HYPERCOAGULABLE STATE AMONG THALASSEMIA MAJOR PATIENTS

BY

DR WAN AMAL HAYATI BINTI WAN HASSAN

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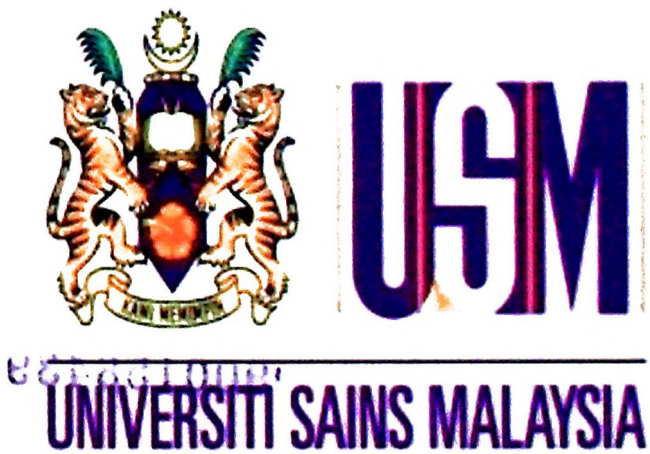

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CD	Cluster of Differentiation
FITC	Fluorescein isothiocyanate
HbE/β	Haemoglobin E/ β
HbH	Haemoglobin H
HCT	Haematocrit
HPLC	High Performance Liquid Chromatography
HUSM	Hospital Universiti Sains Malaysia
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MRI	Magnetic Resonance Imaging
NRBC	Nucleated red blood cell
RBC	Red blood cell
RDW	Red cell distribution width
TF	Tissue factor
TXA	Thromboxane
USM	Universiti Sains Malaysia
WHO	World Health Organization.

7. ABSTRAK

KAJIAN TENTANG STATUS HIPERKOAGULASI DARAH DALAM KALANGAN PESAKIT THALASSEMIA MAJOR.

Insiden tromboembolik yang meningkat dalam kalangan pesakit thalassemia telah mendorong kepada banyak kajian dilakukan untuk menentukan punca keadaan hiperkoagulasi darah dalam kalangan pesakit thalassemia. Banyak faktor penyebab kepada keadaan ini telah dikenalpasti seperti membran sel darah merah yang tidak normal, pengaktifan platelet yang berterusan dan sistem pembekuan darah yang tidak normal.

Objektif kajian ini ialah untuk menentukan paras Protein C, Protein S dan

Antitrombin III dalam kalangan pesakit thalassemia major dan membandingkannya dengan individu normal. Untuk tujuan ini, satu kajian kes kontrol telah dijalankan di Hospital Universiti Sains Malaysia (HUSM). Tiga puluh enam pesakit thalassemia yang datang untuk pemindahan darah secara kerap telah menjadi subjek dalam kajian ini. Dua puluh penderma darah yang sihat diambil sebagai kontrol normal. Sampel darah dianalisa menggunakan mesin koagulometer ACL Elite Pro.

Keputusan menunjukkan purata paras Protein C, Protein S dan Free Protein S adalah rendah dan signifikan secara statistik berbanding kontrol. Purata protein C, Protein S, dan Free Protein S adalah (54.54 ± 13.22 , 94.41 ± 18.73 dan 70.09 ± 12.32) manakala untuk kontrol (94.14 ± 16.34 , 105.12 ± 16.79 dan 99.79 ± 17.33). Keputusan purata antithrombin pula tidak menunjukkan perbezaan yang signifikan secara statistik apabila dibandingkan dengan kontrol. Purata tahap Protein C, Protein S, Free Protein S dan antithrombin tidak menunjukkan perbezaan yang signifikan secara statistik di antara pesakit yang telah menjalani splenektomi dan dengan yang tidak menjalani splenektomi. Selain itu, tiada kes thromboembolik direkodkan.

Kesimpulannya, paras protein C dan protein S yang rendah dalam kalangan pesakit thalassemia menunjukkan keadaan hiperkoagulasi dalam kalangan pesakit ini. Walaubagaimanapun, kajian lanjut perlu dilakukan untuk mengenalpasti parameter lain yang menyumbang kepada keadaan hiperkoagulasi. Paras Antithrombin yang normal adalah mungkin kerana kesan positif daripada pemindahan darah yang kerap. Kajian lanjut perlu dilakukan untuk membuktikan hipotesis ini. Walaupun tiada kejadian thromboembolik berlaku dalam kalangan pesakit, kejadian ini boleh berlaku kemudian atau secara subklinikal. Oleh itu kajian susulan ke atas pesakit dan kajian untuk mengesan trombosis subklinikal perlu dilakukan.

8. ABSTRACT

HYPERCOAGULABLE STATE AMONG THALASSEMIA MAJOR PATIENTS

Increased incidence of thromboembolism among thalassemia patients had triggered various studies to determine hypercoagulable state among thalassemia patients. Several factors for the hypercoagulable state had been identified such as red blood cell membrane disruption, chronic platelet activation and defect in coagulation pathway.

The objectives of this study were to determine the level of Protein C, Protein S and Antithrombin III among thalassemia major patients and to compare the level of these natural anticoagulants between thalassemia major patients and healthy controls. For this purpose, a case control study was conducted in Hospital Universiti Sains Malaysia (HUSM). Thirty six thalassemia patients who came for regular blood transfusion and twenty healthy blood donors for normal control were recruited. Blood samples were collected and analysed for Protein C, Protein S, Free Protein S and Antithrombin III using ACL Elite Pro coagulometer.

The results showed mean Protein C, Protein S and Free Protein S levels were significantly lower in thalassemia patients (54.54 ± 13.22 , 94.41 ± 18.73 and 70.09 ± 12.32 ; respectively) compared to normal controls (94.14 ± 16.34 , 105.12 ± 16.79 and 99.79 ± 17.33 ; respectively).

Mean Antithrombin III showed no significant difference compared to normal controls (116.09 ± 27.28 and 124.36 ± 12.49 respectively). There were no significant differences of mean Protein C, Protein S, Free Protein S and Antithrombin III between splenectomised and non splenectomised patients. No thromboembolic events were documented in this study.

In conclusion, significantly decreased Protein C and Protein S in thalassemia patients suggest hypercoagulable state in those patients. However further studies need to be done to look for other parameters contributing to hypercoagulable state in thalassemia patients. A normal antithrombin level could be due to protective effect of regular transfusion, a further study need to be done to prove this hypothesis. Besides, thromboembolic event can be subclinical, therefore a further study is needed to follow up the patients and to detect subclinical thrombosis.

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

Thalassemia is a growing health problem worldwide. Globally, WHO's South-East Asia Region, is the most severely affected where in this area the thalassaemias and Hb E predominate (Weatherall and Clegg, 2001). Annual HbE/β thalassemia and β thalassemia births over next 20 years was estimated around 400,000 in Malaysia, Thailand, India and Sri Lanka (Vichinsky, 2006).

In Malaysia, latest data from Malaysian Thalassemia Registry 2009, showed there are a total of 4,541 registered thalassemia patients of which 3,310 are transfusion dependent β thalssemia major and HbE/ β thalassemia patients. Thalassemia intermedia accounted for 455 patients and 410 individual having Haemoglobin H disease. The rest are made up by other thalassemia subtype (MOH, 2009).

Currently, due to improvement in the way of managing thalassemia, most of the patients survive longer. A study done among thalassemia major patients showed better survival and complication-free survival for patients born shortly before or after the availability of iron chelation (Borgna-Pignatti *et al.*, 2004). However more new complications had been observed. Complications like hypercoagulable state, osteoporosis, hepatocellular carcinoma and psychosocial problems which were less often described, now have been well recognised (Agarwal, 2009).

Increased prevalence of thromboembolic events had been observed in thalassemia population. OPTIMAL CARE study (Overview on practices in thalassemia intermedia

management aiming for lowering complications across the region) done on 584 patients with thalassemia intermedia revealed thrombosis as the fifth most common complication involving 14% of the patients' population (Taher *et al.*, 2009). The same study which determined the survival of thalassemia major patients showed 1.1 % prevalence of thrombosis in their studied population (Borgna-Pignatti *et al.*, 2004). A study done involving 9 Italian thalassemic centers identified out of 735 subjects , of whom 683 had thalassemia major and 52 thalassemia intermedia, 32 subjects had episodes of thromboembolism corresponding to 3.95 and 9.61%, respectively (Borgna Pignatti *et al.*, 1998). Another multicenter study involving 56 tertiary referral centers in 7 countries involving large group of 8860 patients, found prevalence of thromboembolic event was 0.9% in thalassemia major and 4% in thalassemia intermedia (Cappellini *et al.*, 2006). Besides, a retrospective study of the prevalence of thromboembolic events in 83 adults with thalassaemia intermedia and major during a follow up period of 10 years showed a high prevalence of thromboembolic events particularly in splenectomized patients with thalassaemia intermedia (29%) (Cappellini *et al.*, 2006). In Turkey, the data compiled from the Turkish Thalassemia Study Group from 11 centers revealed thromboembolism in 3.27% of the patients (Akar *et al.*, 1998).

Thrombosis in thalassemia patients involve both arterial and venous thrombosis. A multicentre retrospective study done in Italy estimated the prevalaence of thromboembolic events as 5.6% (33/580). These events consisted of portal vein thrombosis, deep vein thrombosis, intra-atrial thrombosis, central nervous system thrombosis and pulmonary embolism (Iolascon *et al.*, 2001).

Cases of recurrent arterial occlusion, recurrent pulmonary thromboembolism, venous thrombosis and a fatal cerebrovascular event have been reported among thalassemia major patients (Michaeli *et al.*, 1992). Karimi et al reported seven cases presented with the signs of cerebrovascular accident, five ischemic and two with hemorrhage (Karimi *et al.*, 2008). Besides, silent cerebral infarction also detected by brain magnetic resonance imaging (MRI) in splenectomised thalassemia intermedia in Lebanon (Musallam *et al.*, 2012). Priapism as a rare manifestation of thromboembolism was also reported in Hb E/β thalassemia patients (Sharma *et al.*, 2007).

This alarming increase of thromboembolism prevalence in thalassemia patients triggered the study of hypercoagulable state among thalassemia patient. A whole blood thromboelastometry test done in thalassemia patients showed evidence of hypercoagulable state. The clotting time and clot formation time were shortened together with increased maximum clot firmness (Tripodi *et al.*, 2009). Various haemostatic changes that lead to hypercoagulable state have been discovered. There were changes in the coagulation function , coagulation factor inhibitor and component of fibrinolytic system. Presence of increased plasma level of thrombin-antithrombinIII complexes were found in 50% of adults and children of β thalassemia major (Eldor *et al.*, 2001). A study among splenectomised β thalassemia patients whom had pulmonary hypertension also showed significant increased in β_2 thromboglobulin and thrombin-antithrombin III complex levels. These findings together with presence of increased pulmonary vascular resistant indices suggest hypercoagulable state in thalassemia (Atichartakarn *et al.*, 2003b).

Every component of haemostasis is likely to be involved in and contribute to the hypercoagulable state. Though there are various factors involved, Protein C, Protein S and Antithrombin III level are still among important screening marker for thrombophilia. Therefore this dissertation focused on protein C, protein S and antithrombin III level among thalassemia patients to support the evidence of hypercoagulable state among these patients.

CHAPTER 2

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 OVERVIEW OF THALASSEMIA

Thalassemia is a hereditary globin chain disorder that results in diminished rate of synthesis of one or more of the globin chains and subsequently reduced rate of synthesis of the haemoglobin or haemoglobins of which that chain constitutes a part. It may results from deletion of a large part or all of a gene or from a small deletion or other mutation of a gene (Bain, 2006).

This genetic disorder is considered as a heterogenous group of disease with varied ethnicity, phenotypes and treatment (Weatherall DJ et al 2001). The clinical spectrum of thalassemia are varied, from asymptomatic thalassemia minor to thalassemia intermedia and to a more severe transfusion dependent thalassemia major.

Besides, based on clinical presentation, haemoglobin level, dependency of blood transfusion and molecular defect, thalassemia can be divided into thalassemia major, intermedia and thalassemia minor.

Patients with thalassemia trait have haemoglobin level more than 10 g/dL and usually asymptomatic (MOH, 2009). Classical beta-thalassemia trait has hypochromic microcytic red cell indices and a raised Haemoglobin A_2 with values 4% and above

when measured by high performance liquid chromatography (HPLC) (G Elizabeth, 2011).

Thalassemia major or transfusion dependent thalassemia patient is defined as thalassemia patient requiring 8 or more transfusion in 12 months. Thalassemia major patient usually presented at 4 - 6 months or less than 2 years of age and have anaemia, jaundice, hepatosplenomegaly, thalassaemic facies and growth retardation (MOH, 2009).

Thalassemia intermedia is defined when the patient maintains hemoglobin at or above 7–7.5 g/dL (Taher *et al.*, 2006). Presentation of thalassaemia intermedia is at a later age. 59% of patients were diagnosed after the second year of life (MOH, 2009). Several mechanism involved to explain the mild clinical characteristic of thalassemia intermedia. Mild or silent beta chain mutation inheritance , co-inheritance of alpha thalassemia and co-inheritance of determinant asassociated with increased gamma chain production will cause less alpha and beta chains imbalance and therefore lead to less severe phenotype. They presented with wide range of clinical spectrum, from mild anaemia to severe anaemia , hepatosplenomegaly and thalassemia facies. Transfusion in this group of patients may become necessary with advancing age and in conditions, like infection, hypersplenism, and pregnancy (Borgna-• Pignatti *et al.*, 2010).

On the other hand, the major types of thalassemia are classified into α or β thalassemia based on their globin defect and according to their genotype.

2.1.1 Alpha (α) Thalassemia and Haemoglobin H disease (HbH disease)

Alpha (α) thalassemia are a group of condition resulting from a reduced rate of synthesis of α globin (Bain, 2006). The severity of the defect is very variable . At one extreme, deletion or dysfunction of one of four α gene causing a completely asymptomatic condition with trivial abnormality in the blood count and film or no abnormality at all. At the other extreme, deletion of all four α gene causing haemoglobin Bart's hydrops fetalis, a condition that generally not compatible with life (Bain, 2006).

HbH disease is one subtype of α thalassemia and a serious health problem in Southeast Asia and Southern China (Cohen *et al.*, 2004) It is a result of various genetic abnormalities. The most common cause are compound heterozygosity for α^{+} thalassemia and α° thalassemia eg. $\_\ \_^{SEA}$ / $\_\ \alpha^{4.2}$ in South East Asia (Bain, 2006). Excess of β chain production over α chain production leads to abnormal haemoglobin with β chain tetramers referred to as haemoglobin H. HbH is prone to oxidation therefore unstable and precipitates in circulating RBC causing membrane rigidity, red cell fragmentation and chronic haemolytic anaemia. Haemolysis rather than ineffective erythropoiesis is the primary cause of anaemia in HbH disease (Schrier, 2002).

The clinical course may be mild and can be severe depending on type of genetic defect. Seventy five percent (75%) of Hb H mutation involve deletion on chromosome 16 and this defect cause a milder form of the disease. Another twenty five percent (25%) of patients with Hb H disease have two deletions with a point mutation or insertion in the alpha globin gene. The severe form of Hb H disease is usually due to compound

heterozygosity for α° and non deletional α thalassemia (Cohen *et al.*, 2004). Haemoglobin Constant Spring is the most common non deletional alpha thalassemia mutation associated with Hb H disease (Vichinsky, 2006).

Clinically, patients present with symptoms of anaemia and splenomegaly. Laboratory investigation of Hb H disease reveal anaemia with haemoglobin level varying from 3-11g/dl. MCV is around 50-65 fl , MCH is usually 15-20pg and MCHC is of the order of 25-30g/dl. The blood film shows anisopoikilocytosis including target cells, fragments, tear drop cells, hypochromia and microcytosis. Haemoglobin electrophoresis and HPLC reveal HbH comprises 1-40% of total haemoglobin, haemoglobin Bart's comprises around 5% of total haemoglobin in some patients. Heamoglobin A_2 is reduced to 1-2%, Haemoglobin F maybe increased to 1-3%. A haemoglobin H preparation using new methylene blue will show characteristic 'golf ball' HbH inclusion (Bain, 2006)

2.1.2 Beta (β) Thalassemia

Beta (β) thalassemia is another major subtype of thalassemia worldwide. This condition resulting from a reduced rate of synthesis of β globin due to mutation of the β globin gene. Over 200 β thalassemia mutations have been recognized occurring in various ethnic groups (Weatherall and Clegg, 2001).

The homozygous states or compound heterozygous for β thalassemia will lead to phenotype, thalassemia major. Severe anaemia in this group of patient is due to both ineffective erythropoiesis and shortened red cell life span. There is an excess of α chain as the β chain synthesis decreases in this condition. Some of the excess α chain is used

for synthesis of other haemoglobins which do not have β chains, such as haemoglobin F ($\alpha_2\ \gamma_2$) or haemoglobin A2 ($\alpha\ _2\delta_2$) leading to increased level of these haemoglobins. Besides, free α chain left over will form an insoluble tetramer which accumulate and precipitate in the red blood cell (RBC) causing red cell fragility and lysis. Therefore there is ineffective erythropoiesis as the RBC life span is very short and may be destroyed within the bone marrow (Sarnaik, 2005).

Expansion of haemopoietic marrow leads to thalassemic facies while hepatosplenomegaly found in the patients are due to extramedullary erythropoiesis (Sarnaik, 2005). Laboratory investigations show reduced haemoglobin concentration, RBC, haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) while red cell distribution width (RDW) is increased. Haemoglobin range is between 3-7g/dl, MCV 50-60fl and MCH between 12-18 pg. Marked anisopoikilocytosis including teardrop cell, hypochromia microcytosis, and fragmented red cell are seen in blood film. Basophilic stippling, target cells and Pappenhaimer bodies may also be present. Haemoglobin electrophoresis, isoelectric focusing and HPLC will show only haemoglobin F and haemoglobin A_2 (Bain, 2006).

2.1.3 Haemoglobin E/β Thalassemia

Recently, Haemoglobin E/β (HbE/β) thalassemia has replaced β thalassemia as the most common disorder in many regions (Vichinsky, 2006). HbE/β thalassemia maybe the most important haemoglobinopathy because of the high gene frequencies for both HbE and β thalassemia (Weatherall and Clegg, 2001). It occurs as a result of double heterozygote of β thalassemia and Hb E genes. Variable clinical severity are seen in Haemoglobin E- β thalassemia, ranging from a mild form of thalassemia intermedia to transfusion dependence (Fucharoen and Winichagoon, 2000). Half of the patients are phenotypically resembles patients with thalassemia major while the other half have features of thalassemia intermedia (Vichinsky, 2006). Laboratory diagnosis of HbE/β thalassemia is characterized by the presence of haemoglobin E which ranges from 35% to 75% and haemoglobin F (Fucharoen and Winichagoon, 2000).

2.1.4 Treatment

In general, depending on the thalassemia phenotype, blood transfusion is the mainstay treatment. In thalassemia major patients, they require regular transfusion so called hypertransfusion regime. Regular blood transfusion are given in adequate amount every 3 weeks aiming to maintain pre transfusion haemoglobin around 9-10g/dl in order to avoid complications of severe anaemia e.g. growth failure and compensatory bone marrow expansion (Agarwal, 2009). Compared to regular transfusion in thalassemia

major, thalassemia intermedia needs more individualized or tailored transfusion regime that help to prevent transfusion dependency (Borgna-• Pignatti *et al.*, 2010)

However regular transfusion will cause iron overload as every unit of packed red cells contains 200mg iron. A study has shown that the primary long term complication of chronic RBC transfusion is iron overload and the resultant parenchymal organ toxicity (Cunningham *et al.*, 2004) . Iron overload needs to be prevented and controlled as it will lead to many other related complications like endocrinopathies, heart disease and liver disease (Cunningham *et al.*, 2004). Currently, many iron chelation drugs are available. Regular iron chelation with Desferrioxamine (DFO, Desferal), Deferiprone (DFP, L1, kelfer, ferriprox) or Deferasirox (ICL 670, Exjade, Asunra, Desirox) has increased survival free of iron-induced complications (Agarwal, 2009).

2.2 HYPERCOAGULABLE STATE IN THALASSEMIA PATIENTS

2.2.1 Definition of hypercoagulable state

Hypercoagulable state is a condition that predispose individuals to thrombosis. This term is used interchangeably with thrombophilia and prothrombotic state. Rudolf Virchows has postulated three interrelated pathophysiologic causes of thrombosis which involve changes in vessel wall, changes in blood flow and changes in the composition of blood that make blood clot under conditions in which it normally remains fluid (Schafer, 1985). Pathophysiology of hypercoagulable state in thalassemia patients is mainly explained by the third Virchow's triad that suggested changes in coagulability of blood as important factor in thrombogenesis. There are various factors contributing to

hypercoagulable state in thalassemia patients. Combination of these abnormalities will lead to thrombosis.

2.2.2 Overview of Normal Coagulation System

Knowledge on normal haemostasis is important for us before we could understand and further study the underlying pathophysiology of hypercoagulable state in thalassemia. Figure 2.1 and Figure 2.2 showed mechanism of clot formation.

Tissue factor plays a big role in initiating coagulation. In a normal person, tissue factor expressed by vascular smooth muscle cells, pericytes, and adventitial fibroblasts in the vessel wall is physically separated from its ligand FVII/FVIIa by the endothelium. When there is vessel injury, platelets will bind rapidly to the sub endothelium and the coagulation cascade will be activated by tissue factor. Propagation of the thrombus involves intrinsic pathway which amplifies the coagulation cascade and there is recruitment of additional platelets , and possibly by tissue factor-positive microparticles and tissue factor stored in platelets. Finally, fibrin formation stabilizes the clot. De novo synthesis of tissue factor by platelets may also play a role in stabilization of the clot (Mackman *et al.*, 2007)

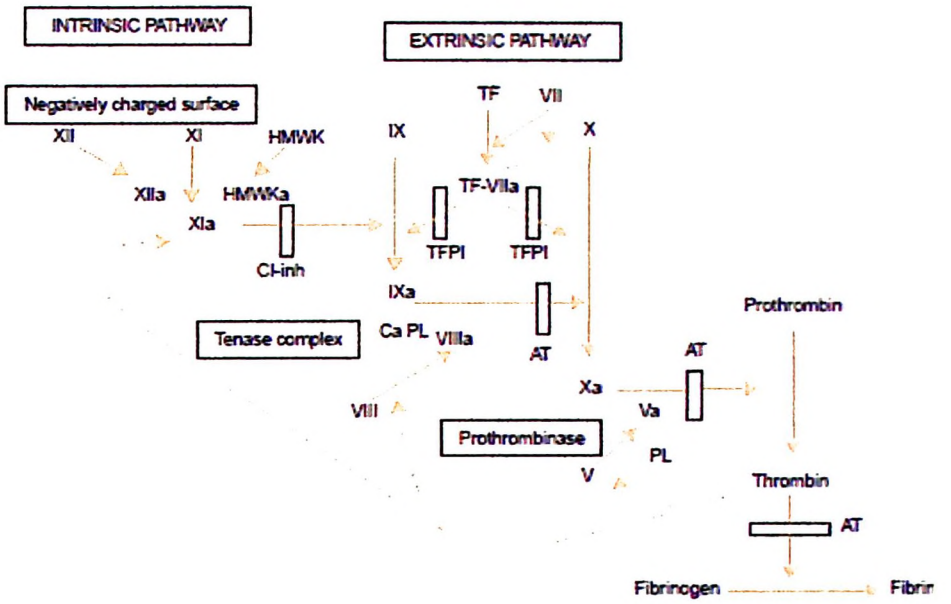

Figure 2.1: The coagulation cascade showing both intrinsic and extrinsic activation, inhibitors and feedback activation (dashed lines). HMWK = high molecular-weight kininogen.,C1-inh = C1-inhibitor. TF = tissue factor. TFPI = tissue factor pathway inhibitor. PL = phospholipids. Ca= calcium. AT = antithrombin. Adapted from (Norris, 2003)

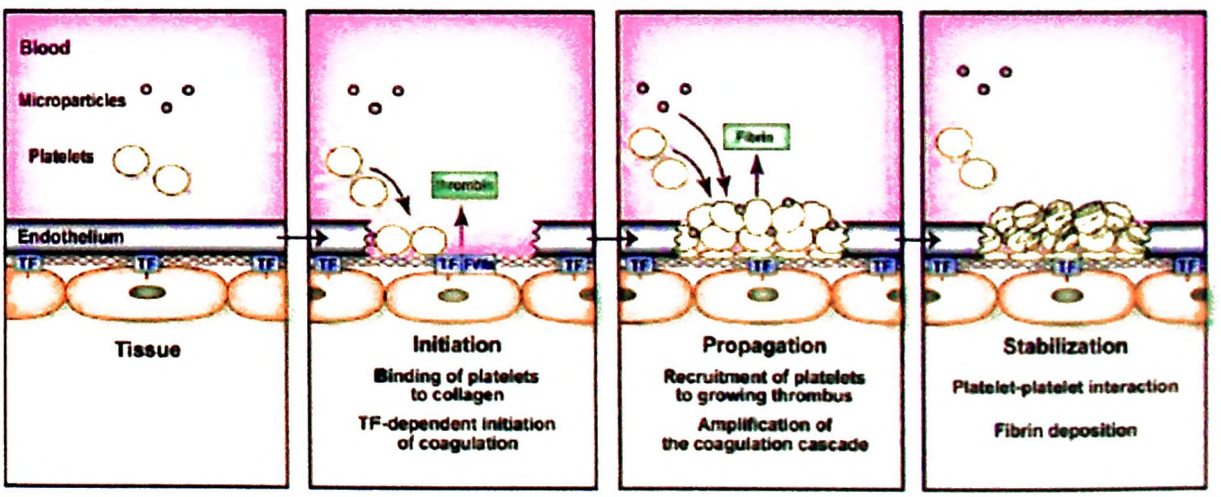

Figure 1.2 : Steps of clot formation. Adapted from (Mackman *et al.*, 2007)

2.2.3 Pathophysiology of hypercoagulable state in thalassemia

Many studies on hypercoagulable state in thalassemia patients had been done. Among factors involved in the pathogenesis are endothelial cells, monocytes and granulocytes activation, platelet activation, red blood cells abnormality, splenectomy, and decreased levels of protein C, protein S and antithrombin III. Several theories are proposed to explain the hypercoagulable state.

Among the possible aetiology is the oxidative state in thalassemia patients. The root cause of this condition is the abnormal haemoglobin itself, together with excess iron which commonly occur in thalassemia patients. In β thalassemia excess α chains form an unstable tetramer then dissociate into monomers which are then oxidized to methaemoglobin and to hemichromes and precipitate with time. Then haem and free iron (labile iron pool) are released and the protein moiety of the globin will precipitate. Formation of reactive oxygen species is enhanced by this chain of events, catalyzed by free iron. In α thalassemia, the excess γ and β chain will form the soluble tetramer , the γ^4 (Hb Bart's) and β^4(HbH). This tetramer are less stable and susceptible to oxidation and hemichrome formation. Another contributor to oxidative stress is iron overload as a result of increased dietary absorption and because of failure to remove excess iron acquired by frequent therapeutic blood transfusions (Fibach and Rachmilewitz, 2008).

Iron overload in transfusion dependent thalassemia patients, generates oxygen-free radicals and peroxidative tissue injury. A study to determine status of oxidant and antioxidant in β thalassemia patients revealed increase in oxidant marker thiobarbaturic acid reactive substances . Antioxidant marker like superoxide dismutase and erythrocyte glutathione peroxidase activities were also significantly increased while total peroxyl radical trapping potential, vitamin E and zinc concentrations were significantly decreased. The findings confirm the peroxidative status generated by iron overload in beta-thalassemia major patients (Kassab-Chekir *et al.*, 2003). Formation of increased reactive oxygen species and oxidative state will further stimulate hypercoagulable state in thalassemia by activating endothelial cells, platelet and lead to changes in red blood cells.

2.2.3.1 Endothelial cells, monocytes and granulocytes activation

Endothelium is a metabolically active interface between the blood and extravascular tissues that produce many active products like prostacyclin, nitric oxide, thrombomodulin, platelet activating factor, von Willibrand factor, thrombomodulin and tissue factor pathway inhibitor to regulate platelet function and blood coagulation. However circulating mediators, damage or disease can disturb the normal antithrombotic and anticoagulant balance of the endothelium (Pearson, 1999).

Endothelial cells can be activated by various stimuli, such as pro-inflammatory cytokines, growth factors, infectious agents, lipoproteins, or oxidative stress (Erdbruegger *et al.*, 2006). Tissue factor is only expressed in activated endothelium

(Pearson, 1999). The activated endothelial cell can participate actively in inflammatory reaction, immunity and thrombosis.

In thalassemia there is increase in circulating endothelial cells which indicate endothelial damage. A study showed increased circulating endothelial cells in both α thalassemia , β thalassemia and HbE/β thalassemia. Good correlation between the number of circulating endothelial cells and low level of Protein C and Protein S were also well demonstrated in the study together with detection of endothelial activation and injury markers like intercellular adhesion molecule-1 [ICAM-1/CD54], E-selectin [ELAM-1/CD62E] and vascular cell adhesion molecule-1 [VCAM-1/CD106] (Butthep *et al.*, 2002). A high circulating endothelial cells count were also found in disease with vascular damage like vasculitis and sickle cell disease (Erdbruegger *et al.*, 2006).

Patients with β-thalassemia major had significantly higher levels of both thrombin – antithrombin complex and ICAM-1 (6.94±3.42 µg/L and 314.11±68.50 ng/ml) compared to control group (3.95±1.69 µg/L and 262.1±41.53 ng/ml) ($p=0.012$ and 0.006 respectively). This findings showed biochemical evidence of hypercoagulable state together with endothelial activation (ME *et al.*, 2008). Furthermore, RBCs from patients with thalassemia major and thalassemia intermedia also showed enhanced adhesion to cultured endothelial cells 10 to 25 fold increase compared to normal RBCs (Hovav *et al.*, 1999). Besides, thrombosis are related to inflammation. Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-1alpha (IL-1alpha) were found

to be above the normal range in HbE/β thalassemia patients (Wanachiwanawin *et al.*, 1999). Monocytes and neutrophils also participate in the haemostatic cascade through expressing or secreting molecules with procoagulant or anticoagulant activity and by causing functional alterations in vascular and perivascular cells like endothelial cells and platelets. Haemostatic process initiated by tissue factor activity are fascilitated by monocytes as monocytes can express tissue factor (TF) at differrent levels of intensity and furthermore Factor Xa and fibrinogen are located on the surface of monocytes (Leone *et al.*, 2001).

A case control study done among β thalassemia major and intermedia using flowcytometry showed significant difference in the mean value of activated monocytes and the monocytes activation markers between the studied groups and within the groups as well (Al-Harbi *et al.*, 2011) This finding suggests the role of monocyte in hypercoagulable state in thalassemia. On the other hand, patients with haemoglobin H disease and β thalassemia major were found to have high level of monocytes colony-stimulating factor and increased monocyte phagocytic activities (antibody-dependent cell cytotoxicity [ADCC]) toward RBCs (Wiener *et al.*, 1996).

2.2.3.2 Platelet activation

Platelets play a major role in coagulation. Activation of platelets is another factor contributing to hypercoagulable state. A study demonstrated the capability of platelets to start and support the entire coagulation process. Tissue factor antigen appeared on the surface of platelets adhering to leucocytes after stimulation with collagen type I. This

tissue factor was competent enough to initiate coagulation cascade (Zillmann *et al.*, 2001).

Evidence of platelet activation using several methods is documented. There are reported studies on platelet aggregation, kinetic studies, measurement of Thromboxane A_2 (TXA_2) and prostacyclin (PGI), and also flowcytometry studies.

A study by Eldor et al showed platelet hypoaggregation among thalassemia patients. This reflect an acquired functional defect that could be due to in vivo activation of platelet causing exhaustion of platelets and refractory to further stimuli (Eldor *et al.*, 1991). However, another study by Atichartakarn showed platelets hyperaggregation in splenectomised HbE/β thalassemia patients in response to adenosine diphosphate, thrombin and ristocetin (Atichartakarn *et al.*, 2003a). This finding was supported by another study by Setiabudy et al that also showed platelet hyperaggregation in splenectomised thalassemia major patients (Setiabudy *et al.*, 2008). Although the result of both platelet aggregation studies is contradicting to each other, both can be the evidence of hypercoagulable state in thalassemia patients.

An interesting study was done in 34 HbE/β thalassemia. Among these, 19 splenectomised patients had partial oxygen pressure (P_aO_2) lower than the normal expected value, while 5 non-splenectomised had P_aO_2 lower than the expected normal value. After Aspirin or Persantin administration there was a definite rise in the PaO_2 in 10 out of 12 patients. The rise of the arterial P_aO_2 after Aspirin administration indicates that the observed hypoxaemia is due to reversible platelet aggregation in the majority of cases (Fucharoen *et al.*, 1981).

A kinetic studies in beta thalassemia major and thalassemia intermedia patients using autologous platelets labeled with indium In 111 oxine showed a significant shortening of platelet life span observed in 13 of 14 patients examined. The mean platelet lifespan of the splectomized thalassemics was 107 ± 36hr (± 1SD) this is significantly shorter than that of the splenectomized normal subjects with no hemoglobinopathy *(P* =0 .0001) investigated with the same techniques. The nonsplenectomized patients with thalassemia also had a significantly shortened mean platelet life span 102 ± 64 hr (± 1 SD) compared to normal *(P* = 0.0001) (Eldor *et al.*, 2006).

Measurement of urinary metabolites of thromboxane A_2 (TXA_2) and prostacyclin (PGI) give another evidence of chronic platelet activation in thalassemia. Thromboxane A_2 (TXA_2) and prostacyclin (PGI) are the major prostanoids formed by platelets and the vessel wall, which involved in the development of cardiovascular and thromboembolic disease. A study of nine splenectomized patients with β thalassemia major, five nonsplenectomized patients with β thalassemia intermedia and twenty healthy individuals found a significant 4 to 10-fold increase in the urinary excretion of the stable hydrolysis products of TXA_2 and PGI_2 in patients with β thalassemia major and β thalassemia intermedia compared to healthy controls. However, the metabolites level between β thalassemia major and β thalssemia intermedia showed no significant difference (Eldor *et al.*, 1991).

In addition, a flowcytometry study also confirmed the existence of chronic platelet activation in thalsssemia by detection of platelet activation marker. There is presence of increased fraction of platelets carrying the activation markers CD62P (P selectin) and CD 63 (Ruf *et al.*, 2003). Elevated plasma levels of platelet factor 3 are also found in patients with β thalassaemia and sickle cell anaemia (Bunyaratvej, 1993).

2.2.3.3 Red blood cells abnormality

Phospholipids in a normal red blood cell membrane are organized in asymmetric pattern. The outer leaflet of plasma membranes consist of predominantly choline phospholipids (sphingomyelin and phosphatidylcholine), whereas majority of the aminophospholipids (phosphatidylserine and phosphatidylethanolamine) are confined to membrane's inner leaflet (Zwaal and Schroit, 1997).

In normal RBC, maintenance of membrane phospholipid asymmetry appears to be provided by the action of an ATP-dependent aminophospholipid translocase (or flipase), that transports phosphatidylserine and phosphatidylethanolamine from the outer to the inner membrane surface. Maintaining a plasma membrane asymmetry is important because presence of phosphatidylserine in the outer membrane leaflet of blood cells will provides a procoagulant surface catalysing the clot formation (Devaux and Zachowski, 1994).

Coagulation process needs a surface containing negatively charged phospholipid, preferably phosphatidylserine. Phosphatidylserine is one of the essential phospholipid cofactors that serve as a surface on which to assemble various complexes to activate clotting factors. This surface is not available in blood under normal circumstances (Yashar *et al.*, 2006). Loss of phospholipid asymmetry, and the exposure of phosphatidylserine , is one of the main factors involved in the red cell pathology in thalassemia patients (Kuypers *et al.*, 1998).

The membrane abnormalities of RBC from the patients with thalassaemia and sickle cell anaemia may result from excessive accumulation of oxidant damage due to excess α or β globin chains which are unstable and tend to oxidize and precipitate within the RBC transforming to hemichromes (Rund and Rachmilewitz, 2005). Peroxidative damage to lipids and proteins is indicated by the increase of about two fold of the serum malondialdehyde, conjugated diene lipid hydroperoxides, and protein carbonyl (Livrea *et al.*, 1996).

A study done on a number of thalassemia patients had shown that there were a subpopulation of circulating red cells exposed phosphatidylserine on their outer surface. The phosphatidylserine exposing cells were identified by binding to fluorescently labelled Annexin V. After analysis by fluorescent microscopy, phosphatidylserine on the outer leaflet of β thalassemic red cells is shown to be distributed either over the entire membrane or localized in areas possibly related to regions rich in membrane-bound α globin chains. The number of such cells can vary dramatically from patient to patient, from as low as that found in normal controls (less than 0.2%) up to 20% (Kuypers *et al.*, 1998).

Another study done among HbE/β thalassemia patients also showed higher phosphatidylserine expression (% of annexin V-positive RBCs) on the outer leaflet of RBC membrane found in splenectomised patients compared to normal control (Atichartakarn *et al.*, 2002). A higher fraction of FITC-Annexin V- labelled RBC were found in thalassemia major and thalassemia intermedia compared to control together with highly significant correlation with CD62(P selectin) and CD63 positive platelet. This finding suggests the procoagulant surface of thalassemic RBC may accelerate thrombin generation and trigger platelet activation (Ruf *et al.*, 2003).

A flowcytometric quantitation of RBC vesicles in thalassemia using Annexin V revealed the numbers of RBC vesicles were significantly higher in thalassemia and most of the RBC vesicles from both normal and thalassemia patients in the study expressed higher percentages of phosphatidylserine positive events than their associated intact RBCs (Pattanapanyasat *et al.*, 2003). Furthermore thalassemia patients have more vesicle level compared to normal person. The vesicle levels were 6-fold greater in sickle cell anaemia and 4-fold greater in thalassemia intermedia than in controls. It is also shown that markers of thrombin generation were significantly related to red blood cell phosphatidylserine . Thrombin generation was promoted by the vesicles in which 40–50% expressed phosphatidylserine (Yashar *et al.*, 2006).

Oxidative stress causes the RBC membrane changes by shedding of phosphatidylserine. This situation is explain by a study using nuclear magnetic resonance spectroscopy, showed 2.6-fold and 1.8-fold more phosphatidylserine and phosphatidylcholine