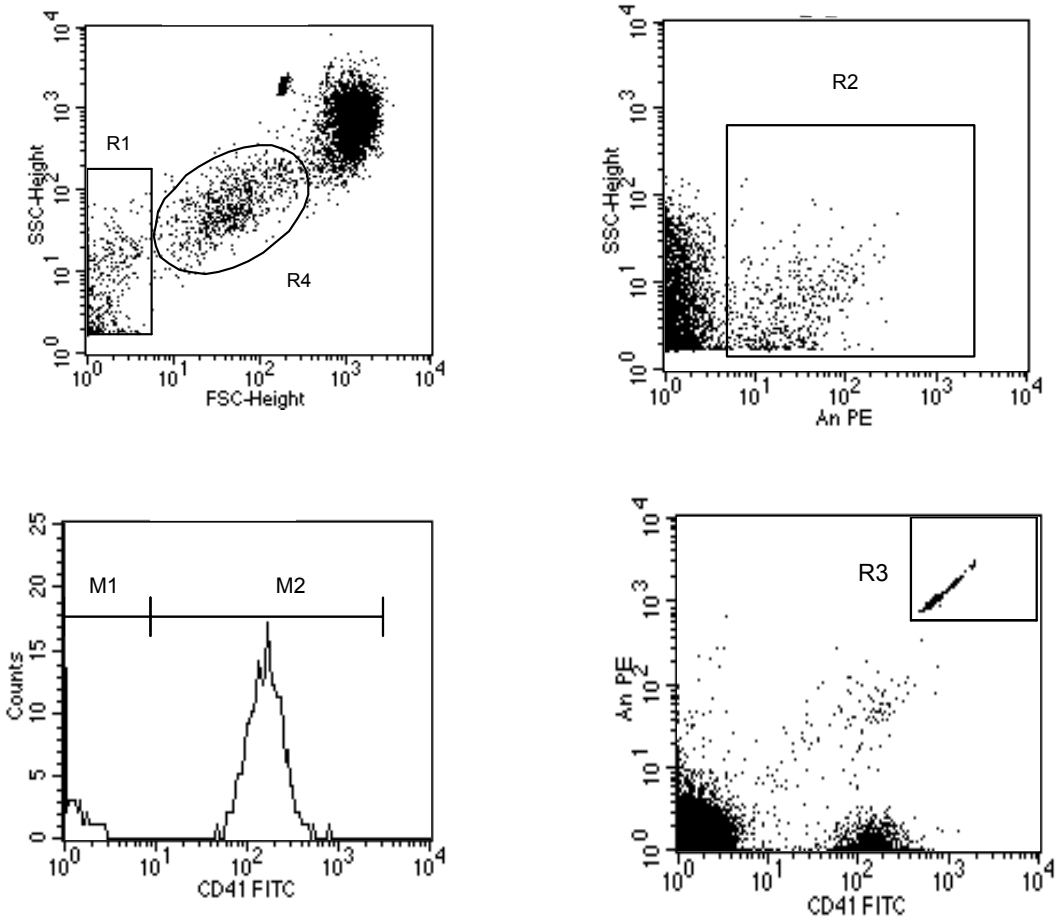


Figure 5 Calculation of absolute annexinV-positive microparticles. The MP population was presented in region R1 and the annexinV-possitive MP population was represented in region R2. The fluorescent bead events were gated by using FL1 and FL2 dot plot as region R3. Absolute platelet was also calculated by using CD41-positive events which were gated on platelet population as region R4 for comparison with platelet count from hematological analyzer.

3.3 Cellular Origin of AnnexinV-Positive Peripheral Blood Microparticles in Peripheral Blood

Two microliters of heparinized blood were stained with 2 µl of annexinV-FITC or annexinV-PE for distinguish MPs from events due to nonspecific noise and 2 µl of monoclonal antibodies directed against cell specific antigen conjugated with PE or FITC for identify cellular origin of MPs, including anti-glycophorinA-PE (DAKO, Glostrup, Denmark) for RBCs, CD105-PE (endoglin, SEROTEC, Oxford, UK) and CD62E-PE (E-selectin, BDB) for endothelial cells, CD66e-PE (BDB) for granulocytes, CD14-PE (BDB) for monocytes, CD45-PE (BDB) for leukocytes, CD62P-PE (P-selectin, BDB), CD61-PE (BDB), CD36-PE (BDB) and CD41-FITC (BDB) for platelets and anti-tissue factor–FITC (American Diagnostica inc.,Greenwich, CT, USA) for tissue factor expression. Then, 94 µl of annexinV binding buffer was added and incubated in the dark at room temperature for 15 minutes. After incubation, 300 µl of annexin binding buffer was added and analyzed by BDB FACSort flow cytometer. Acquisition and data analysis were performed using CellQuest Software (BDB). The light scatter and fluorescence channels were set at logarithmic gain. The MP population was presented in region R1 (Figure 6) and the annexinV-possitive MP population was represented in region R2 as previously mentioned. The annexinV-possitive MP population was further analyzed by histogram plot and event of cell specific marker was represented in positive area of M2 gate and the percent of cell specific marker was obtain by histogram statistics. These percentage were used to calculate the absolute number of annexin V positive MPs in each cell origin by using the following formula:

$$\text{Absolute cell specific annexinV-positive MP} = \frac{\text{Percent of cellular marker positive from annexinV-positive MP population (M2)}}{100} \times \text{Total Absolute number annexinV-positive MP calculate from TruCount tube}$$

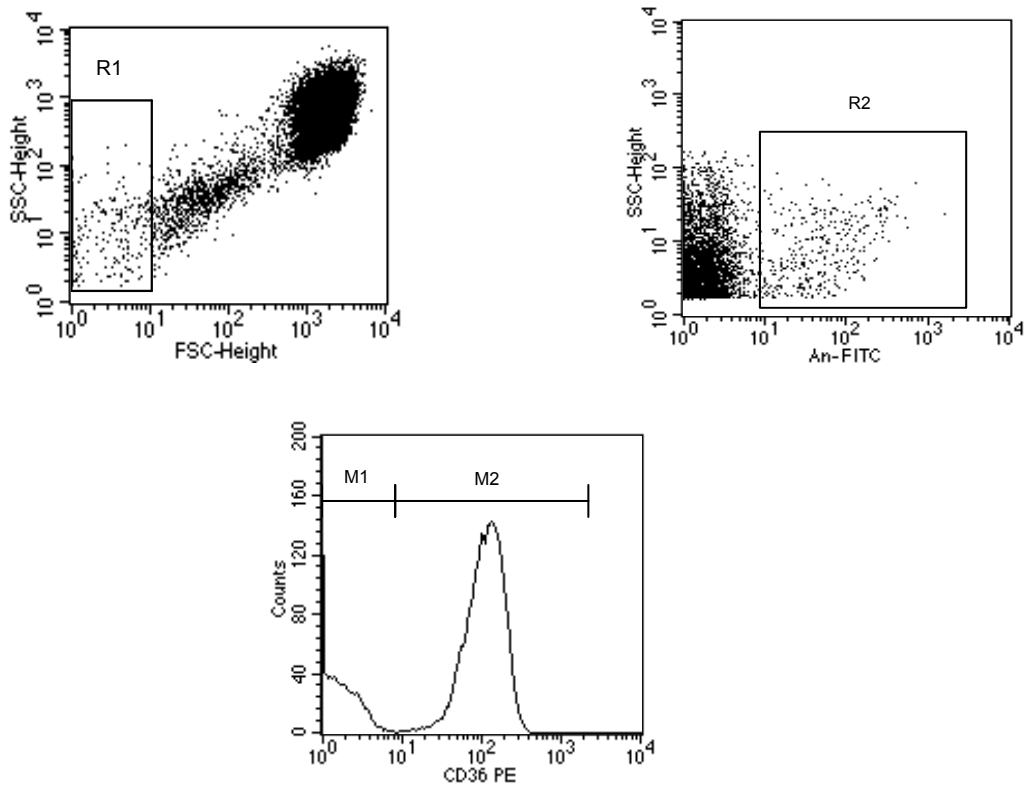


Figure 6 Calculation of cellular origin of annexinV-positive microparticles in peripheral blood. The MP population was presented in region R1 and the annexinV-possitive MP population was represented in region R2. The annexinV-possitive MP population was further analyzed by histogram plot and event of cell specific marker was represented in positive area of M2.

4. Detection of Platelet factor 3 like Activity in Whole Blood

One hundred microliters of 3.8% sodium citrate whole blood was added to a mixture containing 600 µl of Veronal buffer pH 7.35, 100 µl of eligic acid ($1x10^{-4}$M) (Sigma, MO, USA) and 100 µl of MD 805 ($5x10^{-5}$M) for activate contact system. After incubation at 37°C for 5 minutes, 100 µl of $2.5x10^{-3}$M $CaCl_2$ (Sigma, MO, USA) was then added and incubated at 37 °C for 20 minutes. The activation process was stopped by adding 100 µl of $2.5x10^{-3}$ M EDTA (Sigma, MO, USA) and spin down at 3,000 rpm for 3 minutes. The supernatant of thrombin solution was used to assay the thrombin activity by adding 25 µl to the mixture containing 375 µl of Triss-imidazole buffer pH 8.1 and 100 µl of thrombin substrate (S-2238, Sigma, MO, USA) ($2.5x10^{-3}$ M) then incubated at 37 °C for 10 minutes. One hundred microliters of acetic acid was added to stop the hydrolysis of thrombin substrate by thrombin. The absorbance of the reaction mixture was measured at 405 nm with Shimadzu UV-160 spectrophotometer (Shimadzu, Japan).

5. Statistical Analysis

Statistical analysis of quantitative variables was performed using the non-parametric K Independent Sample (Kruskal-Wallis H) test. To compare the mean of each variable using non-parametric Mann-Whitney U Test and to study the linear relationship between variables, Pearson's correlation coefficients were calculated. A p-value less than 0.05 was considered significant. All statistical calculations were performed using SPSS Version 12.0.

CHAPTER V

RESULTS

1. Complete Blood Count

Comparison of hematological parameters which were determined by automated hematologic analyzer Sysmex K800 between nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), HbH, HbH Constant Spring (HbH/CS) and healthy subjects was summarized in Table 1. The decreased mean Hb levels, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of all 4 thalassemia types were significantly different ($p<0.001$) when compared to healthy subjects. The number of RBC was significantly reduced ($p<0.001$) in βE, βES and HbH/CS when compared to healthy subjects. βES group only had a significantly higher level ($p<0.001$) of white blood cell (WBC) and platelet when compared to healthy subjects. Scattergrams and statistical analysis difference of hematological parameters between 4 thalassemia groups and healthy subjects were represented in Figure 7a-h.

Table 1. Mean ± S.D. oerf hematological parameters in nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects determined by automated hematologic analyzer Sysmex K800.

	Healthy subjects	**Patient's type**			
		βE	**βES**	**HbH**	**HbH/CS**
	n=35	n=53	n=38	n=34	n=23
Hb (g/dl)	13.2±1.2	6.8±1.7[a]	6.2±1.3[a]	8.5±1.5[a]	7.7±1.0[a]
RBC (x10^6 µl)	4.6±0.5	3.6±1.0[a]	3.3±1.8[a]	4.3±0.8	3.8±0.5[a]
Hct (%)	39.7±3.7	21.4±5.6[a]	21.9±3.4[a]	29.9±4.8[a]	28.8±3.3[a]
WBC (/µl)	12,543.9± 19,671.8	8,279.4± 3,916.7	55,324.1± 32,531.0[a]	8,065.3± 8,850.5	7,203.9± 1,836.8
MCV (fl)	86.4±5.4	61.7±7.3[a]	73.4±10.3[a]	70.8±8.9[a]	76.2±7.3[a]
MCH (pg)	28.6±2.3	19.1±2.5[a]	20.9±3.8[a]	19.9±1.4[a]	19.7±2.6[a]
MCHC (g/dl)	33.2±1.0	31.5±2.3[a]	28.2±2.5[a]	28.1±2.6[a]	26.7±2.1[a]
Platelet (/µl)	230,894.7± 829,47.7	298,301.9± 290,505.6	651,894.7± 196,893.5[a]	250,000.0± 111,390.9	248,217.4± 111,707.4

a = significantly difference from healthy subjects ($p<0.001$)

Hb = hemoglobin

RBC = red blood cell

Hct = hematocrit

WBC = white blood cell

MCV = mean corpuscular volume

MCH = mean corpuscular hemoglobin

MCHC = mean corpuscular hemoglobin concentration

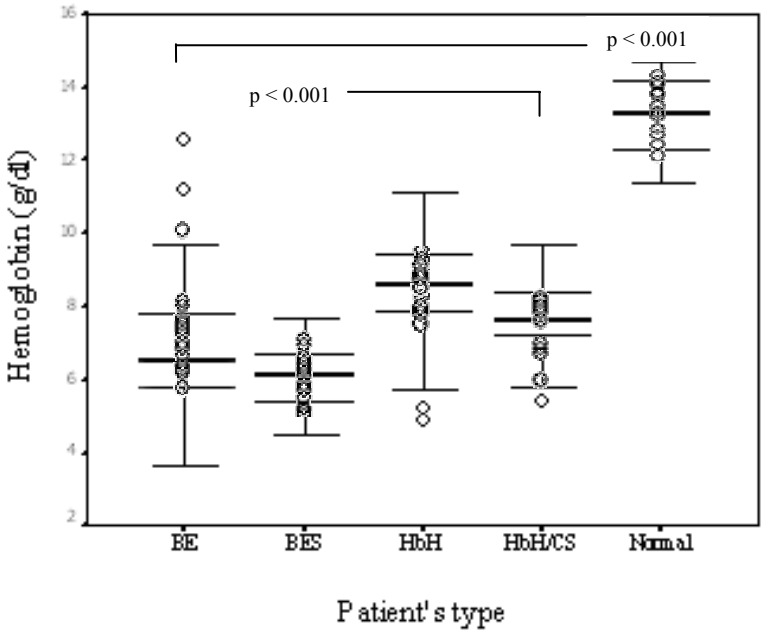


Figure 7a. Scattergram and statistical analysis of difference in hemolgobin concentration (g/dl) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

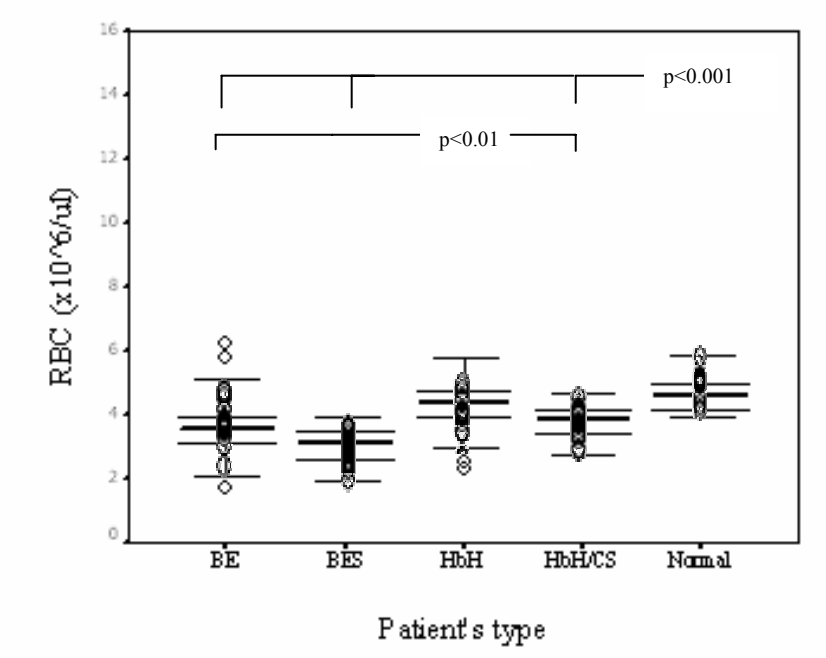


Figure 7b. Scattergram and statistical analysis of difference in RBC concentration ($x10^6/\mu l$) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

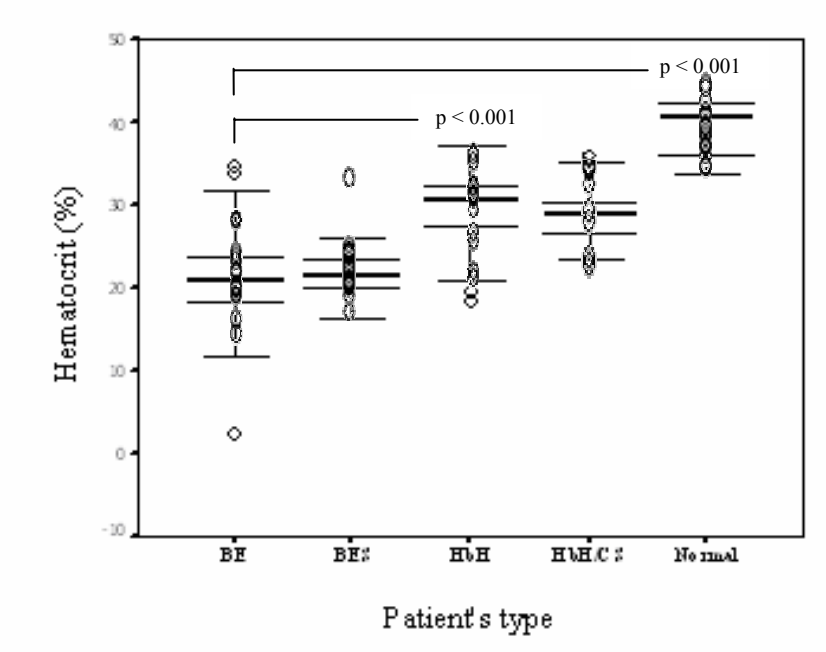


Figure 7c. Scattergram and statistical analysis of difference in hematocrit (%) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

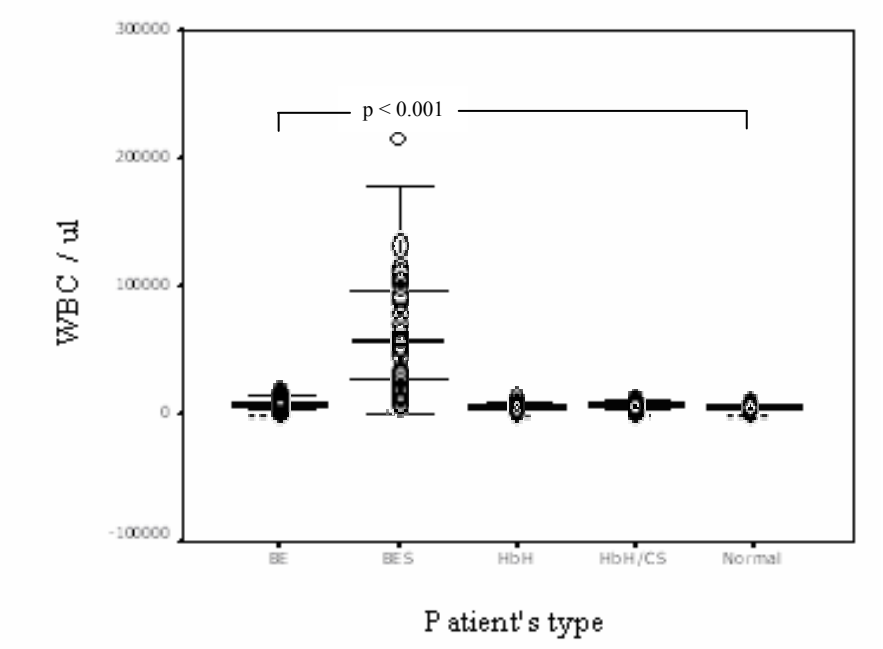


Figure 7d. Scattergram and statistical analysis of difference in WBC concentration (/μl) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

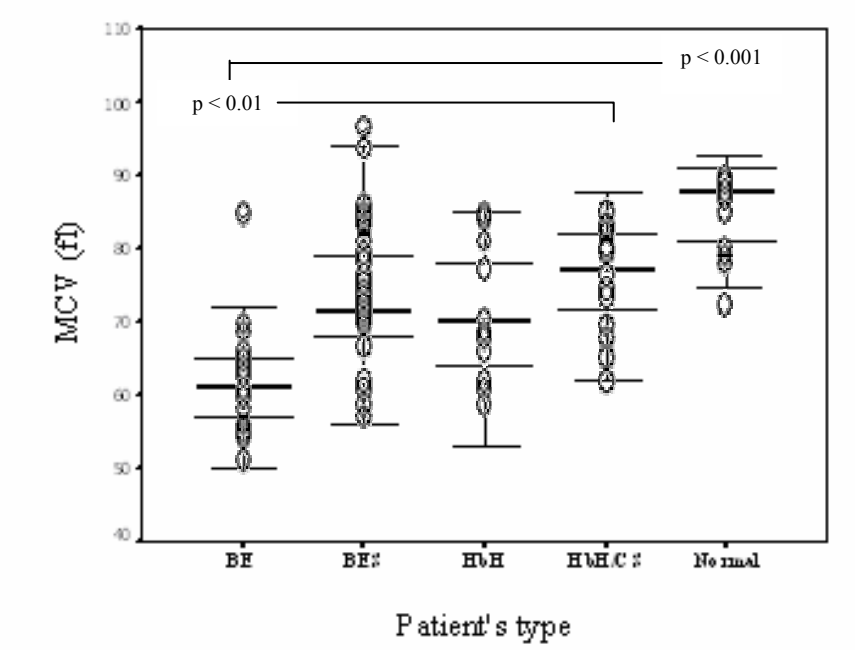


Figure 7e. Scattergram and statistical analysis of difference in mean corpuscular volume (fl) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

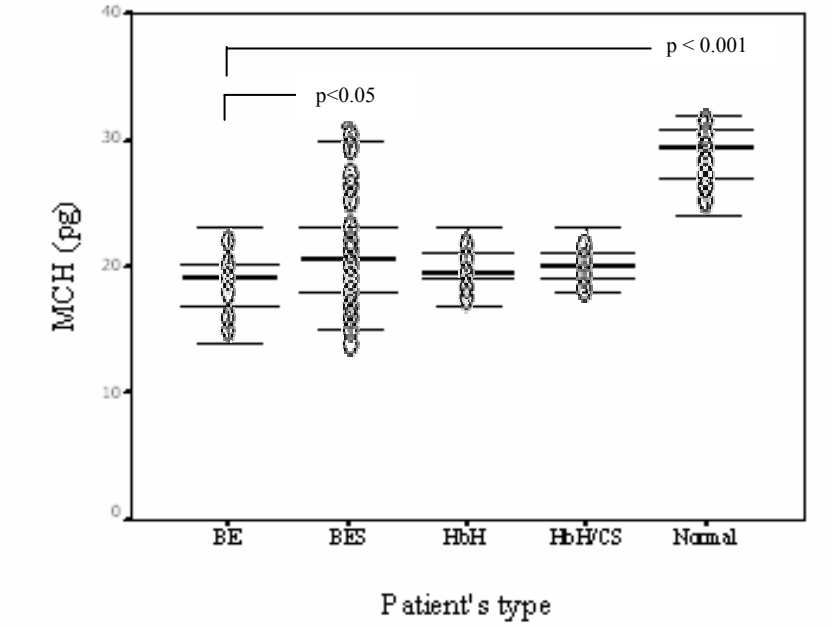


Figure 7f. Scattergram and statistical analysis of difference in mean corpuscular hemoglobin (pg) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

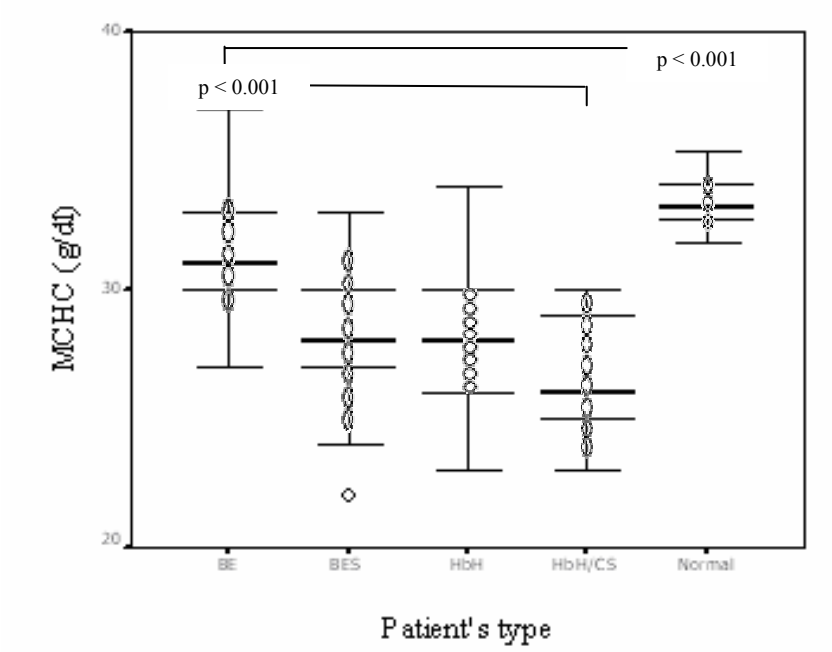


Figure 7g. Scattergram and statistical analysis of difference in mean corpuscular hemoglobin concentration (g/dl) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

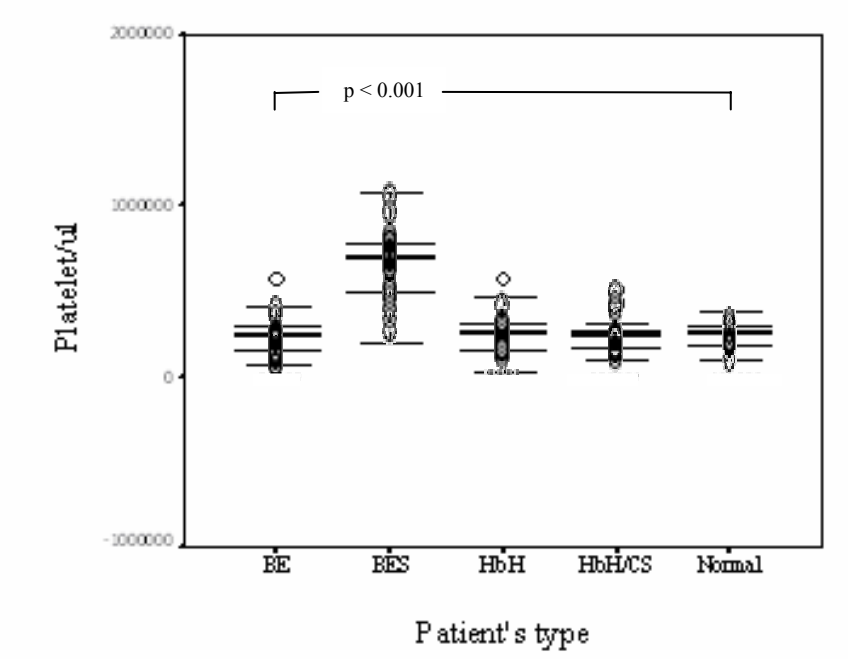


Figure 7h. Scattergram and statistical analysis of difference in platelet (/μl) among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) , hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

2. Time Dependent Test

To determine wheter the time period after blood collection effected the percentage of annexin V-positive MPs, the differncees between percentage of annexin V-positive MPs at various time point were determined. Whole blood samples from 3 thalassemic patients (1 βE, 1 βES, 1 HbH/CS) and 1 healthy subject were labeled and analyzed by flow cytometry as described in "Materials and Methods". Fluorescent thresholds were established by measuring sample in the absence of annexin V and in the presence of anti-mouse IgG1/IgG2 isotype control antibody (Figure 4). These thresholds were also used to identify annexin V-positive MPs population and cellular specific marker antibody in further evaluations. The result was summarized in Table 2 and line plot was represented in Figure 8. In all 4 subjects, percentage of annexin V-positive MPs was slightly increased during 4 hrs although this increase was not significant ($p > 0.05$). However, percent of annexin V-positive MPs was significantly increased at 24 hrs in all 4 subjects ($p < 0.01$) which can be suggested that studying of annexin V-positive MPs should be determined within 4 hours after blood collection.

Table 2. Percentage of annexin V-positive MPs in peripheral blood among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H Constant Spring (HbH/CS) and healthy subject at immediately, 1, 4 and 24 hours after blood colection.

Type / **Time**	**Percent annexin V-positive microparticles**			
	immediately	**1 hr**	**4 hr**	**24 hr**
	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.	Mean ±S.D.
Healthy subject	4.03	4.09	5.24	32.00 [a]
βE	6.00	6.00	6.08	37.00 [a]
βES	23.00	23.02	24.03	46.00 [a]
HbH/CS	4.98	5.06	5.10	49.00 [a]

a = significantly difference from others ($p<0.01$)

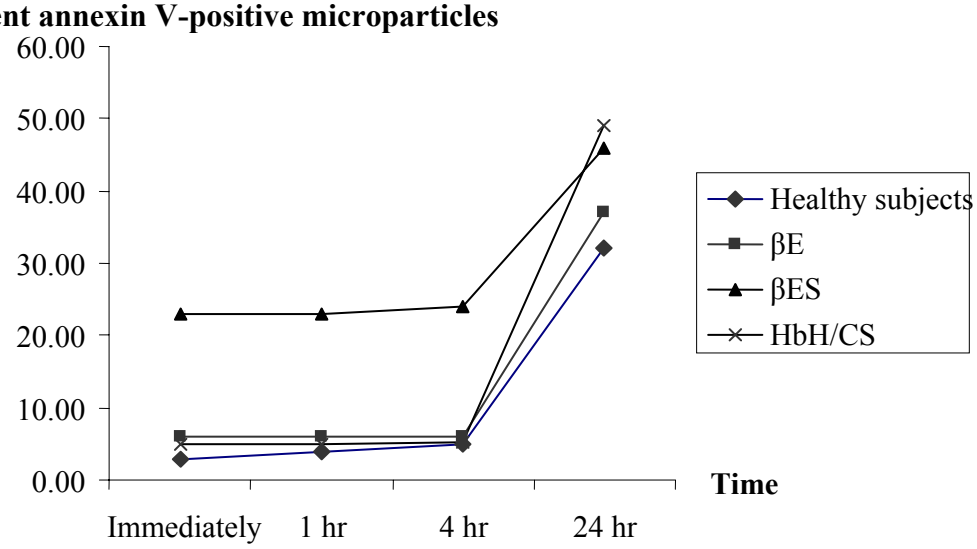


Figure 8. Line plot represents percent annexin V-positive MPs in peripheral blood among patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H Constant Spring (HbH/CS) and healthy subjects at various time.

3. Percentage of Annexin V-positive in Microparticles, Platelets and Red Blood Cells Population in Peripheral Blood.

To determine percentage of annexin V-positive in MPs, Platelets and RBCs population, whole blood samples were stained with annexin V and analyzed by flow cytometry. The results were summarized in Table 3. All thalassemia types and healthy subjects have annexin V-positive in MPs population. Scattergram and statistical analysis using non-parametric Mann-Whitney U Test showed that βES patients have higher mean percentage annexin V-positive in MPs population than other groups (Figure 9a) and healthy subjects ($p<0.05$). The percentage of annexin V-positive in platelet population of βES is also significant different when compared to other thalassemic patients types and healthy subjects ($p< 0.01$) (Figure 9b). βE, βES and HbH thalassemia patients have significantly higher level of annexin V-positive in RBCs population compared to healthy subjects ($p< 0.01$ in βE, βES and $p<0.05$ in HbH). Moreover, βES also have a significant higher level of annexin V-positive in RBCs and platelet population who compared with other thalassemia types ($p<0.05$) as shown in Figure 9b and c.

Correlations between percent of annexin V-positive MPs with percent annexin V-positive in platelet population ($r^2 = 0.259$ in βE, $r^2 = 0.421$ in βES, $r^2 = 0.255$ in HbH and $r^2 = 0.127$ in HbH/CS ($p< 0.05$)) and RBCs population ($r^2 = 0.107$ in βE, $r^2 = 0.305$ in βES, $r^2 = 0.223$ in HbH and $r^2 = 0.352$ in HbH/CS ($p< 0.05$)) were found as represented in Figure 10a-b. Correlation between percent of annexin V-positive in platelet and RBC population was $r^2 = 0.728$ in βE, $r^2 = 0.201$ in βES, $r^2 = 0.273$ in HbH and $r^2 = 0.333$ in HbH/CS ($p< 0.05$) as represented in Figure 10c.

Table 3. Mean±S.D. of percentage of annexin V-positive in Microparticles (MPs), Platelets (Plts) and Red Blood Cells (RBCs) population in peripheral blood of nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

	Percent Annexin V-positive				
	Healthy subjects	**Patient's type**			
		βE	**βES**	**HbH**	**HbH/CS**
	n=35	n=53	n=38	n=34	n=23
	Mean±S.D.	Mean±S.D.	Mean± S.D.	Mean±S.D.	Mean ±S.D.
MPs	10.47±6.87	13.55±9.53	15.99±8.99[a]	15.47±15.63	11.28±7.76
Plts	4.72±2.06	7.76±2.89 [b]	11.62±4.47 [b,c]	6.66±3.53 [b]	7.70±1.70 [b]
RBCs	0.88±0.84	1.57±1.47[b]	3.33±2.80 [b,c]	1.54±1.50[a]	1.09±0.74

a = significantly difference from healthy subjects ($p<0.05$)

b = significantly difference from healthy subjects ($p<0.01$)

c = significantly difference from other thalassemais types ($p<0.05$)

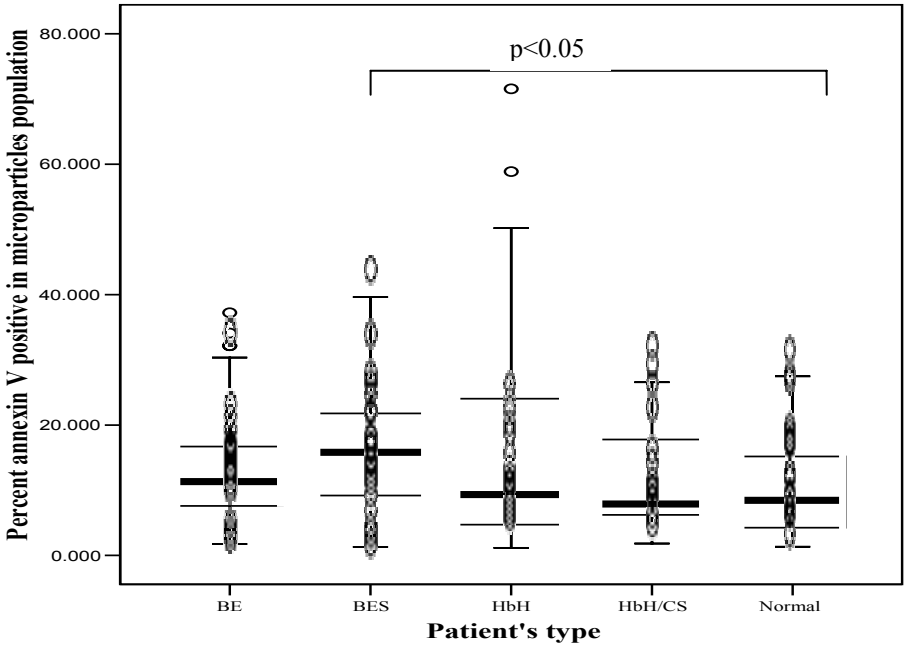


Figure 9a. Scattergram and statistical analysis of percentage of annexin V-positive MPs in peripheral blood of nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

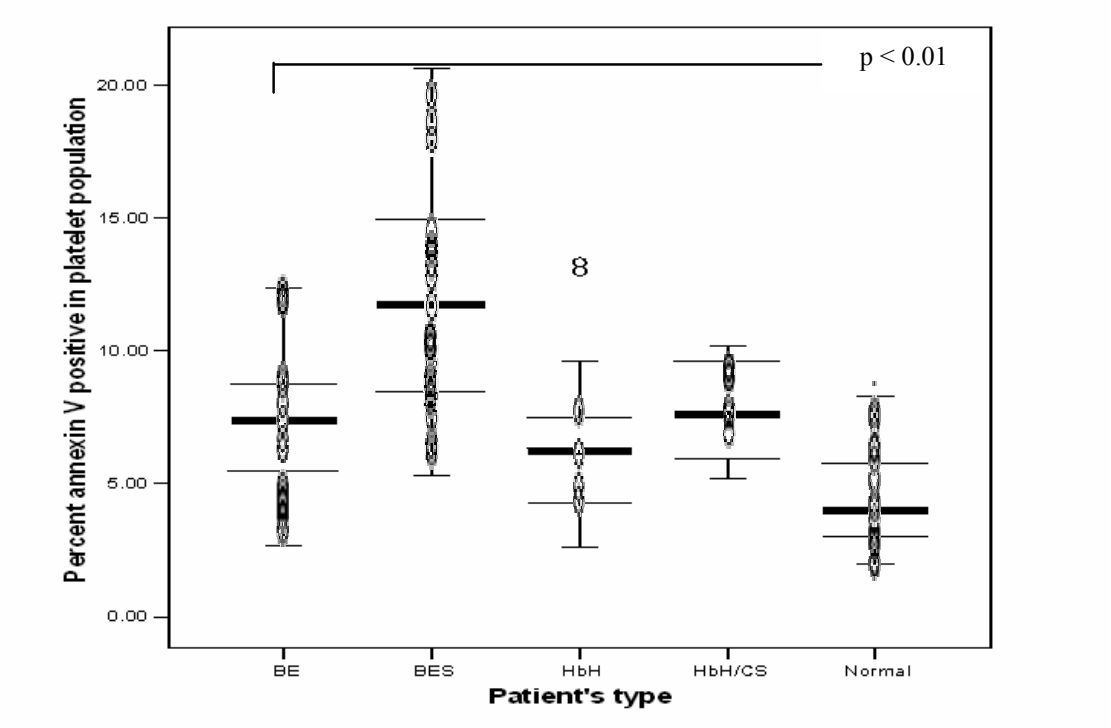


Figure 9b. Scattergram and statistical analysis of percentage of annexin V-positive platelets in peripheral blood of nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

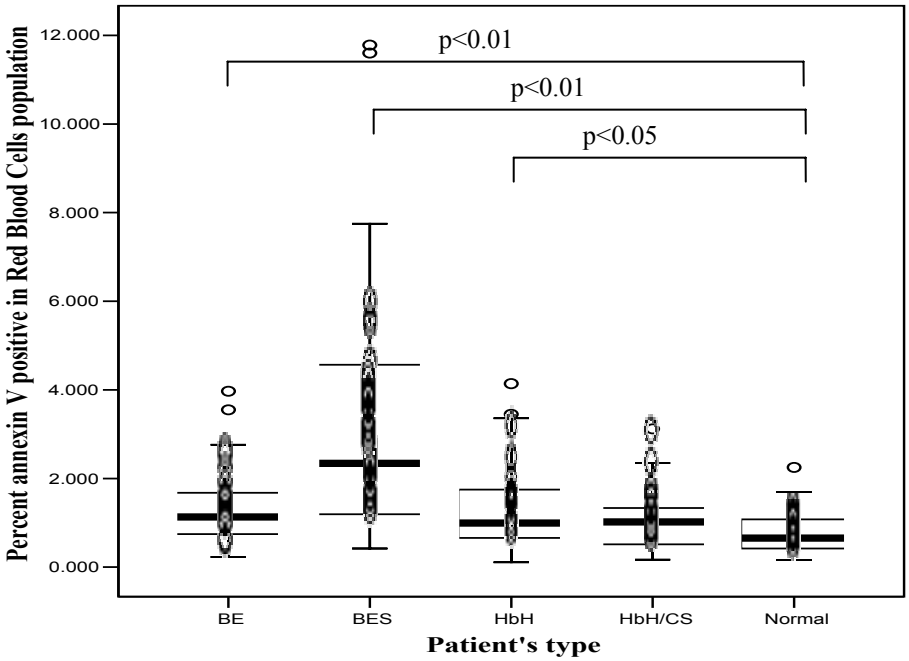
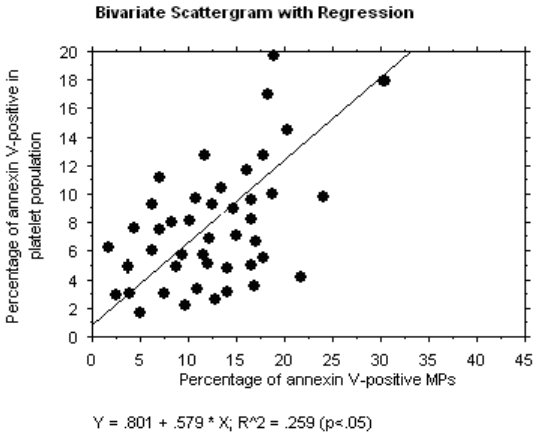
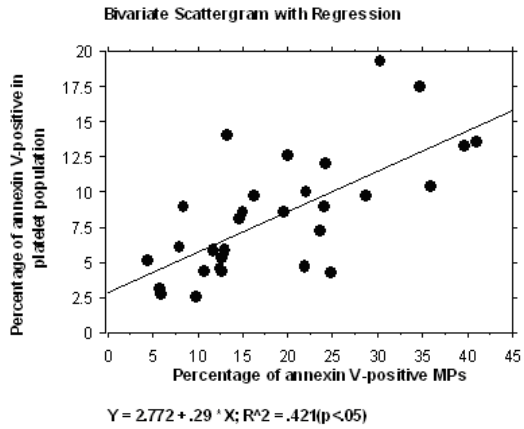


Figure 9c. Scattergram and statistical analysis of percentage of annexin V-positive in RBCs in peripheral blood of nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

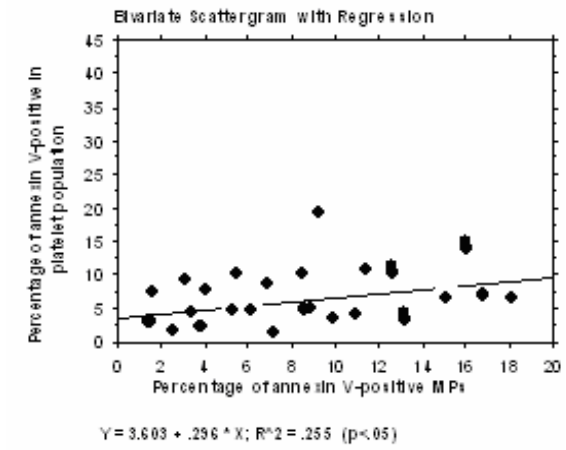
βE



βES



HbH



HbH/CS

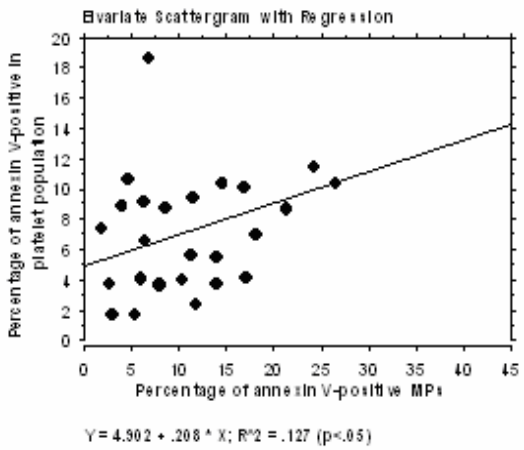
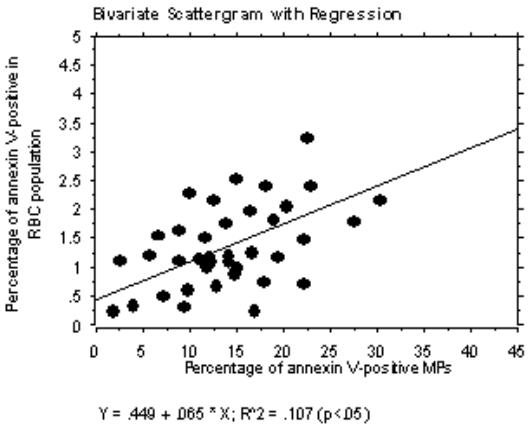
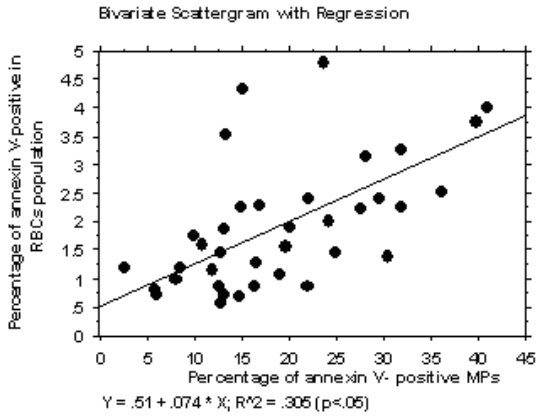


Figure 10a. Relationship between percentage of annexin V-positive MP and platelet population among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

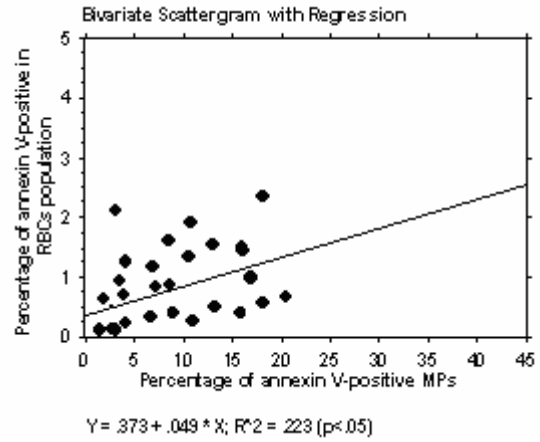
βE



βES



HbH



HbH/CS

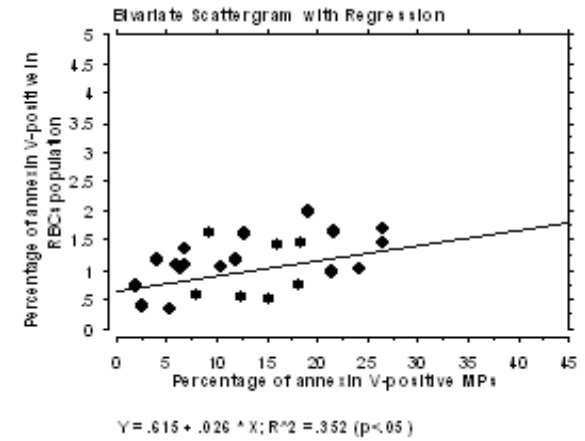
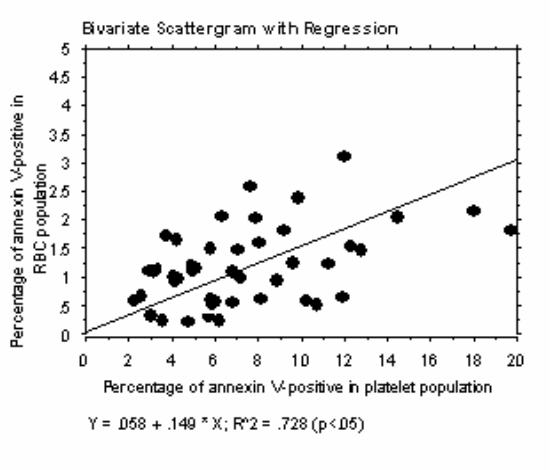
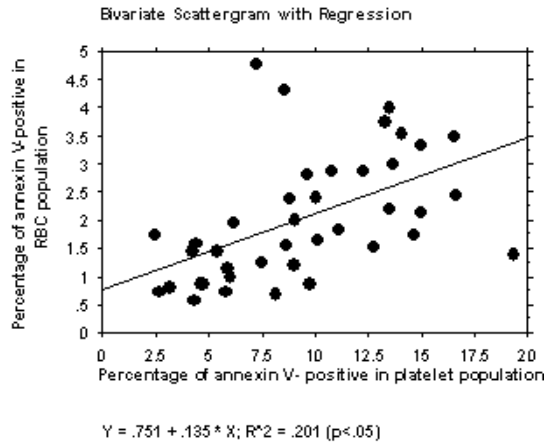


Figure 10b. Relationship between percentage of annexin V-positive in MP and RBC population among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

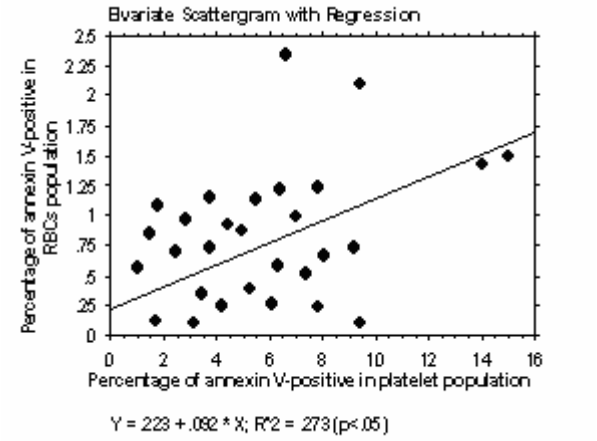
βE



βES



HbH



HbH/CS

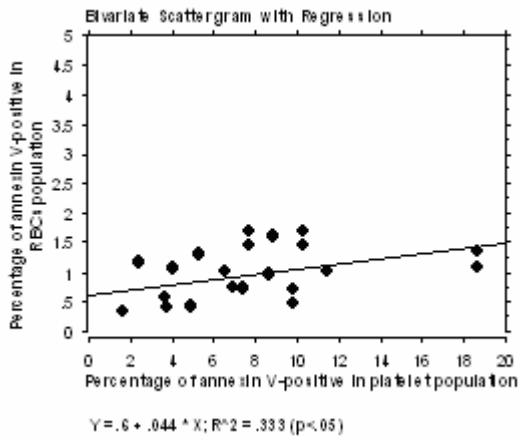


Figure 10c. Relationship between percentage of annexin V-positive in RBC and platelet population among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

4. Calculation of Absolute Number of Annexin V-positive Microparticles in Peripheral Blood

The absolute number of annexin V-positive MPs in peripheral blood sample was determined. Mean ± S.D. of absolute number of annexin V-positive MPs in thalassemic patients and healthy subjects were summarized in Table 5, scattergram and statistical analysis using nonparametric Mann-Whitney U test were shown in Figure 11. The absolute number of annexin V-positive MPs were high in thalassemic patients, particularly in βES and HbH/CS when compared to healthy subjects ($p< 0.01$ and $p< 0.05$, respectively). The highest value was found in βES ($p<0.01$ when compared with βE, HbH and $p<0.05$ when compared with HbH/CS). The correlation was also found between absolute number of annexin V-positive MPs and platelet count in βES ($r^2=0.203$, $p< 0.05$) (Figure 12).

Moreover, the absolute number of platelets was also calculated to compare with the platelets count from automated hematological analyzer. Result showed good correlation with $r^2= 0.412$ and $p< 0.01$ (Figure 13).

Table 4. Mean±S.D. of absolute number of annexin V-positive MPs in peripheral blood among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

	Healthy subjects	**Patient's type**			
		βE	**βES**	**HbH**	**HbH/CS**
	n=31	n=28	n=25	n=24	n=18
	Mean ±S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ±S.D.
Absolute number of annexin V-positive MPs	16,933.81± 10,138.92	24,314.41± 16,917.89	52,959.96± 31,301.78 [a,c]	18,939.29± 16,806.96	23,868.39± 16,956.88 [b]

a = significantly difference from healthy subjects ($p<0.05$)

b = significantly difference from healthy subjects ($p<0.01$)

c = significantly difference from thalassemia groups ($p<0.05$)

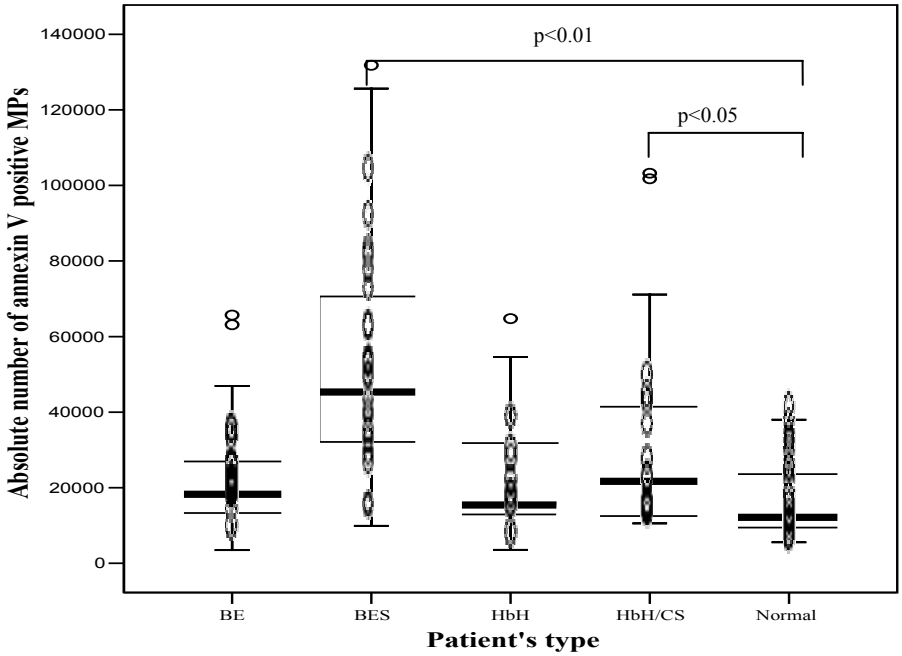
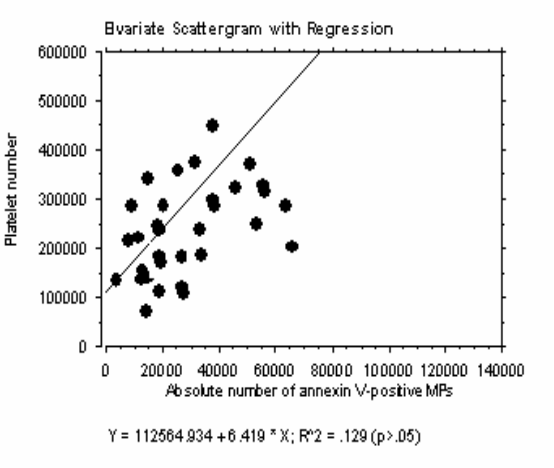
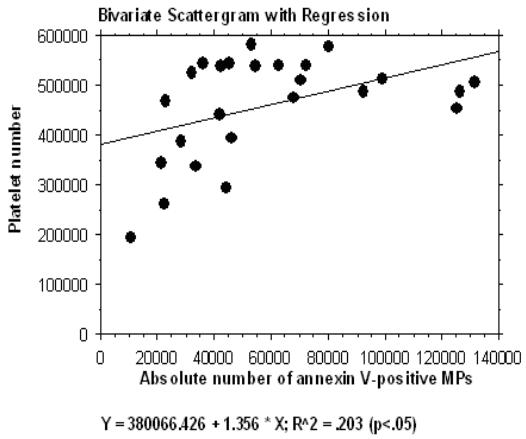


Figure 11. Scattergram represents absolute number of annexin V-positive MPs in peripheral blood among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

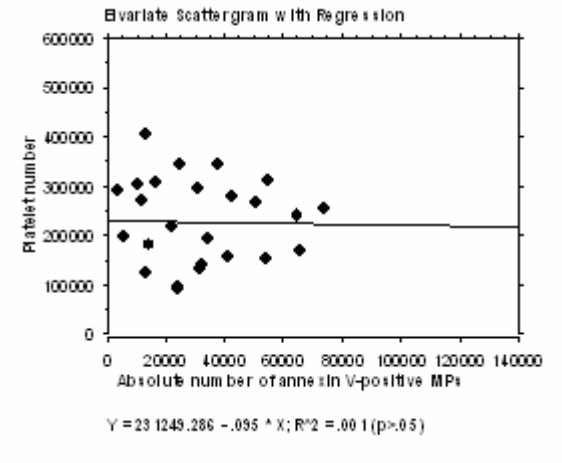
βE



βES



HbH



HbH/CS

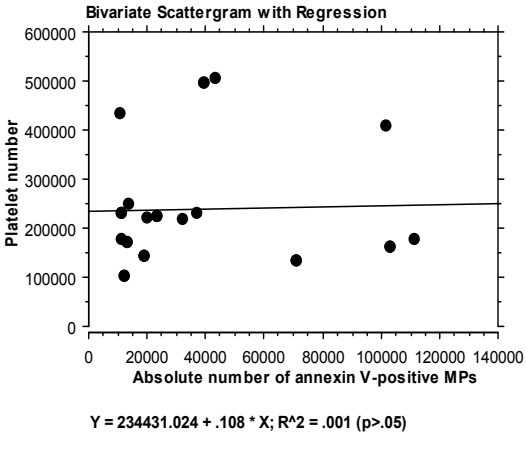


Figure 12. Regression analysis between absolute number of annexin V-positive MPs and platelet number in peripheral blood of nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

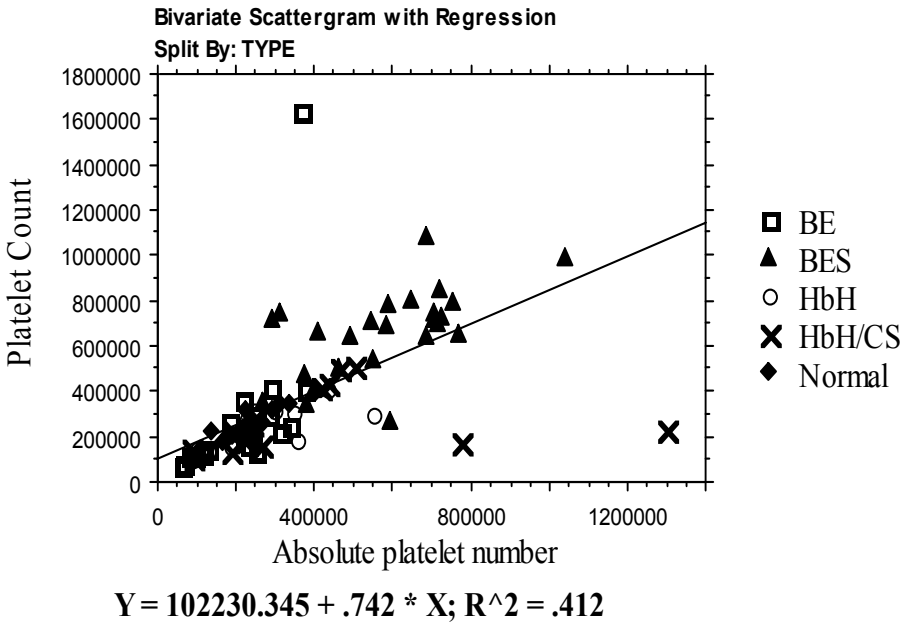


Figure 13. Regression analysis between platelet count using automated hematological analyzer and absolute platelet number calculated from flow cytrometric method.

5. Cellular Origins of Annexin V-positive Microparticles in Peripheral Blood of Thalassemic Patients

To determine cellular origin of annexin V-positive MPs in peripheral blood of thalassemia patients and healthy subjects. Blood samples were stained with annexin V and monoclonal antibody specific to cellular origins and adhesion molecule and analyzed by flow cytrometry. Table 5 showed mean ± S.D. of the percentage of specific antigen and adhesion molecule in annexin V-positive MPs. The major cellular origin of annexin V-positive MPs in healthy subjects was from Platelets (CD36, CD62P, CD41 and CD61) and RBCs (Glycophorin A). Moreover, they are also originated from ECs (CD105, CD62E) and granulocytes (CD66e), respectively. The same results were also found in thalassemia patients but the ratio of the percentage in these markers are varied among the patient's type as shown in Figure 14. Leukocytes (CD45), monocyte (CD14) and tissue factor–derived MPs were also found but in the low level both in thalassemic patients and healthy subjects. The bar plot of the percentage of specific antigen and adhesion molecule in annexin V-positive MPs was represented in Figure 14.

The absolute number of annexin V-positive MP in each cellular origin were determined by calculation from the formular as described in "Materials and Methods". Results were summarized in Table 5. Similar to the percentage, platelet derived MPs have a highest absolute number in both thalassemia patients and healthy subjects especially in βES. RBC-derived MPs also had a high number in βE, βES, HbH and were significantly different when compared to healthy subject ($p<0.05$). MPs originated from ECs, granulocytes, monocytes, and leukocytes also had a significantly high number when compared with healthy subject. The bar plot represented absolute number of annexin V-positive MPs was represented in Figure 15.

Table 5. Mean ± S.D. of the percentage of specific antigen and adhesion molecule in annexin V-positive MPs in peripheral blood among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

Cellular markers	Means ± S.D. of percent cellular markers positive in annexin V-positive MPs				
	Healthy subjects	Patient's type			
		βE	βES	HbH	HbH/CS
	n=35	n=53	n=38	n=34	n=23
RBC					
Glycophorin A	27.64±18.83	36.44±21.02	17.72±14.01	35.86±23.48	23.93±18.88
Leukocyte					
CD 45	5.51±1.36	9.84±2.72	4.29±3.55	7.57±1.85	7.14±1.92
Platelet					
CD 62P	46.95±16.34	35.66±14.19	44.08±21.36	44.28±15.21	41.08±12.08
CD 61	10.23±8.62	12.98±4.62	8.83±10.93	18.21±8.29	24.32±20.16
CD 36	46.14±25.68	48.17±20.99	57.55±20.99	52.74±25.00	53.50±21.20
CD 41	36.25±18.83	32.28±12.83	43.10±20.39	33.94±14.55	36.67±18.27
Granulocyte					
CD 66e	11.09±11.04	11.68±12.74	8.60±5.95	16.58±12.12	13.08±21.36
Monocyte					
CD 14	4.03±4.16	5.60±4.92	7.53±7.89	7.40±7.04	12.28±14.44
EC					
CD 105	16.46±2.55	11.94±3.68	7.00±1.79	17.00±6.51	16.77±9.48
CD 62E	13.65±11.95	9.15±7.80	10.94±4.93	23.73±17.71	12.00±8.21
Anti-TF	4.99±0.36	6.25±3.28	8.53±5.29	8.76±1.03	4.37±2.05

RBC = red blood cell

EC = endothelial cell

TF = tissue factor

Table 6. Mean ± S.D. of calculated absolute number of specific antigen and adhesion molecule in annexin V-positive MPs in peripheral blood among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

Cellular markers	Mean ± S.D. of calculated cellular markers positive in annexin V-positive MPs (particles/µl)				
	Healthy subjects	Patient's type			
		βE	βES	HbH	HbH/CS
	n=31	n=28	n=25	n=24	n=18
Glycophorin A	4,680.28± 3,188.48	8,860.17± 5,110.88[a]	9,384.50± 7,419.69[a]	8,584.63± 1,620.94[a]	5,711.61± 4,506.27
CD 45	933.01± 230.28	2,392.54± 661.35[b]	2,271.98± 1,880.07[b]	1,812.20± 442.87[a]	1,704.18± 458.26[a]
CD 62P	7,950.04± 2,766.85	8,670.52± 3,450.21[a]	23,344.75± 11,312.25[b,c]	10,600.32± 3,641.16[a]	9,804.97± 2,883.25[a]
CD 61	1,732.25± 1,459.62	3,156.01± 1,123.32[a]	4,676.36± 5,788.52[a]	4,359.34± 1,984.56[a]	5,804.70± 4,811.78[a]
CD 36	7,812.89± 4,348.39	11,712.25± 5,103.59[a]	30,478.46± 11,116.30[b,c]	12,625.58± 5,984.82[a]	12,769.38± 5,060.01[a]
CD 41	6,138.21± 3,188.48	7,836.53± 3,119.53[a]	22,825.74± 10,798.54[b,c]	8,125.00± 3,483.16[a]	8,752.40± 4,360.68[a]
CD 66e	1,877.87± 1,869.40	2,839.92± 3,097.65	4,554.56± 1,151.11[a]	3,969.13± 2,901.44[a]	3,121.93± 5,098.20
CD 14	682.40± 304.41	1,361.61± 1,196.26	3,987.88± 1,178.54[b,c]	1,771.51± 1,017.53[a]	2,930.99± 1,446.53[a]
CD 105	2,787.17± 431.79	2,903.14± 894.77	3,707.20± 947.98	4,069.68± 1,558.44[a]	4,002.66± 2,262.68[a]
CD 62E	2,311.35± 1,023.49	2,903.14± 1,896.52	5,793.82± 2,610.92[a]	5,680.79± 1,239.64[a]	2,864.16± 1,959.56
Anti-TF	844.96± 60.95	1,519.65± 797.51	4,517.48± 1,801.58[a]	2,097.08± 246.57	1,043.03± 489.29

a = significantly difference from healthy subjects ($p<0.05$)

b = significantly difference from healthy subjects ($p<0.01$)

c = significantly difference from thalassemia groups ($p<0.05$)

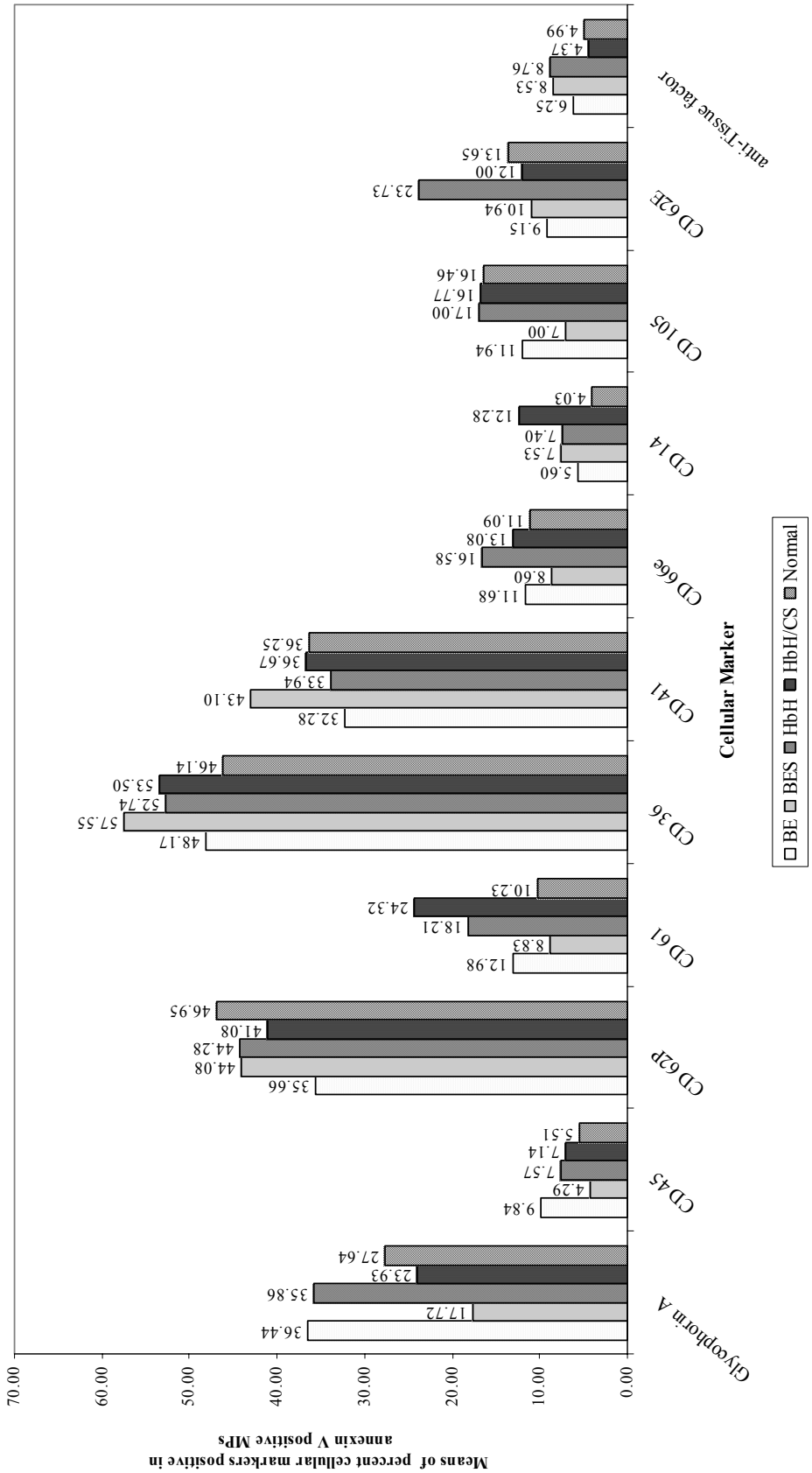


Figure 14. Bar plot represents mean of percent various cellular antigen positive in annexin V MPs in peripheral blood among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

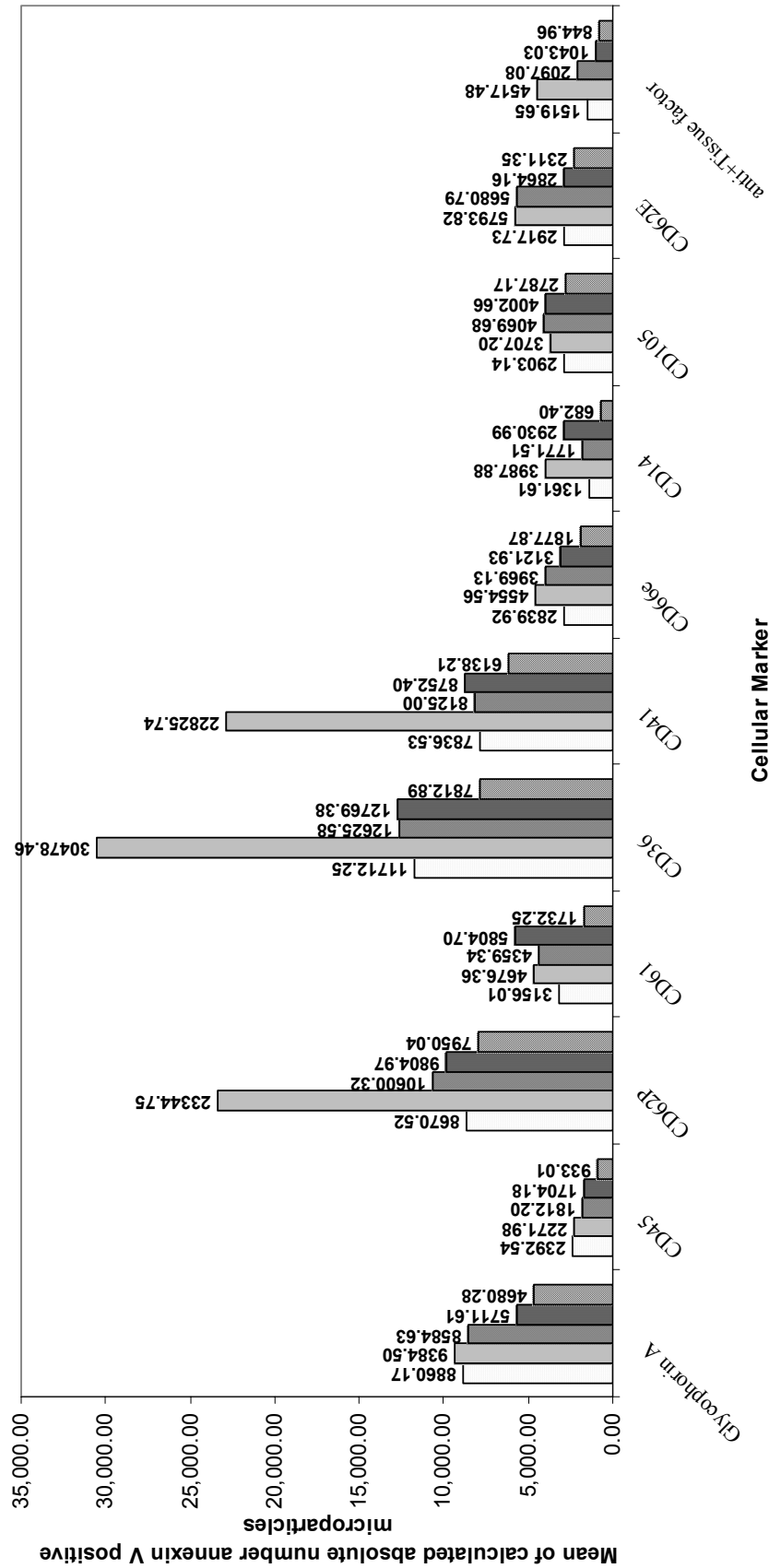


Figure 15. Bar plot represents mean of calculated absolute number of various cellular antigen positive in annexin V MPs in peripheral blood among nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

6. Relationship Between Annexin V-positive Microparticles and Coagulation Factor (Platelet factor 3 like activity)

To investigate whether percent and absolute number of annexin V-positive MPs have a correlation with coagulation factor, the coagulation system capacity were measured. Table 7 showed mean ± S.D. of OD 405 which represented platelet factor 3 like activity. Scattergrams and statistical analysis using nonparametric Mann-Whitney U test was shown in Figure 16. The OD 405 values were highest in βES and significantly high when compared with other thalassemia types and healthy subjects ($p<0.01$). HbH and HbH/CS also had a significant higher level of OD 405 when compared with healthy subjects ($p<0.05$). Although βE had a high level of OD 405, but this was not statistically significant when compared with normal subjects.

The OD 405 value was correlated with absolute number of annexin V-positive MPs ($r^2 = 0.709$ in βE, $r^2 = 0.272$ in βES and $r^2 = 0.38$ in HbH/CS ($p< 0.05$)), percentage of annexin V-positive MPs ($r^2 = 0.342$ in βES, $r^2 = 0.19$ in HbH and $r^2 = 0.142$ in HbH/CS ($p< 0.05$)). The correlation of OD 405 was found with percent annexin V-positive in platelet and percent of annexin V-positive MPs with platete markers, CD 41 ($r^2 = 0.253$ in βE, $r^2 = 0.185$ in βES, $r^2 = 318$ in HbH and $r^2 = 0.275$ in HbH/CS ($p< 0.05$)), CD 36 ($r^2 = 0.273$ in βE, $r^2 = 0.404$ in βES and $r^2 = 0.332$ in HbH/CS ($p< 0.05$)) and CD 62P ($r^2 = 0.332$ in βE, $r^2 = 0.479$ in βES and $r^2 = 0.208$ in HbH/CS ($p< 0.05$)) as represented in Figure 17.

Table 7. Mean ± S.D. of OD 405 of platelet factor 3 like activity in peripheral blood in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

	Healthy subjects	**Patient's Type**			
		βE	**βES**	**HbH**	**HbH/CS**
	n=31	n=28	n=25	n=24	n=18
	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
Platelet factor 3 like activity (OD 405)	**1.13±0.16**	**1.22±0.49**	**2.21±0.76[b]**	**1.48±0.90[a]**	**1.55±0.44[a]**

a = significantly difference from healthy subjects ($p<0.05$)

b = significantly difference from healthy subjects ($p<0.01$)

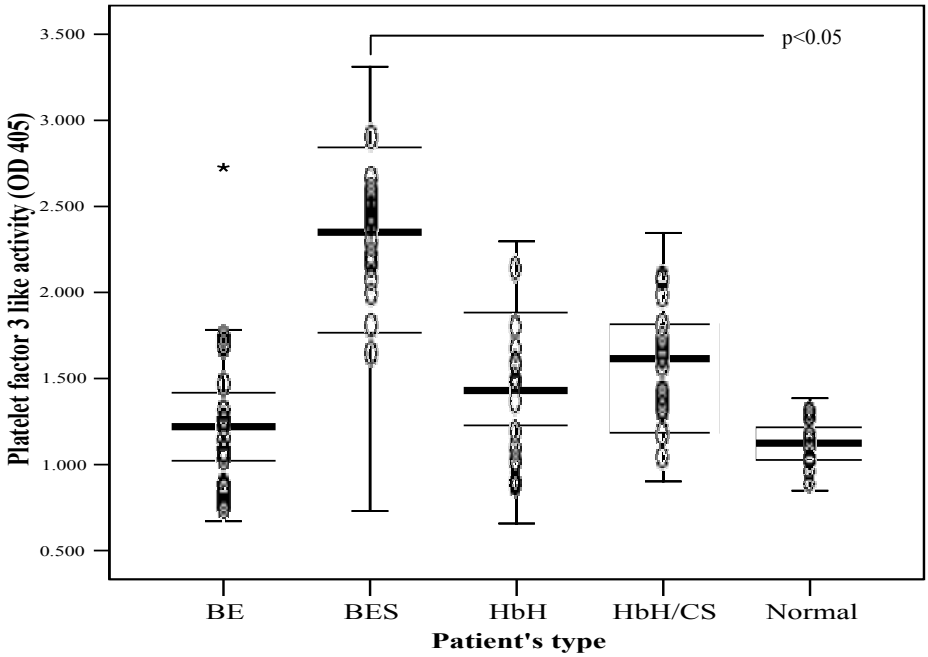
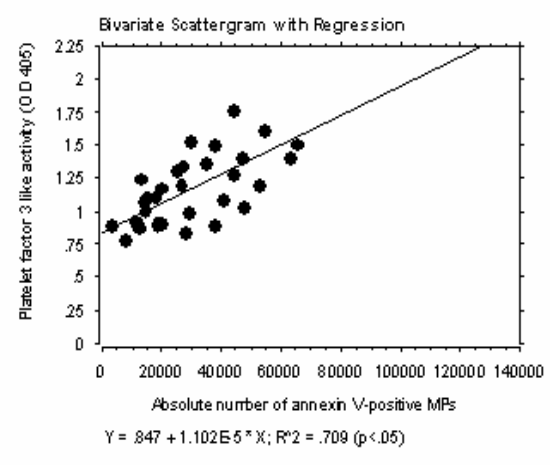
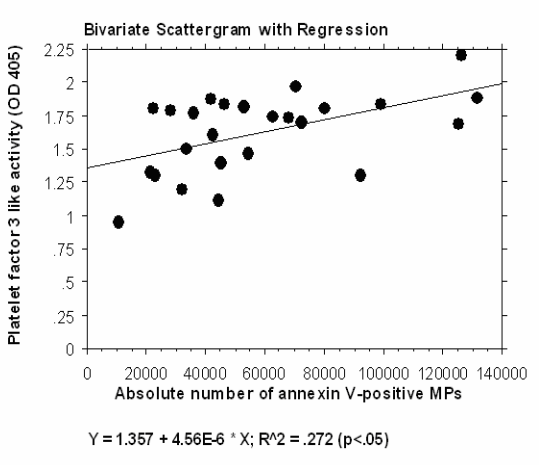


Figure 16. Scattergram shows platelet factor 3 like activity (OD 405) in peripheral blood in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH), hemoglobin H Constant Spring (HbH/CS) and healthy subjects.

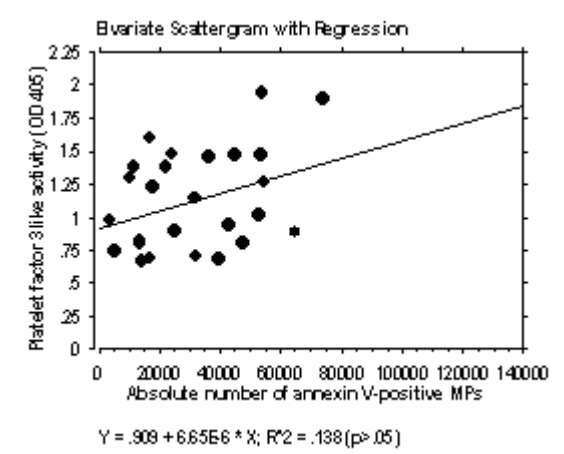
βE



βES



HbH



HbH/CS

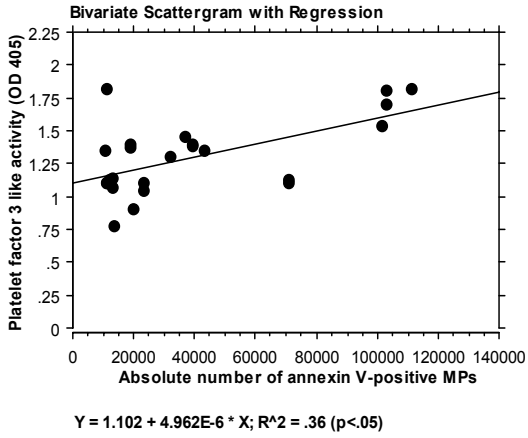
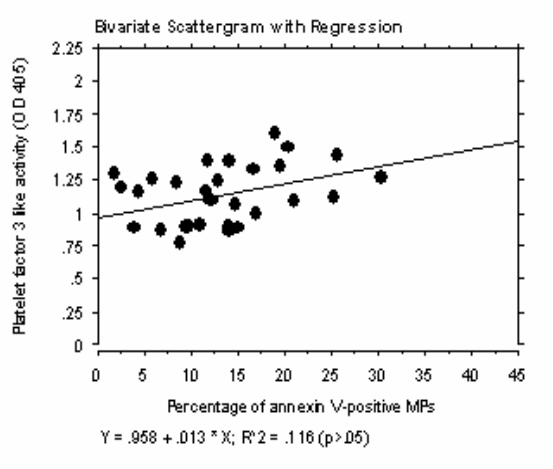
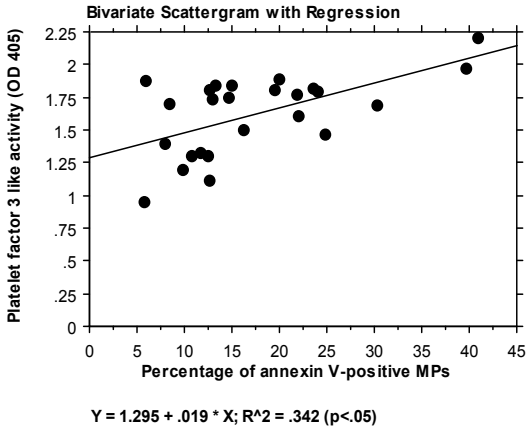


Figure 17a. Regression analysis of platelet factor 3 like activity (OD 405) and absolute number of annexin V-positive MPs in peripheral blood in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

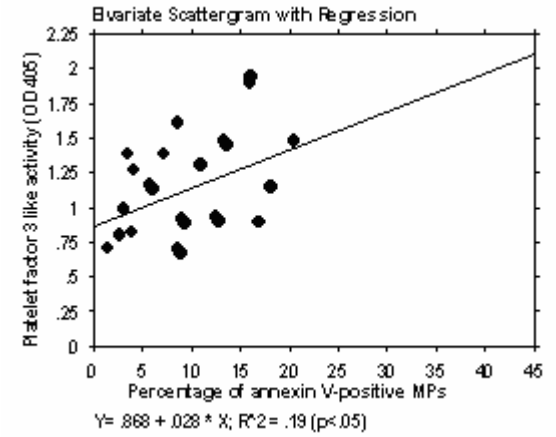
βE



βES



HbH



HbH/CS

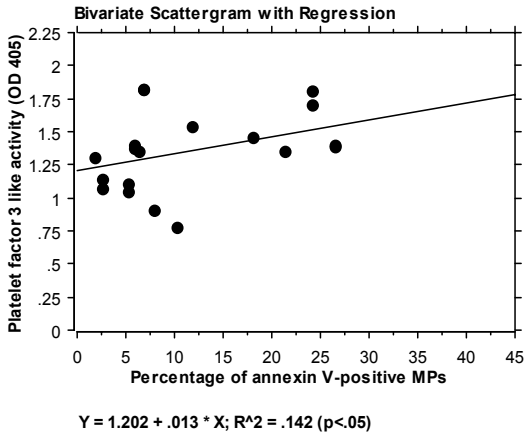
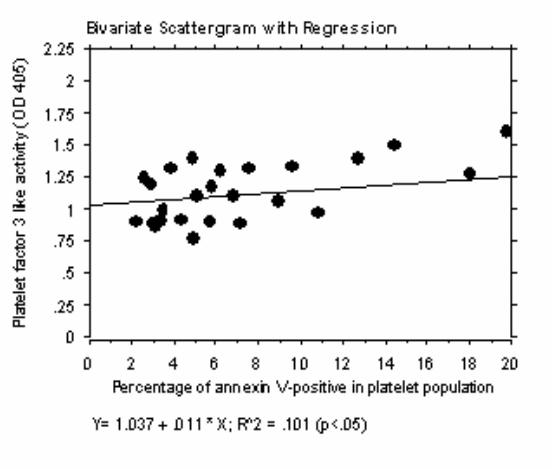
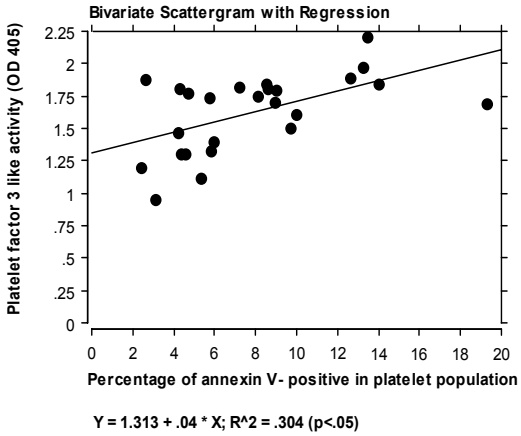


Figure 17b. Regression analysis of platelet factor 3 like activity (OD 405) and percentage of annexin V-positive MPs in peripheral blood in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

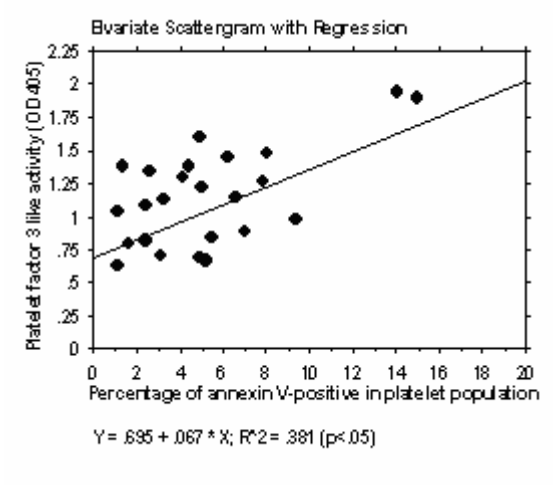
βE



βES



HbH



HbH/CS

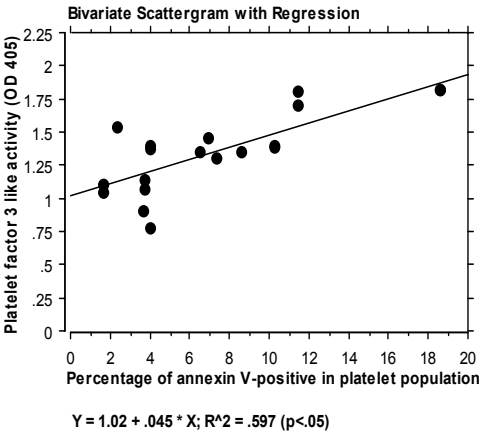
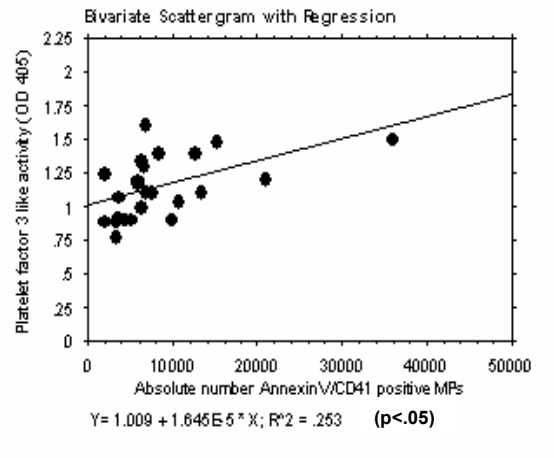
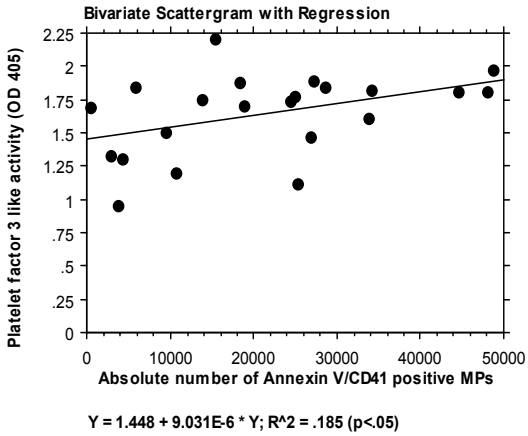


Figure 17c. Regression analysis of platelet factor 3 like activity (OD 405) and percentage of annexin V-positive in pletelet population in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

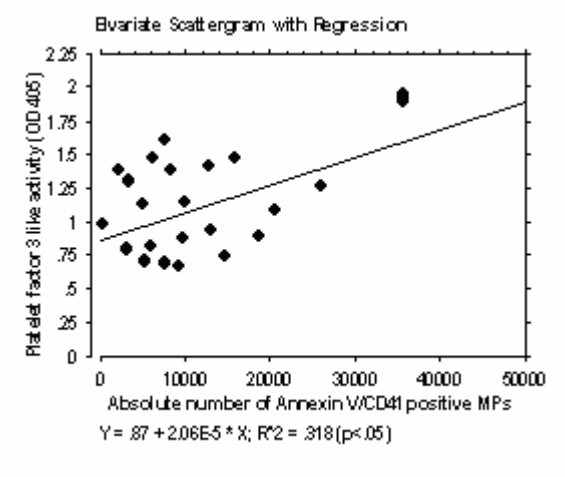
βE



βES



HbH



HbH/CS

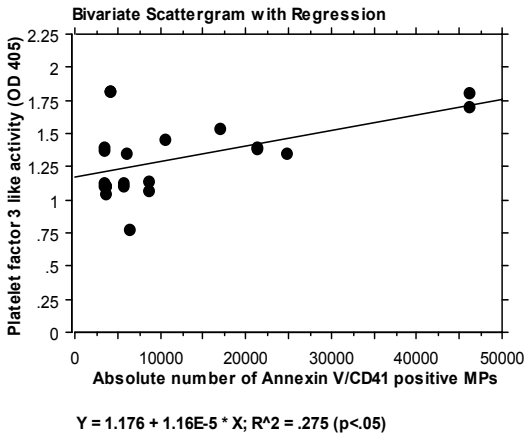
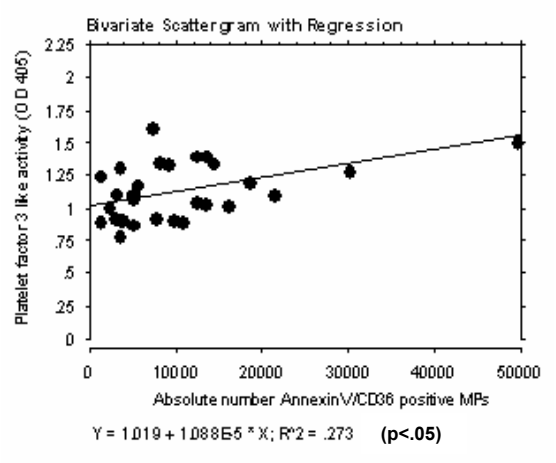
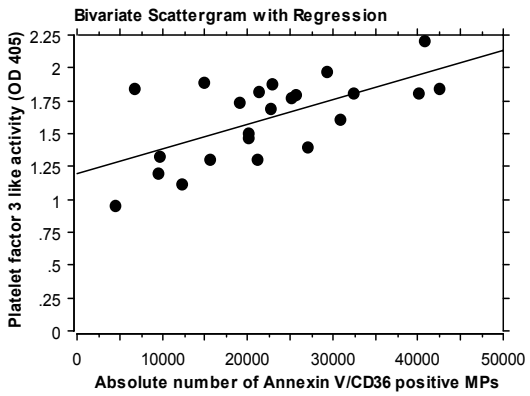


Figure 17d. Regression analysis of platelet factor 3 like activity (OD 405) and absolute number of annexin V with platelet marker (CD41) positive MPs in peripheral blood in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

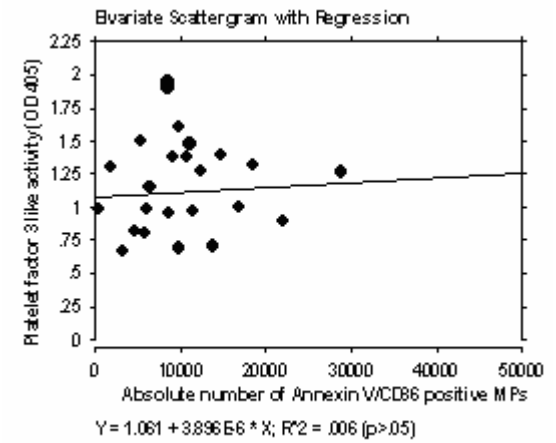
βE



βES



HbH



HbH/CS

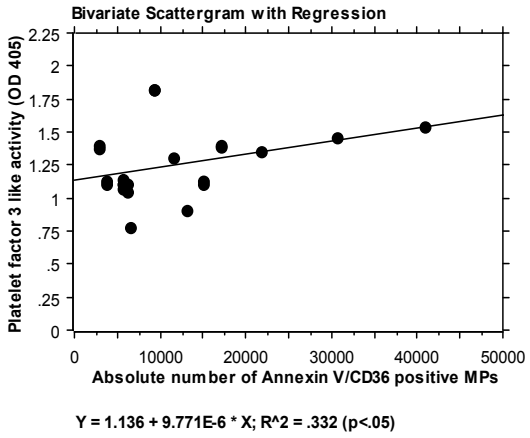
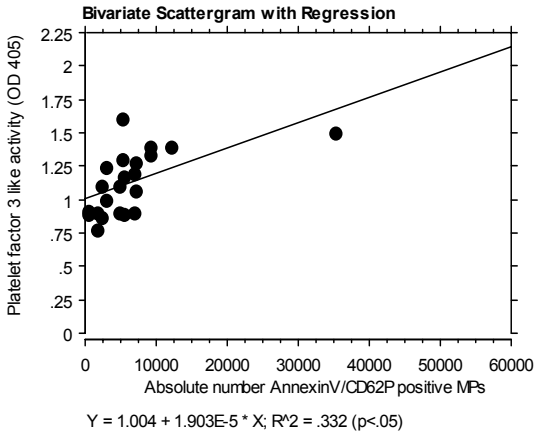
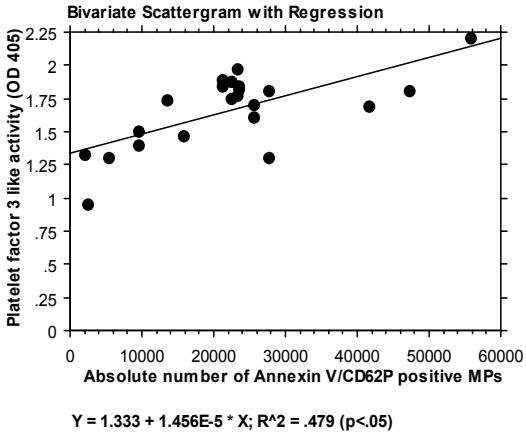


Figure 17e. Regression analysis of platelet factor 3 like activity (OD 405) and absolute number of annexin V with platelet marker (CD36) positive MPs in peripheral blood in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

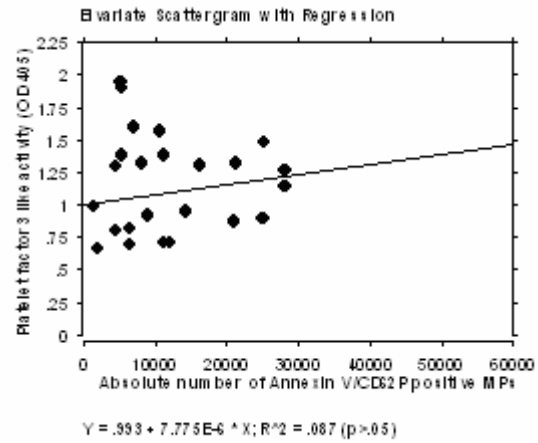
βE



βES



HbH



HbH/CS

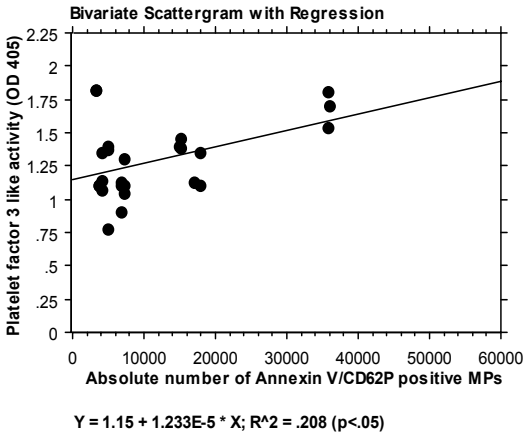
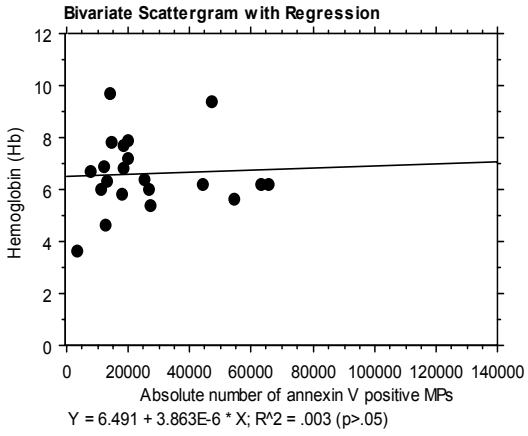


Figure 17f. Regression analysis of platelet factor 3 like activity (OD 405) and absolute number of annexin V with platelet marker (CD62P) positive MPs in peripheral blood in patients with nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

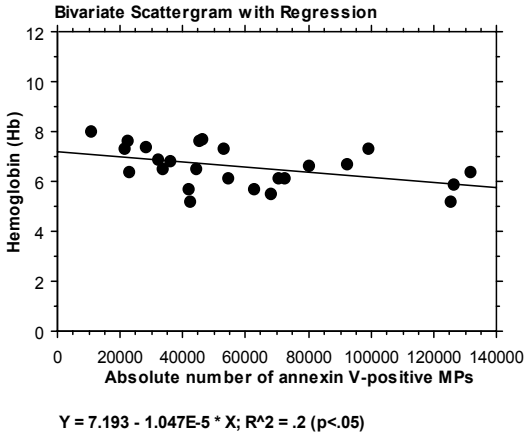
7. Relationship Between Percentage and Absolute Number of Annexin V-positive Microparticles and Hematological Parameter.

Comparison of annexin V-positive MPs and each hematological parameter was performed by using Pearson's correlation coefficient test to study percentage and absolute number of annexin V-positive MPs and their relationship to variety of hematological parameters. A negative correlation was found between the absolute number of annexin V-positive MPs and Hb (r^2=0.2, $p<0.05$) and MCHC (r^2=0.399, $p<0.05$) in βES (Figure 18). In contrast, No correlation between percent of annexin V-positive MPs and hematological parameter was found.

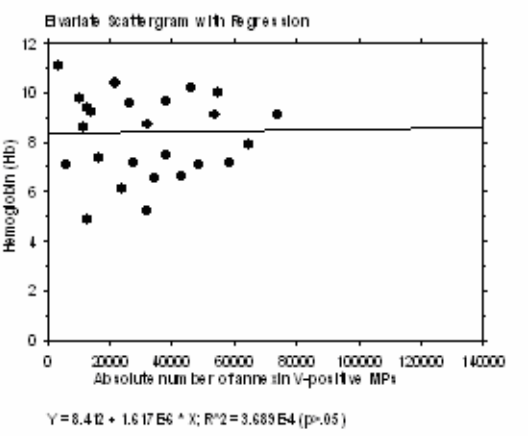
βE



βES



HbH



HbH/CS

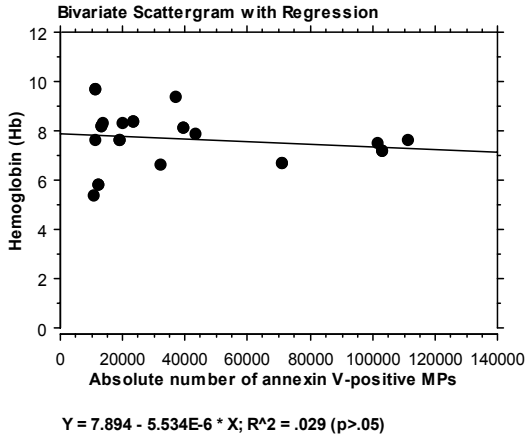
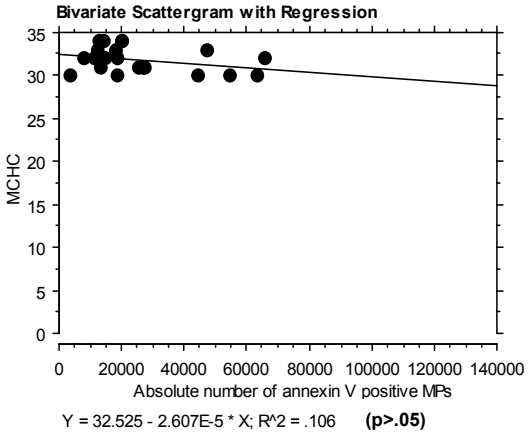
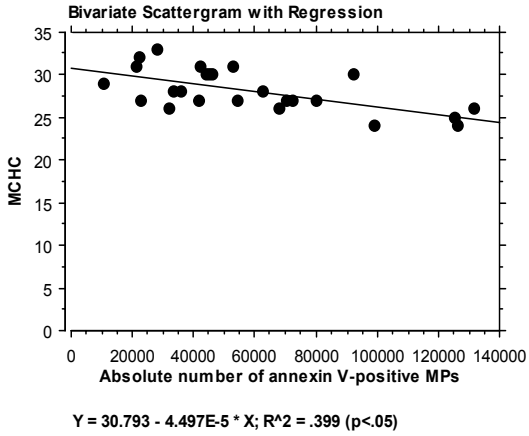


Figure 18a. Relationship between absolute number of annexin V-positive MPs and Hb of nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

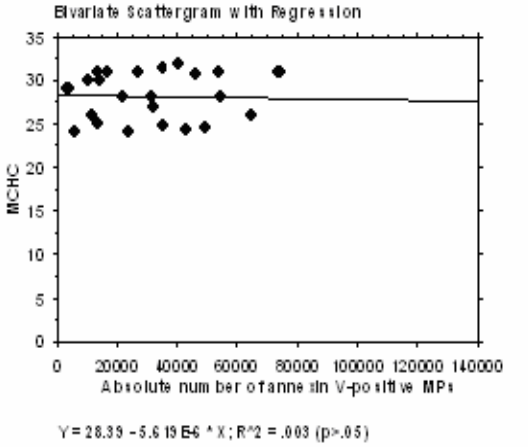
βE



βES



HbH



HbH/CS

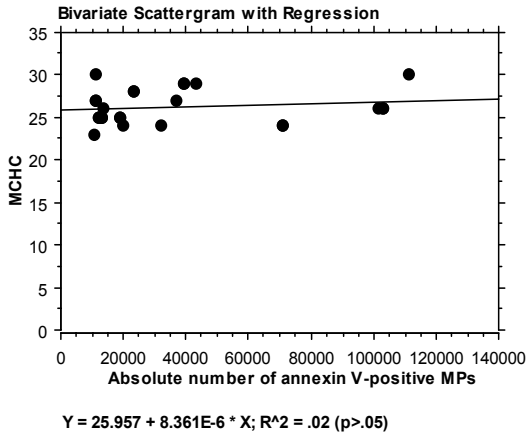


Figure 18b. Relationship between absolute number of annexin V-positive MPs and MCHC of nonsplenectomized β-thalassemia/HbE (βE), splenectomized β-thalassemia/HbE (βES), hemoglobin H (HbH) and hemoglobin H Constant Spring (HbH/CS).

CHAPTER VI

DISCUSSION

Thalassemia is inherited disorder of Hb synthesis. Patient's clinical severity varies widely and caused by an oxidation of excess globin chain on RBCs (2, 3). All thalassemia types show many abnormal hematological parameters such as decreasing in number of RBC, Hb, MCV, MCH, and MCHC. The most severe form is β-thalassemia/HbE especially patients with splenectomy. The reason for its severity is because of the excess α globin chain in β-thalassemia do not form a tetramer like excess β globin in α-thalassemia, which can rapidly react with RBC membrane and cause several damages. These damaged RBCs are destroyed by phagocytic cell causing an ineffective erythopoiesis in bone marrow. Moreover, an ineffective erythrocyte will be destroyed by RE system after circulation in spleen and liver leading to an anemia in β-thalassemic patients (2, 4, 5, 87, 124).

While in α-thalassemias, β-tetramer is formed by unmatched β globin and do not immediately react with RBC membrane which does not lead to an ineffective erythropoiesis but it is unstable and has potential to cause oxidative stress to RBC in α thalassemia afterward (2, 87, 124).

High level of WBC was observed in βES. This effect may be cause by a spleen removal and leading to easy infection in patients (4, 9). Platelet also high in this group. As after usual generation, platelet will generally be incubated in spleen for ½ - 2 days before maturing and circulating in the circulation. When spleen is removed, all platelets generated are therefore found immediately in the circulation (75).

MPs are shed from plasma membrane of most eukaryotic cells undergoing activation or apoptosis. The transverse migration of membrane phospholipid such as PS, which is normally located in the inner leaflet of cell membrane, can be occured in the activation or apoptosis state which is generally coincident with membrane blebbing. Blebs are result from a transient overload of the outer leaflet at the expense of the inner one. When the cytoskeleton is no longer able to counteract the surface tension, then shedding of MPs takes place (28, 96).

There are increasing documented evidences that MPs can interact with neighboring or remote cells, in which case they acquire a pathophysiologic potential (109, 125-128). MPs not only carry accessible PS but also membrane antigens including adhesion proteins or complexes, which can be active, and other procoagulant entities such as tissue factor (29, 32, 96). Several groups have designed methods aimed at measuring MPs by using ultra centrifuges method to collect MPs and then used flow cytometry for their detection based on size and phenotypic analyses (32, 129-131). However, by using ultra centrifuge, the presence of MPs might due to over activation and particles may be loss during complicated process. Pattanapanyasat et al. developed a method for determination of RBC vesicle in thalassemia with non-centrifuge process by using low amount of whole blood sample and using a two-color flow cytometric technique (27). This study we stained the whole blood sample with annexin V conjugated with fluorochome (FITC or PE), which is specifically binding to PS and other fluorochrome-labeled (FITC, PE or PerCP) monoclonal antibodies, that are specific to each cell type to determine number and cellular origins of MPs in peripheral blood from healthy subjects and thalassemia patients.

The MP population is defined by size in forward and side scatter dot plot followed by their morphology which is smaller than platelet and RBC population in the whole blood sample. Flow cytometric and whole blood staining technique have advantages for using low amount of blood sample and using less time as well as less process for rapid measurement which successfully avoid over activation of blood cells after blood collection. Time dependent test showed the percentage of annexin V-positive MPs were not significantly different during laboratory process within four hours, and that comparison of calculated platelet number from this method with platelet number from CBC using hematological analyzer showed a significantly high correlation ($p<0.01$). These results enhance the reliability of the method.

From the whole blood staining technique, percentage of PS exposed on RBCs were also determined. The result supports other previous studies that thalassemia patients have significantly high percentage of PS exposed RBCs when compared with normal individual especially in βES. This is because thalassemic RBCs membrane is damaged by excess globin chain which results in the occurrence of inclusion body and loss of the normal asysmmetrical distribution of membrane phospholipids (5, 25, 26).

β-thalassemia have high percentage of annexin V-positive in RBCs than α-thalassemias. This result could be explained by the excess β-globin chains in α-thalassemias form homotetramers, which are relatively stable and will only damage the RBC membrane when precipitated as inclusion bodies. Whereas, in β-thalassemia, excess α-globin chain do not form such homotetramers and bind to the cytoplasmic surface of the membrane, where they produce oxidative damage leading to rapidly react to cell membrane. Therefore, the degree of membrane damage in β-thalassemia is greater than in α-thalassemia (5).

These PS exposed on RBCs can be recognized by macrophage and removed by RE system in spleen which is one cause of anemia in thalassemia patients. Thus, removal of spleen could cause a decreased removal of these cells which consequently lead to elevated number of PS exposure RBCs in the circulation of βES patients (4, 5).

In this study, we found that PS also exposed on platelet detected by annexin V/CD41 positive in platelet population. Only platelet of βES patients had a significantly high percentage of PS expression. As mentioned above, platelet usually incubated in spleen for ½-2 days before release into the circulation, so platelets in βES patients could be in an immature state which derived immediately from bone marrow to circulation. These immature platelets are prone to activation which leads to PS expression on their membrane. In addition, there are reports suggested that activation of platelet in thalassemia could be activated from thrombin in circulation. Since thrombin is one of platelet agonist. This thrombin might be generated by thalassemic RBCs that have a PS expression which provide a source of negatively charge that provide binding sites of prothrombinase complex. Correlation of the percentage of PS expression on RBCs and platelets also found in this study. At least, these results can support previous evidences about chronic platelet activation in thalaasemia and the relation between PS expression thalassemic RBCs and platelet activation (68, 70).

Some studies reported that thalassemic RBCs have the capacity to shed annexin V-positive RBC vesicles in circulation which related to defective Hb synthesis, RBC perturbation and pathophysiological complications in thalassemia (27, 77). Morover, there are many studies reported that activation of other cells in circulation of thalassemia patients including platelets, ECs, monocytes and granulocytes have a potential ability to shed their MPs in circulation (15, 76, 132). PS

exposured MPs could be derived from these cell types may be involved in pathophysiological complications and hypercoagulation state in thalassemia patients. From these hypotheses, percentage, absolute number and cellular origin of annexin V-positive MPs were determined in peripheral blood from thalassemia patients and were correlated with hematological parameters and coagulation parameter including platelet factor 3-like activity. Results showed a significantly high percentage and absolute number of annexinV positive MPs in thalassemic patients especially in βES when compared with healthy subjects. Most of MPs were originated from platelets. However, some of them may be generated from RBCs or ECs and possibly from granulocytes, monocytes and leukocytes. Tissue facter were also detected on MPs. There was a positive correlation between number of MPs and platelet factor 3 like activity, and correlations were highly seen with platelet activation markers (CD41, CD62P, CD36).

Since, thalassemia have high number of annexin V-positive MPs than normal subjects. This MPs may be initiated from defective RBCs which express PS on their surface. These PS can trigger generation of thrombin even no injury of blood vessel occur. Thrombin can further activate other cells that might induce generation of MPs in the circulation. β-thalassemia especially splenectomized cause have hight level of annexin V-positive MPs than α thalassemias. This could be explained by the different degree of membrane skeleton protien defects and different severity of β-thalassemias versus α-thalassemias that cause more PS expression on β-thalassemia than α-thalassemia (2, 124).

Platelets are major source of MPs in healthy subjects and thalassemia patients becuase they are naturaly easy to be activated by many mechanisms for their specific function in hemostasis. In thalassemia, abnormal RBCs may enhance thrombin generation in vivo and thus trigger platelet activation. Thrombin is the most potent platelet agonist, and its formation in thalassemia has been substantiated by elevated TAT complexes (15, 70).

Oxidative stress, with the generation of reactive oxygen species (ROS), is suspected to play a role in the patho-physiology of thalassemia. Platelets obtained from β-thalassemic patients contain higher ROS and lower glutathione (GSH) levels than normal subject which indicated a state of oxidative stress (5, 24, 87, 133). The

oxidative status of the platelets was also affected by RBCs. There is evidence that higher ROS found in normal platelets when incubated with thalassemic RBCs than with normal RBCs. Thalassemic platelets undergo a state of oxidative stress, leading to their activation and have potential of thromboembolic consequently, including generation of MPs in circulation.

Previous study of MPs in healthy sujects and many patients who are at risk of thrombosis also showed that platelets are the major source of MPs (32, 36, 96). Both platelets and their MPs have potential of being procoagulant and anticoagulant substrate. Nowaday, their functions have been focused on PS molecule that express after activation. In normal state, low amount of PS involve in hematostasis. Lack of PS expression like Scott syndrome lead to decrease capacity to generate MPs and have problem in bleeding (134). However, over generation of them could lead to hypercoagulable as shown in many deseases. Since MPs are hallmark of cell activation. High level of MPs generated from platelet in thalasemia could represent (be index) chronic platelet activation state in patients and support other reports about chronic platelet activation in thalassemia that are involved in thromboembolic complications, a leading cause of morbidity and mortality especialy in β-thalassemia.

Interestingly, defective thalassemia RBCs should have a high shedding MPs since pathology of thalassemia is a consequence from these ineffective RBCs and most of these cell are presented at a high number in the circulation. This effect may be due to a size of membrane vesicles that shed from RBCs called RBCs vesicles. The size of RBC vesicles are larger than other cell types. They are the same size as platelet population and larger than the size of MPs which is about 0.1-1 μm. Comparison between number of RBCs vesicles from previous reports that used techniqe similar to this study showed that the number of RBCs vesicles were higher than MPs detected in this study (RBC vesicles/μl; 30,199±19,686 in healthy subjects; 172,067±220,813 in βE; 261,648±268,690 in βES; 126,489±61,343 in HbH; 114,262±70,340 in HbH/CS) (27). Importantly, this point indicates that shedding of vesicles from defective RBCs is the major source of PS particles in peripheral blood in thalassemia patients, comparison of MPs and RBC vesicles was not performed in this study. However, it remains to be seen if there is any correlation between MPs and RBC vesicles in thalassemia.

Elevated annexin V-positive MPs with adhesion molecules in thalassemia patients may be related to thrombosis. Since circulating MPs are markers of cell activation associated with various prothrombotic states. They are released following cell activation when there is a remodeling of the membrane which leads to externalization of phospholipids, such as PS. MPs in turn lead to increased expression of adhesion molecules which lead to amplifying the procoagulant and/or inflammatory response on the endothelial cell surface.

Percentage and absolute nunber of MPs are significantly correlated with some hematological parameter and coagulation parameter (platelet factor 3 like activity). These results showed the relation between MPs and pathology of patients, especially procoagulant potential. MPs mostly originated from platelet, and so with the, correlation between platelet markers and coagulation parameter. These markers are adhesion molecule such as CD36, CD62P, CD41 which have significant potential to bind with ECs and consequently lead to recruitment of MPs to the growing thrombus. The marker characteristies of MPs could be used to suggest activation or hypercoagulation state in circulation of thalassemic patients. At present, there are many investigators who improve the understanding of the mechanisms governing PS exposure process and offer an opportunity for a new pharmacologic approach to throbotic risk based on the control of PS available at the activated cell surface and on the degree of consecutive membrane vesiculation (29, 45, 135).

In cardiovascular diseases, the inhibition of the shedding of MP may account for the efficacy of various drugs. One benefit of Abciximab, a platelet GPII-IIIa antagonist, may rely on the control of MP release by platelets at high shear stress, a common condition in the vicinity of stenotic plaques (136-138). This process can prove crucial in discriminating the efficiency of anti-GPIIbIIIa treatments. For instance, during myocardial infarction, the initial decrease in platelet-derived MP observed in Abciximab-treated patients could have blunted an amplified inflammatory response (137). Other antiplatelet agents like Ticlopidine were shown to reduce platelet derived MP, circulating chemokine levels or monocyte shedding (139, 140). Furthermore, the study in congestive heart failure, vitamin C could decrease the number of circulating MPs. Since vitamin C is an antioxidant, prevention of generation of oxidized phospholipids in MPs (135). Other amplification loops

promoted by oxidative stress, catecholamine or angiotensin II activation, hyperglycemia or dyslipidemia, can possibly be targets in the pharmacological control of MP release (29, 135).

This study is the first study to determine MPs in thalassemia peripheral blood. By using flow cytometry, the advantage is the need for a small amount of blood volume and does not need a complication process, avoid a loss and over generation of MPs after blood collection. This techniqe can determine PS expresion on MP, platelet and RBC population at the same time. Analysis of peripheral blood MPs appears promissing to provide a useful information on the status of thromboembolic events in thalassemia.

CHAPTER VII

CONCLUSION

Microparticles (MPs) are membrane visicles sheding from many different cell types in circulation when submitted to a number of stress condition. Elevated number of MPs was found in many patients who had a risk of hypercoagulable state. Thalassemia is a hereditary hemolytic disease resulting from unbalanced synthesis of globin chains which also have high incidence of thromboembolic complications. This study uses flow cytrometer to determine number and cellular origin of MPs in peripheral blood of thalassemia patients and healthy subjects. MPs are defined by small particles (0.1-1 μm) that are smaller than RBC and platelet population which also express PS that specifically bind to annexin V and membrane antigen of their cellular origin. Coagulation factors (platelets factor 3 like activity) detected from whole blood were also determined to relate with the number of MPs.

The results showed that thalassemia patients especially with βES have a significantly high percentage and absolute number of annexin V-positive MPs when compared with healthy subjects. Furthermore, significantly high percentage of PS expression in RBCs and platelet population in thalassemia patients were also found. The percentage of annexin V-positive in MPs were correlated well with percentage annexin V-positive in platelet population and the percentage of annexin V-positive in RBCs population. Cellular origin of annexin V MPs are mainly from platelet that were activated with adhesion molecule marker ($AnnexinV^+/CD41^+/CD62P^+/CD36^+$). MPs are also found in RBC origin, ECs, monocytes, granulocytes and leukocytes. Low amount of tissue factor were also detected on MPs. However, these MPs expressing their antigens were low, though high percentage in thalassemia was found when compared with healthy subjects. PS expressed on MPs could provide a binding site to form prothrombinase complex leading to thrombin generation. Thrombin can further activate platelets and other cells in the circulation. This process suggests that the elevated number of MPs may be generated from activated platelet which may be caused from expression of PS on ineffective thalassemic RBCs since positve

correlation between platelet factor 3 like activity and number of annexin V positive in MPs, platelets and RBCs population were found while the highest correlation with MPs that expressed platelet activation markers were seen. This result suggests that MPs may be involed with coagulation state by PS and adhesion molecule expression which could lead to clinical severity in thalassemia patients.

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