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MTHFR C677T, PT G20120A and FV Leiden as Risk Factors for Thrombosis in Egyptian Pediatric ALL Patients

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Dedication

To my Father; I miss you so much, my Mother; thanks for making me who I am, my brother and sisters for always being there for me. To my wife and children the joy and happiness of my life thank you for everything.
Acknowledgment

*Praise is to GOD who granted me this learning opportunity.*

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Abstract

Thrombosis is a well-known side effect associated with Acute Lymphoblastic Leukemia (ALL) treatment leading to significant morbidity rates. Thrombosis occurrence in ALL patients seems to be due to the interaction between the disease, the therapy and the possible inherited genetic defects affecting the hemostatic balance. In this study we aimed to assess the prevalence of prothrombotic defects- FV Leiden, MTHFR (Methylene Tetra Hydrofolate Reductase enzyme) C677T & prothrombin (PT) G20210A mutations in Egyptian pediatric ALL patients and its impact on the risk of thrombosis onset as well as to evaluate the impact of the presence of single versus multiple prothrombotic mutations on thrombosis. Sixty three pediatric ALL patients with thrombotic event treated with ALL protocol adopted from SJCRH (Saint Jude Cancer Research Hospital) study XV at the Children’s Cancer Hospital in Egypt (CCHE) and 63 matched ALL control patients were enrolled in the study. Restriction fragment polymorphism technique was used to assess the prevalence of the FV Leiden and MTHFR C677T while Allele specific PCR was used for Prothrombin G20210A. Our results showed that MTHFR C677T prevalence between the ALL patients with and without thrombosis was 65% and 38.1% respectively p value = 0.002. The FV Leiden prevalence between the ALL patients with and without thrombosis was 17.5% and 15.9 % respectively p value= 0.81. While the prothrombin G20210A prevalence was 3.2% in both groups. In addition, patients who were older than 10 years or on SR/HR treatment protocol or in induction treatment phase were also at high risk of thrombosis. The presence of MTHFR C677T polymorphism can increase the risk of thrombosis 3 folds more than those patients who didn’t have the polymorphism, while FV Leiden and PT G20210A didn’t affect the thrombosis risk. Having more than one mutation didn’t show a significant effect on increasing the risk of thrombus incidence (p= 0.087). We concluded that MTHFR C677T is important risk factor for thrombosis in Egyptian pediatric ALL patients. These results may help in the prediction of the thrombosis susceptibility for ALL patients and a prophylaxis therapy may be considered before having the thrombosis. To the best of our knowledge these findings regarding the thrombosis risk factors in Egyptian pediatric ALL patients are first to be reported.
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Glossary and Abbreviations

ALL: Acute Lymphoblastic Leukemia
APC: Activated Protein C
ASPCR: Allele specific PCR Technique
BFM: Berlin-Frankfurt-Munich protocol
BP: Base pair
CAD: Coronary Artery Disease
CCHE: Children’s Cancer Hospital in Egypt
CI: Confidence Interval
CNS: Central Nervous System
CoALL: Cooperative ALL Study
CVL: Central venous lines
DFCI: Dana Farber Cancer Institute
DIC: Disseminated Intravascular Coagulation
dUMP: Deoxy Uracil Mono Phosphate
DVT: Deep Venous Thrombosis
EFS: Event Free Survival
FIX: Factor Nine
FV: Factor Five
FVIII: factor Eight
FX: Factor Ten
HR: High Risk
IPT: Immunophenotyping
IT: Inherited thrombophilia
LR: Low Risk
MRI: Magnetic Resonance Imaging
MRV: Magnetic Resonance Venography
MTHFR: Methylene Tetra Hydrofolate Reductase enzyme
NCI: National Cancer Institute
OS: Overall Survival
PARKAA: North American Prophylactic Antithrombin Replacement in Kids with Acute Lymphoblastic Leukemia Treated with Asparaginase.
PE: Pulmonary Embolism
PT: Prothrombin
RFLP: Restriction Fragment Length Polymorphism
SJCRH: Saint Jude Cancer Research Hospital
SNP: Single Nucleotide polymorphism
SR: Standard Risk
TLC: Total Leukocyte Count
VTE: venous Thromboembolism
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1. Review of Literature

1.1 Acute Lymphoblastic Leukemia (ALL)

1.1.1 Incidence and Epidemiology

Acute Lymphoblastic Leukemia represent about 81% of pediatric leukemias (Woo, Alberti, & Tirado, 2014). It is the most common cancer in pediatric patients representing 25% of cancer diagnosed in pediatrics. ALL outcome is 90% 5 years overall survival which is very high rate of survival among different types of cancer (Pui et al., 2013). The incidence of ALL in the United States is 35-40 new case/million each year. There is a gradual increase in ALL incidence in the previous 25 years (Howlader et al., 2013).

At National Cancer Institute (NCI), Egypt, ALL constitutes 19.6% of all childhood malignancies (Elattar et al., 2005), while the percentage of ALL cases treated in Children’s Cancer Hospital of Egypt (CCHE) in the period between 2007-2012 was reported as 20% (1305/6240) of all pediatric cancer patients (CCHE 57357 Registry, 2012). There is a sharp increase in ALL incidences in age 2-3 years about 90 new case/million each year worldwide. This rate decreases to 30 case/million per year by age 8 years. This high incidence of ALL among 2-3 years aged patients is 4 to 5 folds greater than that of infants and 10 years or older aged children (Howlader et al., 2013). The incidence of ALL among white children is higher than that in black children. There is about three-fold increase in ALL incidences in 2 - 3 years aged white children than in black children. There is also a slight male predominance in all age groups (Howlader et al., 2013).

1.1.2 Risk Factors for Developing ALL

There are varieties of risk factors associated with increased ALL risk; these include X-rays exposure during prenatal stage, exposure to pesticides and ionizing radiations. In addition, ALL occurs with higher frequency in patients with Bloom syndrome, neurofibromatosis type I, ataxia-telangiectasia and Down syndrome. For
example, Down syndrome patients have a 20 fold increase in risk of ALL (Onciu, 2009).

On the other hand, there are factors with limited evidence to be a risk factor for ALL. Few studies suggest a higher risk (1.5 fold) in case of paternal smoking before pregnancy. Although some studies have reported increase in the ALL risk for children who live near high voltage; other studies reported no risk association. In addition to that few studies were done on the meat consumption in diet and showed association with the increase in risk (Blot et al., 1999; Howlader et al., 2013).

1.1.3 Pathogenesis

ALL originates in the bone marrow from either the T or B lymphoblasts (Figure 1). About 85% of pediatric ALL is B cell origin. The B cell origin ALL is classified into several subtypes: pre-B ALL, common ALL and pro-B ALL. On the other hand, T cell ALL represents 15% of pediatric ALL patients. Although T cell ALL patients are characterized by resistance to chemotherapy in comparison with the B cell ALL patients, the outcome of the ALL T cell approaches the B cell using risk adapted therapy in many study groups (Pieters & Carroll, 2010).

Risk adapted therapy is therapy modification done according to the patient predicted risk for relapse. Patients who have high risk criteria receive aggressive therapy to prevent recurrence of the disease. While patients with good prognosis receive effective therapy with fewer side effects and at the same time less aggressive than that received by high risk (HR) patients. The factors used for the patients stratification into different treatment arms are clinical and biological factors (Pieters & Carroll, 2010).
Figure 1: A diagram showing the normal pathway for the differentiation of **blood stem cell**. In normal healthy child, the Bone marrow produce immature cells called blood stem cells, it can differentiate to either a lymphoid stem cell or a myeloid stem cell. Myeloid stem cell differentiates into one of three blood cells which are; red blood cells, platelets and white blood cells. While lymphoid stem cell can differentiate into one of three types of white blood cells which are: B lymphocytes, T lymphocytes and Natural killer cells.
1.1.4 Clinical Presentation and Patients’ Outcome

Acute Lymphoblastic Leukemia is a bone marrow disease. The onset of ALL is acute in most of the cases, although there are few cases that may evolve over several months. Doctors can suspect ALL when there are symptoms of pancytopenia (bone marrow failure). The most Common ALL signs and symptoms include bone pain, pallor, bleeding, easy bruising and fatigue. Signs of extra medullary involvement may appear in signs of intracranial pressure increase including headache. In addition, physical examination may show hepato-splenomegaly and lymphadenopathy (Woo et al., 2014). Laboratory abnormalities may include hyperleukocytosis, thrombocytopenia, leucopenia or neutropenia. Hyperleukocytosis appears in 15% of pediatric ALL patients. Another important laboratory abnormality is hyperuricemia that can result from tumor lysis (Onciu et al., 2009).

The pediatric ALL prognosis has improved over the last decades, where therapy is adapted according to the patient’s level of relapse risk (low, standard and high risk groups) and the patient’s response to chemotherapy. In addition, the continuous appearance of new chemotherapeutic agents had a great role in improving the outcome. The pediatric ALL patients’ outcome has dramatically increased from 10% in 1960 to a current 90% in many developed countries (Figure 2). The precise risk assessment, improved supportive care and the optimal use of chemotherapeutic agent have improved the 5 years disease free survival and the 5 years overall survival rates to more than 85% and 90 % respectively in several treatment protocols (Pui et al., 2013).
Figure 2: Survival assessed by Kaplan Mayer analysis for 2852 newly diagnosed pediatric ALL patients’ in St. Jude children cancer hospital from 1962 to 2007. During this time period, the patients have been treated by 15 different consecutive treatment plans. Ten year free survival rates are shown in the above curve. The results show a drastic improvement in patient outcome from 11.1% in the 1960’s to 91.1% in the early 20th century.
1.2 Treatment

The ALL protocols of treatment consist of 3 main phases: induction of remission, consolidation phase and maintenance or continuation phase. The typical duration of the treatment is 2-3 years, according to which protocol is used. The goal of the induction stage is to induce remission and restore the normal blood cell production in bone marrow while the goal of the consolidation and the maintenance phases are to eliminate residual disease and maintain the patient in remission. In addition, chemotherapy can be injected intrathecally for central nervous system (CNS) prophylaxis or treatment from the disease. There are different types of antileukemic agents that are used for ALL treatment such as methotrexate, 6-mercaptopurine, L-asparaginase, vincristine and glucocorticoids (Pieters & Carroll, 2010).

1.2.1 Asparaginase

In 1953, it was noted by Kidd that the serum of guinea pig has an antileukemic effect in mice. Then it was discovered that the reason of the antileukemic effect was the L-asparaginase enzyme in the serum, which leads to the asparagine amino acid depletion and tumor regression. In 1970s, the L-asparaginase became one of the important chemotherapeutic agents in ALL treatment protocols and the scientists started to use it in the treatment protocols of ALL patients to achieve remission (Rytting, 2012).

The use of L-asparaginase depends on the fact that the leukemic lymphoblasts lack asparagine synthetase enzyme, which is essential for the synthesis of asparagine. Therefore, it depends entirely on external asparagine supplies to grow. L-Asparaginase enzyme results in the hydrolysis of asparagine amino acid producing ammonia and aspartic acid. In effect, asparaginase enzyme results in the selective death of cancer cells only due to depletion of asparagine amino acid. However, normal cells can synthesize their own asparagine using asparagine synthetase enzyme. The asparagine depletion causes an arrest in the cell cycle at the G1 phase as well as an impairment in the cancer cell’s DNA, RNA and protein synthesis resulting in cancer cell death (Mu
& Boos, 1998). Therefore, L-asparaginase enzyme results in the selective death of cancer cells (Figure 3).

Asparaginase is given in induction and maintenance phases of total XV protocol. In induction phase, the dose is 10,000 U/m² taken intramuscular at days 6, 8, 10, 12, 14 and 16 of the induction phase. Three extra doses can be given on days 19, 21 and 23 if the patient residual leukemia cells at day 19 in the bone marrow are greater than or equal 1%. While in the continuation phase, dose and regimen differs according to the patient risk group. Low risk patients receive asparaginase on weeks 7-9 and 17-19, three doses of 10,000 U/m2/dose weekly. On the other hand, standard and high risk patients receive 19 doses from week 1 till week 19, 1 dose weekly. Each dose is 25,000 units /m² taken intramuscular (Pui et al., 2009).

![Image](critical_reviews_hematology.png)

Image adopted from Critical Reviews in Oncology: Hematology 28 (1998) 97–113

**Figure 3: L- asparaginase Mechanism of Action.** L-asparaginase hydrolyzes asparagine amino acid into ammonia and aspartic acid. In normal healthy cells, asparagine can be synthesized from aspartic acid and glutamine using asparagine synthetase enzyme. This enzyme is absent in Lymphoblastic leukemia cells so it depends on external asparagine amino acid. Using L- asparaginase results in depletion of asparagine amino acid and inhibition of protein synthesis in cancer cells.
1.2.2 Types of L-Asparaginase

There are three types of L-Asparaginase commercially available in the market to date: asparaginase isolated from *E.coli* bacteria, Erwinia asparaginase extracted from *Erwinia chrysanthemi* bacteria and the PEG asparaginase which is the peglated form of the *E.coli* asparaginase (containing polyethylene glycol) (Mu & Boos, 1998). L- Asparaginase hypersensitivity manifests in the form of anaphylactic or allergic reactions and can lead to the change to another asparaginase formulation in order to achieve the best outcome and the least side effects.

The *E.coli* asparaginase is the native form of asparaginase as it is the oldest one in the market. Its half-life is 1.2 days, which is a moderate half-life in comparison with PEG and Erwinia asparaginase. If the patient has an allergic reaction from *E.coli* asparaginase, he should be shifted to the Erwinia asparaginase.

The PEG asparaginase has a longer half-life than the native asparaginase. This means a lower number of doses will be given to the patient and therefore better patient compliance. Patients who have an allergic reaction from the peglated asparaginase should be shifted to the Erwinia asparaginase (Asselin et al., 1993).

Erwinia asparaginase is used in patients have an allergy from both the pegelated or the native asparaginase its half-life is 0.65 day which is shorter than the peglated and the *E.coli* asparaginase. This short half-life result in increase the number of asparaginase doses taken by the patient to get the asparagine depleted (National Cancer Institute US, 2014; Rytting, 2012).

It has been shown that the use of Asparaginase in ALL protocols increase the event free survival by 10% - 15% (Tong et al., 2014). Although the L-asparaginase is considered an important antitumor drug in the treatment protocol of ALL patients as it kills cancer cells without harming the normal body cells, L-asparaginase cause a wide spectrum of adverse reactions to the patients. The L- asparaginase side effects include severe allergic reactions, pancreatitis, hepatic dysfunction, hyperglycemia, hemostatic system alterations which appear in the form of thrombosis, bleeding and disseminated intravascular coagulation (DIC) (Pieters et al., 2012). In most of the protocols, L – asparaginase is left out from the consolidation phase and administrated
only in the induction and maintenance phases. Asparaginase can be administered either as an intramuscular injection or intravenous infusion (Rytting, 2012).

1.2.3 L-Asparaginase Side effects

**Hypersensitivity**

Hypersensitivity to asparaginase is one of the serious side effects that can take place after asparaginase administration. The risk of hypersensitivity incidence depends on prior administration of asparaginase and concomitant drugs in the treatment regimen (for example, steroids). The incidence of hypersensitivity reaction in ALL treatment protocols that include high dose corticosteroids in the induction phase is not frequent (Rytting, 2012). On the other hand, hypersensitivity is more frequent upon repeated administration of asparaginase without receiving steroids and its occurrence ranges from 5%–10% (Rytting, 2012; Storring et al., 2009; Vrooman et al., 2010). Although PEG-asparaginase cause less hypersensitivity than E.coli asparaginase, it still can cause allergic reactions associated with repeated doses of administration (Douer et al., 2007). Erwinia asparaginase can be used for patients having hypersensitivity from E.coli and PEG- asparaginase, where there is no cross reaction between E.coli and PEG- asparaginase antibodies and Erwinia asparaginase (Rytting, 2012).

**Pancreatitis**

The three types of L-asparaginase preparations can result in pancreatitis. A randomized clinical trial done on pediatric ALL patients using E.coli L- asparaginase versus PEG L- asparaginase for standard risk patients showed no difference in pancreatitis incidence rate between the two groups (Avramis et al., 2002). It was shown that pancreatitis incidence associated with asparaginase in pediatric ALL patients increase with patient age. This can give a hint that the pancreatitis incidence in Adult will be higher than in pediatric patients (Kearney et al., 2009). Generally, the pancreatitis incidence associated with asparaginase administration ranges from 5%–10% (Avramis et al., 2002; Fu & Sakamoto, 2007).
Thrombus Formation

Hemostasis is the mechanism where the body can control the bleeding from injured blood vessels by clot formation. Then the body dissolves clots that no longer needed. Blood clot is initiated from injury of wall vessel. Platelets accumulate rapidly at the injury site followed by accumulation of coagulation factors forming a clot. The coagulation factors are produced from a cascade of reactions to finally produce fibrin. Fibrin will form a mesh between the platelets aggregation causing clot stabilization. Any disturbance in the hemostatic system can result in excessive thrombosis or bleeding (Horne, 2005).

Thrombus formation is a severe side effect that can takes place due to asparaginase administration. It usually takes place in the early phases of treatment and is related to different factors, such as central lines placement and hypercoagulability due to leukemia activity. The incidence of thrombus formation varies from 10% to greater than 30%. The incidence of thrombus increases with older age. It was reported that the incidence of thrombosis is the same when *E.coli* and Erwinia asparaginase are compared (Grace et al., 2011). The type of steroids used in the induction therapy also can affect the incidence of thrombus where it was reported that prednisolone is associated with higher incidence of thrombus (Durden et al., 1983; Hernández-Espinosa et al., 2009; Nowak-Göttl et al., 2003).

Once thrombus diagnosis is confirmed, low molecular weight heparin is used in the treatment of thrombus. Some studies showed the use of anti-thrombin III infusion as a supportive coagulation therapy, but its role in decreasing the thrombus incidence is not clear till now. A study was done on adult ALL patients showed a decrease in thrombotic events when anti-thrombin III was used as prophylactic infusion (Hunault-Berger et al., 2008). In order to avoid the L-asparaginase side effects and improve the treatment outcome for ALL patients different trials were tried, including using L-asparaginase from different sources and treatment schedules modifications to optimize the L-asparaginase use in the therapy.
1.3 Thrombosis in ALL

Although ALL disease has a very good outcome reaches to 90% 5 years overall survival, the morbidity and mortality that result from toxicities of ALL therapy are significant. These toxicities can lead to therapy modifications that will affect the cure rate. Thrombosis is a well-known side effect associated with ALL treatment protocol leading to significant morbidity rates. The fatality rate is about 15% in those patients affected from thrombosis. The development of thrombosis can interfere with the treatment plan of ALL patients thus affecting the ultimate outcome. There are several studies that correlate the concomitant use of glucocorticoids and asparaginase with increasing incidence of the thrombosis (Pui et al., 2013).

1.3.1 Incidence of Thrombosis in Children with ALL

Thrombosis in children in general is an infrequent incidence. In general healthy pediatric population the incidence of pulmonary embolism (PE) and deep venous thrombosis (DVT) is 0.7–14 events / 100,000 children (Monagle et al., 2001; Van Ommen et al., 2001). In comparison to the general estimates of thrombosis, the ALL pediatric patients are considered to be at much higher risk for thrombosis. The incidence of thrombosis in pediatric ALL patients ranges from 1.1% to 36.7% with an average of 3.2%. This wide range of variation in the reported thrombosis incidence is due to difference in the reported thrombosis definitions (asymptomatic vs. symptomatic), different treatment protocols used and diagnostic tools used for diagnosis and detection of the thrombosis (Athale & Chan, 2003a).

The designed studies for evaluating asymptomatic thrombosis reported much higher thrombosis incidence when compared with other studies reporting symptomatic thrombosis only. For example, a PARKAA study whose objective was to compare ultrasonography to venography in the diagnosis of asymptomatic DVT in pediatric ALL patients reported a 36.7% incidence of thrombosis (Male et al., 2002; Mitchell et al., 2003). On the other hand, other studies reported symptomatic thrombosis incidence ranges from 2.8% to 14.3%. This shows the importance of highlighting the thrombosis definition in the reported studies (Athale & Chan, 2003a).
In addition to the previous discussed factors, the chemotherapy schedule has an important role in affecting the incidence of thrombosis incidence in pediatrics. When 2 different chemotherapy protocols were compared, Berlin-Frankfurt-Munich (BFM) and COALL protocols, it was found that although they treat the same ethnic population and in the same time frame, the thrombosis incidence in patients receiving BFM 90/95 protocol was 10 times higher than those receiving COALL 92/97 protocol. This difference in thrombosis incidence was due to difference of the chemotherapy schedule in the 2 protocols (Mauz-Körholz et al., 2000; Nowak-Göttl et al., 1999, 2001).

1.3.2 Locations of the Thrombotic Events in Children with ALL

The majority of thrombosis events in children are of venous origin; however there are reports for thrombosis events of arterial origin. A large prospective meta-analysis of 17 studies examined the venous thromboembolism (VTE) in ALL pediatric patients. The meta-analysis showed that symptomatic VTE was diagnosed in about 50% of the patients in the central nervous system (CNS), where infarction or stroke represents 18% and cerebral venous sinus thrombosis 28.6%. On the other hand, the study reported other locations for VTE including right atrium (1%), pulmonary embolism (1%), superficial VTE (2.2%) and the lower limbs (7.7%) (Caruso et al., 2006).

Central venous lines (CVL) are great tools that have been used for more than 20 years for children. It is used to maintain a venous access that facilitates the administration of blood products, chemotherapy and other supportive care medications. Central venous lines greatly improve the quality of life in pediatric cancer patients. Although there are several advantages for the use of CVL, its use is associated with both thrombosis and infections. Most thrombosis associated with CVL is asymptomatic and located at the catheter entry site into the vein. The CVL associated thrombosis occurs primarily in the upper venous system. It was reported in a meta-analysis that the incidence of upper limb thrombus and CVL was 27.5% (Caruso et al., 2006; Kenet et al., 2009; Mitchell et al., 2003).
1.3.3 Effect of Age and Gender on the Development of Thrombosis

The incidence of VTE in ALL patients is high in patients greater than 1 year and the incidence trend increase towards older children. On contrast, the VTE incidence in the normal pediatric population is higher in the neonatal period and less than 1 year of age (Athale et al., 2008; Caruso et al., 2006).

The gender effect on the development of thrombosis is unclear. While some of the studies reporting gender distribution show female predominance or male predominance other studies show equal distribution (Athale & Chan, 2003a). (Pui et.al, 1985) reported male predominance in development of VTE. In contrast (Gugliotta et al., 1992), (Priest et al., 1982) and (Nowak-Göttl et al., 1999) reported female predominance.

1.3.4 The Main Risk Factors that Affect Thrombus Formation in ALL Patients

The Thromboembolism occurrence in ALL patients seem to be due to the interaction between the ALL disease, the therapy and the possible inherited genetic defects affecting the hemostatic balance (Athale & Chan, 2003b) (Figure 4).

Risk Factor 1: Effect of Disease on Thrombosis

At diagnosis, there is evidence of increased thrombin generation in children with ALL, the etiology of which is unclear. However, thrombosis in children with ALL is most commonly reported after the initiation of anti-leukemic therapy indicating a possible interaction of the disease and therapy (Athale & Chan, 2003b).
Figure 4: shows the 3 main factors involved in thrombosis pathogenesis in ALL patients. Development of thrombosis in ALL patients depends on the interaction between three main factors. The disease itself can produce a procoagulant state cause the patient at high risk of thrombus. The therapy can cause alteration in coagulation factors, in addition the patient himself may have an inherited prothrombotic disorders that can affect the thrombosis incidence susceptibility. In addition, other factors like having inflammation or central venous line may affect the thrombosis incidence.
Risk Factor 2: Effect of ALL Therapy on Thrombus Formation

Asparaginase

Asparaginase is a corner stone in most of contemporary All treatment protocols. However, L-asparaginase results in asparagine amino acid depletion, which can impair the coagulation cascade by reduction in coagulation factors and or inhibitors (Rizzari et al., 2014).

Glucocorticoids

It was found that glucocorticoids administrated concomitantly with asparaginase in ALL patients can affect the inflammatory reactions by inhibitory effects. The inhibitory effects can be divided into early and late effects. The glucocorticoids early effects are inhibition of capillary dilatation, inhibition of developing edema, as well as deposition of fibrin while late effects are proliferation of fibroblasts, capillary proliferation and collagen deposition. On the other hand, it was found that dexamethasone is more efficient in protection against thrombotic event versus prednisolone and that was explained by a stronger anti-inflammatory glucocorticoid effect (Goodman A & JG, 2001).

A prospective multicenter study was done on pediatric ALL patients treated according to BFM protocol to assess the risk of symptomatic thrombus incidence in patients receiving either prednisolone or dexamethasone. The thrombus frequency in the prednisolone group was 10.4% while in the dexamethasone group was 1.8%. This study concluded that the use of dexamethasone instead of prednisolone in the induction phase can significantly reduce the thrombus onset in ALL pediatric patients (Nowak-Göttl et al., 2003).
Risk Factor 3: Effect of Inherited Thrombophilia on the Development of Thrombosis

Blood coagulation is an important mechanism that protects our body from bleeding. The key enzyme of the coagulation system is the thrombin enzyme. It converts fibrinogen to fibrin to form the fibrin mesh which occludes the vascular injury and has a feedback effect for the amplification of the coagulation process. The adequate amount of thrombin at injury sites results from a cascade of reactions called coagulation cascade. It is triggered by endothelium injury which led to blood exposure to the extravascular tissue. In normal conditions, there is a harmony between the procoagulant system, which induce coagulation and the anticoagulant system which controls its effect. Any disturbance in this natural balance due to genetic or acquired reasons may lead to thrombotic or bleeding diseases (Dahlbäck, 2000). Most of the factors that alter an individual’s thrombosis risk are related to an alteration in the normal balance that exists between the procoagulant and the anticoagulant state.

Regulation of the blood coagulation process is important in order to prevent its generalized or continuous activation, which can lead to thrombus formation. The coagulation system must be active only in case of injury and for sufficient time to control bleeding by the formation of a fibrin clot. In order to achieve this balance, there are a number of regulatory mechanisms that are activated in order to control the effect of activated coagulation factors such as FIXa, FXa, FVa and FVIIIa. There are anticoagulant cofactors and proteins responsible for binding to the activated coagulation factors to limit their activity, for example, protein C, protein S and anti-thrombin. Anti-thrombin inactivates many activated coagulation factors such as thrombin, FIXa, FXa and TF-VIIa complex. Protein S acts as a cofactor for activated protein C (APC) forming a protein S/APC complex. This complex is able to inactivate FVa and FVIIIa, which are responsible for the conversion of the inactive prothrombin to thrombin, and thus in effect the S/APC complex controls thrombin production (Dahlbäck, 2000; Norris, 2003).
Thrombophilia refers to a group of conditions that make an individual more susceptible for having clots than normal. Inherited thrombophilia (IT) can affect ALL patients’ thrombus incidence risk and may be used as a predictive tool for thrombus prevention. Although it is an important risk in thrombus formation; however it is still not clear till now how the thrombophilia screening will help in decreasing the thrombus incidence rate (Revel-Vilk et al., 2003).

A classification system for inherited prothrombotic defects divided these conditions into two groups. Group 1 disorders: hereditary deficiencies of anticoagulation factors (Anti thrombin III deficiency, protein C deficiency and protein S deficiency). Group 2 disorders: hereditary disorders associated with the increase in the levels or function of coagulation factors (Factor V Leiden, PT gene mutation, elevated level of homocysteine, elevated levels of lipoprotein (a), elevated levels of factors VIII, IX and XI) (Athale & Chan, 2003b).

It was reported in literature that inherited thrombophilia has an important role in increasing thrombus risk in ALL patients. A prospective multicenter study was done on patients treated on treatment protocol BFM 90/95 to assess the risk of thrombus in pediatric ALL patients. The study showed that the thrombus risk was much higher in patients with inherited thrombophilia defect (46.5% vs. 2.2%, P < 0.001). In addition, thrombus risk was higher in patients with multiple inherited thrombophilia defects when compared to those with single inherited thrombophilia defect (P =0.009) (Nowak-Göttl et al., 1999). In contrast, PARKAA study reported no correlation between inherited thrombophilia defects and thrombus formation (Mitchell et al., 2003).

A meta-analysis concluded that inherited thrombophilia can increase the risk of thrombosis by 8 folds (Caruso et al., 2006). There is a current debate in the field regarding the role of inherited thrombophilia (IT) on the incidence of thrombosis in ALL patients and its importance as a predictive tool for asparaginase-related thrombus formation. This debate and difference in results is due to different treatment protocols used and the different populations with ethnic variability. That is why it is important to screen ALL pediatric patients in the Egyptian population for
inherited thrombophilia in order to examine its role on asparaginase-related thrombus formation.

**IT Factor 1: Factor Five Leiden**

Factor V is a coagulation factor whose active form is known as FVa. FVa has an important role in the production of thrombin from the inactive prothrombin. Therefore, an increase in the levels of FVa will lead to an increase in thrombin production resulting in a hyper coagulopathy state. Normally, Activated protein C (APC) has an inhibitory effect on factor V and thus an inhibitory effect on the conversion of prothrombin to thrombin. However, it was found that in some patients, factor V will show resistance against APC and will not be inactivated. This was later found out due to a point mutation in the FV gene, referred to as FV Leiden mutation (Norris, 2003). This mutation results in the replacement of arginine with glutamine at codon 506 and thus a loss of one of the three APC cleavage sites in factor Va which leads to an impaired ability of APC to degrade factor Va resulting in the Activated protein C resistance phenomena (Dahlbäck, 2000).

In 1993, it was reported as unusual phenomena while studying the effect of adding external activated protein C (APC) to venous thromboembolism (VTE) patient’s plasma. They were expecting slowing down in the coagulation process upon adding the APC, as FV will be inactivated. However, they noticed that in some patients the slowdown of the coagulation process did not occur and they called this phenomena as APC resistance (Carlsson & Svenssont, 1993). After one year, another team of researchers discovered a point mutation in FV gene where Guanine is replaced by Adenine at position 1691 of exon 10 of the gene. This mutation was called FV Leiden according to the city they were doing their research in. This point mutation causes a change in the amino acid coding for arginine at codon 506 changing it to glutamine (Bertina et al., 1994). Because of this change in the FV gene the APC enzyme cannot recognize the FV effectively and the FV remains active causing hyper coagulopathy state. This lead to interruption for one of the most important regulatory pathways in the coagulation cascade which may be a risk factor for VTE (Jadaon, 2011a).
Since the discovery of the FV Leiden, many studies were done to know the prevalence of FV Leiden in patients with VTE and healthy populations. It was found that Factor V Leiden has high prevalence in Europe especially in the Caucasian population. Factor V Leiden prevalence in healthy Caucasian population is (1-15%) while in patients with VTE is (15-65%). On the other hand, FV Leiden prevalence in other ethnic groups like Africans, Japanese, Chinese, and South-East Asians is almost rare. It was suggested that the FV Leiden mutation first occurred in an old ancestor of the European Caucasian population (Jadaon, 2011a). The highest prevalence of the FV Leiden mutation is found in Europe; especially in Germany, Sweden and Cyprus. It is also common in Saudi Arabia (Rees, 1996).

**IT Factor 2: Prothrombin Gene Mutation**

Prothrombin has an important role in the coagulation cascade. Blood coagulation is initiated by a blood vessel injury then a biochemical cascade starts to block the injured vessel by a blood clot. The coagulation cascade consists of a large number of proteins and enzymes called coagulation factors. Thrombin is an important coagulation factor produced in the liver in an inactive form, known as prothrombin. Prothrombin circulates in the blood stream till it is activated, as in the case of a blood vessel injury for example. Prothrombin at this point is activated to thrombin by a coagulation factor called factor X. Thrombin is essential for the formation of a fibrin clot by converting fibrinogen (factor I) to fibrin, thus leading to the blockage of the injured blood vessel by a mesh like structure (Jadaon, 2011b).

In 1996, it was reported by Poort et al. that the prothrombin gene G20210A mutation is a cause for venous thrombosis. Prothrombin G20210A mutation results in increased levels of prothrombin in plasma causing a higher risk of thrombus formation (Poort et al., 1996). Prothrombin G20210A gene mutation is a point mutation. This point mutation involves a replacement of an adenine with guanine at 20210 position on prothrombin 3’ untranslated region (3’ UTR) (Pollak, Lam, & Russell, 2002). A study investigated the effect of the G20210A mutation on the mRNA and protein expression of prothrombin. The study reported that this mutation affects the polyadenylation site of the prothrombin gene, increasing prothrombin mRNA and protein synthesis, with a subsequent increase in prothrombin plasma
levels (Ceelie et al., 2004). An increase in prothrombin production thus leads to a higher tendency for clot formation. This condition is called hyper coagulopathy, whereby a patient will be at a higher risk of thrombus formation than normal, by 2 folds (Poort et al., 1996).

Prothrombin G20210A mutation has a prevalence of 2% in white Caucasian population (Atasay et al., 2003). It is more prevalent in Southern European than in Northern European. It is rare in people from Asian and African descent (Rosendaal et al., 1998). Individuals with a G20210A mutation suffer from an increased risk of thrombosis by 2-3 folds (Bounameaux & Rosendaal, 2011).

**IT Factor 3: Elevated Level of Homocysteine**

Homocysteine (Hcy) is an intermediate product produced from methionine conversion to Cysteine. Homocysteine in normal circumstances is catabolized into cysteine by transulfuration pathway or can be processed back to produce methionine amino acid by remethylation pathway (Figure 5). Hyper homocysteinaemia results from MTHFR polymorphism. Methylene tetra hydrofolate Reductase (MTHFR) is an important enzyme in folate metabolism. In addition, it is a cofactor in homocysteine re-methylation process to produce methionine. Therefore, reduction in MTHFR enzyme cause increase in homocysteine plasma level. There are 2 common genetic polymorphisms associated with low function of MTHFR gene: MTHFR A1298C and MTHFR C677T (Athale & Chan, 2003b). The MTHFR C677T polymorphism takes place in exon 4 resulting in substitution of Alanine to Valine at codon 222 (Tantawy et al., 2010).

Hyper homocysteinaemia can also result from other factors like methotrexate chemotherapy (antifolate medication), vitamin B12, folate, vitamin B6 deficiencies (Girling, 2001). It was found that an elevated homocysteine plasma level is an independent risk for venous thrombosis and arteriosclerotic vascular disease. The exact mechanism of thrombosis associated with high homocysteine plasma level is unknown (Athale & Chan, 2003b; Crowther & Kelton, 2003).

MTHFR C677T polymorphism is associated with high levels of fasting Hcy plasma levels. This mutation is a risk factor for thrombosis development especially in
case of folate deficiency (Frosst et al., 1995). There is a high variation in MTHFR polymorphism among different population. In Asia and Europe, there is a north to south increase gradient, while in the sub Saharan African population the mutation prevalence is very low. On the other hand, there is a high prevalence of this mutation in the Mediterranean region (Bauduer & Lacombe, 2005). The prevalence differs in different ethnic groups and ranges from 2% to 54.5% (G.Pepe et al. 1998).

1.3.5 Inherited Thrombophilia in Cancer Patients

There are several studies done on FV Leiden, MTHFR C677T and Prothrombin G20210A mutations to investigate their role as a risk factor for thrombus in cancer patients but the results appear controversial (Akın et al., 2012; Haim et al., 2001; Mitchell et al., 2003; Otterson et al., 1996; Ramacciotti et al., 2003; Ravin et al., 2002). This difference in results between different studies may be due to a difference in population under study and/or there may be other factors in the coagulation cascade having a role in synergistic the hyper coagulopathy effect of the studied mutations.
Figure 5: Shows the normal mode of action of MTHFR enzyme. In normal metabolism, homocysteine (Hcy) is converted to methionine via a trans-methylation reaction in the presence of methionine synthetase, which catalyses the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine forming methionine and tetrahydrofolate. On the other hand, Hcy is converted in a certain proportion to Cysteine through transsulfuration pathway. MTHFR convert 5, 10- methylene-tetrahydrofolate to 5 methyl tetrahydrofolate (5MTHF) ensuring continuous supply of 5MTHF and thus allowing the production of methionine. MTHFR polymorphism affects the Hcy re methylation process and result in increase in homocysteine plasma level. In addition, 5,10- methylene-tetrahydrofolate act as methyl group donor which allow the conversion of (dUMP) to deoxthymidine monophosphate (dTMP) in the presence of thymidylate synthase enzyme result in DNA synthesis.
1.4 Aim of the Work

As more insight should be gained in the field of thrombophilia, it becomes important to re-examine this old problem in the context of the pediatric Egyptian ALL patients. This will enable us to identify patients at risk for thrombosis, and may help to develop therapeutic and/or preventive strategies.

The main objectives of this study were:

1. To assess the prevalence of prothrombotic defects (FV Leiden, MTHFR C677T and prothrombin G20210A mutations) in Egyptian pediatric ALL patients.
2. To estimate the impact of inherited Thrombophilia (IT) on the risk of thrombosis onset in pediatric ALL patients that can guide therapy modification and thromboprophylaxis, if indicated.
3. To evaluate the impact of the presence of single versus multiple IT prothrombotic mutations on thrombosis.
2. Subjects and Methods

2.1 Subjects

Sixty three pediatric ALL patients with thrombotic event (age more than 1 year & less than 18 years old at diagnosis) treated with ALL protocol adopted from SJCRH study XV for low or standard/high risk at the Children’s Cancer Hospital in Egypt (CCHE) during period between August 2009 and September 2013. While the control group included 63 ALL patients without thrombotic event treated at CCHE with same protocol and matched for age, gender, IPT and risk stratification.

Subjects were excluded if they were:
- Non-Egyptian ALL patients.
- Their ages were less than one year or greater than 18 years at diagnosis.
- Patients with Down syndrome or having other syndromes to avoid any other factors that may affect their risk of thrombosis.

The ethical committee of CCHE and the American university in Cairo approved the study and a written informed consent was obtained for each patient’s guardian according to the guidelines of the Helsinki declaration.

After the patient had got thrombosis, he is treated with Low molecular weight heparin with a therapeutic dose till the thrombus become stationary or recanalized followed by prophylactic dose. Radiological diagnosis and assessment is done by using Doppler flow in cases of DVT & MRI +/- MRV in case of brain thrombosis to check thrombosis status. Clinical data collected included: patient’s age at diagnosis, gender, risk stratification, Immunophenotyping, initial TLC and patients’ outcome. Number of L-asparaginase doses before event, treatment modifications, site of thrombus, symptoms and complications associated with thrombus and either the patient was rechallenged with L-asparaginase after the event or not was also collected.

Acute Lymphoblastic Leukemia patients were treated according to Saint Jude Total XV protocol without the up-front window phase (Pui et al., 2009). They were subjected to initial work-up to confirm ALL diagnosis which included: CBC at diagnosis, bone marrow examination, immunophenotyping (IPT), DNA index,
molecular translocations, cytogenetic karyotyping (Coustan-smith et al., 2000). The treatment protocol started with remission-induction phase (42 days) in which patients received prednisone, L-asparaginase (*E.Coli*), cytrabine, doxorubicin, vincristine, 6-mercaptopurine and cyclophosphamide. According to patient initial characteristics and response at remission date (Day 42), each patient was assigned to low risk (LR), standard risk (SR), or high risk (HR) at the end of induction. Consolidation phase (8 weeks) followed the induction period, consisted of four cycles of HD-MTX given every other week. Then L asparaginase continued again in continuation phase, where for SR/HR patients they receive weekly L Asparaginase till Week 19, While LR patients received L asparaginase every other day (3 doses /week) on weeks7-9 and weeks 17-19. Continuation phase lasted for 146 weeks for boys and 120 weeks for girls (Pui et al., 2009).

### 2.2 Blood sampling

All the forthcoming procedures were performed at the molecular biology unit at the laboratory department, CCHE-57357. Blood samples (2-5ml each) were withdrawn from patients. Tubes containing disodium ethylene diamine tetra-acetic acid (EDTA) were used (Diagnostics, Franklin Lakes, NJ, USA).

### 2.3 DNA Extraction

DNA was isolated from peripheral blood to study the selected genes. DNA was extracted using Gene JET Genomic DNA Purification Kit (Thermo scientific, #K0721) in accordance to the manufacturer instructions. The isolated DNA concentration was measured using NanoDrop spectrophotometer (NanoDrop 2000, USA). The O.D (optical density) was measured at wavelength A$_{260nm}$ and A$_{280nm}$.

### 2.4 MTHFR C677T gene genotyping

Detection of MTHFR C677T mutation was performed by PCR-RFLP technique (restriction fragment length polymorphism). Primers, restriction enzyme and fragments obtained are presented in table 1 (Tantawy et al., 2010).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Restriction enzyme</th>
<th>Restriction condition</th>
<th>Fragment Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR C677T</td>
<td><strong>Forward primer:</strong> 5'TGAAGGAAGAAGGTGCTCGGGGA3'</td>
<td>HinfI fast digest</td>
<td>1 µl at 37°C for 10 minutes.</td>
<td>Wild type: 198 base pair.</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse primer:</strong> 5'AGGACCGGTGGCGGTGAGAGTG 3'</td>
<td>(10U/1µl) (Thermo scientific, FD0804)</td>
<td></td>
<td>Heterozygous: 198, 175, and 23 base pair.</td>
</tr>
</tbody>
</table>

The amplification was done using [8 x 11 cm (Bio-Rad mini sub cell® GT 712Br)]. The total volume for the PCR reaction was 25 µl, and the components of each reaction were: 1 µl extracted DNA (50 ng/1µl), 0.25µl (1 unit) DFS-Taq DNA Polymerase, (BIORON, Germany), 2.5 µl10X Buffer, 2.5 µl (10 mM) Deoxynucleotide triphosphates (dNTPs) (Thermo Scientific, #R0181), 1.25µl (10 µM) MTHFR F primer, 1.25µl (10 µM) MTHFR R primer (Table 1), then 16.25 µl PCR Water was added to complete the reaction volume to 25µl.

The PCR conditions for MTHFR gene amplification were: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of: denaturation (94°C for 15 sec), annealing (72°C for 15 sec) and extension (72°C for 30 sec). Then final extension step at 72°C for 1 minutes. An aliquot (5 µl) from the PCR product was run on 2% agarose gel (Lonza) at 100 volt for 20 minutes against a DNA ladder Gene Ruler 100 bp (Thermo Scientific, #SM0241) to check the presence of the amplified product.

The MTHFR amplicon was 198 bp fragment. The amplicon was digested using sequence depending endonuclease fast digest enzyme [HinfI] (1 µl/10 U, 37°C for 10 minutes). Digested fragments were visualized on UV trans illuminator after vertical electrophoretic separation (Bio-Rad Mini-PROTEAN® 3 cell 525 BR) at 100 V for 120 min. on a 15% acrylamide gel. Digestion of the amplicon for MTHFR C677T gene polymorphism yielded bands of 198 bp in CC wild type, 175 bp, 23bp in TT homozygotes, and all 3 bands (198, 175, and 23 bp) in CT heterozygotes (Figure 6).
UNDIGESTED

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td></td>
<td>198 bp</td>
</tr>
<tr>
<td>Homozygous</td>
<td></td>
<td>175bp 23bp</td>
</tr>
<tr>
<td>Heterozygous</td>
<td></td>
<td>198 bp 175bp 23bp</td>
</tr>
</tbody>
</table>

Figure 6: The digestion of DNA by Hinfl enzyme in case of normal and mutant MTHFR C677T alleles. The MTHFR C677T polymorphism creates a restriction site for Hinfl digest enzyme. The digestion of the PCR product showed production of 198 bp in CC wild type. Heterozygous allele produced 3 bands: 198, 175 and 23 bp. Homozygous allele produced 2 bands: 175 bp, 23bp.

2.5 Factor V Leiden gene genotyping

Detection of Factor V Leiden was performed by PCR-RFLP technique (Abdullah et al., 2010). Primers, restriction enzyme and fragments obtained are presented in Table 2.

Table 2: Primers, restriction enzyme and fragments produced from FV Leiden gene digestion.

<table>
<thead>
<tr>
<th>Gene Mutation</th>
<th>Primer sequence</th>
<th>Restriction enzyme</th>
<th>Restriction condition</th>
<th>Produced Fragments size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden G1691A</td>
<td>Forward primer: 5’ GGA ACA ACA CCA TGA TCA GAG CA -3’ &lt;br&gt; Reverse primer: 5’ TAG CCA GGA GAC CTA ACA TGT TC -3’</td>
<td>MnII fast digest (10U/1µl) (Thermo scientific, FD1074)</td>
<td>1µl at 37°C for10 minutes.</td>
<td>Wild type: 157, 93 and 37 bp &lt;br&gt; Heterozygous: 157, 130, 93 and 37 bp. &lt;br&gt; Homozygous: 157 and 130 bp</td>
</tr>
</tbody>
</table>
The amplification was done using [8 x 11 cm (Bio-Rad mini sub cell® GT 712Br)]. The total volume for the PCR reaction was 25 µl, and the components of each reaction where: 2.5 µl 10X Buffer, 2.5 µl (10 mM) Deoxynucleotide triphosphates (dNTPs) (Thermo Scientific, #R0181), 1.25µl (10 µM) FV F primer, 1.25µl (10 µM) FV R primer (Table 2), 1 µl extracted DNA (50ng/1 µl), 0.25µl (1 unit) DFS-Taq DNA Polymerase (BIORON, Germany) then 16.25 µl PCR Water was added to complete the reaction volume to 25µl.

The PCR conditions for FV gene amplification were: initial denaturation at 94 ºC for 2 minutes, followed by 40 cycles of: denaturation (94 ºC for 15 sec), annealing (55ºC for 15 sec) and extension (72 ºC for 30 sec). Then final extension step at 72 ºC for 1 minutes. An aliquot (5 µl) from the PCR product was run on 2% agarose gel (Lonza) at 100 volt for 20 minutes against a DNA ladder Gene Ruler 100 bp (Thermo Scientific, #SM0241) to check the presence of the amplified product.

The presence of FV Leiden mutation removes the MnII digest enzyme restriction site. The FV amplicon size was 287 bp fragment. The amplicon was digested using sequence depending endonuclease fast digest enzyme [MnII] (1 µl/10 U, 37ºC for 10 minutes). Digestion of the amplicon for FV Leiden gene polymorphism yielded bands of 3 fragments: 157 bp, 93 bp, and 37 bp in GG wild type while 157bp, 130 bp in AA homozygotes, and all 4 bands 157, 93, 130and 37bp in GA heterozygotes (Figure 7). The digested fragments were visualized on UV Trans illuminator after vertical electrophoretic separation (Bio-Rad Mini-PROTEAN® 3 cell 525 BR) at100 V for 120 min. on a 15% acrylamide gel.
<table>
<thead>
<tr>
<th>Undigested</th>
<th>287 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>157 bp 93 bp 37 bp</td>
</tr>
<tr>
<td>Homozygous</td>
<td>157 bp 130 bp</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>157 bp 93 bp 37 bp</td>
</tr>
<tr>
<td></td>
<td>157bp 130 bp</td>
</tr>
</tbody>
</table>

**Figure 7:** The digestion of DNA by MnII in case of normal and mutant FV alleles. The presence of FV Leiden mutation removes the MnII digest enzyme restriction site. The PCR product digestion showed production of 157, 93 and 37 bp bands in case of wild type. In case of heterozygous alleles, 157, 130, 93 and 37 bp bands were produced, while homozygous allele showed 157 and 130 bp bands.

### 2.6 Prothrombin G20210A genotyping

Detection of PT G20210A mutation was performed by Allele specific PCR Technique (ASPCR). For each patient 2 reactions were performed one reaction containing the mutation primer while the other reaction contains wild type primer. FIX primers were used in both reactions to detect factor IX gene as an internal control (Ranguelov et al., 2002). The primers used in amplification of the PT gene are shown in Table 3.

The ASPCR is a modified technique of standard PCR that allows efficient SNPs discrimination. Where 2 forward (Mutation or wild type primer) and 1 reverse primers (common) are used in 2 different tubes. Where Tube M contains the entire reaction component without the wild type primer and it is supposed to detect if the patient has SNP. Normally, if the patient is normal only the internal control band will appear at 219 bp in this lane. While tube N contains the entire reaction components except the Mutation primer, this reaction is supposed to detect if the patient has a wild type allele or not. If the patient is heterozygous or wild type a band will appear at 340 bp.
plus the internal control band. While if the patient is homozygous only the internal control band will appear at 219 bp (Figure 8) (Rangelov et al., 2002).

### Table 3: Primers used in PT G20210A Allele Specific PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT Reverse (Common)</td>
<td>5’-TCT AGA AAC AGT TGC CTG GCA G-3’</td>
</tr>
<tr>
<td>PT Forward (WT)</td>
<td>5’-GCA CTG GGA GCA TTG AGG ATC-3’</td>
</tr>
<tr>
<td>PT Forward (Mutant)</td>
<td>5’-GCA CTG GGA GCA TTG AGG ATT-3’</td>
</tr>
<tr>
<td>FIX-Forward</td>
<td>5’-CTC CTG CAG CAT TGA GGG AGA TGG ACA TT-3’</td>
</tr>
<tr>
<td>FIX-Reverse</td>
<td>5’-CTC GAA TTC GGC AAG CAT ACT CAA TGT AT-3’</td>
</tr>
</tbody>
</table>

The amplification was done using [8 x 11 cm (Bio-Rad mini sub cell® GT 712Br)]. The total volume for the PCR reaction was 25 µl, and the components of each reaction where: 1 µl extracted DNA (50ng/1 µl), 0.25µl (1 unit) HOT FIRE POL hot start DNA Polymerase (SolisBioDyne,01-02-00500 ), 2.5 µl 10X Buffer B1 (Without MgCl$_2$ or detergent), 2.5 µl (10mM) Deoxynucleotide triphosphates (dNTPs) (Thermo Scientific , #R0181), 2.5 µl (25 mM) MgCl$_2$, 1.25µl (10µM) PT Common primer Table 3, 1.25µl (10µM) PT wild primer (N reaction only), 1.25µl (10µM) PT Mutation primer (M reaction only), 1.25µl (10µM) FIX- Forward primer, 1.25µl (10µM) FIX- Reverse primer then 11.25 µl PCR Water was added to complete the reaction volume to 25µl.

The PCR conditions for PT gene amplification were: initial denaturation at 95 °C for 10 minutes, followed by 10 cycles of: denaturation (94 °C for 30 sec), annealing (60°C for 30 sec) and extension (72 °C for 60 sec). Then repeat the following steps for 25 cycles: 30 seconds at 94 °C, 30 seconds at 55 °C and 1 minute 72°C. Then final extension step was run at 72 °C for 7 minutes. Twelve µl from each PCR product was run on 2% agarose gel (Lonza) at 100 volt for 30 minutes against a DNA ladder Gene Ruler 100 bp (Thermo Scientific, #SM0241) to check the presence of
the amplified product.

<table>
<thead>
<tr>
<th>Allele</th>
<th>N Lane</th>
<th>M Lane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>340 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>219 bp</td>
<td>219 bp</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>340 bp</td>
<td>340 bp</td>
</tr>
<tr>
<td></td>
<td>219 bp</td>
<td>219 bp</td>
</tr>
<tr>
<td>Homozygous</td>
<td>219 bp</td>
<td>219 bp</td>
</tr>
</tbody>
</table>

Figure 8: Prothrombin Allele Specific PCR expected bands in both M and N lanes. M lane represents the PCR product when the mutant forward primer was used, while N lane represents the PCR product when the wild type allele forward primer was used. The wild type allele shows 340 and 219 bp bands in the N lane while 219 bp band only produced in the M lane. The heterozygous allele shows 340 and 219 bp bands in both lanes N and M. The homozygous allele shows 219 bp band in N lane while 340 and 219 bp bands produced in M lane.

2.7 Statistical Methods

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher’s exact test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Odds ratio (OR) with it 95% confidence interval (CI) were used for risk estimation. All tests were two-tailed. A p-value < 0.05 was considered significant.
3. Results

One hundred twenty six ALL patients were enrolled in this study and divided into 2 groups. The 1\textsuperscript{st} group included patients with thrombus and the 2\textsuperscript{nd} group is the control group. Patients descriptive data were collected from the hospital medical records (archived files and electronic medical records) for both control and thrombus groups.

Out of a total 889 Egyptian patients treated with ALL protocol (age more than 1 year and less than 18 years old at diagnosis) adopted from SJCRH study XV for low or standard/high risk at the Children’s Cancer Hospital in Egypt (CCHE) during period between August 2009 and September 2013, 84 patients had thrombus. There were 8/84 patients died with no sample, 6/84 patients refused to participate, 1/84 lost follow up, 1/84 had Marfan syndrome and 5/84 were down syndrome. The final number of thrombus patients enrolled in the study was 63/84.

3.1 Patients' characteristics

In both groups the patients age, Gender, initial total leukocyte count (TLC), immunophenotyping (IPT) and risk at diagnosis were comparable (Table 4). The patients were divided according to age into 3 subgroups, from 1 to less than 5 years, from 5 to less than 10 years and from 10 to 18 years.

In thrombus group, the patients’ age trend was higher in the sub group from 10-18 years (52.4%) in comparison to the other 2 sub-groups. Most of the thrombus patients were diagnosed as standard/high risk (80.9%) while the low risk patients were 19%. The Male patients represented 69.8% of the total thrombus group. The initial TLC was less than 100 x10\textsuperscript{3}/ml in 85.2% and the immunophenotype was (68.3%) of B cell origin.
Table 4: Clinical characteristics of the enrolled patients with and without thrombus

<table>
<thead>
<tr>
<th></th>
<th>Cases N (%)</th>
<th>Control N (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at diagnosis</strong> (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-&lt;5</td>
<td>11 (17.5)</td>
<td>11 (17.5)</td>
<td>0.536</td>
</tr>
<tr>
<td>5-&lt;10</td>
<td>19 (30.2)</td>
<td>19 (30.2)</td>
<td></td>
</tr>
<tr>
<td>10-18</td>
<td>33 (52.4)</td>
<td>33 (52.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Initial TLC (10³/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 100</td>
<td>52 (85.2%)</td>
<td>51 (81%)</td>
<td>0.524</td>
</tr>
<tr>
<td>&gt;=100</td>
<td>9 (14.8%)</td>
<td>12 (19%)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>2</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td>0.847</td>
</tr>
<tr>
<td>Male</td>
<td>44 (69.8%)</td>
<td>43 (68.3%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>19 (30.2%)</td>
<td>20 (31.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Risk</strong></td>
<td></td>
<td></td>
<td>0.744</td>
</tr>
<tr>
<td>Low Risk</td>
<td>12 (19%)</td>
<td>15 (23.8%)</td>
<td></td>
</tr>
<tr>
<td>Standard Risk</td>
<td>44 (69.8%)</td>
<td>40 (63.5%)</td>
<td></td>
</tr>
<tr>
<td>High Risk</td>
<td>7 (11.1%)</td>
<td>8 (12.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Immunophenotype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cell</td>
<td>43 (68.3%)</td>
<td>44 (69.8%)</td>
<td></td>
</tr>
<tr>
<td>B Precursor</td>
<td>0 (0%)</td>
<td>1 (1.6%)</td>
<td></td>
</tr>
<tr>
<td>undifferentiated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-ALL</td>
<td>26 (41.3%)</td>
<td>28 (44.4%)</td>
<td></td>
</tr>
<tr>
<td>Pre-B</td>
<td>14 (22.2%)</td>
<td>14 (22.2%)</td>
<td></td>
</tr>
<tr>
<td>Pro-B</td>
<td>3 (4.8%)</td>
<td>1 (1.6%)</td>
<td></td>
</tr>
<tr>
<td>T cell</td>
<td>20 (31.7%)</td>
<td>19 (30.2%)</td>
<td>0.847</td>
</tr>
<tr>
<td>T early</td>
<td>11 (17.5%)</td>
<td>7 (11.1%)</td>
<td></td>
</tr>
<tr>
<td>T intermediate</td>
<td>7 (11.1%)</td>
<td>11 (17.5%)</td>
<td></td>
</tr>
<tr>
<td>T late</td>
<td>0 (0%)</td>
<td>1 (1.6%)</td>
<td></td>
</tr>
<tr>
<td>T cell</td>
<td>2 (3.2%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>undifferentiated</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2 Thrombus Timing

In 42/63 patients (66.7%) the incident took place during the induction phase while 21/63 patients (33.3%) had the incident during the continuation phase. The incident here is thrombus ± infarction (Table 5). Thrombosis that occurred between the first dose of asparaginase and the last dose of asparaginase at week 19 was included in the analysis.

3.3 Number of Doses Before the Thrombosis

The L-asparaginase doses taken before the incident were classified into 3 groups: 1-4 doses, 5-9 doses and greater than or equal 10 doses. Seventeen patients (26.9%) had from 1 to 4 doses before the event, 26/63 patients (41.2%) had from 5 to 9 doses before the event, 20/63 patients (31.7%) had greater than 10 doses before the event (Table 5).

3.4 Treatment Modifications

Out of the 63 patients with thrombosis 47 patients (74.6%) had treatment modifications and the rest continued the treatment protocol smoothly. The number of patients who skipped L-asparaginase doses was 43 (68.3%) while four patients had delayed treatment without omitting doses (Table 5).

3.5 Rechallenge with L - Asparaginase After Thrombosis

In the thrombosis group, 56/63 patients (88.9%) were rechallenged (restarted) with L- asparaginase after the event and 7/63 (11.1%) patients stopped L-asparaginase for the rest of the protocol. Progression took place in 15 rechallenged patients (15/56 = 26.7%), while 41/56 patients (73.2%) continued the L – asparaginase smoothly. Asparaginase was rechallenged after diagnosis of thrombosis with a median duration of 10 weeks (Table 5).
<table>
<thead>
<tr>
<th>Type of incidence</th>
<th>Cases N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombus only</td>
<td>58 (92.1%)</td>
</tr>
<tr>
<td>Thrombosis with infarction</td>
<td>5 (7.9%)</td>
</tr>
<tr>
<td>Hemorrhagic infarction</td>
<td>2/5</td>
</tr>
<tr>
<td>Non hemorrhagic infarction</td>
<td>3/5</td>
</tr>
<tr>
<td><strong>Treatment phase at time of the event:</strong></td>
<td></td>
</tr>
<tr>
<td>Induction</td>
<td>42 (66.7%)</td>
</tr>
<tr>
<td>Continuation</td>
<td>21 (33.3%)</td>
</tr>
<tr>
<td><strong>Number of L-Aspara doses before event</strong></td>
<td>Median =6 (Min=1&amp; Max=28)</td>
</tr>
<tr>
<td>1-4 doses</td>
<td>17 (26.9%)</td>
</tr>
<tr>
<td>5-9 doses</td>
<td>26 (41.2%)</td>
</tr>
<tr>
<td>≥10 doses</td>
<td>20 (31.9%)</td>
</tr>
<tr>
<td><strong>Treatment Modifications</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16 (25.4%)</td>
</tr>
<tr>
<td>Yes</td>
<td>47 (74.6%)</td>
</tr>
<tr>
<td>Delayed Aspara doses</td>
<td>4/63 (6.3%) (Median =14, Min.= 4 &amp; Max=49 )</td>
</tr>
<tr>
<td>Number of patients</td>
<td></td>
</tr>
<tr>
<td>Duration (days)</td>
<td></td>
</tr>
<tr>
<td>Omitted Aspara doses</td>
<td>43/63 (68.3%) (Median =3.5, Min=1 &amp; Max=17 )</td>
</tr>
<tr>
<td>Number of patients</td>
<td></td>
</tr>
<tr>
<td>Number of doses omitted:</td>
<td></td>
</tr>
<tr>
<td>1-4 doses</td>
<td>28/43 (65.1%)</td>
</tr>
<tr>
<td>5-9 doses</td>
<td>7/43 (16.2%)</td>
</tr>
<tr>
<td>10-17 doses</td>
<td>8/43 (18.6%)</td>
</tr>
<tr>
<td><strong>Rechallenge of L-asparaginase</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>7 (11.1%)</td>
</tr>
<tr>
<td>Yes</td>
<td>56 (88.9%)</td>
</tr>
<tr>
<td>Progression on rechallenge:</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41/56 (73.2%)</td>
</tr>
<tr>
<td>Yes</td>
<td>15/56 (26.8%)</td>
</tr>
</tbody>
</table>
3.6 Outcome and prognosis

Symptoms associated with the thrombus included headache 52%, convulsions 21.3%, blurred vision (8%), vomiting (8%), lower limb swelling and pain (9.3%) and dysphagia due to laryngeal paralysis (1.3%) (Table 6). On the other hand, about 10% of the thrombus patients were associated with comorbid complications such as hemiplegia, hypotonicity and tremors that lasted with the patient for 18 months (Table 5). Those patients with comorbid complications were associated with CNS thrombosis. The Event free survival (EFS) is the duration from the start of treatment till relapse or death, while the overall survival (OS) is the duration from the start of treatment till death. With a median follow up of 22.4 months, the 36 months OS was 89.8% ± 10.2 and 85.4 ± 13.5 for patients with and without thrombosis respectively, while the EFS was 86.7% ± 10.3 and 81.7 ± 13.3 for patients with and without thrombosis respectively. There was no significant difference in the OS (P = 0.91) and EFS (P =0.85) when comparing patients with and without thrombosis (Figure 9 and 10).

Table 6: Symptoms and comorbidities in the studied pediatric ALL thrombus patients

<table>
<thead>
<tr>
<th>Symptom or complication</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symptoms</strong> *</td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>39/75 (52%)</td>
</tr>
<tr>
<td>Convulsions</td>
<td>16/75 (21.3%)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>6/75 (8%)</td>
</tr>
<tr>
<td>Blurred vision</td>
<td>6/75 (8%)</td>
</tr>
<tr>
<td>Laryngeal paralysis</td>
<td>1/75 (1.3%)</td>
</tr>
<tr>
<td>Swelling &amp; pain</td>
<td>7/75 (9.3%)</td>
</tr>
<tr>
<td><strong>Comorbidity</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>57 (90.5%)</td>
</tr>
<tr>
<td>Yes</td>
<td>6 (9.5%)</td>
</tr>
<tr>
<td>Hemiplegia</td>
<td>3 (4.8%)</td>
</tr>
<tr>
<td>Hypotonia</td>
<td>1 (1.6%)</td>
</tr>
<tr>
<td>Tremors</td>
<td>1 (1.6%)</td>
</tr>
<tr>
<td>Unstable convulsions</td>
<td>1 (1.6%)</td>
</tr>
</tbody>
</table>

*Most of patients had more than 1 clinical symptom associated with the incident, where 53 patients had 75 symptom associated with the thrombosis. No data for 10 patients regarding symptoms associated with event.*
Figure 9: Overall survival (OS) of the thrombosis group compared to non-thrombotic group. The 3 years OS for the non-thrombus and thrombus patients was 85.4 ± 13.5 and 89.8% ± 10.2, respectively. No significant difference in OS between the 2 groups ($P = 0.91$).
Figure 10: Event free survival (EFS) of the thrombosis group compared with the non-thrombotic group. The 3 years EFS for the non-thrombus and thrombus patients was 81.7 ± 13.3 and 86.7% ± 10.3, respectively. No significant difference in EFS between the 2 groups (P = 0.85).
3.7 Site of thrombosis

Most of the patients had more than 1 clot at different sites. A total of 79 clots were diagnosed in the 63 patients with thrombosis. The most common sites of thrombosis were central nervous system (CNS) (n=55), upper venous system (n=17) and lower limb (n=7). Patients with a CNS site of thrombosis had either a cerebral venous sinus thrombosis or infarction. On the other hand, those with an upper venous system site of thrombosis had the clot in either the internal jugular vein and/or the superior vena cava (Table 7).

Table 7: Site of thrombus in 63 pediatric ALL patients

<table>
<thead>
<tr>
<th>Site of VTE</th>
<th>Thrombosis sites N=79*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td>55 (69.6%)</td>
</tr>
<tr>
<td>Sinus venous thrombosis</td>
<td>50 (63.3%)</td>
</tr>
<tr>
<td>Thrombosis with infarction</td>
<td>5 (6.3%)</td>
</tr>
<tr>
<td>Upper venous system:</td>
<td>17 (21.4%)</td>
</tr>
<tr>
<td>Internal jugular vein</td>
<td>16 (20.2%)</td>
</tr>
<tr>
<td>Superior Vena Cava</td>
<td>1 (1.2%)</td>
</tr>
<tr>
<td>Lower limb</td>
<td>7 (8.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>79 (100%)</td>
</tr>
</tbody>
</table>

*79 clots were diagnosed in 63 patients
3.8 MTHFR C677T Genotyping

Presence of a C677T mutation creates a new restriction site for HinfI in the amplified MTHFR PCR product resulting in 2 fragments (175 bp, 23 bp). On the other hand, its absence results in the PCR product remaining intact (198 bp). The digestion of the PCR product showed production of 198 bp in CC wild type. Heterozygous allele produced 3 bands: 198, 175 and 23 bp. Homozygous allele produced 2 bands: 175 bp, 23bp (Figure 11). Our results showed that, in the case group 22/63 patients (34.9 %) were wild type allele, 36/63 patients (57.1%) were heterozygous allele and 5/63 patients (7.9 %) were homozygous allele (Table 8). On the other hand, in the control group 39/63 patients (61.9%) were wild type allele, 18/63 patients (28.6 %) were heterozygous allele and 6/63 patients (9.5 %) were homozygous allele. There was a significant difference between patients with and without thrombosis (P=0.002) with odds ratio 3.028 and 95% confidence interval (CI) (1.465-6.258).

Figure 11: MTHFR PCR product digestion using HinfI Fast digest enzyme on 15 % acrylamide gel. Patients can either be wild type (one band; 198 bp) (lane 4-9), heterozygous (3 bands; 198, 175, 23) (lane 10) or homozygous C677T (2 bands; 175, 23 bp) (lane 3), in addition the 23 bp band not detected on the gel.
3.9 FV Leiden Genotyping

Presence of a FV mutation abolishes a restriction site for MnII enzyme in the amplified FV PCR product resulting in 2 fragments (157 bp, 130 bp). On the other hand, its absence results in 3 fragments (157, 93 and 37 bp). The PCR product digestion showed production of 157, 93 and 37 bp bands in case of wild type. In case of heterozygous alleles 157, 130, 93 and 37 bp bands were produced, while homozygous allele showed 157 and 130 bp bands as shown in figure 12. Our results showed that in thrombus patients, 52/63 patients (82.5%) were wild type allele, 10/63 patients (15.9%) were Heterozygous allele and 1/63 (1.6%) was homozygous allele. On the other hand, in the control group 53/63 patients (84.1%) were wild type allele, 9/63 patients (14.3%) were heterozygous allele and 1/63 patient (1.6%) was homozygous allele. Comparing the 2 groups showed no significance difference ($P = 0.811$) (Table 8).

![Figure 12: FV Leiden PCR product digestion using MnII Fast digest enzyme on 15 % acrylamide gel. Patients can either be wild type (3 bands; 157, 93 and 37 bp) (lane 2-5&7), homozygous (2 bands; 157 and 130 bp) (not shown in this figure) or heterozygous (4 bands; 157, 130, 93 and 37 bp) (lane 1, 6).](image-url)
3.10 Prothrombin G20210A Genotyping

The expected produced bands in case of wild type allele G/G will be 219 bp in M lane while in N lane will has 219 bp and 340 bp in N lane, the Homozygous A/A allele will produce 219 bp and 340 bp in M lane and 219 bp band in N lane, while the heterozygous allele G/A will produce 219 and 340 in both lanes (Figure 13). Our results showed that out of the 63 patients with thrombus 61 patients (96.8 %) were wild type allele, 2 patients (3.2%) were heterozygous allele and there was no homozygous allele detected. On the other hand, in the control group 61 patients (96.8 %) were wild type allele, 2 patients (3.2 %) were heterozygous allele and there was no homozygous allele detected. There was no significant difference detected between patients from both groups (Table 8).

![Figure 13: Prothrombin G20210A allele specific PCR products on agarose 2% gel.](image)

Each patient had 2 adjacent lanes (M and N). The M lane represents the PCR product containing the Mutant allele primer, while N lane represents the PCR product containing the wild type allele primer. Wild Type allele (lane 2, 3, 5, 6) showed 340 and 219 bp bands in the N lane while 219 Bp band only produced in the M lane. The heterozygous allele (lane4) showed 340 and 219 bp bands in both lanes N and M.
3.11 Combined Genes

Having more than one mutation didn’t show a significant effect on increasing the risk of thrombus incidence (p= 0.087) Table 8.

Table 8: Shows the prevalence of the studied genes among patients in the case and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Control</th>
<th>P Value</th>
<th>OR (CI 95 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor V Leiden</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>52 (82.5%)</td>
<td>53 (84.1%)</td>
<td>0.811</td>
<td>1.121 (0.439 - 2.86)</td>
</tr>
<tr>
<td>Mutant:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>10/63 (15.9%)</td>
<td>9/63 (14.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>1/63 (1.6%)</td>
<td>1/63 (1.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MTHFR C677T</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>22/63 (34.9 %)</td>
<td>39/63 (61.9 %)</td>
<td>0.002</td>
<td>3.028 (1.465 - 6.258)</td>
</tr>
<tr>
<td>Mutant:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>36/63 (57.1%)</td>
<td>18/63 (28.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>5/63 (7.9 %)</td>
<td>6/63 (9.5 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PT G20210A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild</td>
<td>61/63 (96.8 %)</td>
<td>61/63 (96.8 %)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Mutant:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>2/63 (3.2 %)</td>
<td>2/63 (3.2 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Combined genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Multiple traits)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No mutation</td>
<td>17/63 (27%)</td>
<td>32/63 (50.8%)</td>
<td>0.087</td>
<td>3.12 (0.852 - 10.647)</td>
</tr>
<tr>
<td>Single mutation</td>
<td>38/63 (60.3 %)</td>
<td>26/63 (41.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mutation</td>
<td>8/63 (12.7%)</td>
<td>5/63 (7.9 %)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* No p value because of small number of cases within sub groups.
4. Discussion

The thrombus risk in ALL patients is thought to arise from idiopathic generation of thrombin at diagnosis associated with decrease in the inhibitory effect of anti-thrombin due to depletion by asparaginase in addition to other risk factors (Payne & Vora, 2007). In this study, we examined 63 ALL pediatric patients with thrombosis and 63 matched non-thrombus patients. Our main objective was to identify the main risk factors for thrombosis in Egyptian pediatric ALL patients. Up to our knowledge there were no reports about the risk factors for thrombosis in this population of patients. Since the genetic factors mainly depend on ethnicity so it is important to study those risk factors in Egyptian population in addition to the clinical factors.

4.1 Patients Gender, Age, IPT and Risk Stratification as Risk Factors for Thrombosis

The current study showed that the thrombosis incidence was higher in SR and HR patients than LR patients. Out of the 63 patients with thrombosis, 81% were SR/HR while 19% were LR. These results are consistent with other investigators reports, in a study conducted on patients treated with Total XV study protocol thrombosis incidence was reported to be higher in SR and HR patients. Out of 36 patients with thrombosis 86% of the patients were standard and high risk while 13.8% of the patients were low risk (Pui et al., 2009). In addition, it was reported that the frequency of SR/HR patients among pediatric ALL patients in Egypt was 57.4% (Sidhom et al., 2013). There was a higher frequency of SR/HR patients in the current study thrombus patients when compared to what was reported by Sidhom et al 2013. This higher frequency can implicate the importance of considering the Risk stratification as a risk factor for thrombosis incidence.

In addition, it was found in the current study that patients greater than 10 years old had a higher incidence of thrombosis. Where the percentage of patients with age greater than 10 and less than or equal 18 was 52.4%, while the percentage of patients from 1 year to less than 5 years and from 5 years to less than 10 years was found to be 17.5% and 30.2% respectively (Table 1). Similarly, it was reported that age group greater than 10 years was associated with higher risk of thrombus in Total
XV study protocol (Pui et al., 2009). The same results were reported by Dana-Farber cancer institute protocols results from 1991 to 2008 (Grace et al., 2011). This was explained by the delayed dexamethasone clearance in older patients. In addition, it had been shown that older pediatric ALL patients have both decreased fibrinolysis and decreased anticoagulant factors when compared to younger patients receiving the same therapy (Appel et al., 2008; Pui et al., 2009; Yang et al., 2008). On the other hand, it was reported that the percentage of Egyptian pediatric ALL patients with age greater than 10 years was 25% (Sidhom et al., 2013). This difference in patients’ age distribution between the current study thrombus patients and what was reported by Sidhom et.al 2013 indicates that there may be an important role for age as an important risk factor for thrombosis.

The patients gender was analyzed in the current study thrombus group, the males’ frequency in the thrombus group were 70% while 30% were females. These results are consistent with what was reported before regarding male predominance in acquiring thrombosis in ALL patients (Pui et al., 1985). On the other hand, Sidhom et al. 2013 reported male frequency was 60% while female was 40% in pediatric ALL patients. The current study showed a slight higher frequency of males in the thrombus patients when compared to what was reported by Sidhom et.al 2013. This indicates that males may have higher risk for thrombosis.

The immunophenotyping results of the current study thrombus patients showed 68.3% of the patients were B cell and 31.7% T cell precursor. This is consistent with what was reported regarding a higher frequency of T cell precursor in the thrombus ALL patients in comparison with those without thrombosis (26% vs. 10%) (Grace et al., 2011). On the other hand, Sidhom et al. 2013 reported 75.8% of the studied patients were B cell precursor while 24.2% were T cell precursor. This slight increase in T cell frequency in thrombus patients may indicate it as risk factor for thrombosis.

Based on what we demonstrated in this study, it is clear that both the age greater than 10 years and SR/HR patients have a significant higher risk of thrombus incidence more than others. While both males and T cell patients have a slight higher risk than the other patients.
4.2 Number of L-Asparaginase Doses Given Before Thrombosis

In the current study, patients were divided into 3 groups according to the number of L-Asparaginase doses received before the thrombosis incidence. The number of patients who had thrombus after receiving 5 to 9 L-Asparaginase doses (41.2%) was higher than the other 2 groups. This may have an implication of increase thrombosis risk after the 5\textsuperscript{th} to the 9\textsuperscript{th} dose of L-Asparaginase due to the accumulation of doses till the end of the induction phase which contains 6 or 9 doses of L-Asparaginase according to the disease risk. In addition to the number of accumulated asparaginase in induction phase, there are other thrombosis risk factors as the prednisolone glucocorticoids and the hyper-coagulopathy associated with the disease. On the other hand, the patients who received L-Asparaginase greater than 9 doses have dexamethasone glucocorticoid administrated concomitantly which is characterized by less risk of inducing thrombosis than prednisolone. Furthermore, the hyper-coagulopathy state of the patient from the disease becomes lower than in the induction phase (Nowak-Göttl et al., 2003).

4.3 Time of Thrombus Incidence as a Risk Factor for Thrombosis

The current study results showed that 66.3% of the thrombosis incidence took place in induction phase while 33.3% of the patients had the thrombosis in continuation phase. Similarly, other investigators reported that the majority of thrombosis incidents took place in the induction phase of the treatment protocol (Payne & Vora, 2007). In a retrospective study, it was reported that 90% of the thrombosis incidence in ALL patients treated on BFM-90 German protocol occurred during the induction phase.

The higher incidence of thrombosis during the induction phase in comparison with the rest of treatment phases was explained by different factors, where the disease is still highly active with high thrombin production and a high burden of lymphoblast cytolysis. In addition, the treatment is highly intense during induction containing a combination of concurrent administration of glucocorticoids and L-
asparaginase (Sutor et al., 1999). On the other hand, patients in the post induction phases have less cell lysis and treatment intensity (Caruso et al., 2006). Another reason for high incidence of thrombosis in induction is the type of treatment used in the protocol. Total XV treatment protocol used in this study is applying prednisolone glucocorticoids in the induction phase. It was reported in a study comparing 2 BFM studies that the risk of thrombosis during induction was much higher with prednisolone than dexamethasone (thrombosis frequency in BFM90/95 prednisolone =10.4% while BFM 2000 dexamethasone =1.8 % p=0.028) and this is consistent with the current study results (Nowak-Göttl et al., 2003). This higher incidence of thrombosis in induction phase indicates the importance of considering patients in induction phase at higher risk of thrombosis than others.

4.4 Rechallenge with L – Asparaginase After Thrombus Incidence

In the current study, 88.9% of the patients with thrombus were rechallenged with L – Asparaginase; out of those patients 28.3% were progressed. Grace et al. 2011 confirmed that L -Asparaginase can be rechallenged in cancer patients with thrombosis. The rechallenging of thrombus patients with L - Asparaginase might be needed to achieve high EFS and OS in these patients. In DFCI protocols (1991-2008), Grace et al. 2011 reported that out of the 27 pediatric patients who got thrombus 74% of patients were rechallenged. The progression on rechallenge was 20% of the rechallenged patients in that study and this is comparable with what was demonstrated by our study. The guidelines used by Grace et al. 2011 to rechallenge patients with L-Asparaginase were to closely monitor anti-FXa levels and imaging should demonstrate thrombus improvement or stabilization before rechallenging. In addition, he recommended 4 weeks from incidence before rechallenging patients with the L-Asparaginase. He reported median of 9 weeks from incidence before rechallenging patients with the L-Asparaginase for pediatrics and 4 weeks for adults in DFCI protocols. While in the current study, the median duration from thrombus till rechallenge was 10 weeks that is comparable with DFCI protocols results. It is important to mention that the thrombus patients in the current study was rechallenged
with L-Asparaginase once the thrombus is stationary or showed partial recanalization in order to achieve the best patients’ outcome.

### 4.5 Prognosis and Outcome of Patients with Thrombosis in Comparison to Control Patients

In the current study, there was no significant difference between EFS (\(P = 0.856\)) or OS (\(P = 0.912\)) of the thrombus patients and those patients in the control group. This comparable EFS and OS between the 2 groups may be explained by the high percentage of rechallenged patients (88.9%) in the current study, which means less number of missed L - Asparaginase doses and better adherence to the treatment protocol. In addition, a study was done by Grace et al. 2011 presenting the results of Dana-Farber protocols from 1991 to 2008 showed that the thrombus history didn’t affect the patient prognosis. In his study, the EFS and OS rates in patients with thrombus were similar to those in patients treated with the same treatment protocol but without thrombus. This was explained by the guidelines followed to rechallenge asparaginase and avoid asparaginase discontinuation which negatively affect the EFS and OS. On the other hand, it has been reported that thrombus patients had a lower EFS than those patients treated with the same protocol and did not have thrombosis. This was explained by early asparaginase discontinuation (Hunault-Berger et al., 2008; Ku et al., 2009). Based on the similar OS and EFS rates in patients from the thrombus and the non-thrombus group of the current study, we can conclude that following a strict guidelines in resuming L- Asparaginase after thrombosis incidence is very important for enhancing thrombus patients survival.

### 4.6 Site of Thrombosis

It was found in the current study that the most common thrombus site was the cerebral sinuses (69.6%) followed by upper venous system (21.4%) while the CVL use did not have an effect on the thrombus formation in the current study. Similarly, it was reported that cerebral venous sinus thrombosis has high prevalence in ALL pediatric patients followed by upper venous system thrombosis and lower limb (Payne & Vora, 2007). A review done on symptomatic thrombosis in pediatric ALL between 1966 and 2003 reported that 50% of the incidence was CNS and 50% non-
CNS (Athale & Chan, 2003a). In addition, a meta-analysis done on 17 studies having 1752 pediatric ALL patients reported that out of 91 events 53.8% were in CNS (Caruso et al., 2006). However, it was reported in the results of DFCI that the most common site is the upper limb and the CVL associated thrombus followed by the sinus venous thrombus and lower limb (Grace et al., 2011). This difference in results between the current study and the DFCI results can be explained by our limited use of CVL when compared to the other institution in ALL patients.

4.7 Comorbidities and Symptoms Associated with Thrombosis in ALL Patients

The present study showed that the most common clinical symptoms associated with sinus thrombus were headache (52%), convulsions (21.3%). On the other hand, the lower limb DVT presented commonly with pain and swelling (9.3%). These symptoms are similar to those described in previous studies (Grace et al., 2011; Payne & Vora, 2007).

Comorbidity is a long lasting complication associated with the patient, in the current study it is associated with the patients due to thrombosis. We found in the current study that 10.7% of patients with CNS thrombosis suffered from comorbidities in the form of hemiplegia, long lasting tremors, unstable convulsions and hypotonicity. Usually the comorbidity of thrombosis is associated with CNS thromboembolism. It was reported that 15–20% of patients with CNS thrombosis are associated with comorbidities which appear in the form of hemiparesis, aphasia or having residual neurological deficits (Athale & Chan, 2003a). It is important to notice that comorbidities associated with thrombosis did not affect the patients’ treatment plan only but also it affects the patients’ quality of life as it causes long term complications to the patients.

4.8 Inherited Thrombophilia as a Risk Factor for Thrombosis

The inherited thrombophilia role as risk for thrombosis in cancer patients is controversial. There are studies that reported no association between the FV Leiden,
Prothrombin G20210A and MTHFR C677T and risk of thrombosis, while others reported an association between those mutations and thrombosis incidence in cancer patients. The variability in association of those mutations to thrombosis risk factors was a great motivation to start the current study in Egyptian pediatric ALL patients.

4.8.1 FV Leiden and PT G20210A

The current study was a case control one and it was done on 2 groups of Egyptian pediatric ALL patients. Each group had 63 patients matched in sex, disease risk and age group. The results showed that the prevalence of FV Leiden mutation in thrombus group was 17.5 % while it was 15.9 % in the control group. There was no significant difference between both groups in FV Leiden mutation $P$ value = 0.81.

The prothrombin G20210A mutation prevalence in the current study showed no significant difference between the 2 groups. The 2 groups almost have the same PT G20210A prevalence which was 3.2 %. No homozygous allele was detected for PT G20210A only heterozygous. These results are consistent with other investigators findings, where a study done to evaluate the VTE risk on gynecologic oncology patients concluded that FV Leiden is not a risk factor for VTE incidence (Ravin et al., 2002). Another study done on 135 Turkish pediatric leukemia patients showed that 10.3% of the patients had FV Leiden mutation while 5% had prothrombin G20210A mutation. Out of the 135 patients 3 had thrombosis and none of the 3 patients had FV or prothrombin mutation. This study suggested that there is no association between FV and Prothrombin G20210A mutation and risk of thrombosis in leukemia pediatric patients (Akin et al., 2012). In addition, a prospective study done on 211 cancer patients in Brazil, patients were divided into 2 groups with and without thrombosis to investigate the role of FV Leiden and PT G20210A in thrombosis risk. FV Leiden was found with a frequency of 2.7% and 1.5% in the control and thrombus group respectively. Prothrombin G20210A was found in 1.3% and 1.5% of control group and the thrombus group respectively. This study concluded that FV Leiden and PT G20210A don’t have a significant role in the risk of thrombosis in patients with different types of cancer (Ramacciotti et al., 2003). Finally, a prospective cohort study done by PARKAA group on ALL pediatric
patients suggested that there was no correlation between the FV Leiden or prothrombin G20210A and the risk of thrombosis (Mitchell et al., 2003).

In contrast to the previous studies, other studies showed that FV Leiden, Prothrombin G20210A mutations have an important role in increasing the risk of thrombus in cancer patients. As example, a study done on cancer patients showed that PT G20210A was a significant risk factor of VTE (Kennedy et al., 2005). Another study was done on 80 ALL pediatric patients reported the presence of FV Leiden and prothrombin 16.3% and 4% respectively in patients without thrombosis. While 50% of the patients with thrombosis had Prothrombin G20210A mutation (Harlev et al., 2010). In a prospective study, researchers showed that there was a significant thrombosis risk associated with inherited thrombophilia traits in pediatric ALL patients. This prospective study was done on 289 ALL patients. It was found that 58/289 patients were associated with inherited thrombophilia trait. While 27 out of the 58 patients (46.5%) with at least 1 inherited thrombophilia trait developed thrombosis compared to 5/231 (2.2%) patients without inherited thrombophilia trait (Nowak-Göttl et al., 1999).

4.8.2 MTHFR C677T

In the current study, MTHFR C677T mutation prevalence was found to be highly significant in the thrombus group when compared to the control group with $P$ value = 0.002. The prevalence of MTHFR C677T mutation in the thrombus group was 65.1% vs. 38.1% in the control group. The results showed that the presence of MTHFR C677T mutation in ALL pediatric patients increases the risk of having thrombosis by 3 folds (Table 8). These findings presented here confirm other studies which reported the increase of thrombosis risk in patients with MTHFR C677T mutation.

In agreement with our results, it was reported that MTHFR C677T mutation is a risk factor for thrombosis in Indian patients (Kumari et al., 2014). In a case control study done in Japan, it was found that MTHFR gene mutation was an important risk factor for thrombosis when combined with other prothrombotic defect (Fujimura et al., 2000). In Russia, it was reported that MTHFR C677T mutation was
a risk factor for pulmonary artery thromboembolism (Avdonin et al., 2006). A study done on 298 Chinese patients diagnosed with: deep venous thrombosis (DVT), cerebral haemorrhage and cerebral infarction and coronary artery disease (CAD) reported that MTHFR C677T mutation was an important risk factor for DVT and cerebral infarction while the CAD was less associated with the mutation (Zheng et al., 2000). Another study done on Chinese patients also reported that the MTHFR C677T mutation was a risk factor for DVT (Guo et al., 2002). A meta-analysis included 24 studies with 4048 controls and 2339 patients with thrombosis reported that MTHFR C677T polymorphism was a risk factor for thrombosis in Chinese population (Zhang et al., 2014). However, it was reported by Ramacciotti et al. 2003 that MTHFR C677T is not a significant risk factor for thrombosis. Where MTHFR 677T was found in 60.5% and 53.1% of control group and thrombus group respectively. The 2 groups of patients were comparable to each other. Another study done on Chinese patients reported the same results (Lu et al., 2002). In a case control study done on Brazilian patients it was reported that the presence of MTHFR C677T mutation was not a significant risk factor for thrombosis (Morelli et al., 2002)

Based on what was demonstrated in the current study, it is clear that MTHFR C677T is an important risk factor for thrombosis in Egyptian pediatric ALL patients while FV Leiden and PT G20210A are not.

4.9 Multiple prothrombotic Defects vs. Single and Risk of Thrombosis

Our study showed that having more than one mutation didn’t have a significant effect on thrombus incidence. This may be due to the small sample size of the study group (63 patients) and studying 3 prothrombotic risk factors only. In contrast to our results a German study showed a higher risk for thrombosis in patients had more than 1 prothrombotic defect. The German study was done on 289 pediatric ALL patients treated on Berlin-Frankfurt-Munster (BFM) 90/95 protocol to assess the risk of thrombosis in patients having prothrombotic defects (Nowak-Göttl et al., 1999). This can be due to the large number of prothrombotic defects studied by the German than in our study. In addition to FV Leiden, MTHFR C677T, prothrombin G20210A mutations, they also studied the lipoprotein (a) concentration,
deficiencies of anti-thrombin, protein S and protein C. The possibility to have significant prothrombotic defects associated with risk of thrombosis will be higher in case of studying larger number of parameters for each patient.

4.10 Prevalence of Inherited thrombophilia in Egyptian Pediatric ALL patients

4.10.1 FV Leiden and PT G20210A prevalence in Egyptian Pediatric ALL patients

The current study showed that FV Leiden prevalence in the control patients was 14.3% and 1.6% for heterozygous and homozygous alleles respectively, while in the thrombus patients was 15.9% and 1.6% for heterozygous and homozygous alleles respectively. There was no significant difference in FV Leiden prevalence between the patients with and without thrombosis. On the other hand, the PT G20210A prevalence in the control patients and the thrombus patients was the same (3.2%) for heterozygous and no homozygous was detected in both groups. To the best of our knowledge, this is the 1st study on the prevalence of FV Leiden and prothrombin G20210A in Egyptian pediatric ALL patients.

Association between ALL risk and FV Leiden and PT G20210A mutation

The prevalence of FV leiden and PT G20210A mutation in our control patients was comparable to the healthy Egyptian population. It was reported that the prevalence of FV leiden and prothrombin G20210A mutations in healthy Egyptian population was 16.5% and 1% respectively (Ulu et al., 2006), while ours was 15.9 % and 3.2 % respectively. These results indicate that those 2 mutations could not be associated with ALL risk. On contrary, it was reported in Iran that there was an association between pediatric ALL risk and FV Leiden when compared with healthy population (Rahimi et al., 2012), while the prothrombin G20210A mutation was not a risk factor for pediatric ALL (Rahimi et al., 2013).
4.10.2 MTHFR C677T point mutation in Egyptian pediatric ALL patients

Our results showed that MTHFR C677T mutation prevalence in the control patients was 28.6% and 9.5% for heterozygous and homozygous alleles respectively, while in the thrombus patients was 57.1% and 7.9% for heterozygous and homozygous alleles respectively. Several studies were done on Egyptian pediatric ALL patients to assess the association between MTHFR polymorphism and several toxicities. A study done to assess the high dose methotrexate toxicity in relation to the prevalence of MTHFR C677T reported that it was 35%, 10% for heterozygous and homozygous alleles, respectively (El-Khodary et al., 2012). While another study reported MTHFR C677T prevalence as 27.5% and 40% for heterozygous and homozygous alleles, respectively (Tantawy et al., 2010). There is a higher percentage of homozygous allele reported by Tantawy et al when compared to EL-Khodary et al. and the current study results.

Association between ALL Risk and MTHFR C677T

A previous study done on Egyptian population to evaluate the association of MTHFR C677T on the pediatric ALL patient, recruited 88 ALL pediatric patients and 311 healthy Egyptian individuals. The study reported prevalence of MTHFR C677T of 47.7 % and 8% for heterozygous and homozygous allele in the ALL patients, respectively. While the control healthy Egyptian patients showed MTHFR prevalence of 43.4% and 6.4% for heterozygous and homozygous alleles, respectively. The study concluded that MTHFR C677T polymorphism lack protective effect against ALL risk (Kamel et al., 2007). On the other hand, the present study results showed that the prevalence of MTHFR C677T in the control group was 28.6 % and 9.5 % for heterozygous and homozygous alleles, respectively. When we compare these results with the prevalence of MTHFR C677T in healthy Egyptian population reported in Kamel et.al 2007 study we found that the healthy Egyptian population had a higher incidence of MTHFR C677T than the ALL patients. This may indicate a protective role for MTHFR C677T mutation against ALL disease risk in Egyptian population. The current study results are in line with other studies done on the MTHFR and its role in ALL risk. They suggested that
increase in the MTHFR variants is associated with decreased risk of ALL, which means that MTHFR variants had a protective role against ALL (Skibola et al., 1999; Tone et al., 2001).

A meta-analysis included 12 articles with 4146 controls and 1803 pediatric ALL patients reported that MTHFR C677T mutation TT genotype had a protective role against ALL in the Chinese children (Lin et al., 2014). Another meta-analysis included 11 articles with 2,438 controls and 1,738 ALL patients found that MTHFR polymorphism had a protective role from ALL (Xiao et al., 2014). In Brazil, a study done on pediatric ALL patients found that MTHFR C677T polymorphism was associated with a lower risk of ALL risk (Tone et al., 2001). The MTHFR polymorphism protective role against ALL was explained by the following mechanism. Leukemia arises commonly as a result of DNA deletions, inversions and translocations in the genes responsible for the regulation of homeostasis or blood cell development. It was reported that Folate deficiency is associated with mis-incorporation of Uracil into DNA. During uracil excision repair DNA double strand breaks and lead to increase of chromosomal aberrations risk. These breaks could contribute in increasing cancer susceptibility (Robien et al., 2003).

Normally, MTHFR enzyme directs 5, 10-methylenetetrahydrofolate towards the synthesis of methionine at the expenses of DNA synthesis. Methylene- THF is a methyl donor, which converts deoxyuracil monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) in the presence of thymidylate synthase enzyme. Increase of 5, 10 - methylene - THF plasma levels, leads to increasing of dUMP conversion to dTMP. Which means a reduction in uracil mis incorporation rate and optimal DNA synthesis (Figure 5) (Robien et al., 2003). In case of MTHFR polymorphism, the decrease in the enzyme activity leads to decrease in the conversion of 5, 10-methylene- THF to 5-methyl-THF and increase homocysteine levels takes place. It is thought that MTHFR polymorphism can lead to optimal DNA synthesis and less Uracil mis incorporation. This means less chromosomal aberration during Uracil mis incorporation excision repair process (Robien et al., 2003).
5. Conclusion

We found that MTHFR C677T is important risk factor for thrombosis in Egyptian pediatric ALL patients. The presence of this polymorphism can increase the risk of thrombosis 3 folds more than those patients who did not have the polymorphism. In addition, the patients who are older than 10 years, on SR/HR treatment protocol or in induction treatment phase are also at high risk of thrombosis than others who are less than 10 years, treated on LR protocol or in any treatment phase other than induction. Having one or more of those factors can increase the risk of thrombosis in pediatric ALL patients. Those factors can be used as important indicator for the risk of thrombosis in ALL patients. Using those factors will help in the prediction of the thrombosis susceptibility for ALL patients and a prophylaxis therapy can be given before having the thrombosis. Prediction of thrombosis is crucial in enhancing the patient quality of life by preventing the complications associated with the thrombosis that represent 10% of patients in our study. We also found that there was no significant role for FV Leiden and PT G20210A in risk of thrombosis.

Rechallenging patients with asparaginase as soon as possible after the thrombus total recanalization took place is very essential as it can affect the patient survival. We found that the EFS and the OS of the patients were the same in the 2 studied groups. That’s why we recommend restarting the L Asparaginase once the thrombus is stationary or showed partial recanalization in order to keep the EFS and the OS of thrombus patients as those without thrombosis. In addition, it was found that there was no significant association between the thrombosis risk and the combined mutations. Most probably this finding was due to the small sample size and studying 3 prothrombotic factors only.
6. Future recommendations

A routine screening for MTHFR polymorphism should be done in ALL new patients before starting L-asparaginase and prednisolone to evaluate the risk of thrombosis especially for those who have other risk factors such as age greater than 10 years or SR/HR patients. Further prospective study is recommended to evaluate the importance of using anticoagulant as prophylaxis therapy in ALL pediatric patients having high risk factors for thrombosis.

Multicenter study with larger sample size is recommended to evaluate the role of FV Leiden, PT G20210A as a thrombosis risk factor in Egyptian pediatric ALL patients.

In the present study, we looked at genetic factors only, where other possible risk factors such as protein S, protein C, homocysteine, anti-thrombin levels were not evaluated. Evaluation of large number of factors will clarify whether the risk of thrombosis was affected by the presence of combined risk factors or not.

A large case control study is recommended to evaluate the protective role of MTHFR C677T against ALL.
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Appendix 1: IRB Approval

To: Mohamed Nagy  
cc: Hind Al Helaly  
From: Atta Gebril, Chair of the IRB  
Date: January 11, 2013  
Re: Approval of study

This is to inform you that I reviewed your revised research proposal entitled “MTHFR C677T, PROTHROMBIN G20210A and Factor V Leiden mutation and Risk of Thrombosis in Pediatric Acute Lymphoblastic Leukemia,” and determined that it required consultation with the IRB under the “expedited” heading. As you are aware, the members of the IRB suggested certain revisions to the original proposal, but your new version addresses these concerns successfully. The revised proposal used appropriate procedures to minimize risks to human subjects and that adequate provision was made for confidentiality and data anonymity of participants in any published record. I believe you will also make adequate provision for obtaining informed consent of the participants.

Please note that IRB approval does not automatically ensure approval by CAPMAS, an Egyptian government agency responsible for approving much off-campus research involving surveys and interviews. CAPMAS issues are handled at AUC by the office of the University Counsellor, Dr. Amr Salama. The IRB is not in a position to offer any opinion on CAPMAS issues, and takes no responsibility for obtaining CAPMAS approval.

This approval is valid for only one year. In case you have not finished data collection within a year, you need to apply for an extension.

Thank you and good luck.

Atta Gebril

IRB chair, The American University in Cairo  
2046 HUSS Building  
T: 02-26151919  
Email: agebril@aucegypt.edu
Appendix 2: Consent Form

الموافقة المسؤولة للمشتركة في مشروع بحث

اسم المشروع: "MTHFR C677T وعامل تأثين الخمسة وخطر الإصابة بنتج في سرطان الدم الليمفاوي الحاد لدى الأطفال."

المؤسس: مكتبة الأطباء المصريون

الباحث الرئيسي: محمد ناجي

المؤسس: مكتبة الأطباء المصريون

تم تقديم مجلس إخلاقيات البحث العلمي من مكتبة الأطباء المصريون، مسؤولية مراجعة البحوث التي تجري ب นอกจาก الاتصال بمكتب مجلس إخلاقيات البحث العلمي من مكتبة الأطباء المصريون. إنه يمنح إخلاقيات البحث العلمي من مكتبة الأطباء المصريون، مسؤولية مراجعة البحوث التي تجري ب

الغرض / الهدف: سرطان الدم الليمفاوي الحاد (ALL) هو أكثر أنواع السرطان شيوعًا في الأطفال الذين يعانون من سرطان الدم الليمفاوي الحاد. ويؤكد على أن الخطر هو في الضعفاء في الأطفال الذين يعانون من سرطان الدم الليمفاوي الحاد. ويؤكد على أن الخطر هو في الضعفاء في الأطفال الذين يعانون من سرطان الدم الليمفاوي الحاد.

الإجراءات:

إذا وُقعت على المشارك، سوف يتم تصويرهم لمدة 5 دقائق لشرح الدراسة. سوف تحتاج إلى ذلك عينة من الدم لإجراء الدراسات. سيتم الحصول على عينة الدم في المختبر لمعرفة تطور الإناث أو الأمراض الجينية وحث الأطباء في مرضى سرطان الدم الليمفاوي الحاد. سوف يتم إجراء الدراسة على مجموعة من المرضى:

المجموعة 1: مرضى سرطان الدم الليمفاوي الحاد (ALL) الذين يتلقون من حالة الجلطة.

المجموعة 2: مرضى سرطان الدم الليمفاوي الحاد (ALL) الذين لا يعانون من حالة الجلطة (المجموعة الضابطة)

CCHIE - IRB Approval
Serial no: 6/2/2021
Exp. no: 16/2/2021
الموافقة / القوانين:

القوانين:

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المشاركة الممكنة:

قد يحدث تزويج طيف أو كميات مختلفة من المواد إذا أثر ذلك الحدوث. الجذور متخصص مدربي جدًا سيقوم بسحب الحبوب من طلباتك، ولكن فإن فرص هذه المشاركات تكاد تكون محدودة.

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إذا كان لديك أسرة أخرى يمكنك الاتصال بالباحث الرئيسي (السيد محمد خليفة الصيدلاني، مستشار سرطان الأطفال من شارع الهاير) 0123296589600 253351905 (414).

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