INTRODUCTION

Hemophilia is an X-linked bleeding disorder caused by mutations in coagulation factor VIII (hemophilia A) or IX (hemophilia B). Severe disease is defined by protein levels below 1% of normal, and typically results in frequent spontaneous joints and soft tissue bleeding. Bleeding into critical closed spaces (e.g. intracranial) can be fatal ***(Herzog et al., 2006).***

The optimal treatment is recombinant factor replacement to prevent bleeding; however, this treatment has many barriers. The most serious complication of treatment is the development of inhibitors to factor products ***(Zimmerman and Valentino, 2013).***

In protein therapy, the overall incidence of inhibitor formation is ~3–4% in hemophilia B and 20%–30% in hemophilia A (10–20% and 30–40% in severe disease, respectively). The risk in gene therapy remains to be defined, but is in part influenced by the gene transfer vector and target organ. Formation of inhibitors is dependent on T help, and subjects with gene deletion or nonsense mutations are more likely to form inhibitors compared with missense mutations. Protocols exist to treat inhibitors using frequent intravenous infusion of high dose factor, often in combination with immunoglobulin infusion and immune suppression ***(Cao et al., 2009).***

It has become obvious that immune regulation is an important and integral component of tolerance to self-antigens and of many forms of induced tolerance ***(Cao et al., 2009)***.

Establishment of long-term immune tolerance to FVIII is essential to ensure the success of gene therapy for hemophilia A. Development of tolerance is a dynamic process involving several mechanisms, including the limitation or deletion of alloreactive T-cell pools and immune regulation ***(Wood and Sakaguchi, 2003).***

$CD4^{+}CD25^{+}$ Forkheadboxes P3 (FOXP3) + regulatory T cells (Tregs) play a critical role in the regulation and suppression of autoimmune and alloimmune responses. T-cell homeostasis can be achieved by balancing Treg and effector T-cell (Teff) populations. Regulation of T-cell function and tolerance induction can be accomplished by inducing a shift in the balance between effector and regulatory cells ***(Read et al., 2009).***

Recent studies provide a rapidly growing body of evidence that Tregs play a crucial role in tolerance to coagulation factors delivered by means of gene transfer. Evidence for involvement of Tregs in controlling the pathogenesis of inhibitor formation in patients has also been provided ***(Cao et al., 2009).*** However, the clinical relevance of Tregs in patients with hemophilia A remains to be fully elucidated.

Aim of the Work

The aim of this study was to determine the frequency of regulatory T cells (Tregs) in children and adolescents with Hemophilia A and to assess their relation to the clinical characteristics of patients as well as Factor VIII inhibitors.

Chapter One

HEMOPHILIA A

Hemophilia A (deficiency in factor FVIII) and hemophilia B (deficiency in FIX) are the most common serious congenital coagulation factor deficiencies. Hemophilia A is an X-linked, recessive disorder caused by deficiency of functional plasma clotting factor VIII (FVIII), which may be inherited or arise from spontaneous mutation ***(Herzog et al., 2006)***.

Epidemiology of hemophilia:

Hemophilia is prevalent worldwide and occurs in all racial and socioeconomic groups. The incidence of Hemophilia A and Hemophilia B is about 15-20 per 100 000male born worldwide. Hemophilia A is also known as 'Classical hemophilia' and account about 80% of cases of hemophilia and occurs 1 in 10,000 male births ***(Kulkarni and Soucie, 2011).***

According to the Report of the annual global survey 2009, the 11th survey by World federation of Hemophilia (WFH) with a participating 105 countries, total number of hemophilia patients is 153,253 of which 115,209 is Hemophilia A and 24,038 is Hemophilia B. Number of Hemophilia A and Hemophilia B patients with clinically identified inhibitors was 5013 & 363, Reported number of Hemophilics infected with HIV and HCV was 5,665 & 24,340 ***(World Federation of Hemophilia Annual Global Survey, 2009).***

However, these figures are an underestimate than actual ones. Because as per estimation of WFH, with a prevalence of HA and HB of 135 per million male child (world population being 6 billion), there would have been 399,000 hemophilia worldwide. So, the majority of the patients remain under diagnosed and it is true that most of them are living in the developing countries ***(Manony and Black, 2005).***

Basics in Bleeding and Clotting *(Zimmerman and Valentino, 2013)*:

The primary function of the coagulation system is to maintain the integrity of the endothelium while preserving vasculature patency. The basal state of the coagulation system is nonthrombogenic for 2 main reasons: the coagulation factors circulate in their inactivated forms and the endothelium is nonthrombogenic. Disruption of the endothelium causes exposure of the thrombophilic subendothelium and initiation of the hemostatic mechanism. The classic pathway of coagulation has been replaced by a cell-based model of coagulation (Figure 1) in which tissue factor (TF), platelets, and thrombin play key roles in initiating, amplifying and propagating clot formation.

Homeostasis involves the complex interaction of platelets with the vessel wall mediated primarily by collagen in low-shear (venous) and VWF in high-shear (arterioles) circuits, both serving an adhesive function in bridging platelets to the subendothelium, where the coagulation proteins dock to

phospholipids sites on the surface of activated platelets, resulting in fibrin-generating concentrations of thrombin.

Coagulation is typically triggered after the exposure of TF, found in the subendothelium, which binds circulating activated (a) FVII. The TF-FVIIa complex activates zymogen FX and FIX to FXa and FIXa, respectively. FXa converts prothrombin (FII) to thrombin (FIIa). The picomolar quantities of FIIa generated on the TF-bearing cell surface releases FVIII from its carrier protein VWF and activates it to FVIIIa. Thrombin also activates platelets, exposing a negatively charged phospholipid-rich surface capable of binding coagulation proteins, including FIXa, previously generated on TF-bearing cell surface. FIXa along with cofactor FVIIIa, calcium, and phospholipids form the Tenase complex, recruit FX to the complex, and activate it to FXa. FXa along with calcium and phospholipids form the prothrombinase complex, which in turn converts large quantities of prothrombin to thrombin, such that fibrinogen is converted to fibrin monomers. Thrombin also activates FXIII, which cross-links the fibrin monomers to stabilize the clot, and thrombin-activatable fibrinolysis inhibitor, which prevents clot breakdown, both improving clot firmness.

The intrinsic system consists of FXII and FXI, which along with high-molecular-weight kininogen and prekallikrein form the contact pathway, and FVIII and FIX. The extrinsic

system is composed of TF and FVII. The common pathway includes FX, FV, thrombin, and fibrinogen.

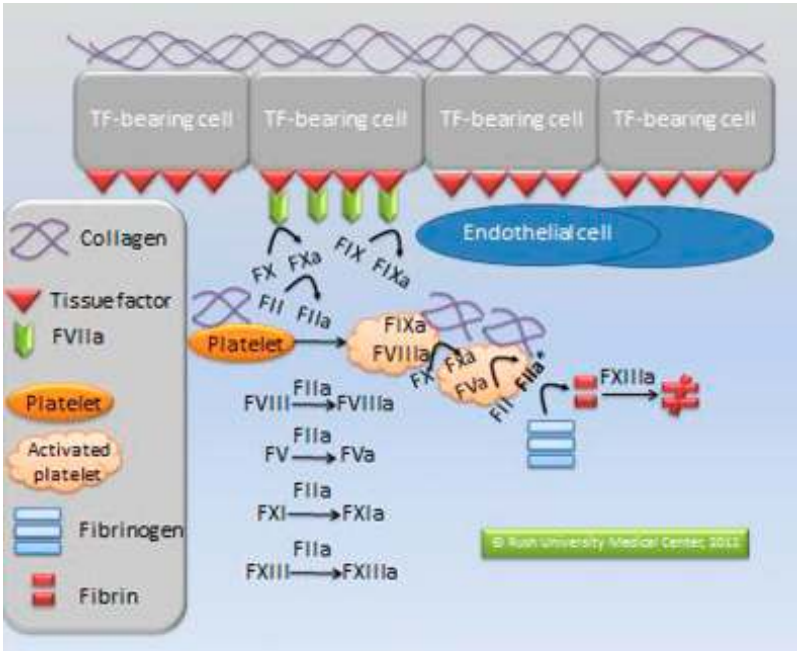

Figure (1): Cell-based model of blood coagulation. F =factor; TF =tissue factor ***(Zimmerman and Valentino, 2013)***.

Genetics of hemophilia:

The genes encoding FVIII and FIX are on the long armof the X chromosome. Hemophilia A and B are the onlyhereditary clotting diseases inherited in a sex-linked recessive pattern. All female children of a father with hemophilia will be carriers, whereas none of his sons will be affected. Further, the sons of a carrier mother have a 50% chance of disease, whereas the daughters will have a 50% chance of being a carrier (Figure 2) ***(Mannucci and Tuddenham, 2001)***.

The genetic mutations cause a quantitative decrease in protein expression, a qualitative decrease in protein activity, or both. Approximately 5% to 10% of patients with hemophilia A and 40% to 50% of patients with hemophilia B make a dysfunctional protein, which results in decreased protein activity without a quantitative decrease. More than 1000 mutations in either the factor VIII or factor IX genes have been identified to cause clinical hemophilia. There is a high rate of spontaneous mutation (approximately one-third of cases) such that even in the absence of a family history, hemophilia should be suspected in a newborn with bleeding and a prolongation in the PTT. Through lyonization of the X chromosome, females with Turner syndrome or X chromosomal mosaicism may have bleeding disorders secondary to reduction in either FVIII or FIX activity ***(Zimmerman and Valentino, 2013)***.

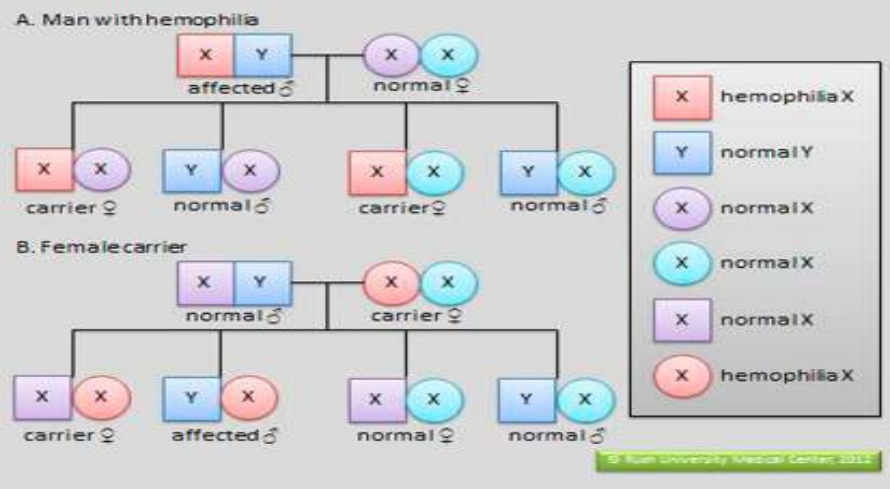

Figure (2): Inheritance of hemophilia
(Zimmerman and Valentino, 2013).

Clinical manifestation of hemophilia:

Hemophiliacs have the heterogeneous phenotypic presentation depending upon its severity described in the Table 1. Severe hemophilia usually present in neonatal period and early infancy, while moderate hemophilia in toddlers and mild hemophilia in late childhood or adolescent and adult often incidental or following major trauma. Bleeding is the hallmark of hemophilia, sites and pattern of bleeding varies over life time. Table 2 shows the common site of hemorrhage in hemophilia ***(Lanzkowsky, 2011).***

Table (1): Relationship of factor level to the severity of clinical presentation in hemophilia

Types	% of FVIII	Type of hemorrhage
Severe	<1	Spontaneous; hemarthrosis and deep tissue hemorrhages.
Moderate	1-5	Gross bleeding following mild to moderate trauma; some hemarthrosis; seldom spontaneous hemorrhages.
Mild	>5-<40	Severe hemorrhages only following moderate to severe trauma, spontaneous bleeding is rare.

(Anwarul and Chowdhry, 2013)

Table (2): Common sites of hemorrhage in hemophilia

Hemarthrosis Intramuscular hematoma hematuria Mucous membrane hemorrhage: Mouth Dental Epistaxis **High risk hemorrhage :** Central nervous system Intracranial extracranial	Retropharyngeal Retroperitoneal Hemorrhage causing compartmental Mouth syndrome/nerve compression Femoral (ilispoas), Sciatic (buttock), Tibial (calf muscle), Perineal (anterior compartment of leg), Median and Ulnar nerve (flexor muscles of fore arm).

(Anwarul and Chowdhry, 2013)

In neonatal period and infancy:

Newborns with hemophilia have distinctive different pattern of as compared with older children and adults and it could be misdiagnosed especially in the setting of negative family history. Though hemarthrosis is rare, iatrogenic and cranial bleeding are common ***(Kenet et al., 2010)***.

Beyond neonatal period most hemophilic seldom have bleeding episode requiring treatment unless accidental injury occurs. Beginning around 1 year of age when the child learns to walk has frequent fall or bumps into the furniture, acute hemarthrosis may occur. Soft tissue bruising and laceration is common in this age group and in a family with unaware of the disease status, he or she might have been suspected as child abuse. Tongue and mouth laceration may occur around this time ***(Anwarul and Chowdhury, 2013).***

Older children and adolescent:

- **Hemarthrosis:**

It is the leading bleeding symptoms in older children and adolescent. Recurrent bleedings causes pathological changes leading to 'target joint' and eventually further bleeding into the joint occur without trauma, overtime this cycle of bleeding causes erosion of joint cartilage resulting in arthritis and the crippling deformities of hemophilic arthropathy (***Anwarul and Chowdhury, 2013).*** Radiological joint damage might appear by 6 years in subjects with no or minimal episode of hemarthrosis ***(Manco-Johnson et al., 2007)***. Eighty percent of bleeding occurs in knee, ankles, and elbow; however involvement of other joints is not unusual.

- **Muscular hemorrhage:**

Muscular bleeding occurs in 10-25% of all bleeds in severe hemophilia and bleeding may become limb threatening, recovery and rehabilitation may be protracted. In contrast to hemarthrosis, muscle bleeds are mostly associated with trauma ***(Beyer et al., 2010).***

Affected muscles become swollen, painful and stiff. There may be bruising on overlying skin if the bleeding occurs in superficial muscle. Deeper muscle bleeding causes pressure on nerves leading to numbness and tingling sensation and if not properly treated by factor replacement leads to compartmental syndrome ***(Mannucci and Tuddenham, 2001)*** and insufficiently

treated muscle bleed may also result in several other complications like irreversible damage to muscle, reduce range of movement, loss of function, myositis ossificans and damage to tendon (Volksmann's ischemic contractures). Iliopsoas bleeding might be life threatening as large volume of blood is accumulated causing shock. Signs of iliopsoas bleeding include upward flexion and discomfort on passive extension of thigh, tenderness on palpation on the lower quadrant and parasthesias just below the inguinal ligament from femoral nerve compression ***(Beyer et al., 2010).***

- **Intracranial hemorrhage:**

It is the serious complication of hemophilia with a significant cause of disability and long term neurological sequelae ***(Stieltjes, 2005)***. Severity of the disease, trauma to head, mode delivery and presence of inhibitor are the risk factors for developing ICH. Beyond neonatal period, ICH is 20-50 times more frequent in hemophiliac than general population ***(Ljung, 2008).***

In children <2 years of age most frequent first documented symptom were apathy and or unusual tears, Vomiting and coma. In those of 2 years most frequent symptoms were headache, coma and vomiting and Irrespective of age coma was observed during the course of ICH in 2/3rd of the cases ***(Kulkarni and Soucie, 2011).***

- **Hematuria:**

Hematuria usually results from blow to the flank, renal calculi and rarely, it may be spontaneous and asymptomatic. It is an infrequent occurrence before 12 years of age. Every patient with haemophilia will present at least one episode of haematuria in his lifetime. In general, haematuria episodes have a short duration and do not cause any severe sequel, except in patients with high titer inhibitors and in HIV patients with bone marrow hypoplasia ***(Cermelj, 2002).***

Diagnosis of hemophilia *(Revel-Vilk, 2011):*

At birth: Hemophilia is diagnosed either due to known family history or after presentation with bleeding.

Collection of blood sample: Arrangement of collection of blood sample from fetal side of placenta should be done if there is possible family history or mother of a male fetus is known or possible carrier. If any newborn child presents with unusual prolonged bleeding is subjected for basic screening test.

Screening tests:

- ***Complete blood count***- remains normal other than anemia.
- ***Bleeding time:*** remains normal.
- ***Prothrombin time (PT)****:* also normal in hemophilia.

- ***Activated partial thromboplastin time (APTT)****:* usually increased by one and half fold to more than 2 fold.

Normal hemogram, bleeding time and PT with prolonged APTT leads to the suspicion of hemophilia which warrants specific factor analysis.

Specific study: Correction study with deficient plasma might identify the types of hemophilia and with normal plasma might suggest presence of inhibitors. Quantitative assay of FVIII, FIX helps in identify the types of hemophilia and its severity.

Molecular genetic testing:

1. Sequence analysis.
2. Targeted sequence analysis.
3. Deletion and duplication analysis and Linkage analysis for:
    - Tracking an unidentified mutation.
    - Identifying the origin of de novo mutation.

Carrier detection:

a) Factor assay- usually lower than normal.

b) DNA analysis- identifies the genetic abnormality.

Antenatal diagnosis: by collecting sample of chorionic villous (CVS) in10-12 weeks of pregnancy and amniotic fluid (amniocentesis) in 16-20 weeks of pregnancy for molecular genetic testing.

Complications of hemophilia:

The development of neutralizing antibodies remains a frequent and serious complication of hemophilia replacement therapy. Most patients with hemophilia do not mount a clinically measurable immune response toward FVIII. In ~30% of patients, however, such FVIII antibodies develop, rendering FVIII treatment ineffective and impairing the functional status of patients ***(Lorenzo et al., 2001; Maclean et al., 2011).***

- ***FVIII inhibitors background:***

Inhibitors of factor VIII (FVIII) have been recognized for more than half a century. They are of two types: those that develop in haemophilics exposed to exogenous FVII (alloantibodies) and those that appear in persons with normal F8 genes (autoantibodies). Hemophilic inhibitors were characterized as antibodies in1963.The inactivation of FVIII was noted to be time, temperature and pH dependent. Factor VIII inhibitors are usually composed of IgG1 and IgG4 heavy chains and kappa light chains, and do not fix complement ***(Shapiro, 1967; Roberts et al., 1975).***

Antigens located in the A2,C2 or A3-CI domains of FVIII can elicit antibody formation, but most alloantibodies are directed against epitopes in the A2 and A3-C1 domains ***(Prescott et al., 1997).*** Binding at these sites interferes with the assembly of the FVIII–FIX complex, inhibiting thrombin generation ***(Fay and Scandella, 1999).***