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Sudan University of Science and Technology

College of Graduate Studies

Detection of Tissue Factor Pathway Inhibitor-2 Gene Mutation and its Correlation with Coagulaton Parameters in Sudanese Pediatric Patients with Leukemia

الكشف عن الطفرات الوراثية لمثبط مسار العامل النسيجى -2 المانع للتخثر وعلاقته باختبارات التجلط لدي الأطفال السودانيين المرضى بسرطان الدم الأبيض

A thesis submitted for fulfilment of the requirements for the degree of Doctorate of Philosophy in Haematology & immunohaematology

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بِسْمِ اللّهِ الرّحْمَنِ الرّحِيمِ

قال الله تعالى:

((إِنّا خَلَقْنَا الْإِنسَانَ مِن نُّطْفَةٍ أَمْشاجٍ نّبْتَلِيهِ فَجَعَلْنَاهُ سَمِيعًا بَصِيرًا))

صدق الله العظيم

سورة الإنسان (الآية2)

DEDICATION

Iwould like to dedicate this study to:

- My father soul, which it was always with me,
- My family for their constant love and understanding,
- To all of the teachers, who have taught me hematology as well as many friends, colleagues, students, archivists, and other librarians who assisted, advised, and supported my research and writing efforts over the years.
- All Sudaneae leukemic childrens and their families challenge the journey difficulties with the disease from diagnosis to treatment.

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ABSTRCT

This is analytical case control study was carried out in radio isotope center Khartoum (RICK) to detect TFPI-2 gene mutation and its correlation with coagulation parameter in Sudanese pediatric patients with leukemia. Ninety-eight patients less than 15 years with mean age for all (9.03 ± 4.058) where diagnosed with leukemia of both gender 52 (53.1%) were males and 46 (46.9%) were females .The center administration and the relatives of the patients were informed about the study and agreement of participation was obtained.

In additive ninety-seven healthy children, less than 15 years 53 (54.6%) were males and 44 (45.4%) were females with mean age for all (8.08 ± 4.033) selected as a control group. A structured questionnaire used to collect information about patient age, sex, and patient history of disease. Five ml of venous blood was collected, three ml in EDTA and 1.8 ml in trisodium citrate anticoagulant container from all patients and controls.

Reverse transcription polymerase chain reaction followed by restriction-fragment length polymorphism (RFLP) analysis of the TFPI-2 was, carried out for all patients and controls samples.

TFPI-2 gene was investigated in all samples, there were 14(14.3%) of the patients group have mutation, 10(71.4%) of them were homozygous and 4 (28.6%) were heterozygous.

No participant among control group (0%) had TFPI-II gene mutation; there was significant association between patients, which have TFPI-2 gene mutation, and control group, P value 0.0258.

Automated hematological analyzer (Sysmex KXN-21) used to measure platelet count for the patient and control group.

Automated coagulation tests (PT, PTT, fibrinogen, protein C and D-dimer level) were measured using coagulation analyzer (sysmex CA 500) for all patient and control group.

Statistical package for social science (SPSS) computer program was used for data analysis

In patients group with TFPI-2 gene mutation the result of PT mean level was (17.05 ± 2.974). PTT mean level was (39.564 ± 6.809), fibrinogen mean level (2.759 ± 0.630), protein C mean level (0.845± 0.198) and platelet count mean (288.21 ± 155.797), while in the patients group without mutation the result of, PT mean was (17.689 ± 6.741), PTT mean was (40.555 ± 11.551), fibrinogen mean (2.659 ± 0.681), protein C mean level (0.822 ± 0.037) and platelet count mean was(289.67 ± 136.85).

There were insignificant variations in coagulation tests parameter (platelet count, PT, INR, APTT, protein C and fibrinogen) between patients with TFPI-2 gene mutations and those without TFPI-2 gene mutations. There were only two patients had high D-dimer level which excludes disseminated intravascular coagulation.

ملخص الدراسة

هذه دراسة مقارنة تحليلية أجريت في مركز الإشعاع والنظائر بالخرطوم للكشف عن الطفرات الوراثية لمثبط مسار العامل النسيجى -2 المانع للتخثر وعلاقته باختبارات التجلط فى الأطفال السودانيين المرضى بسرطان الدم الابيض. شملت الدراسة ثمانية وتسعين مريض اعمارهم أقل من 15 عاما مع متوسط العمر للجميع (9.03 ± 4.058) تم تشخيصهم بسرطان الدم الابيض من الجنسين 52 (53.1٪) ذكور و 46 (46.9٪) من الإناث. تم الحصول على اذن باجراء الدراسة من إدارة المركز وأقارب المرضى.

بالاضافة لعدد سبعة وتسعين من الأطفال الأصحاء اعمارهم أقل من 15 سنة 53 (54.6٪) من الذكور و 44 (45.4٪) من الإناث مع متوسط العمر للجميع (8.08 ± 4.033) تم اختيارهم كمجموعة ضابطة. تم ملءاستبيانات لجمع المعلومات حول عمر المريض، والجنس، والتاريخ المرضى للمرض. تم جمع خمسة مل من الدم الوريدي، ثلاثة مل في EDTA و 1.8 مل في ثلاثى الصوديوم سترات فى حاوية مضادة للتخثر من جميع المرضى والمجموعة الضابطة.

تم عمل الاختبار الجزيئى للحامض الرايبزومى ثم اختبارات بواسطة الانزيم القاطع للحامض النووى لمثبط مسار العامل النسيجى -2 المانع للتخثر لجميع المرضى والمجموعة الضابطة.

تم اختبار جين العامل النسيجى -2 المانع للتخثر فى جميع العينات، كان هناك 14 (14.3٪) من مجموعة المرضى لديهم طفرة جينية ، 10 (71.4٪) منهم متماثلة و 4 (28.6٪) غير متماثلة بينما لاتوجد اى طفرات فى المجموعة الضابطة (0%) هنالك علاقة احصائية بين مجموعة المرضى الذين لديهم طفرة جينية فى مسار العامل النسيجى -2 المانع للتخثر والمجموعة الضابطة القيمة الاحتمالية = 0.0258

تم استخدام جهاز(SYSMEX KXN 21) الآلى لقياس الصفائح الدموية لمجموعة المرضى والمجموعة الضابطة بالاضافة لجهاز(SYSMEX CA 500) الآلى لقياس اختبارات التخثر(زمن البروثرومبين, زمن البروثرومبين الجزئى المحفز, الفبرينوجين , بروتين سى و الدى دايمر) لجميع المرضى والمجموعة الضابطة

تم استخدام برنامج الحاسب الآلى(الحزم الإحصائية للعلوم الاجتماعية SPSS) لتحليل البيانات.

في مجموعة المرضى الذين لديهم طفرة جينية لمثبط مسار العامل النسيجى-2 المانع للتخثر كانت نتيجة متوسط زمن البروثرومبين (17.05 ± 2.974),متوسط زمن البروثرومبين الجزئى المحفز (39.564 ± 6.809). متوسط مستوى الفيبرينوجين (2.759 ± 0.630), متوسط بروتين سى (0.845± 0.198) ومتوسط عدد الصفائح الدموية (288.21 ± 155.797) بينما في مجموعة المرضى الذين ليس لديهم طفرة جينية كان متوسط زمن البروثرومبين (17.689 ± 6.741)، متوسط زمن البروثرومبين الجزئى المحفز (40.555 ± 11.551)، متوسط مستوى الفيبرينوجين (2.659 ± 0.681) , متوسط بروتين سى (0.822 ± 0.037) ومتوسط عدد الصفائح الدموية (289.67 ± 136.85).

ليس هنالك اختلافات مؤثرة في اختبارات التخثر (متوسط زمن البروثرومبين ,متوسط زمن البروثرومبين الجزئى المحفز , متوسط مستوى الفيبرينوجين , متوسط بروتين سى ومتوسط عدد الصفائح الدموية) بين المرضى الذين لديهم طفرة جينية لمثبط مسار العامل النسيجى-2 المانع للتخثر وباقى المرضى الذين ليس لديهم طفرة جينية.

لا يوجد سوى اثنين من المرضى كان لديهم مستوى عالى من قطع البروتينات الناتجه عن تحلل الفيبرين من ما يستبعد وجود تخثرللدم منتشر داخل الأوعية لدى المرضى.

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LIST OF ABBREVIATIONS

AIHA	Autoimmune hemolytic anemia
ALL	Acute lymphoblastic leukemia
ALL-B cell	Acute lymphoblastic leukemia subclass B
ALL-T cell	Acute lymphoblastic leukemia subclass T
AML	Acute myeloid leukemia
AML(M2)	Acute myeloid leukemia subclass M2
AML(M3)	Acute myeloid leukemia subclass M3
APL	Acute promyelocytic leukemia
APTT	Activated partial thromboplastin time
AT	Antithrombin
BMT	Bone marrow transplantation
cDNA	Double-stranded DNA synthesized from a single stranded RNA
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
CNS	Central nervous system
COG	Children's oncology group
GM-CSF	Granulocyte and monocyte colony stimulating factor
CpG	Cytosine-phosphorothioate-guanine
DVT	Deep vein thrombosis

DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic Acid
EPCR	Endothelial cell protein C receptor
ECM	Extracellular matrix
EPCR	Endothelial protein C receptor
ET	Essential thrombothenia
FDPs	Fibrin degradation products
HBS	Heparin binding site
INR	International normalize ratio
JMML	Juvenile myelomonocytic leukemia
LPS	Lipopolysaccharide
MDS	Myelodysplastic syndrome
MPD	Myeloproliferative disaese
MRD	Minimum residual disease
MMPs	Matrix metalloproteinases
NCR	National population-based cancer registry
PCR	Polymerase chain reaction
Plts	Platelets
Ph	Philadelphia chromosome
PRV	Polycythaemia ruberavera

PT	Prothrombin time
PE	Pulmonary embolism
RA	Refractory anemia
RFLP	Restriction fragment length polymorphism
RICK	Radio isotope center Khartoum
RNA	Ribonucleic acid
RT- PCR	Reverse transcriptase polymerase chain reaction
TFPI-2	Tissue factor pathway inhibitor two
TAT	Thrombin-anti thrombin III complex
tPA	Tissue plasminogen activator
VTE	Venous thromboembolism
WHO	World Health Organization

CHAPTER-ONE

Introduction and literature review

Chapter one

Introduction and literature review:

1.1 Introduction:

Cancers form one of the major causes of death in children between the ages of one and 15 years. They differ markedly from adult cancers in their nature, distribution and prognosis. The patterns of childhood cancers in America and Europe are almost the same, with leukemia and central nervous system tumors accounting for over one-half of the new cases. In contrast, lymphoma is the most common prevailing cancer of this age group in Africa (Haroun, *et al.*, 2006).

Cancer in children less than 15 years old constituted about, 486 case 7% of the cancer cases recorded by the national population-based cancer registry (NCR) in Khartoum State, the most common cancer in children were leukemia, lymphoma, and cancer of the eye, bone, kidney, brain, breast, oral, liver, and stomach. Lymphomas were mostly non-Hodgkin's lymphoma and eye tumors were mostly retinoblastoma (Intisar, *et al.*, 2014).

Abnormalities of the coagulation system have long recognized in cancer patients, in whom the plasma levels of several coagulation factors have observed, altered. Moreover, thromboembolism have been reported as being the second most frequent cause of death in cancer patients, even without obvious thrombosis. However, it is still unclear whether such abnormalities in coagulation bear any significance in the pathogenesis or progression of the malignant process. Mean while, the extent of activation of coagulation and fibrinolysis, as measured via plasma levels of antithrombin (AT) III and D-dimer, has been reported to correlate with tumor stage, aggressiveness, and prognosis in various malignancies (Sung, *et al.*, 2010).

Several mechanisms, including degradation of the extracellular matrix (ECM), are involved in the spread of cancer cells from the primary tumor. Among the variety of proteinases acting in proliferation and invasion processes, serine proteinases and matrix metalloproteinases (MMPs) have been shown to be highly expressed and activated in the tumor microenvironment especially in highly aggressive malignant tumors (Marion,*et al*.,2013).

One potent inhibitor of proteinases is tissue factor pathway inhibitor-2 (TFPI-2), a 32 kDa Kunitz-type serine proteinase inhibitor secreted within the ECM and considered

as candidated tumor suppressor gene. In particular, it inhibits plasmin, which is involved in matrix metalloproteinases (MMPs) activation and could thus regulate ECM degradation and tumor cell invasion.TFPI-2 is,down regulated in most aggressive tumors such as glioma, non-small cell lung cancer (NSCLC), breast cancer, melanoma, colorectal cancer, pancreatic cancer and hepatocellular carcinoma. Such silencing is mainly due to epigenetic changes induced by TFPI-2 promoter hypermethylation and histone deacetylation. Aberrant methylation of TFPI-2 studied to differentiate benign from malignan diseases and to evaluate disease progression. Tumour cells are also able to synthesize an untranslated splice variant of TFPI-2 transcript that correlates with a low level of full-length TFPI-2 transcript (Marion, *et al.,* 2013).

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy, and relapsed B-lineage ALL remains a leading cause of cancer death in young people , B-progenitor acute lymphoblastic leukemia (B-ALL) is characterized by recurring chromosomal abnormalities, including aneuploidy and chromosomal rearrangements (Kathryn, *et al*., 2012).

The improving survival rate for children with acute lymphoblastic leukemia (ALL) is one of the greatest success stories of cancer treatment. However, a small proportion of patients will relapse; relapsed disease has a poor response to current therapy, resulting in reduced survival. Thus, to ensure optimum treatment, it is essential that patients at high-risk of relapse are identified at the time of diagnosis. The prognostic relevance of specific chromosomal abnormalities in B-lineage ALL has known for some time. To date, this information remains an important factor in risk stratification of these patients for treatment (Moorman *et al.*, 2010).

To my knowledge, no study has yet demonstrated TFPI-2 at genetic level, therefore detection of tissue factor pathway inhibitor-2 gene mutation and its correlation with coagulaton parameter (platelet count, PT, APTT, fibrinogen level, D-dimer level and protein C) in Sudanese pediatric patients with leukemia, the study may be useful in the improvement of oncological care of pediatric patients with leukaemia.

1.2 Literature review:

Cancers of different origins may have very different characteristics, cancer patients are at a clear risk of thrombosis and idiopathic thrombosis may be a marker for underlying undetected cancer. One large study showed a clear association between idiopathic venous thrombosis and development of cancer, an association that extended for 10 years or more after the thromboembolic event, it is very clear that cancer patients have an increased risk of thrombosis, there are a number of studies that examine the role of tissue factor in alterations of hemostasis in cancer. As is known from in vitro assays and even relatively small amounts of tissue factor can promote rapid clotting in a test tube. Also injection of sufficiently large amounts of tissue factor into animals can induce fibrinogen depletion and fibrin deposition, sometimes resulting in thrombosis, so a number of studies have hypothesized that tissue factor expression might contribute to thrombosis in cancer patients (David and Hau 2009).

1.2.1 Cancer and aging:

Increasing age is one of the strongest risk factors for cancer development. There is a marked increase in epithelial carcinomas from ages 40 to 80 years. Interestingly, beyond age 80 the incidence of cancers levels off. The link between cancer and aging is complex. In some instances, such as cellular senescence or telomere shortening, strategies that protection from cancer may increase the rate of aging. However, cancer and aging also seem to share common etiologies such as genomic instability and reduced rate of autophagy (Dirk, *et al.*, 2010).

Frequent germ line cells mutations previously demonstrated to be associated with aging. This suggests a higher incidence of childhood cancer among children of older parents. A population-based cohort study of parental ages and other prenatal risk factors for five main childhood cancers performed with the use of a linkage between several national based registries. This findings indicate that advanced parental age might be associated with an increased risk of early childhood cancers.For children <5 years of age, maternal age were associated with elevated risk of retinoblastoma and leukaemia (Benjamin, *et al*., 2006).

1.2.2. Differences between adults and children cancer:

The types of cancers that develop in children are often different from the types that develop in adults. Childhood cancers are often the result of DNA changes that take place in cells very early in life, sometimes even before birth. Unlike many cancers in

adults, childhood cancers are not strongly, linked to lifestyle or environmental risk factors,there are exceptions, but childhood cancers tend to respond better to treatments such as chemotherapy. Children's bodies also tend to tolerate chemotherapy better than adults bodies do, but cancer treatments such as chemotherapy and radiation therapy can have some long-term side effects, so children who survive their cancer need careful attention for the rest of their lives. Since the 1960s, most children and teens with cancer treated at specialized centers designed for them. Being treated in these centers offers the advantage of a team of specialists who know the differences between adult and childhood cancers, as well as the unique needs of children with cancer and their families. These centers also have psychologists, social workers, child life specialists, nutritionists, rehabilitation and physical therapists, and educators who can support and educate the entire family. Most children with cancer in the United States treated at a center that is a member of the children's oncology group (COG). All of these centers are associated with a university or children's hospital learned more about treating childhood cancer, it has become even more important that experts in this area give treatment, any time a child or teen diagnosed with cancer, and it affects every family member and nearly every aspect of the family's life (American Cancer Society, 2014).

1.2.3. Types of childhood cancer:

The incidence of childhood cancer is lower than in western countries. Respective overall survival for ALL, lymphoma, renal tumors, liver tumors, retinoblastoma, soft tissue tumors is lower than those reported in developed countries while for CNS tumors, neuroblastoma and germ cell tumors the figures are comparable (Wiangnon,*et al.*, 2011).

Leukemias were the most commonly diagnosed cancer, accounting for 32% of all childhood cancers. Cancers of the central nervous system (CNS) were the second most commonly diagnosed childhood cancer group (19%). CNS tumours begin when normal cells in the brain or the spinal cord change and grow uncontrollably, forming a mass. The third most commonly diagnosed group of childhood cancers were lymphomas (11%). Lymphomas begin when cells in the lymphatic system (a system of the body responsible for fighting infection or other diseases) change and grow uncontrollably (Lawrence and Teresa, 2015).

1.2.4 Hematological malignancies :

Hematological malignancies includes leukemias,lymphomas,multiple myeloma, myelodysplastic syndrome (MDS) and myeloprolifrative disorders which includes, polycythaemia ruberavera (PRV), idiopathic myelofibrosis, undifferentiated myeloproliferative disaese (MPD), essential thrombothenia (ET) (Catovsky, *et al*., 2005).

Hematologic malignancies are a diverse group of blood cancers with various etiology, incidence, prognosis and survival, in population studies, hematological malignancies grouped into four broad categories including leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma and myeloma. World health organization (WHO) has developed a consensus-based classification in which hematologic malignancies categorized according to their lineage (myeloid and lymphoid) and cell maturity, this classification utilizes morphology, immunophenotype, genetic and clinical criteria for further subdivide each category (Moustapha and Manuchehr 2014).

1.2.5 Myelodysplastic syndrome in children:

Myelodysplastic syndrome (MDS) comprises a group of clonal haematopoietic disorders characterized by peripheral blood cytopenias, bone marrow hypercellularity, and abnormal blood cell differentiation (Disperati, *et al*., 2005).

MDS is another group of hematopoietic disorders, which are rare in children and correspond to less than 3% of hematologic malignancies in this age group. The majority of pediatric patients with diagnosed MDS are, assigned to aggressive and poor prognosis FAB subtypes such as RAEB and RAEB-t (Locatelli, *et al*., 1995).

The estimated incidence of pediatric MDS in Europe and Canada varies from 1 to 4 cases per million per year (Passmore, *et al*., 2003).

It is equal in males and females. The median age at presentation in children is 6.8 years (Jackson, *et al*., 1993).

The most common is JMML belonging to the MDS/MPN group according to the WHO classification. Very similar features characterized it as CMML, which refers to adult patients in the FAB classification. There are many mutations connected with JMML have been discovered in genes coding for the RAS protein family, protein tyrosine phosphatase (PTPN11) and neurofibromin 1 (NF1) – components of the RAS/MAPK pathway. Activation of signal transduction and hypersensitivity of myeloid precursor cells to granulocyte and monocyte colony stimulating factor (GM-

CSF) lead to excessive proliferation. Cytogenetic analysis is a crucial component for diagnosis and disease differentiation, e.g. between MDS and AML with low blast count. Reported in MDS, chromosome 7 and 5 abnormalities and phenotypic effects caused by genes localized on their long arms prove that in MDS pathogenesis an essential role played by hematopoietic growth factors and their receptors. Karyotype disruption may have a complex nature and involve more than one chromosome. A significant diagnostic point is to exclude *BCR-ABL1* fusion. The proliferative advantage of altered cells and genetic instability determine the ability of syndromes to progress to AML (Martinez-Climent. 1997).

1.2.5.1 Children myelodysplastic syndrome transformation :

The myelodysplastic syndrome (MDS) represents a spectrum of stem cell malignancies that manifests dysplastic and ineffective hematopoesis, which is associated with a variable risk of transformation to acute leukemia. Patients have a deteriorating course with 30% evolving into acute leukemia usually of myeloid phenotype (Tilak, *et al*., 2008).

Evolution into acute lymphoblastic leukemia (ALL) is rare and seen in <1% adult cases and extremely rare in pediatric population. There is only one other case report where a child with refractory anemia (RA) transformed into ALL (Goel, *et al*., 2007).

Approximately 30% of cases of MDS eventually progress to acute myelogenous leukemia (AML), while progression of MDS into acute lymphoblastic leukemia (ALL) is rare (Disperati, *et al*., 2005).

1.2.6 Leukaemias:

The leukaemias are a group of disorders characterized by the accumulation of malignant white cells in the bone marrow and blood. These abnormal cells cause symptoms because of bone marrow failure (i.e. anaemia, neutropenia, and thrombocytopenia) and infiltration of organs (e.g. liver, spleen, lymph nodes, meninges, brain, skin or testes) (Hoffbrand, *et al.,* 2011).

Leukaemias and lymphomas are clonal disorders of the haematopoietic cell characterized by somatically acquired genetic alterations. The discovery that molecular abnormalities are involved in their pathogenesis has greatly improved understanding of these diseases. In leukaemias, more than 300 chromosomal rearrangements have detected and more than 100 of these have cloned and characterized. Molecular abnormalities in haematologic malignancies are diverse but

can be grouped in two categories abnormal rearrangemments caused by chromosome translocations, inversions and duplications which usually result in oncogene activation; the result of these rearrangements can be either fusion proteins or deregulated expression of genes mutations and deletions of tumour suppressor genes (Renata. 2010).

1.2.6.1 Diagnosis and classification:

Precise diagnosis and classification are essential to the successful treatment and biologic study of the childhood leukemias. In broadest terms, the leukemias classified as acute versus chronic and as lymphoid versus myeloid. The terms acute and chronic originally referred to the relative durations of survival of patients with these diseases when effective therapy was not available. With improvements in treatment, they have taken on new meanings. Acute currently refers to leukemia characterized by rapid tumor cell proliferation and a predominance of blast cells, while chronic leukemia encompasses a variety of myeloproliferative and lymphoproliferative disorders in which the predominant tumor cells show variable degrees of differentiation beyond the blast stage. The vast majority of childhood leukemia cases are acute, unlike those in adults. The most common subtype is acute lymphoblastic (also termed lymphocytic or lymphoid) leukemia (ALL) accounts for 75% to 80% of all childhood cases; while acute myeloid (also termed myelocytic, myelogenous, or nonlymphoblastic) leukemia (AML) comprises approximately 20%. By contrast chronic myeloid leukemia (CML) represents only approximately 2% of childhood leukemias and chronic lymphocytic leukemia (CLL) is reported only rarely in children. Finally, myelodysplastic syndrome (MDS) designates a heterogeneous group of clonal diseases related to a subset of AML. MDS characterized by peripheral blood cytopenias, normocellular or hypercellular but nonproductive bone marrow (inefficient hematopoiesis) and dysmorphic maturation of hematopoietic precursors. It may evolve into frank AML or result in death due to cytopenic complications. The modern approach to leukemia classification incorporates morphologic findings, immunophenotype and genetic lesions, in an attempt to delineate homogeneous and clinically and biologically relevant disease categories.Current principles and techniques used for the diagnosis and classification of the childhood leukemias is the basis for treatment assignment and biologic study (Ching-Hon, 2006).

For the analysis of a suspected hematological malignancy, a complete blood count and blood film are essential, as malignant cells can show in characteristic ways on light microscopy. When there is lymphadenopathy, aspiration from lymph node is generally undertaken surgically. In general, a bone marrow biopsy is part of the "work up" for the analysis of these diseases. All specimens examined microscopically to determine the nature of the malignancy (Mohammad, 2009) .

A cute myeloid leukemia (AML) is a molecularly and clinically heterogeneous disease. Nucleophosmin (NPM1) gene mutations resulting in cytoplasmic delocalization of nucleophosmin (NPMc+) are the most common genetic alteration in AML, being, detected in about one-third of cases. Because of its unique molecular, genotypic, immunophenotypic and prognostic features, AML with mutated NPM1 was included as a separate provisional entity in the 2008 World Health Organization (WHO) classification of myeloid neoplasms, under the heading of “AML with recurrent genetic abnormalities (Brunangelo,*et al.*, 2010).

Immunocytochemical study was required especially in diagnosing of AML with minimal differentiation, acute megakaryoblastic leukaemia, acute erythroid leukaemia and acute leukaemias of ambiguous lineage. Acute lymphoblastic leukaemias was broadly classified into B-lineage and T-lineage ALL. According to the degree of B-lymphoid differentiation of the blast cells four subtypes of B-lineage ALL were established. T-lineage ALL observed in patients and divided into four subtypes (Gluzman, *et al.*, 2010).

Evaluation of the bone marrow by microscopy is often relatively insensitive, and complemented or displaced, by evaluation of minimum residual disease (MRD). This technique uses flow cytometry or the polymerase chain reaction (PCR) to assess for disease at a significantly lower limit of detection (1 leukemic blast in 10,000–100,000 cells).(Cooper and Brown. 2014).

1.2.6.2. Types of leukemia in children:

The most common cancers in children are (childhood) leukemia (34%), brain tumors (23%), and lymphomas (12%) (Kaatsch P, *et al*., 2010).

Leukaemia is the most common childhood cancer and acute lymphoblastic leukaemia (ALL) is the most common subtype, accounting for 75 – 80% of all cases. Childhood ALL comprises different biological subtypes defined by cell morphology, immunophenotype, gene expression features and genetic abnormalities, some of

which are associated with disease aggressiveness and treatment response. Progress in the treatment of childhood ALL over the last four decades has steady, with cure rates, surpassing 80%.This advance attributed to three main factors: recognition of reliable prognostic factors leading to increasingly refined risk – directed therapies, development of clinical trials designed to gain substantial increments in knowledge, and improvements in supportive care (Hoffbrand, *et al.,* 2011).

1.2.6.2.1 Acute leukemias:

Acute leukemia characterized by a rapid increase in the number of immature blood cells. The crowding that results from such cells makes the bone marrow unable to produce healthy blood cells. Immediate treatment is required in acute leukemia because of the rapid progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body. Acute forms of leukemia are the most common forms of leukemia in children (Nagham and Fatema, 2015).

The main types of acute leukemia are:

1.2.6.2.1.1. Acute lymphocytic (lymphoblastic) leukemia (ALL):

Acute lymphoblastic leukaemia (ALL) seen in both children and adults, but its incidence peaks between ages 2 and 5 years. The causation of ALL considered as multi-factorial, including exogenous or endogenous exposures, genetic susceptibility, and chance. The survival rate of paediatric ALL has improved to approximately 90% with risk stratification by biologic features of leukaemic cells and response to therapy (Inaba, *et al.,* 2013).

ALL arises from hematopoietic precursors of the lymphoid lineage. It is the most common leukemia in pediatrics, accounting for up to 80% of leukemias in this group and 20% of leukemias in adults. With the advances in cytogenetic (and especially molecular), the understanding of the biology and pathogenesis of leukemia has progressed tremendously.This progression in understanding of the process of lymphoid progenitors, pro–B cells, pre–B cells, and mature B cells. This maturation process is tightly, controlled by the hierarchical activation of transcription factors and selection through functional signal transduction (Zhou, *et al.,* 2012).

1.2.6.2.1.2. ALL epidemiology:

ALL is the most common childhood malignancy, accounting for close to 25% of all cancers in children and 72% of all cases of pediatric leukemia (Scheurer, *et al.,* 2011).

ALL occurs at an annual rate of 3 to 4 cases per 100.000 children less than 15 years of age (Ribera and Oriol.2009).

The American cancer society provides estimates the number of new cancer cases and deaths for children and adolescents in the United States and summarizes the most comprehensive data on cancer incidence, mortality and survival from the national cancer institute. The centers for disease control and prevention and the North American association of central cancer registries with high-quality data from 45 states and the District of Columbia, covering 90% of the US population. In 2014, an estimated 15,780 new cases of cancer diagnosed and 1960 deaths from cancer will occur among children and adolescents aged birth to 19 years. The annual incidence rate of cancer in children and adolescents is 186.6 per 1 million children aged birth to 19 years, approximately 1 in 285 children diagnosed with cancer before age 20 years (Elizabeth, *et al.*, 2014).

A sharp peak in incidence observed among children aged 2 to 5 years. Males affected more often than females except in infants, the difference being greater among pubertal children. There is a geographic variation in the frequency of ALL. The incidence is lowest in North Africa and the Middle East, and highest in the industrialized Western countries, suggesting that this may reflect more exposure to environmental leukemogens (Margolin, *et al.*, 2011).

Numerous investigators have reported the occurrence of leukemic clusters in different geographic areas, thus pointing towards infectious and/or environmental causes of at least some cases of ALL (Nyari, *et al.*, 2013).

Several studies have suggested a link between maternal reproductive history and the risk of ALL. Fetal loss is associated with a higher risk for ALL in subsequent children (Rość, *et al.*, 2007).

There is evidence that increased in utero growth rates and Insulin Growth Factor (IGF) pathways play a role in the development of ALL (Caughey and Michels.2009).

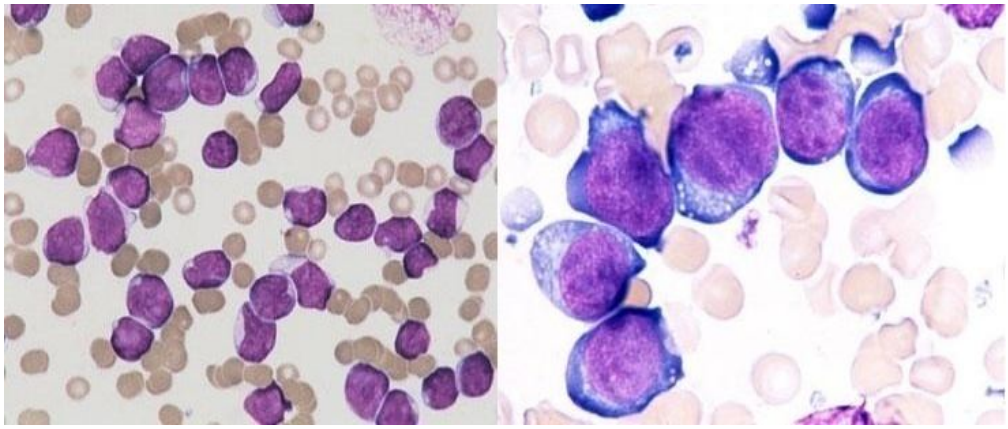


Figure 1.1: Pediatric acute lymphoblastic leukemia (Vikramjit, 2014)

1.2.6.2.1.3. Acute myelogenous leukemia (AML):

Acute myeloid leukemia (AML) accounts for approximately 15–20% of all childhood leukemias, and despite dramatic improvements in treatment outcome, only approximately 70% of children with AML cured. AML results from collaborating genetic aberrations in at least two different classes; type-I aberrations, inducing uncontrolled cell proliferation and or survival, and type-II aberrations inhibiting cell differentiation (Valerio DG, *et al*., 2014).

All subtypes of AML probably share abnormalities in common pathways that regulate proliferation, differentiation, and cell death. These include mutations that impart proliferative and survival signals, and mutations that lead to differentiation arrest or enhanced self-renewal (Pui, *et al*, 2011).

Infants <1 year of age have a high prevalence of prognostically unfavorable leukemias and a presumed susceptibility to treatment-related toxicities.

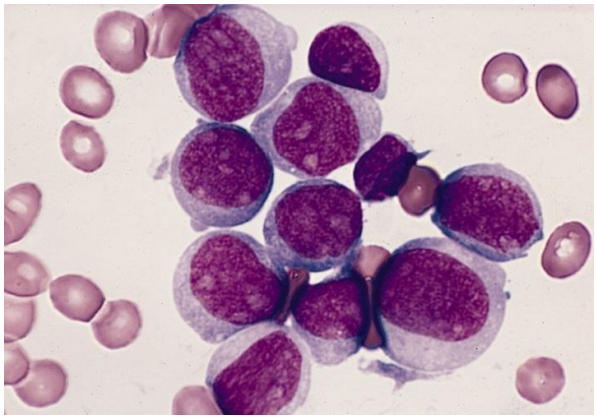


Figure 1.2: Acute myeloid leukemia (AML) (Steve. 2014)

1.2.6.2.1.4. Hybrid or mixed lineage leukemia:

Biphenotypic acute leukemia (BAL) is an uncommon clinical entity. It is a type of acute leukemia with features characteristic of both the myeloid and lymphoid lineages and for this reason designated as mixed-lineage, hybrid or biphenotypic acute leukemia, the precise incidence among acute leukemia is likely to account for approximately less than 5% of all acute leukemia. BAL collectively considered as "mixed phenotype acute leukemia" (MPAL) (Anupam S, *et al.*, 2013).

Mixed-phenotype acute leukemia (MPAL) encompasses a heterogeneous group of rare leukemias in which assigning a single lineage of origin is not possible. Varieties of different terms and classification systems used historically to describe this entity (Ofir and Richard, 2015).

Acute leukemia with a mixed phenotype is a rare disease and comprises 2–5% of all acute leukemias. These disorders have been known historically by a variety of names, such as mixed lineage leukemia, bilineal leukemia and biphenotypic leukemia, and the criteria for diagnosis have often been arbitrary (Weinberg and Arber, 2010).

1.2.6.2.2 Chronic leukemias:

Chronic leukemias are much more common in adults than in children. They tend to grow more slowly than acute leukemias, but they are also harder to cure. Chronic leukemias can be dividing in to 2 types.

1.2.6.2.2.1. Chronic myelogenous leukemia (CML):

The median age at diagnosis of chronic myelogenous leukemia, (CML) is 60 to 65 years in Western registries and CML is rare among children and adolescents. CML constitutes 2% of all leukemias in children younger than 15 years and 9% of all leukemias in adolescents between 15 and 19 years, with an annual incidence of 1 and 2.2 cases per million in these 2 age groups, respectively (Gugliotta ,*et al.*, 2014).

The same biology as in adults, but there is data indicate that some genetic differences exist in pediatric and adult CML (Nobuko, *et al.*, 2015).

1.2.6.2.2.2. Chronic lymphocytic leukemia (CLL):

CLL was, characterized by the gradual accumulation of malignant lymphocytes in the bone marrow, blood, lymph nodes, liver, and spleen. Thus, the major clinical features are the sequelae of bone marrow failure and the compressive syndromes that result from gross enlargement of lymphoid organs. In addition, CLL is associated with humoral and cell-mediated immunodeficiencies as well as a greatly enhanced risk of

autoimmune cytopenias-particularly autoimmune hemolytic anemia (AIHA) (Gary J.S, 2003).

Chronic lymphocytic leukemia and lymphoma (CLL) is an extremely rare disease during childhood (Demir, *et al*., 2014).

1.2.6.2.2.3 Juvenile myelomonocytic leukemia (JMML):

Juvenile myelomonocytic leukemia (JMML) is a lethal myeloproliferative disease (MPD) of young childhood characterized clinically by overproduction of myelomonocytic cells and by the in vitro phenotype of hematopoietic progenitor hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF). In contrast to normal subjects, the morphological composition of progenitor colonies from JMML patients is predominantly macrophages and monocytes. It is notable, however, that progenitor colonies from JMML patients contain monocytic cells along the full spectrum of differentiation, including blast forms, promonocytes, monocytes, and macrophages. This distinct characteristic indicates that JMML is not a disease induced by a complete block in differentiation as observed in acute leukemias, but instead results from shunting of hematopoietic differentiation toward the monocytic pathway, similar to increased granulocytic differentiation in the chronic phase of chronic myeloid leukemia (Chan, *et al*., 2009).

1.2.6.2.2.4 Congenital leukemia:

Congenital leukemia is a rare condition that is, diagnosed from birth to 6 weeks of life. It occurs at a rate of one per 5 million births and represents less than 1% of all childhood leukemia. Leukemia cutis is, typified by cutaneous infiltrates of leukemic cells and is an uncommon manifestation of leukemia. It has been, documented in 25～30% of patients with congenital leukemia. In childhood and adult leukemia, leukemia cutis typically develops late during the disease course and is strongly associated with the presence of extramedullary disease at other sites (Choi, *et al*., 2009).

1.2.6.3. Molecular genetics change in acute leukemia:

Improved techniques in identifying the chromosome changes and the affected genes that are involved in acute leukemias have led to improved treatments for these diseases. Identification of consistent chromosomal changes has allowed us to target the location of particular genes and has enabled us to focus on treatments more specifically to certain subtypes of leukemia. Translocations, in particular, are common cytogenetic abnormalities in human leukemia, and the prevalence of certain types of

translocations varies with age. Cancers, lymphomas and leukemias, known to be genetic diseases and it recognized that genotype-specific therapies should be used that take into account the genetic alterations of the particular leukemia (Rowley, 2000).

Infants with ALL represent a particularly high-risk subclass, with higher risks of both treatment failure and treatment complications. The highest rates of treatment failure are seen in infants diagnosed before 6 months of age, those with high initial WBC count, or those with MLL gene rearrangements (which occur in 70%–80% of infants diagnosed with ALL) (Cooper and Brown. 2015).

Rearrangement of the mixed lineage leukaemia (MLL) gene, which occurs in the 11q23 translocation, leads to aggressive acute leukaemia and may be present in both AML and ALL (Bas,*et al.*, 2011).

It occurs in 34% of this subgroup,on the other hand, spontaneous remission can occur in a subset of neonates affected by AML M4 with t(8;16)(p11;p13). Nevertheless, recurrence can occur in almost half of the cases in the first year of life (Coenen, *et al.*, 2013).

1.2.6.4. Clinical presentation:

Children with ALL often present with signs and symptoms that reflect bone marrow infiltration with leukemic blasts and the extent of extramedullary disease spread. The duration of symptoms may vary from days to months, frequently accumulating in a matter of days or weeks, and culminating in some event that brings the child to medical attention. Most of children have 3 to 4 weeks history of presenting symptoms. The initial presentation includes manifestations of the underlying anemia – pallor, fatigue, exercise intolerance, tachycardia, dyspnea, and sometimes congestive heart failure; thrombocytopenia , petechiae, purpura, easy bruising, bleeding from mucous membranes; neutropenia , fever whether low or high-grade, infection, ulcerations of buccal mucosa. Anorexia is common, but significant weight loss is infrequent. Bone pain is present in one-third of patients, particularly affects long bones, and may lead to a limp or refusal to walk in young children. Bone pain reflects leukemic involvement of the periosteum, bone infarction, or expansion of marrow cavity by lymphoblasts. Joint pain and joint swelling are rarely seen (Winick, *et al.*, 2010).

Physical examination may show enlarged lymph nodes, liver and spleen. It is a common misperception that a significant lymphadenopathy and hepatosplenomegaly

are hallmarks of childhood ALL. In rare cases, predominantly in patients with T-cell ALL, respiratory distress or signs of superior vena cava syndrome due to enlargement of mediastinal lymph nodes may be presenting symptoms. CNS involvement occurs in less than 5% of children with ALL at initial diagnosis. It usually presents with signs and symptoms of raised intracranial pressure (headache, vomiting, and papilledema) and parenchimal involvement (seizures, cranial nerve palsies). Other rare sites of extramedullary invasion include heart, lungs, kidneys, testicles, ovaries, skin, eye or gastrointestinal tract (Margolin, *et al*., 2011).

Increased suspicion is also, warranted for conditions associated with a higher risk of childhood cancer, including immunodeficiency syndromes and previous malignancies, as well as with certain genetic conditions and familial cancer syndromes such as Down syndrome (Ioanna, *et al*., 2013).

Such involvement usually occurs in refractory or relapsed patients (Pui. 2012).

1.2.6.5. Effects of malignancy on platelets:

The general notion that functional platelets are important for successful hematogenous tumor metastasis has been, inaugurated more than 4 decades ago and has corroborated in numerous experimental settings. Thorough preclinical investigations have at least in part clarified some specifics regarding the involvement of platelet adhesion receptors, such as thrombin receptors or integrins, in the metastasis cascade. Pivotal preclinical experiments have demonstrated that hematogenous tumor spread dramatically diminished when platelets depleted from the circulation or when functions of platelet surface receptors were, inhibited pharmacologically or genetically. Such insight has inspired researchers to devise novel antitumoral therapies based on targeting platelet receptors. In addition, there are results suggesting that targeted inhibition of certain platelet surface receptors may even result in enhanced experimental tumor metastasis have demonstrated vividly that the role of platelets in tumor metastasis is more complex than has been anticipated previously (Luise and Schön. 2010).

Platelet contents, may be released into the peritumoral space following platelet activation and enhance tumor cell extravasation and metastases. An important step in metastatic dissemination is the breakdown of vessel basement membrane. By releasing proteolytic enzymes such as gelatinase, heparanase and various matrix metalloproteinases (MMPs), activated platelets can directly degrade structural

components, or alternatively, support this process by activating other proteinases and/or enabling tumor cells and endothelial cells to do the same. Moreover, modulation of proteolytic activity is accomplished by growth factors released by platelets.Tumor cells have the ability to aggregate platelets a finding first reported in 1968, and referred to as tumor cell-induced platelet aggregation (TCIPA), this aggregation correlates with the metastatic potential of cancer cells in both in vitro and in vivo models of experimental metastasis. The mechanisms by which tumor cells induce platelet aggregation may differ by cancer type, but have in common the theme of conferring survival advantage. In turn, platelets can protect tumor cells in at least two ways by coating them and thereby directly shielding them from physical stressors within the vasculature and by permitting evasion from the immune systems effecter cells. For example, platelets have been, shown to protect tumors from NK cells and TNF-cytotoxicity (Bambace and Holmes, 2011).

1.2.6.6. Hereditary and acquired disorders of platelet function:

The diagnostic evaluation of platelet disorders is complex, poorly standardised and time consuming. This coupled with the wide spectrum of a known range of disorders some of which are very rare, presents a significant challenge to even the best diagnostic laboratory (Paul, *et al.*, 2011).

Abnormalities of platelet function diagnosed by a platelet count and direct smear adhesion. Platelet function testing is important for the diagnostic evaluation of common and rare bleeding disorders (Hayward, *et al.*, 2010).

The bleeding time (BT) test has also been widely utilized as a means of accessing primary hemostatic response (platelet-injured vessel wall interaction (Mielke.1984).

Platelet aggregation is an important component of laboratory testing in a patient with clinical findings suggestive of a primary hemostatic abnormality (Achim, *et al.*, 2010).

Patients with afibrinogenemia lack both primary and secondary responses to various platelet agonists (Elaine M, *et al.*, 2012).

1.2.6.7. Fibrinogen:

Fibrinogen is a plasma protein critical to hemostasis and clot formation. The blood plasma concentration of fibrinogen ranges between 1.5 and 4.0 g/L but it can be higher, particularly in certain conditions such as pregnancy. Structurally, human fibrinogen comprises of two outer D domains, which are both-linked by a central E

domain. Each D domain is made up of three polypeptide chains (α, β and γ), which together form a coiled-coil configuration. These domains linked at the N-terminus to the central E domain via a series of disulfide bonds. Thrombin cleavage occurs at specific amino-acid sequences present on α and β polypeptide chains, removing the N-terminal peptides (fibrinopeptides) and exposing the polymerization sites. Fibrin polymerization then occurs via noncovalent interaction of the exposed polypeptide chain with complementary binding sites present on the D domain of a neighboring molecule (Kollman, *et al*., 2009).

There are data have suggested that fibrinogen may be heme associated and could play a role in carbon monoxide sensing (Nielsen, *et al*., 2011).

Some studies have demonstrated the importance of thrombin generation and haemostatic activation for clot formation (Bolliger, *et al*., 2010).

Functionally, fibrinogen molecules act during both cellular and fluid phases of coagulation, in the cellular phase; it facilitates the aggregation of platelets via binding of glycoprotein IIb/IIIa receptors on platelet surfaces. In the fluid phase, it cleaved by thrombin to produce fibrin monomers, which polymerize to form the basis of the clot. Fibrinogen also plays other important roles, functioning in vivo as an acute phase reactant, helping modulate inflammatory cellular reactions and also increasing in plasma concentration after injury, when acute hemorrhage occurs, the resulting blood loss and consumption of procoagulants combine to reduce the circulating concentration of multiple clotting factors. Derangement in common measures of coagulation (prothrombin time and activated partial thromboplastin time) can develop in cases of acute trauma, before administration of fluid therapy (Jerrold, *et al.*, 2012).

Macromolecular plasma proteins such as fibrinogen cause red blood cells to form large aggregates, called rouleaux, which are usually, assumed to be disaggregated in the circulation due to the shear forces present in bulk flow. This leads to the assumption that rouleaux formation is only relevant in the venule network and in arterioles at low shear rates or stasis (Brust, *et al*., 2014).

Thrombocytopenia and low fibrinogen levels increase the risk of bleeding and haemorrhagic complications of ALL therapy and, in particular, of mini-invasive procedures (lumbar puncture, bone marrow aspirate and biopsy) (Paola, *et al.*, 2014).

1.2.7. The extrinsic coagulation pathway:

Extrinsic coagulation pathway considered as the first step in plasma mediated haemostasis.It activated by TF, which is expressed in the subendothelial tissue.Under normal physiological conditions, normal vascular endothelium minimises contact between TF and plasma procoagulants, but vascular insult expose TF that binds with factor VIIa and calcium to promote the conversion of factor X to Xa (Owens and Mackman 2010).

Complex, interrelated systems exist to maintain the fluidity of the blood in the vascular system while allowing for the rapid formation of a solid blood clot to prevent hemorrhaging subsequent to blood vessel injury.These interrelated systems are collectively, referred to as haemostasis. The components involved in the haemostatic mechanism consist of vessel walls, platelets, coagulation factors, inhibitors, and the fibrinolytic system. In the broadest sense, a series of cascades involving coagulation proteins and enzymes, as well as cell surfaces (platelets and endothelial cells), work together to generate thrombin, the key enzyme in coagulation, subsequently leading to the formation of a fibrin clot. However, there also exist direct and indirect inhibitors of thrombin to ensure that clot formation does not go ucontrolled once the fibrin clot formed; the fibrinolytic system ensures that the clot lysed so that it does not become a pathological complication. Taken together, the systems exist to balance each other and maintain order. The balance of coagulation and fibrinolysis keeps the haemostatic system functioning efficiently (Anthony, *et al*., 2013).

The extrinsic pathway initiated by the entry of tissue thromboplastin into the circulating blood. Tissue thromboplastin derived from phospholipoproteins and organelle membranes from disrupted tissue cells. These membrane lipoproteins, termed tissue factors, are normally extrinsic to the circulation. Platelet phospholipids are not necessary for activation of the extrinsic pathway because tissue factor supplies its own phospholipids.Factor VII binds to these phospholipids in the tissue cell membranes and activated to factor VIIa, a potent enzyme capable of activating factor X to Xa in the presence of ionized calcium. The activity of the tissue factor–factor VII complex seems to be largely dependent on the concentration of tissue thromboplastin. The proteolytic cleavage of factor VIIa by factor Xa results in inactivation of factor VIIa. Factor VII participates only in the extrinsic pathway. Membranes that enter the circulation also provide a surface for the attachment and activation of factors II and V.

The final step is the conversion of fibrinogen to fibrin by thrombin (Mary and Turgeon 2012).

1.2.7.1 Inhibitors of coagulation:

A number of mechanisms exist to ensure that the production of the fibrin clot is limited to the site of injury and not allowed to propagate indefinitely. First there are a number of proteins that bind to and inactivate the enzymes of the coagulation cascade. Probably the first of these to become active is TFPI, which rapidly quenches the factor VIIa–TF complex that initiates coagulation. It does this by combining first with factor Xa, so that further propagation of coagulation is dependent on the small amount of thrombin that has been, generated during initiation being sufficient to activate the intrinsic pathway.The principal physiological inactivator of thrombin is antithrombin (AT, formerly ATIII), which belongs to the serpin group of proteins. This binds to factor IIa forming an inactive thrombin–antithrombin complex (TAT), which subsequently cleared from the circulation by the liver. This process greatly enhanced by the presence of heparin or vessel wall heparan. AT is responsible for approximately 60% of thrombin-inactivating capacity in the plasma; the remainder provided by heparin cofactor II and less specific inhibitors such as a2 macroglobulin. AT is also capable of inactivating factors X, IX, XI and XII but to lesser degrees than thrombin. As thrombin, spreads away from the area of damage it also bound by thrombomodulin on the surface of endothelial cells. In these way it is changed from a primarily procoagulant protein to an anticoagulant one. Although remaining available for binding to AT, thrombin bound to thrombomodulin no longer cleaves fibrinogen. It has a greatly enhanced preference for PC as a substrate. PC is presented to the thrombin–thrombomodulin complex by the endothelial protein C receptor (EPCR) and when activated by thrombin cleavage acts to limit and arrest coagulation by inactivating factors Va and VIIIa. This action further enhanced by its cofactor, protein S, which does not require prior activation. The role of EPCR is particularly important in larger vessels, where the effective concentration of thrombomodulin is low. PC subsequently inactivated by its own specific inhibitor (Dacie and Lewis. 2011).

It is important that the effect of thrombin is limited to the site of injury. The first inhibitor acts is tissue factor pathway inhibitor (TFPI), which is synthesized in endothelial cells and it is present in plasma and platelets and accumulates at the site of injury caused by local platelet activation. This inhibits Xa and VIla and tissue factor

to limit the main ill vivo pathway by forming the quaternary complex. There is direct inactivation of thrombin and other serine protease factors by other circulating inhibitors of which antithrombin is the most potent. It inactivates serine proteases by combining with them by peptide bonding to form high molecular weight stable complexes (Hoffbrand, *et al.*, 2011).

1.2.7.1.1. Protein C:

Protein C is a vitamin K-dependent anticoagulant serine protease zymogen in plasma, which upon activation by the thrombin-thrombomodulin complex down-regulates the coagulation cascade by degrading cofactors Va and VIIIa by limited proteolysis (Alireza, 2010).

During biosynthesis, the primary translation product proteolytically processed releasing the dipeptide Lys156Arg157. In order to exert its anticoagulant effect, PC first must be activated to APC. This is chieved by the action of thrombin, which cleaves the heavy chain to release a 12 - residue (Gly158 – Arg169) activation peptide, revealing the active site by the usual chymotrypsin - like mechanism. Thrombin activation of PC is slow in free solution but markedly accelerated by specific endothelial cell receptors for both thrombin (TM) and PC (EPCR), which coordinate the assembly of a membrane complex for PC activation. APC interacts with PS bound to the phospholipid surface of activated platelets, enhancing the anticoagulant activity of APC against FVa and FVIIIa. These procoagulant cofactors are inactivated by APC on the platelet surface by specific cleavage in their A domains, terminating the activity of the tenase and prothrombinase complex by disrupting their binding sites for FIXa and FXa respectively. In the absence of PS, this reaction is inefficient. The most common form of familial thrombosis is FV Leiden, which caused by mutation of Arg506 to Gln, which makes the molecule resistant to cleavage by APC. It appears that APC inactivation of FVa is a prerequisite for efficient inactivation of FVIIIa invivo, as FVIIIa inactivation is also impaired in these patients, although there is no abnormality in the FVIII molecule. Since the discovery of FV Leiden, other less common FV mutants have been, found with a similar phenotype. None of the mutations reported in the FVIII molecule have been, shown to cause a similar prothrombotic phenotype. PC synthesized in the liver, and being vitamin K dependent is often low in the newborn. Even though its substrates (FV and FVIII) are normal at birth, this deficiency compensated for by the reduction in plasma

of the vitamin K - dependent procoagulant factors (prothrombin, FVII, FIX and FX). Because warfarin treatment initially decreases PC levels faster than FVII, FIX, FX and prothrombin levels, it can paradoxically increase the procoagulant tendency when anticoagulant treatment is first begun (many patients starting on warfarin are given heparin in parallel to combat this). Disruption of the murine PC gene results in lethal perinatal consumptive coagulopathy, whereas in human homozygous PC deficiency is associated with lethal purpura fulminans (in the absence of PC replacement therapy) and heterozygous individuals have a high risk of venous thrombosis. There is increasing appreciation of the role of the PC pathway in regulation of inflammation and the concept of signaling mechanisms allowing 'cross - talk' between the haemostatic and inflammatory networks. The survival benefits that APC concentrate provides in this situation have subsequently been seen in patients with sepsis and multiorgan failure.Variant recombinant PC molecules have been produced that appear to have either anticoagulant or anti - inflammatory effects independent of each other (Hoff brand, *et al.*, 2011).

Some studies showed phosphatidylcholine as the major phospholipid bound to human EPCR, which could be, exchanged with lysophosphatidylcholine and platelet activating factor, facilitated by the enzymatic action of secretory group V phospholipase A2.The placement of lysophosphatidylcholine and platelet-activating factor in the hydrophobic groove impaired the ability of EPCR to bind protein C (López-Sagaseta J, *et al.*, 2012).

1.2.7.1.2. Protein S:

Protein S is a vitamin K-dependent protein, which is a co-factor to protein C. Protein S in plasma linked to a considerable extent to the C4b binding protein. This makes it easy to differentiate between free and total Laboratory investigations 21 protein S. The free protein S fraction is physiologically active. Hereditary protein S deficiency generates an increased risk of thrombosis. Decreased levels observed in vitamin K deficiency, liver damage, AVK treatment, DIC and in pregnancy and hormonal treatment (replacement and/or contraceptives). Standardized methods are available only for detecting free and total protein S antigen. An analysis of free protein S is sufficient to determine whether an individual is sick. Functional methods are being developed (Margareta and Jovan. 2010).

Hereditary protein S deficiency is a haploinsufficiency disorder associated with an increased risk of venous thrombosis (Castoldi, *et al*., 2010).

The genetic basis of protein S deficiency is heterogeneous. More than 200 mutations in *PROS1* have been described, the vast majority missense or nonsense mutations (Stenson, *et al*., 2009).

Protein S homozygous deficiency is a state associated with severe life-threatening neonatal purpura fulminans or massive venous thrombosis. While in the heterozygous deficiency of Protein S also increases the risk for developing thrombosis (Marlar and Gausman. 2011).

1.2.7.1.3. D-dimer and cancers:

D-dimer, a high molecular weight fibrinogen derivative derived from the cleavage of cross-linked fibrin, reflects both thrombin production and activation of fibrinolysis. Among healthy individuals, there is significant between-person variability of D-dimer concentration within the normal range (Gorog DA. 2010).

Elevated D-dimer levels occur in various disorders in which the coagulation system activated such as acute venous thromboembolism, ischemic cardiovascular disease and cancer (Augusto, *et al*. 2013).

D-dimer is a biomarker that globally indicates the activation of hemostasis and fibrinolysis. It is a degradation product of fibrin, which produced when cross-linked fibrin is degraded by plasmin-induced fibrinolytic activity. As D-dimer, plasma levels are elevated after clot formation, the measurement of D-dimer routinely used in conjunction with clinical parameters in the initial assessment of suspected acute VTE (Cihan, *et al.,* 2012).

D-dimer levels also been detected in patients with disseminated intravascular coagulation, vaso-occlusive crisis in sickle cell disease, thromboembolic events, and myocardial infarction. Accummulating evidence suggests that critical oncogenic events may also trigger activation of the coagulation cascade, leading to a prothrombotic environment that not only manifest as venous thromboembolic disease but also promotes the growth and progression of the malignancy.Various solid tumor patients, including lung, prostate, cervical, and colorectal cancer patients found with elevated D-dimer level in the plasma. In patients with colorectal cancer, D-dimer level has been shown to correlate with depth of tumor invasion at the time of surgical excision. Plasma D-dimer level has also been shown to directly correlate with other

tumor markers, including CA-125 and carcinoembryonic antigen. In one study D-dimer was not associated with cancer-associated thrombosis, although other studies found solid tumor frequently followed thrombotic events (Ketut and Bakta.2011).

D-dimer is a global indicator of coagulation activation and fibrinolysis and, therefore, an indirect marker of thrombotic activity. The utility of D-dimer measurement has been evaluated in several clinical situations including the exclusion of venous thromboembolism (VTE), prediction of future risk of VTE, and the diagnosis and monitoring of disseminated intravascular coagulation (DIC). Assay standardization remains problematic and clinicians need to be aware of variability in D-dimer assay performance and the characteristics of their institution's test when making clinical decisions. This article will review the available evidence for the utilization of D-dimer antigen measurement in the management of thrombotic and bleeding disorders (Bates. 2012).

1.2.7.1.4. Antithrombin III:

Antithrombin III (AT III) is a single chain glycoprotein consisting of 432 amino acids, synthesized in the liver, and of molecular weight 5800 Daltons. The normal plasma AT III concentration is 100-150 μg/ml and its biological half-life is 68 hours in normal subjects. Persons predisposed to thrombosis found to have elevated concentration of thrombin-anti thrombin III (TAT) complex and decreased plasma AT III activity (Ehab, *et al.,* 2013).

Antithrombin III (ATIII) is a potent inhibitor of the coagulation cascade. A nonvitamin K-dependent protease inhibitor inhibits coagulation by lysing thrombin and factor Xa. Antithrombin III activity markedly potentiated by heparin; potentiation of its activity is the principle mechanism by which both heparin and low molecular weight heparin result in anticoagulation (Gaman and Gaman. 2014).

Antithrombin is a natural anticoagulant that circulates in the plasma at a concentration of 112–140 mg/L with a half- life of two to three days. It is a serine protease inhibitor (serpin), which inhibits not only thrombin and factor Xa, but also factors IXa, XIa, XIIa, kallikrein, and plasmin. Like other serpins, antithrombin acts as a suicide substrate inhibitor, covalently binding to and inactivating thrombin. Antithrombin's activity is greatly, accelerated by interaction with the heparan sulfate family of glycosaminoglycans, including heparin. In vivo, heparan sulfate found on the endothelial cell surface, thus localizing antithrombin activity. The interaction of

antithrombin with heparan sulfate on the endothelial cell surface also appears to result in release of prostacyclin, a platelet inhibitor.Significant advances have been maded in understanding the molecular mechanism of antithrombin activity. The gene that encodes antithrombin, SERPINC1, comprises seven exons spanning 13.5 kb on chromosome 1. The 1392 bp mRNA encodes a 432 amino acid, 58 kDa glycoprotein that contains 3 β-sheets and 9 α-helices with an active site region and a heparin binding site (HBS). Heparin binds to the D-helix of antithrombin, exposing antithrombin reactive center and accelerating its inhibitory activity ~1,000-fold. While inhibition of thrombin requires the formation of a trimolecular complex between antithrombin, thrombin, and a heparin longer than 18 saccharides (including a specific pentasaccharide sequence), and inhibition of factor Xa by antithrombin can be accelerated by just the pentasaccharide of heparin (Bernard, *et al.*, 2010).

Measurements of antithrombin III (AT III), the primary inhibitor of serine protease of coagulation proteins in the blood have obtained and analyzed in various disease states. One large important group of patients in whom coagulation, alterations have been postulated is patients with solid neoplasms (Rubin, *et al.*, 1980).

1.2.7.1.5. Tissue factor pathway inhibitor-2 (TFPI-2):

Human tissue factor pathway inhibitor-2 (TFPI-2), is an extracellular matrix-associated Kunitz-type serine proteinase inhibitor that inhibits the plasmin- and trypsin-mediated activation of matrix metalloproteinases and inhibits tumor progression, invasion and metastasis. Previous studies have shown that TFPI-2 was down regulated in the progression of various tumors (Yong, *et al*, 2011).

Tissue factor- pathway inhibitor (TFPI) is an important regulator of the extrinsic pathway of blood clotting through its ability to inhibit factor Xa and factor Via-tissue factor activity. The molecular cloning and expression of a full-length cDNA that encodes a molecule described, designated TFPI-2, which has a similar overall domain organization and considerable primary amino acid sequence homology to TFPI. After a 22-residue signal peptide, the mature protein contains 213 amino acids with 18 cysteines and two canonical N-linked glycosylation sites. The deduced sequence of mature TFPI-2 revealed a short acidic amino-terminal region, three tandem Kunitz-type domains, and a carboxyl-terminal tail highly enriched in basic amino acids. Northern analysis indicates that TFPI-2 transcribed in umbilical vein endothelial cells, liver, and placenta. TFPI-2 was expressed in hamster baby kidney cells and purified

from the serum-free conditioned medium by a combination of heparin-agarose chromatography, Mono Q FPLC, Mono S FPLC, and Superose 12 FPLC. Purified TFPI-2 migrated as a single band in SDS/PAGE and exhibited a molecular mass of 32 kDa in the presence and absence of reducing agent. The amino-terminal sequence of recombinant TFPI-2 was identical to that predicted from the cDNA. Despite its structural similarity to TFPI, the purified recombinant TFPI-2 failed to react with polyclonal anti-TFPI IgG. Preliminary studies indicated that purified recombinant TFPI-2 strongly inhibited the amidolytic activities of trypsin and the factor Vil-tissue factor complex. In addition, the inhibition of factor Vila-tissue factor amidolytic activity by recombinant TFPI-2 was markedly, enhanced in the presence of heparin. TFPI-2 at high concentrations weakly inhibited the amidolytic activity of human factor Xa, but had no measurable effect on the amidolytic activity of human thrombin (Cindy, *et al.,* 1994).

Activation of the extrinsic pathway of blood coagulation plays the key role in the process of blood coagulation. The haemostasis influenced by the activity of procoagulant factors and its inhibitors. TFPI and TFPI-2 have structural similarity; they exhibit many differences in synthesis, distribution and mechanism of action. TFPI inhibits the activity of factor Xa and the complex of tissue factor and factor VIIa (TF/VIIa). Conversely, TFPI-2 does not inhibit factor Xa, but it is a strong inhibitor of factor XIa, plasma kallikrein, plasmin and trypsin (Sierko. *et al*, 2002).

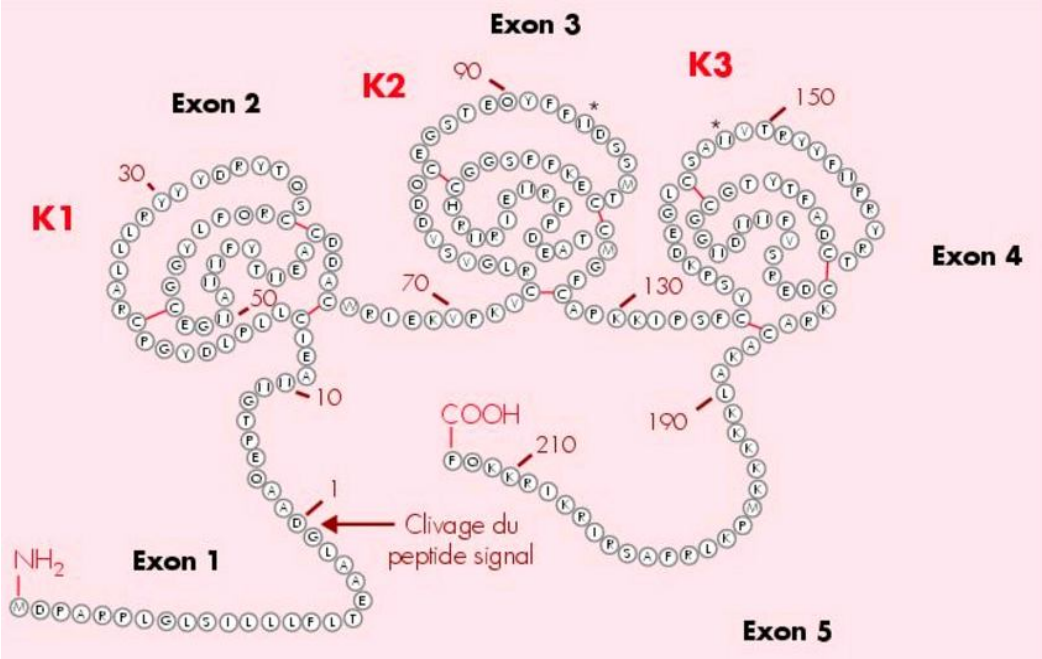
Human TFPI-2, previously designated as placental protein 5, inhibits a broad spectrum of serine proteinases almost exclusively through its first Kunitz-type domain, and is thought to play an important role in the regulation of extracellular matrix digestion and remodeling (Chand, *et al.,* 2005).

1.2.7.1.5.1. Synthesization and localization of TFPI-2:

TFPI-2 synthesized in endothelial cells (ECs) of different blood vessels (venous, arterials, and capillaries) but predominately by ECs of small vessels and abundantly secreted (60 to 90 %) in to the extracellular matrix (ECM). Furth more, expression of the gene encoding TFPI2 was detected in human ciliary epithelium, mature placenta, heart, liver, kidney, and skeletal musceles.TFPI-2 mRNA was also demonstrated in cyncytiotrophoblasts, whereas the product of gene found both in cyncytiotrophoblasts and cytotrophoblast.TFPI-2 protein was also immunohistochemcally detected in seminal vessels, colon, breast, pancreas, larynx, kidney, endometrial, and brain tissue.

The plasma concentration of TFPI-2 men and non-pregnant women is very low (0.46 to 0.49 ng/ml) Moreover; TFPI-2 is present in seminal plasma, preovulatory follicular fluid, and in the mucous of the uterus (Sierko, *et al.*, 2010).

It is likely that during pregnancy PP5/TFPI-2 may be retained on the surface of placental villi via proteoglycans, and may play an important role to maintain intervillous blood flow and the patency of microvasculature in feto-maternal blood system mediated by the inhibition of serine proteinases involved in the blood coagulation (Udagawa, *et al*., 2002).



(Figure 1.3) Structure of protein TFPI-2, the three Kunitz domains, K1, K2 and K3 each contain three disulphide bridges (Pascale, *et al*., 2003).

1.2.7.1.5.2 The role and function of TFPI-2 in cancer:

Down regulation of TFPI-2 suggested being involved in tumor invasion and metastasis in some cancers.

TFPI-2 plays an important role in normal ECM remodeling, and becoming increasingly recognized as a tumor suppressor gene. In several types of malignancies, such as choriocarcinoma, glioma, prostate cancer, pancreatic carcinoma and lung cancer, TFPI-2 has significantly demonstrated tumor-suppressive functions during tumor cell invasion, metastasis, apoptosis, proliferation and angiogenesis. It was

reported that, TFPI-2 showed high frequency of CpG islands aberrantly methylated in both cervical cancer specimens and cell lines (Qiao, *et al.*, 2012).

Tissue factor pathway inhibitor-2 (TFPI-2) has recognized as a new tumour suppressor gene. Low expression of this protein in several types of cancers allows for enhanced tumour growth, invasion and metastasis (Chundi, *et al.*, 2012).

The expression of TFPI-2 in tumors was inversely, related to an increasing degree of malignancy, which may suggest a role for TFPI-2 in the maintenance of tumor stability and inhibition of the growth of neoplasms. TFPI-2 inhibits the tissue factor/factor VIIa (TF/VIIa) complex and a wide variety of serine proteinases including plasmin, plasma kallikrein, factor XIa, trypsin, and chymotrypsin. Aberrant methylation of TFPI-2 promoter cytosine-phosphorothioate-guanine (CpG) islands in human cancers and cancer cell lines was widely documented to be responsible for diminished expression of mRNA encoding TFPI-2 and decrease or inhibited synthesis of TFPI-2 protein during cancer progression. Furthermore, an aberrantly spliced variant of TFPI-2 mRNA (designated asTFPI-2), was detected, which represents an untranslated form of TFPI-2. The levels of asTFPI-2 were very low or undetectable in normal cells but markedly upregulated in neoplastic tissue. TFPI-2 functions in the maintenance of the stability of the tumor environment and inhibits invasiveness and growth of neoplasm's, as well as metastases formation. TFPI-2 has also shown to induce apoptosis and inhibit angiogenesis, which may contribute significantly to tumor growth inhibition. Restoration of TFPI-2 expression in tumor tissue inhibits invasion, tumor growth, and metastasis, which creates a novel possibility of cancer patient treatment. However, more information still needed to define the precise role of TFPI-2 in human tumor biology (Sierko. *et al.*, 2007).

TFPI-2 expression strongly augmented in smooth muscle cells exposed to shear stress and that TFPI-2 co-localizes with caspase-3 in vivo. In addition, TFPI-2 inhibits smooth muscle cell proliferation and induces apoptosis in vitro. The adaption of smooth muscle cells to shear stress is of interest in understanding the pathophysiology behind intimal hyperplasia and restenosis (Johan, *et al.*, 2010).

Antimicrobial peptides (AMP) provide a first line of defense against invading microbes, at epithelial surfaces as well as in blood. Many antimicrobial peptides (AMPs), such as defensins and cathelicidins, are multifunctional and may exert immunomodulatory functions, and are therefore often-denoted host defense peptides (HDP). HDPs have been, shown to encompass not only the “classical” antimicrobial

peptides, but also various bioactive peptides and proteins with antimicrobial activities, e.g, chemokines, ribonucleases. Calgranulins and complement C3a. Furthermore, several proteins of the coagulation system, such as thrombin, kininogen, protein C inhibitor , fibrinogen , and TFPI-1, may either as intact molecules or after proteolysis, act in host defense involving killing of bacteria and immunomodulation. Tissue factor pathway inhibitor 2 (TFPI-2) is a 32 kDa matrix-associated Kunitz-type serine protease inhibitor. The molecule contains a highly negatively charged N-terminus, three tandemly-linked Kunitz-type domains, and a highly positively charged C-terminus. TFPI-2, was synthesized and secreted by many cells, including skin fibroblasts, endothelial cells, smooth muscle cells, dermal fibroblasts, keratinocytes, monocytes, and macrophages. In vitro, TFPI-2 weakly inhibits coagulation induced by the tissue factor (TF)-VII complex, and inhibits a wide range of proteases like trypsin, chymotrypsin, plasmin, matrix metalloproteinases, factor XIa and plasma kallikrein. Notably, stimulation of human umbilical vein endothelial cells with inflammatory mediators such as phorbol myristate acetate, lipopolysaccharide (LPS), and TNF-α significantly increases TFPI-2 expression. Analogously, in a murine model, TFPI-2 expression is dramatically upregulated in the liver during LPS stimulation.Of note is that ADAMTS1 (Adisintegrin and metalo- proteinase with thrombospondin motifs 1), plasmin and thrombin have been, shown to cleave TFPI-2 at its C-terminus in vitro. The involvement of TFPI-2 in inflammation, as well as the presence of a highly cationic, protease release the present results demonstrate the presence of TFPI-2 in skin and wounds, as well as its up-regulation during wounding. The fact that neutrophil elastase generated a C-terminal TFPI-2 fragment, which interacted with bacteria in vitro, and that similar fragments found in vivo and in association with bacteria, indicated a plausible antimicrobial role of the C-terminal region of TFPI-2.able region, a feature typical for many host defense peptides (Papareddy, *et al.*, 2012).

1.2.7.1.5.3 TFPI-2 gene polymorphisms:

There is a study that investigated the link between TFPI-2 gene polymorphisms and the severity of coronary atherosclerosis, a subgroup analysis based on Gensini score (GSS) was performed. The results showed that the patients with rs59805398 CC genotype, rs34489123 AA genotype, Hap3 (GGA), Hap6 (AGG), Hap7 (GAA), or Hap8 (GAG) were at higher risk of developing coronary atherosclerosis even after sub

analysis based on (GSS). It was strengthened that TFPI-2 gene polymorphisms were associated with coronary atherosclerosis (Jia, *et al.*, 2015).

Although, previous research indicated that in early phase of acute coronary syndrome (ACS), elderly patients had low levels of TFPI-2 protein and high levels of tissue factor and matrix metalloproteinases MMP-1, implying that the lack of TFPI-2 may be related to ACS (Zhao, *et al.*,2012).

polymorphism TF -603 A/G was associated with the endometrial protein level in infertile women, being highest in women with GG genotype, and variation TFPI -287 T/C was associated with unexplained infertility (Altmae, *et al.*, 2011).

The transcription of TFPI-2 changed by single nucleotide polymorphisms and that the sequence variations in transcription factor binding sites of the TFPI-2 promoter may influence the regulation of this gene. The variation −18C>A, where a putative binding site of the transcription factor Sp-1 is located, had the strongest effect on transcriptional activity (Sabine, *et al.*, 2006).

1.2.7.1.5.4 TFPI- 2 and leukaemia:

Human tissue factor pathway inhibitor-2 (TFPI-2), has been implicated as a metastasis-associated gene in many types of tumors. There is a study wich investigated whether TFPI-2 was inactivated epigenetically in Chinese pediatric acute myeloid leukemia (AML).Methylation status was investigated by methylation-specific polymerase chain reaction, and bisulfate genomic sequencing. TFPI-2 aberrantly methylated in 50% (3/6) of AML cell lines. Aberrant methylation of TFPI-2 promoter detected in 71.6% (48/67) of the Chinese pediatric AML patients. TFPI-2 transcript was significantly lower in AML group compared with controls (3.44 vs. 32.8, $P<0.001$). Patients with methylated TFPI-2 gene had significantly lower TFPI-2 transcript, than those, patients without methylated TFPI-2 ($P=0.04$). Promoter hypermethylation of TFPI-2 is frequent and specific event in pediatric AML (Jian, *et al.*, 2012).

It is widely recognized that a strong correlation exists between cancer and aberrant hemostasis. Patients with various types of cancers, including pancreatic, colorectal, and gastric cancer, often develop thrombosis, a phenomenon commonly referred to as Trousseau syndrome. Reciprocally, components from the coagulation cascade also influence cancer progression. The primary initiator of coagulation, the transmembrane receptor tissue factor (TF), has gained considerable attention as a determinant of

tumor progression. On complex formation with its ligand, coagulation factor VIIa, TF influences protease-activated receptor- dependent tumor cell behavior, and regulates integrin function, which facilitate tumor angiogenesis both in vitro and in mouse models. Furthermore, evidence exists that an alternatively spliced isoform of TF also affects tumor growth and tumor angiogenesis. In patient material, TF expression and TF cytoplasmic domain phosphorylation correlate with disease outcome in many, but not in all, cancer subtypes, suggesting that TF dependent signal transduction events are a potential target for therapeutic intervention in selected types of cancer (Yascha, *et al.,* 2012).

1.2.7.1.5.5. TFPI-2 Gene:

This gene encodes a member of the Kunitz-type serine proteinase-inhibitor family. The protein can inhibit a variety of serine proteases including factor VIIa/tissue factor, factor Xa, plasmin, trypsin, and chymotryspin and plasma kallikrein.This gene has been, identified as a tumor suppressor gene in several types of cancer. As alternative splicing results in multiple transcriptional variants (Refseq. 2012).

The human TFPI-2 gene is located on chromosome 7q22 and encodes a 32-kDa Kunitz-type serine protease inhibitor that negatively regulates the enzymatic activity of trypsin, plasmin, and VIIa-tissue factor complex TFPI-2 found abundantly expressed in various normal human tissues. Several studies have provided suggestive evidence that TFPI-2 is inactivated or absent during tumor progression (Shumin, *et al.,* 2010).

1.2.8. Treatment of leukaemia and coagulation system:

Optimal use of existing antileukemic agents and improved supportive care in contemporary clinical trials have improved the 5-year survival rate of childhood acute lymphoblastic leukemia (ALL) above 85% in developed countries, further advances in survival and quality of life required a better understanding of ALL pathobiology, the mechanisms of drug resistance(Ching-Hon *et al*.,2012).

Extraordinary advances in the treatment outcome of childhood acute lymphoblastic leukemia (ALL) rank as one of the most successful stories in the history of oncology, with the current rate of approximately 80% of children being cured (Paul , *et al.*, 2010).

Asparaginases are a cornerstone of treatment protocols for acute lymphoblastic leukemia (ALL) and are used for remission induction and intensification treatment in all pediatric regimens and in the majority of adult protocols (Rob, *et al.,* 2011).

Glucocorticoids and L-asparaginase (L-ASP) are essential elements of contemporary chemotherapy of childhood acute lymphoblastic leukemia (ALL). they affect the inhibitors of coagulation including antithrombin III (AT III), protein C and protein S. Sixteen patients treated according to the Berlin-Frankfurt-Münster (BFM) ALL protocol 2000. The result showed decreased plasma AT III and fibrinogen concentrations during induction therapy (after the fifth L-ASP dose), as compared to previous BFM protocols which used the Medac L-ASP. The results confirmed that following a mono-therapy with glucocorticoids the AT III, protein C and protein S levels increased while the fibrinogen level decreased.As the D-Dimers remained within the normal range during the 3 weeks of L-ASP combination chemotherapy and decrease of anticoagulant proteins (Attarbaschi, *et al.,* 2003).

L-asparaginase is a chemotherapeutic agent commonly used in the treatment of both adult and pediatric acute lymphoblastic leukemia (ALL). A major complication is thrombosis, resulting from reduced synthesis of proteins such as antithrombin III. Hypofibrinogenemia, also a side effect, may be a marker of thrombosis and decreased protein synthesis (Beinart and Damon 2004).

Improved treatment regimens for acute lymphoblastic leukaemia (ALL) continue to improve survival future; therapy must also take into consideration the many secondary problems. Most of these are the direct result of combination chemotherapy and L-asparaginase (ASP), is an example of a highly effective chemotherapeutic agent with serious side-effects such as thromboembolic events (Andrew, *et al.,* 1994).

Patients with hematologic malignancies often present with a hypercoagulable state or chronic disseminated intravascular coagulation (DIC) in the absence of active thrombosis and/or bleeding (Falanga and Rickles. 2007).

Acute promyelocytic leukemia (APL) once fatal has emerged as the curable subtype of acute myeloid leukemia in adults. Cure expected in approximately 70 to 90% of patients when treatment includes all-trans retinoic acid (ATRA) combined with anthracycline-based chemotherapy. Early mortality most often is due to a severe and often catastrophic bleeding, often intracerebral in location, and remains a major cause of treatment failure. Thrombosis either in diagnosis or during the course of treatment may be unrecognized and reflects the complexity of the coagulopathy. The dual

phenomenon of bleeding and thrombosis is attributable to at least three processes: disseminated intravascular coagulation; fibrinolysis (generated in part by expression of annexin-II on the APL cell surface); and direct proteolysis of several proteins including fibrinogen and von Willebrand factor (Tallman, *et al.,* 2007).

Since the initial description of the disease, the life-threatening coagulopathy associated with acute promyelocytic leukaemia (APL) has been the defining clinical characteristic. Historically, this uncommon subtype of acute myeloid leukaemia has been associated with a high mortality rate during induction therapy, most frequently attributable to haemorrhage. Since the introduction of all-trans retinoic acid (ATRA) into the therapy of all patients with APL, disease-free survival and overall survival have improved dramatically (Stein, *et al.,* 2009).

The patients with acute promyelocytic leukemia (APL) are at high risk for the development of life-threatening thrombotic and hemorrhagic complications, particularly during induction chemotherapy. This propensity has been, attributed to the release of tissue factor (TF)-like procoagulants from the leukemic cells leading to disseminated intravascular coagulation (DIC) (Martin, *et al.,* 1993).

Developing new therapeutic agents to destroy these resting cells is a major challenge for the improvement of cancer therapy in the future (Mauerer and Gruber. 2012).

1.2.9. Hypercoagulable state in leukaemia:

The concept of a state of hypercoagulability dates back to 1854, when German pathologist Rudolph Virchow postulated that thrombosis resulted from, and in turn precipitated, three interrelated factors decreased blood flow (venous stasis), inflammation of or near the blood vessels (vascular endothelial injury), and intrinsic alterations in the nature of the blood itself. These “blood changes” alluded to in Virchow's triad have become what are, known as, hypercoagulable states, or thrombophilias (Steven. 2010).

VTEs are common among patients with acute leukemia. They are more frequently associated with ALL than with AML, develop within a few months after leukemia diagnosis during periods of treatment-related thrombocytopenia, are usually central venous catheter associated, and often recur (Khanh, *et al.,* 2015).

Hypercoagulability is defined as, an increased tendency to thrombosis. It is may be acquired or inherited arterial or venous.Venous thromboembolism (VTE) is the commonest manifestation of a thrombophilic state and approximately 25% of

thrombophilia is detected in over 50% of cases following a first clinical episode of VTE. Inherited hypercoagulable states may be secondary to deficiency of natural clotting inhibitors, elevated procoagulants, or decreased fibrinolytic factors. Among these, activated protein C (APC) resistance, is the commonest underlying cause and seen in 20-50% of patients with inherited thrombophilia (Renu and Monica. 2009).

1.2.9.1. Incidence of thrombosis and leukemia:

The close relationship between cancer and thrombosis has been, known since the days of Armand Trousseau, who first described the clinical association between idiopathic venous thromboembolism (VTE), and occult malignancy in 1865. Cancer is associated with a hypercoagulable state and a four-fold increase in thrombosis risk, with chemotherapy elevating this risk even more (Anna. 2011).

Patients with malignant tumor often have systemic blood coagulation dysfunction, the relationship between cancer and coagulation was, characterized, by several mechanisms pointing that tumour biology and coagulation are closely linked processes (Yusheng, *et al.,* 2014).

Among the malignant hematologic disorders, the incidence of thrombosis is higher in patients with lymphoma or with acute leukemia. Significant morbidity and high mortality in acute leukemia due to complications of bleeding and infection frequently overshadow thromboembolic events. Case-controlled studies of patients with cancer revealed a fourfold increase in thromboembolic occurrence in acute leukemia, with about the same rate in acute myelogenous leukemia (AML) and in acute lymphocytic leukemia (ALL). Among patients with acute leukemia, thrombosis has the highest incidence in acute promyelocytic leukemia (APL) (Mohren, 2006).

Cancer favors the activation of blood coagulation with the appearance of a hypercoagulable state or chronic DIC in these patients. The results of laboratory tests demonstrate that a process of fibrin formation and fibrinolysis parallels the development of malignancy, increasingly in those with metastases. Particularly, subtle haemostatic alterations are detected, such as high levels of plasma by-products of clotting reactions (prothrombin fragment, fibrinopeptide A, thrombin–antithrombin complex (TAT) and D-dimer), or an acquired protein C resistance (Falanga and Vignoli. 2013).

1.2.9.2. Laboratory evaluation of coagulation pathways:

The modern hemostasis laboratory performs a large number of distinct tests, often using a variety of methodologies. All hemostasis laboratories perform routine coagulation tests comprising the prothrombin time (PT), international normalized ratio (INR) and the activated partial thromboplastin time (APTT), sometimes supplemented by specific fibrinogen assays, and occasionally thrombin time (TT) assays. Most routine test laboratories also perform D-dimer assays. These tests are variably performed to investigate hemostasis in patients suspected of having a potential dysfunction in the secondary hemostasis pathway, either congenital like hemophilia) or acquired like DIC. This is because PT/INR, APTT, and TT are sensitive to deficiencies or defects in various procoagulant factors. Thus, the PT/INR is sensitive to factors (F) I, II, VII, V, and X, and the APTT to F I, II, V, VIII, IX, X, XI, and XII (Emmanuel, *et al.*, 2012).

1.3. Rationale and hypothesis:

Several studies approved that there was strong relation between tissue factor pathway inhibitor-2 (TFPI-2) and many types of cancers, include leukemia, which is play an important role in maintenance of the stability of the tumor environment and inhibit invasiveness and growth of neoplasm's, as well as metastases formation. TFPI-2 has been, shown to induce apoptosis and inhibit angiogenesis, which may contribute significantly to tumor growth inhibition (Hisashi, *et. al.*, 2010).

The Study of TFPI-2 gene mutation and its correlation with coagulaton parameter (platelet count, PT, APTT, protien C, fibrinogen and D dimer) in Sudanese pediatric patients with leukemia not done previously in Sudan and there are no clear databases on it. This study will be a base line.

The study may be useful in the improvement of oncological care of pediatric patients with leukaemia through supporting the consideration that, TFPI-2 is a putative tumor suppressor gene and can use as prognostic marker, cancer screening, also tissue inhibits invasion tumor growth and metastasis, which creates a novel possibility of cancer patient treatment. However, more information still needed to define the precise role of TFPI-2 in human tumor and accurate knowledge of all significant complications in these patients regarding to (TFPI-2) gene mutation.

1.4 Objective:

1.4.1 General objective:

To detect, tissue factor pathway inhibitor-2 (TFPI-2) gene mutation in Sudanese pediatric patients with leukaemia.

1.4.2. Specific objectives:

1. To detect TFPI-2 gene mutation in Sudanese children with leukaemia using PCR-RFLP.
2. To measure haemostatic tests (Platelet count, PT, APTT, protein C and fibrinogen level) in patients and control groups.
3. To determine the relationship between (TFPI-2) gene mutation and coagulation tests (platelet count, PT, APTT, protein C and fibrinogen level) in Sudanese pediatric patients with leukaemia.
4. To estimate D-dimer level in patients and control group.

CHAPTER-TWO

Materials and Methods

CHAPTER-TWO

2. Materials and Methods:

2.1. Materials

2.1.1. Study area:

Radiation and isotopes centre - Khartoum (RICK)

2.1.2. Study design:

A prospective analytical case control study.

2.1.3. Study population:

Sudanese pediatric patients with leukaemia less than 15 years old

2.1.4. Sample size:

Ninety-eight Sudanese pediatric patients with leukaemia less than 15 years old selected which are all patient admitted to RICK at the period of collection depending on statistical office in the centre and supported by the last published data from national population-based cancer registry (NCR) which estimate cancer in children less than 15 years old constituted about, 486 (Intisar, *et al.,* 2014).

So all pediatric male and female patients during the period of collection (2014-2015) admitted in Khartoum radiation and isotopes centre (RIC) are includes in this study, nevertheless, the represents samples should be, selected according to the fallowing equation:

$n= N /1 +N (e)^{2}$

n= sample

N= population size

e= precision

2.1.5 Controls:

Ninety-seven individual pediatric less than 15 years, male and female appeared healthy from primary, kindergartens and nurseries schools matched in their ages and sex selected as control group.

2.1.6. Inclusion criteria:

- Sudanese pediatric patients with leukaemia less than 15 years old.

2.1.7. Exclusion Criteria:

- Other patients aged more than 15 years.

- Patients with known coagulation disorder history.

2.1.8. Questionnaire:

-Include general information

- History information

-Laboratory investigations

2.2 Method

2.2.1 Sample collection:

-5.0 ml of whole blood was collected from each patient and control group.

- 3.0ml of whole blood was collected in EDTA tube, for PCR

-1.8ml of whole blood was collected in tube containing 0.2ml of 3.2% sodium citrate; (1 part sodium citrate solution (0.11 mol/L) with 9 parts venous blood), for coagulation tests. Collected samples centrifuged immediately at 3000-x g for 15 minutes. The stability of the sample was 15 to 25 C° 4 hours.

2.2.2. Platelet count by using sysmex machine:

2.2.2.1. Principle:

The Sysmex XS-1000i counts and sizes of platelets (PLT) using electronic resistance detection enhanced by hydrodynamic focusing (Sysmex, 2012).

2.2.3 Hemostatic analysis:

2.23.1. Plasma sample preparation:

Citrated blood specimens, centrifuged at 3000 g for 15 minutes at room temperature, and then the plasma separated into two plain containers. Fresh samples analyzed for PT, PTT, and fibrinogen level. The plasma was frozen at (-70 C°) for one month then assayed for protein C.

A fully automated coagulation analyzer (sysmex CA 500) used for measurement of PT, PTT and fibrinogen level. (Appendix2).

2.2.3.2 Prothrombin time (PT) (Automated), using coagulometer:

2.2.3.2.1 Principle:

Prothrombin activated into thrombin when prothrombin activator acts on it. From here, thrombin then activates fibrinogen into fibrin. These steps are essential for blood clotting. Fibrin is the long protein strands that form a mesh network trapping blood cells and other blood components in order to form a blood clot.

The PT test measures the clotting time of re calcified plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system. Although originally thought to be measure prothrombin, the test is now known to depend also on reactions with factors V, VII and X and on the fibrinogen concentration of the plasma.

2.2.3.2.2 Reagents:

Thromborel® S Reagent Thromborel S Reagent was prepared from human placental tissue factor combined with calcium chloride and stabilizers. The reagent contains minimal residual clotting factors, such as prothrombin or factors VII or X, for clear definition of factor deficiencies and steep factor assay curves. Because of its high sensitivity to these coagulation factors, the reagent is suitable for monitoring oral anticoagulant therapy. Thromborel S Reagent exhibits good correlation with the WHO international reference thromboplastin preparation. With the Thromborel S Reagent and the appropriate deficient plasma, it is possible to determine activity of coagulation factors II, V, VII, and X. The reagent differentiates abnormal plasmas, even in the mildly pathological range.

2.2.3.2.3 Storage and stability:

Store Thromborel® S Reagent unopened at 6 C° and use by the date given on the label.

2.2.3.2.4 Stability after reconstitution:

8 hours at 37 °C (opened vial) 2 days at 15 to 25 °C (opened vial) 5 days at 2 to 8 C° (closed vial) Note on reagent expiration (Marion, 2012).

2.2.3.2.5 Expression of results:

The results expressed as the mean of the duplicated readings in seconds. The control plasma obtained from 20 normal men and women (not pregnant and not taking oral contraceptives) and the geometric mean normal PT (MNPT) is calculated. Because of the importance of the PT in oral anticoagulant control, the results can reported as an international normalized ratio (INR) (Jackson, *et al.*, 2003).

2.2.3.2.6 Internal quality control:

Normal range: control plasma N, Ci-Trol® Level 1 therapeutic range:

Control plasma P, control plasma U, Ci-Trol® level 2, Ci-Trol® and level 3

2.2.3.2.7 Normal values:

Normal values depend on the thromboplastin used, the exact technique and whether visual or instrumental endpoint reading is used. With most rabbit thromboplastin the normal range of the PT is between 11 and 16 seconds for recombinant human thromboplastin, it is somewhat shorter (10–12 s). Each laboratory should establish its own normal range (Dacie and Lewis, 2011).

2.2.3.3 Activated partial thromboplastin time (APTT), using coagulometer:

2.2.3.3.1 Principle:

The APTT is functional determination of the intrinsic pathway of coagulation factors XII, XI, IX, VIII, V, II, I, prekallikrein and high molecular weight kininogen. This pathway was, intimated by the interaction of factor XII with a negatively charged surface. A cascade mechanism results in fibrin production and clot formation (Dacie and Lewis. 2011).

2.2.3.3.2 Expression of results:

The results were expresd as the mean of the paired clotting times.

2.2.3.3.3 Normal range:

The normal range is typically 26–40 s. The actual times depend on the reagents used and the duration of the pre incubation period, which varies in manufacturer's recommendations for different reagents. These variables also greatly alter the sensitivity of the test to minor or moderate deficiencies of the contact activation system. Laboratories can choose appropriate conditions to achieve the sensitivity they require. Each laboratory should calculate its own normal range

2.2.3.4 Fibrinogen level (automated), using coagulometer:

2.2.3.4.1 Principle:

The fibrinogen-assay based on the Claus method. In the presence of a high concentration of thrombin, the time required for clot formation in diluted plasma is inversely proportional to fibrinogen concentration.

2.2.3.4.2 Reagents of the instrument:

- PT reagent: Dia-plastin, the liquid calcium and thromboplastin (rabbit brain) (Diamed Co Ltd).
- PTT reagent: Dia-celin: and the liquid cephalsoplastin (activated with complexed kaolin), (Diamed co ltd), with expiry date 7/2017.

- Fibrinogen assay kit (REF 5376) from Helena was used, with expiry date 7/2017

2.2.3.4.3 Coagulation detection principles:

The CA-500 employs the photo-optical clot detection methods.by using a red light (660 nm)to illuminate the sample plasma/reagent mixture, the CA-500 detects the change in scattered light intensity due to increased turbidity as fibrinogen changes to fibrin. The coagulation curve is drawn by taking the time and scattered light intensity as the X-Y axis respectively. The coagulation time is determined by a percentage detection methods.

Tests were measured using scatter-light end-point detection. The light source and wavelength at 660nm.

2.2.3.4.4 Instrument setting for the standard curve:

1. The standard curve data of the desired parameter was, displayed.
2. [Manual Entry] key on the standard curve data screen was, pressed and the manual entry screen displayed.
3. The standard curve data on the numeric keys pressed by [↑] and [↓] keys.
4. [Graph] key was pressed for the confirmation.

Normal PT, PTT, fibrinogen value and ISI value entered manually and standard curve data was, entered manually by the numeric keys.

2.2.3.4.5 Instrument setting for the reagent name:

[Manual entry] key and the reagent name-setting screen was, pressed and the reagent name entry screen appeared.The reagent name entered will be set on the specified reagent holder, then [Quit] key was, pressed after the reagent name entered.

2.2.3.5 CA-500 extra reagents:

-Sample cup conical (4 mL):
-Approx. 0.2 ml Dade
-Behring 4 ml vial
-Rinse solution
-Distilled water
-Printer paper

2.2.3.6 PT, PTT and fibrinogen method:

1. The power of (CA-500) switched on the left side of the instrument was, turned on and the system automatically performed in 10-second self checked, and the rooted menu screen appeared.

2- The rooted menu screen displayed “Ready”, when the detector and cooler reached an analysis-permitting temperature.

3- Prepared plasma inserted to the test tube securely to the bottom of the instrument rack.

4- The samples setted on the root menu screen specified by [↑] and [↓] keys to move the cursor to a sample to be setted pressed.

5- An analysis parameter keys (PT, APTT, Fibrinogen and their controls were pressed and signed.

6- Sample information screen when [←] key was, pressed on the first page of the analysis result screen, the sample information screen was displayed.

7- Start key was, pressed and all result printed (Marion, 2012).

2.2.3.7. Protein C assays:

2.2.3.7.1 Principle:

A Chromogenic assay - protein C is activated using (commonly) Protac™, an extract of the venom of Akistrodon contortrix and the concentration of Protein C is determined from the rate of color change in the test sample due to cleavage of a Chromogenic substrate.

2.2.3.7.2 Protein C measurement:

For protein C level in plasma, Biosystems BTS-330 used (Appendix 3).It is a Chromogenic assay method

2.2.3.7.3 Principle of protein C measurement:

Protein C in plasma was, activated by a specific fraction from the Agkistrodon contortrix snake venom. The amount of activated protein C (APC) is determined by monitoring the rate of hydrolysis of Protein C specific chromogenic substrate; the release of PNA measured at 405 nm and is proportional to the protein C level.

2.2.3.7.4 Protein C reagent:

(Helena Biosciences Ltd) with expiry date 2/2017

1-Protein C substrate lyophilized pyro - Glu-Pro-Arg-pNA-HCl.

2. Protein C activator: activator from snake venom

3. Protein C diluents:

4. Buffer with sodium preservative

2.2.3.7.5 Procedure of protein C measurement:

The frozen samples warmed until it reached the room temperature.

- Plasma standards and patient's plasma sample were prepared as follow:

For STD:

- 1ml from SARP (special assayed R plasma) +3ml normal saline
- For the Test :

  1ml patient plasma + 3ml normal saline
- 100 microliter from STD was pipetted in test tube, then incubated at 37 C° for 2minutes
- 100 microliter from patient's plasma was pipetted in test tube, then incubated at 37 C° for 2minutes
- 200 microliter activator was added to both STD and Test , then mixed and incubated at 37 C° for 5minutes
- 200 microliter protein c substrate was added to both STD and test, then mixed and incubated at 37 C° for 10 minutes
- 200 microliter GAA (glacial acetic acid) was added to both STD and test then mixed then 200-microliter water was, added to both STD and Test.
- Absorbance was, measured at 405 nm in 1cm semi-micro cuvette against blank prepared with deionised water.

2.2.3.7.6 Quality control of protein C measurement:

Protein C deficient plasma (REF 5346806) was lyophilized immunodepleted human plasma with a protein C content < 1 %, used in combination with (Protac®) in the modified aPTT-based protein C determination.

2.2.3.8. Determination of D-dimer:

The D-dimer is a rapid assay used for the qualitative and semi-quantitative measurement of cross-linked fibrin degradation products. During clot formation, these cross-linked products or FDPs formed from the conversion of fibrinogen to fibrin by thrombin. Once a clot formed, it triggers the production of plasmin. Then, plasmin

starts to degrade the cross-linked fibrin, forming fragments.D-dimer levels were, measured by a quantitative latex assay (STA-LIAtest D-DI; diagnostica - Stago, Asnieres, France).

2.2.3.8.1 Procedure: Semi-quantitative:

1. A dilution series maded when the undiluted sample was positive for an estimation of the level of D-dimer.

Tube	**No. 1**	**No. 2**	**No. 3**
Diluent	0.1 ml	0.1 ml	0.1 ml
Sample	0.1 ml		
Dilution	1:2	1:4	1:8

2. For the dilution protocol, 0.1 ml from No. 1ml was taken and transfered to no.2, then 0.1 ml from no. 2 transfered to No. 3.
3. Using the droppers, one drop of each starting at No. 3 and moved backwarded, each drop on a separate circle on the reaction card labeled with the dilution was, placed.
4. The latex reagent resuspended by swirling and the bottle inverted several times, then 20µl of the suspension added to the reaction circle with out touch the tip of the latex nozzle to the sample.
5. A mixing stick used for latex/sample mixture over the entire area of the circle.
6. Manually reaction card rotated for exactly 3 minutes.
7. The circle, macroscopically examined, after rotated the card for 3 minutes with out magnifying lens.
8. A positive reaction indicated when visible clumps of latex were, formed within 3 minutes of testing.
9. A negative reaction indicated when the milky appearance remains unchanged throughout, the 3-minute test.

2.2.3.8.2 Expression of results:

The results were expresd used the following guidelines:

Neat	1:2	1:4	1:8	D-dimer level (ng/ml)
-	-	-	-	<200
+	-	-	-	200-500
+	+	-	-	500-1000
+	+	+	-	1000-2000
+	+	+	+	>2000

2.2.4 Molecular analysis:

2.2.4.1. Reverse transcriptase polymerase chain reaction (RT-PCR):

2.2.4.1.1 Blood collection and RNA extraction:

Three ml venous blood collected from each participant into EDTA tubes after consent obtained from each participant.

Leukocytes prepared from peripheral blood samples after the addition of erythrocytes lysis buffer (150mM NH4Cl, 1mM KHCO3 and 0.1mM EDTA) (pH 7.3). Total RNA was, extracted from mononuclear cells by TRIzol reagent (Invetrogen, California. USA).

2.2.4.1.2 cDNA synthesis:

For cDNA synthesis, 5µl of total RNA reversely transcribed in a reaction mixture containing reverse transcriptase (RT) and buffer. (20 mM Tris HCl, 50 mM KCl (PH 8.3), 5mM MgCl2, 10 mM DTT, 5mM random hexamers, 20 units RNAase inhibitor, 10 units RT enzyme, 1mM dNTP and H2O to a total volume of 20 µl at 42 C° for 60 minutes). RT enzyme denatured by incubating the reaction at 99°C for 5 minutes.

2.2.4.1.3 Reverse Transcriptase-PCR:

Primers specific for the (TFPI-2) used to amplify the cDNA as follows

A 406 bp fragment of the full-lengthTFPI-2coding sequence was, amplified from primers

5`-GGGGTACCG CTT TCT CGG ACG CCT TG-3` (for-ward) and 5`-CGG GAT CCT GAT TTG TTT CCTCAT GCT GTC-3` (reverse)

For the first round of nested PCR, 3µl of cDNA product amplified in a reaction mixture containing 0.2 mM dNTP mix, 1.9µM MgCl2, 0.5u Taq polymerase, 1X PCR buffer, and 0.5 mM of each primer and H2O to 25 µl. The PCR cycling condition was 94°C for 30s, 64°C for 60s, 72 °C for 60s for 35 cycles and 72°C for 10 minutes final extension . For the second round of nested PCR, 1µl aliquot of the first round of nested PCR product was, amplified with specific primers using the same PCR reaction mixture and the PCR cycling condition of the first round of nested PCR.

2.2.4.1.4 Agarose gel electrophoresis:

From the PCR products, 5µl Aliquots was, separated by electrophoresis on a 1.8% agarose gel and was visualized with ethidium bromide staining.

2.2.4.1.5 Restriction fragment length polymorphism (RFLP) analysis of the TFPI-II:

PCR was, carried out according to the above protocols, using mismatched primers as shown. Having checked PCR products by running 5ul on 1% agarose gel, they were incubated over night with 1.5 U of Mae III (Boehringer Mannheim) at 55 C° in a total volume of 20µl. After loading the samples on 2.5% agarose gel, products were analysed under UV-light. The designed mismatched primer creates a cutting site at the position of GTNAC which recognized by Mae III endonuclease. The normal TFPI-II (235 bp fragment) was cutted in to three smaller fragments of 145, 64 and 26 bp. In the presence of mutation (G:A transition in exon 2 the fragment was cut into two smaller fragments of 171 and 64 bp owing to the absence of the GTNAC cutting site in the mutant TFPII . Finally, in the case of heterozygous DNA, there were four fragments of 171, 145, 64 and 26 bp.

2.3. Data analysis:

Data entered and analyzed by (SPSS) prograrmme (version: 17.0).

All demographic data of the study population presented as mean and SD in the text and P.value used for detecting the power of relationship between the determinant and the outcome and 95% confidence interval calculated. Data were analyzed using the Chisquare test for comparison the prevalence of TFPI-2 gene mutation between patients with TFPI-2 gene mutations and those without TFPI-2 gene mutations (The test considered significant when P.value <0.05).

2.4. Ethical cl earance:

Research ethics approval obtained from RICK administration, the relatives of the patients informed about the study, and agreement of participation was, obtained before starting the research and collection of data and samples from patient and control groups.

2.5 Privacy and confidentiality:

All information and results of the investigations obtained and kept in top confidentiality.

CHAPTER-THREE

Results

CHAPTER THREE

3. Results:

The participants included 195 children aged less than 15 years. 98 out of them were already diagnosed with leukemia and 97 healthy children as control.

In the patient group males were 52(53.1%) and females were 46(46.9%) with age for all (9.03 ± 4.058) .In the control group males were 53 (54.6%) and females were 44 (45.4%) with age for all (8.08 ±4.033)

The patient group in this study divided in to three subgroups based on type of leukemia ALL, AML and CML. The frequency of ALL was 81(82.7%), AML 12(12.2%) and CML 5 (5.1%), (Table 3.2).

TFPI-II gene investigated in all samples, there were 14(14.3%) in the patients group have mutation, 10(71.4%) out of them were homozygous and 4 (28.6%) were heterozygous. No participant among control group (0%) had TFPI-II gene mutation (Table3.3).

There was insignificant variations between the patients which have TFPI-II gene mutation and those without TFPI-2 gene mutations, ALL (OR = 1.3, P-value = 0.744), AML (OR = 1.23, P-value = 0.801) and CML (OR= 0, P-value = 0.349) (Table3.5).

In the patients group with TFPI-2 gene mutation the result of PT mean level was (17.05 ±2.974), PTT mean level was (39.564±6.809), fibrinogen mean was (2.759 ± 0.630), protein C was (0.845±0.198) and platelet count mean was (288.21 ± 155.797). While in the patients group without mutation, the result of PT mean was (17.689 ± 6.741), PTT mean was (40.555 ± 11.551), fibrinogen mean was (2.659 ± 0.680), protein C mean was (0.822 ± 0.037) and the platelet count mean was (289.67 ±136.85) .

There were insignificant variations in coagulation tests parameter (platelet count, PT, APTT, protein C and fibrinogen) between patients with TFPI-2 gene mutations and those without TFPI-2 gene mutations, P value >0.5 (Table3.6).

There are only two patients which there D-dimmer level were more than 200 ng/ml (high) and all control group and other patient were normal (less than 200 ng/ml) (Table3.7).

RT.PCR results:

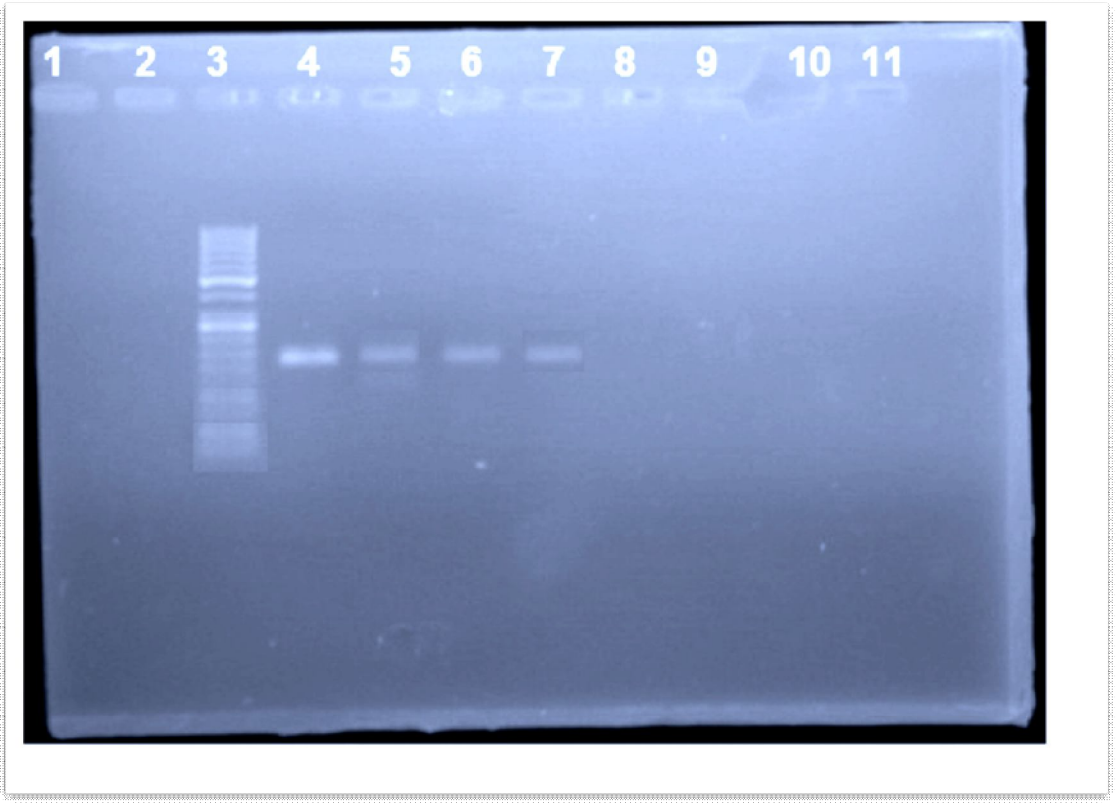


Fig (3.1) Control result for TFPI-II restriction fragment length polymorphism (RFLP) analysis:

1. Negative control.
2. TFPI-2 negative sample.
3. 100 pb DNA marker.
4. 4 to 7- positive sample for TFPI-2.
5. 8 To 11 – negative sample for TFPI-2.

Figure (3.2) Patient Result for TFPI-II restriction fragment length polymorphism (RFLP) analysis:

Restriction fragment length analysis of the TFPII polymorphism using MaeIII (lanes numbered from left to right).

1- Lanes number M: DNA Marker 25 bp to 350 bp
2- Lanes number (1) Negative Control.
3- Lanes number (2,3) heterozygous DNA, there are 4 fragments of 171, 145, 64 and 26 bp
4- Lanes number (4,5) homozygous DNA two fragments of 171 and 64 bp
5- Lanes number (6,7) normal TFPI-2 is digested into three fragments of 145, 64 and 26 bp

Table (3.1) Gender frequency and percentage in case and control group:

Sex		Frequency	Percent
Patient group	Male	52	53.1 %
	Female	46	46.9 %
	Total	98	100 %
Control group	Male	53	54.6 %
	Female	44	45.4 %
	Total	97	100 %

In the patient group, males were 52(53.1%) and females were 46(46.9%) while in the control group, males were 53 (54.6%) and females are 44 (45.4%)

Table (3.2)

Distribution of patients according to type of leukemia:

Case group	Frequency	Percent
ALL	81	82.7 %
AML	12	12.2 %
CML	5	5.1 %
Total	98	100 %

The patient group divided in to three subgroups based on type of leukemia ALL, AML and CML. The frequency of ALL was 81(82.7%), AML 12(12.2%) and CML 5 (5.1%).

Table (3.3)

Frequency and percentage of TFPI-2 gene mutation type:

TFPI-2 gene mutation	Frequency	Percent
Homozygous mutation	10	71.4 %
Heterozygous mutation	4	28.6 %
Total	14	100 %

There were 14(14.3%) in the patients group have TFPI-2 gene mutation, 10(71.4%) out of them were homozygous and 4 (28.6%) were heterozygous.

Table (3.4)

Correlation of TFPI-2 gene mutation between case and control group:

TFPI-2 gene	Patients N (%)	Controls N (%)	OR (95%CI)	P-value
Without mutation	84 (85.7%)	97(100%)	0	0.0258
With Mutation	14(14.3%)	0		
Total	98	97		

- OR = 0 means no association between patient and control groups in TFPI-2 gene mutation with risk of leukemia.
- P-value < 0.05 means there was significant variation between the patient and control groups in TFPI-II gene mutation.

Table (3.5)

Association of TFPI-2 gene mutation with risk of leukemia:

Patient group	Patient without mutation	Patient with mutation	Total	OR (95%CI)	P-value
ALL	69	12	81	1.3 (0.264 - 6.446)	0.744
AML	10	2	12	1.233 (0.24 - 6.333)	0.801
CML	5	0	5	0	0.349
Total	84	14	98		

- OR > 1 means TFPI-2 gene mutation associated with ALL and AML
- OR < 1 means TFPI-2 gene mutation not associated with CML
  TFPI-2 gene mutation
- P-value > 0.05 means there was insignificant variation between the patients which have TFPI-II gene mutation and those without TFPI-2 gene mutations

Table (3.6)

Association between patients with TFPI-2 gene mutations and those without TFPI-2 gene mutations in coagulation tests parameter:

Coagulation variant	Patients with Mutation Mean ± SD	Patients without Mutation Mean ± SD	P-value
PT	17.05 ± 2.9739	17.689 ± 6.7413	0.729
APTT	39.564± 6.8091	40.555± 11.5507	0.756
Fibrinogen	2.7593± 0.63034	2.6588± 0.68072	0.607
Protein C	0.845± 0.19779	0.8217± 0.03697	0.803
Platelets	288.21± 155.797	289.67± 136.85	0.971

Independent T test

-Sig. value less than 0.05, concider as statistical significant

-Sig. value 0.05 and more concider as statistical insignificant

There were insignificant variations in coagulation tests parameter (platelet count, PT, APTT, protein C and fibrinogen) between patients with TFPI-2 gene mutations and those without TFPI-2 gene mutations, P value > 0.5.

Table (3.7)

D. Dimer results in the patient and control groups:

	Case		Control	
D. Dimmer	Frequency	Percent	Frequency	Percent
(< 200) Normal	96	98 %	97	100 %
> 200) (High	2	2 %	0	0 %
Total	98	100 %	97	100 %

There are only two patients which there D-dimmer level were more than 200 ng/ml (high) and all control group and other patient were normal (less than 200 ng/ml)

CHAPTER-4

Discussion, Conclusion and Recommendation

CHAPTER FOUR

Discussion, Conclusion and Recommendation:

4.1Discussion:

In this study the patient group males were 52(53.1%) and females were 46(46.9%) and this in agreement with findings from Bhopal who concluded that sex-specific differences in the incidence of pediatric malignancies are consistent globally. Male predominance is a common phenomenon for many childhood cancers in developed countries (Bhopal, *et al*., 2012).

It also agree with study done in Bangladesh which done for some cancers (leukaemias and hepatoblastoma), the male predominance was noted to be more than three times higher among Bangladeshi boys (Mohammad, *et al*., 2016).

Our finding also in agreement with study done by Edgren who has been recognized that males are more prone to develop cancer, and particularly hematologic malignancies (Edgren, *et al*., 2012).

In this study, the commonest type of leukaemia was acute lymphoblastic leukaemia (ALL), which accounted 81(82.7%), followed by acute myeloid leukaemia (AML), (12.2%), while chronic myeloid leukaemia accounted for (5.1%). This was supported by a study done by Coebergh, who reported from the automated childhood cancer information system project, that done in Europe, acute lymphoblastic leukaemia (ALL) accounts for around 80% of leukaemia among children aged 0– 14 years (Coebergh, *et al*. 2006).

This also goes in line with literature written by Samia, who concluded that the majority of childhood leukaemia being 75% (ALL) followed by 25% (ANLL) and (CGL) 5% in USA and Europe (Samia, 2015).

TFPI-2 gene investigated in all samples, there were 14(14.3%) of the patients group have mutation, 10(71.4%) of them were homozygous and 4 (28.6%) were heterozygous, while TFPI-2 gene mutation absent among control group. There was insignificant association between patients, which have TFPI-2 gene mutation, and those without TFPI-2 gene mutation.

Our result cannot be hypothesized due to monosomy 7 or deletions of 7q, which studied by Heerema who examined complete or partial loss of chromosome 7, predominantly monosomy 7 or deletions of 7q, is associated with a variety of myeloid

disorders, including acute myelogenous leukemia (AML) in children. (Heerema, *et al*. 2004)

Or other study by Saito who conclude that Several highly aggressive cancers delete the locus for the TFPI-2 gene in chromosome 7q region, which results in a complete lack of TFPI-2 protein expression in these cells (Saito, *et al.,* 2005).

In addition, other study stated by Dong, 2001 that TFPI-2 transcription could regulated by polymorphisms in the promoter sequence affecting transcription factor binding sites and absence of TFPI-2 expression could occur through deletion of the chromosomal region 7q22, as in prostate cancer (Dong.2001).

Based on that we mentioned above, TFPI-2 gene detected in all samples of the patients and control group.

In addition, there were insignificant variations in coagulation tests parameter (platelet count, PT, INR, APTT, protein C and fibrinogen) between patients with TFPI-2 gene mutations and those without TFPI-2 gene mutations.

As we mention before this the first study, there was no study like this done before so we cannot compare our finding with other studies.

In our study there were only two patients which their D-dimer level more than 200 ng/ml and all control group and other patient with normal range (less than 200 ng/ml). Our findings agree with Wei who measured the level of D-dimer in different phrases of patients with acute leukemia and to explore its significance in the progress and curative effect of leukemia after complete remission (CR) plasma levels of D-dimer had no significant difference in all patients (26 cases) vs control group (Wei, *et al.,* 2011).

Our finding does not agree with Krzysztof in their study which carried out in 70 patients including 49 with AML and 21 with ALL, he concluded that the level of TAT, DD and PAP was elevated (Krzysztof, *et al.,* 2000).

4.2 Conclusions:

It concluded that:

- ALL is the most common among Sudanese pediatric children with leukemia
- Leukemia occur equally in Sudanese pediatric males and females
- 14.3% of Sudanese pediatric patients with leukemia have TFPI-II gene mutation and no mutant gene detected among the controls group.
- There was an insignificant variation between the patients with TFPI-II gene mutation and and those without TFPI-2 gene mutations.
- There were insignificant variations in coagulation tests parameter (platelet count, PT, INR, APTT, protein C and fibrinogen) between patients with TFPI-2 gene mutations and those without TFPI-2 gene mutations.
- There were only two patients which their D-dimer level more than 200 ng/ml and all control group and other patients with in normal range (less than 200 ng/ml).

4.3 Recommendations:

1. As findings of the current study seems to indicate limited importance of genetic changes of TFPI-2 ,further studies in epigenetic silencing of TFPI-2 in Sudanese pediatric patient with leukemia were required to assess correlation between leukemia and TFPI-2 gene expression.
2. More prospective studies are required to explain the relationship betweenTFPI-2 gene mutation and deletion of chromosome 7 on the same patients.
3. Further studies on Sudanese pediatric patient with leukemia needed to assess correlation between cancer and tumor suppressor genes like TP53.

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APPENDICES

Questionnaire

Sudan University

College of graduate studies

Questionnaire on Sudanese Pediatric Patients with leukemia

Questionnaire on patients group

نموذج استبيان

Questionnaire

General information: **المعلومات العامة:**

الاسم :.....Name

رقم السجل:.....ID No

العمر:.....Age

النوع :.....Gender

رقم الهاتف :.....Phone

Medical information : **المعلومات الطبية.**

حالة المريض العامة:.....Physical state

نوع سرطان الدم الابيض :.....Type of leukemia

العلاج المتبع :.....Treatment

Questionnaire on control group

نموذج استبيان

Questionnaire

General information: **المعلومات العامة:**

الاسم :.....Name

العمر:.....Age

النوع :.....Gender

رقم الهاتف :..... Phone

Medical information : **المعلومات الطبية.**

حالة المشارك العامة:.....Physical state

هل يوجد تاريخ اصابة بسرطان الدم :.....Is there a history of leukemia

Coagulation Investigations:

Coagulation results in control group :

No	Age months	Gender	PT	INR	APTT	Fibrinogen	Protien C	D.Dimer	Pletelets count
1	84	F	14.1	1.01	26.4	2.1	0.89	<200	320
2	42	F	13.6	0.97	27.3	2.3	0.91	<200	232
3	16	M	13.9	0.99	22.9	2.0	0.79	<200	289
4	48	F	13.8	0.99	24.3	3.0	0.96	<200	393
5	45	M	13.6	0.97	28.1	2.8	0.77	<200	348
6	44	M	14.0	1.0	27.3	2.6	0.86	<200	324
7	72	F	14.3	1.02	26.1	2.4	0.75	<200	242

Coagulation results in patients group :

No	Age month	Gender	Type of leukemia	PT	INR	APTT	Fibrinogen	Protien C	D. Dimer	Pletletes count
1	24	F	ALL	14.0	1.0	37.0	1.21	0.73	200<	250
2	84	F	ALL	13.5	0.96	26.9	1.12	0.67	200<	732
3	156	F	ALL	13.5	0.96	40.1	1.31	0.95	200<	255
4	120	M	ALL	12.8	0.91	36.3	3.5	0.91	200<	567
5	84	M	AML(M2)	12.4	0.88	32.4	2.62	1.14	200<	260
6	156	F	ALL	12.5	0.89	38.2	2.82	1.07	200<	392
7	50	M	ALL	11.1	0.79	29.7	2.31	1.09	200<	601
8	156	M	ALL	14.1	1.01	34.6	2.21	1.12	200<	290
9	158	F	CML	15.9	1.13	24.4	2.09	1.1	<200	315

RT-PCR Investigations:

RT-PCR result of control group:

No	PCR result	RFLP	No	PCR result	RFLP	No	PCR result	RFLP
1	Positive	Normal	9	Positive	Normal	17	Positive	Normal
2	Positive	Normal	10	Positive	Normal	18	Positive	Normal
3	Positive	Normal	11	Positive	Normal	19	Positive	Normal
4	Positive	Normal	12	Positive	Normal	20	Positive	Normal
5	Positive	Normal	13	Positive	Normal	21	Positive	Normal
6	Positive	Normal	14	Positive	Normal	22	Positive	Normal
7	Positive	Normal	15	Positive	Normal	23	Positive	Normal
8	Positive	Normal	16	Positive	Normal	24	Positive	Normal

RT-PCR result of patients group:

No	PCR result	RFLP	No	PCR result	RFLP	No	PCR result	RFLP
1	Positive	Normal	35	Positive	Normal	**69**	**Positive**	**Mutation**
2	Positive	Normal	36	Positive	Normal	70	Positive	Normal
3	Positive	Normal	**37**	**Positive**	**Mutation**	71	Positive	Normal
4	Positive	Normal	38	Positive	Normal	72	Positive	Normal
5	Positive	Normal	39	Positive	Normal	73	Positive	Normal
6	Positive	Normal	40	Positive	Normal	74	Positive	Normal
7	Positive	Normal	41	Positive	Normal	75	Positive	Normal
8	Positive	Normal	42	Positive	Normal	76	Mutation	Normal
9	Positive	Normal	**43**	**Positive**	**Mutation**	77	Positive	**Mutation**
10	Positive	Normal	44	Positive	Normal	78	Positive	Normal
11	Positive	Normal	45	Positive	Normal	79	Positive	Normal
12	Positive	Normal	46	Positive	Normal	80	Positive	Normal
13	Positive	Normal	47	Positive	Normal	81	Positive	Normal
14	Positive	Normal	48	Positive	Normal	82	Positive	Normal
15	Positive	Normal	49	Positive	Normal	83	Positive	Normal
16	**Positive**	**Mutation**	50	Positive	Normal	84	Positive	Normal
17	Positive	Normal	51	Positive	Normal	85	Positive	Normal
18	Positive	Normal	52	Positive	Normal	**86**	**Positive**	**Mutation**
19	Positive	Normal	53	Positive	Normal	87	Positive	Normal
20	Positive	Normal	54	Positive	Normal	88	Positive	Normal
21	Positive	Normal	55	Positive	Normal	89	Positive	Normal
22	Positive	Normal	56	Positive	Normal	90	Positive	Normal
23	Positive	Normal	57	Positive	Normal	91	Positive	Normal
24	**Positive**	**Mutation**	58	Positive	Normal	92	Positive	Normal
25	**Positive**	**Mutation**	59	Positive	Normal	93	Positive	Normal
26	**Positive**	**Mutation**	**60**	**Positive**	**Mutation**	**94**	**Positive**	**Mutation**
27	**Positive**	**Mutation**	61	Positive	Normal	95	Positive	Normal
28	**Positive**	**Mutation**	62	Positive	Normal	96	Positive	Normal
29	Positive	Normal	63	Positive	Normal	97	Positive	Normal
30	Positive	Normal	64	Positive	Normal	**98**	**Positive**	**Mutation**
31	Positive	Normal	65	Positive	Normal			
32	Positive	Normal	66	Positive	Normal			
33	Positive	Normal	67	Positive	Normal			
34	Positive	Normal	68	Positive	Normal			

- Homozygous
- **Heterozygous**

Appendix1

F**igure (6.2):** RT-PCR (reverse transcription-polymerase chain reaction) is a sensitive technique for mRNA detection

Master Mix 1 (for one reaction)

Components (Mix 1) [a]	**Volume**	**Final conc.**
PCR Grade Water (vial 6 from kit)	variable	to make total vol = 25 µl
PCR Nucleotide Mix, 10 mM (vial 2)	4 µl	200 µM (each nucleotide)
DTT solution (supplied in kit)	2.5 µl	5 mM
RNase Inhibitor (vial 3) [b]	1 µl	5 U/reaction
Downstream primer [c]	variable	0.4 µM
Upstream primer [c]	same as downstream primer	0.4 µM
Template RNA (isolated total RNA) [c]	variable	1 pg–1 µg total RNA
Final volume (Mix 1)	**25 µl**	

Appendix2

Figure (6.2) Sysmex KX -21N: Used for platelate count

KX-21N reagent System

Cellpack: Dilution

Stromatolyser-WH

Appendix 3

Figure (6.3) sysmex CA 500

The CA-500

The sysmex automated blood coagulation analyser CA-500 is acompact fully-automated instrument capable of 5-parameter random analysis.

This instrument is able to analyze 5 parameters simultaneously.In addtion, it has a number of functions including preferential processing of STAT samples and built-in quality control function. Moreover, it allows analyzed data to be displayed and printed out together with coagulation curves,thus making it possible to obtain highly reliable analysis result.

Used of PT, PTT and fibrinogen level measurements

Appendix 4

Figure (6.4) Biosystems BTS-330

Chromogenic assay method used for protein C measurment

Reagents system:

1. Protein C substrate

Each vials contained 2.75-µmol lypholised pyro-Glu-Pro-Arg-pNA. HCL.

Preparation for use: each vial reconstituted with 2.0 ml of protein C diluent.

2. Protein C activator: each vial contains 0.8 units of activator from snake venome (protac ®

3. Protein C diluents: each vial contains tris buffer with sodium azide as a preservative, Ready for use.

4. SARP

5. Spectrophotometer (Biosystem BTS- 302. Spain). Capable for reading to 405 nm

6. Glacial acetic acid 33% and timer for manual