Quantitative Analysis of Kallikrein-Related Peptidases *KLK6*, *KLK10*, and *KLK14* Gene Expression in Childhood Acute Lymphoblastic Leukemia

A Thesis Submitted to the Council of the College of Medicine-University of Sulaimani in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Clinical Chemistry

By

Shwan Majid Ahmad

MSc in Chemistry with Medicinal Chemistry

Supervised by

Assistant Professor Dr. Basima S. Ahmed

Ph.D. in Clinical Biochemistry

Co-supervised by

Assistant Professor Dr. Karzan G. Khidhir

Ph.D. in Molecular Biology and Genetics

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أعوذ بالله من الشيطان الرجيم

بسم الله الرحمن الرحيم

الحمد لله رب العالمين ۝ الرحمن الرحيم ۝ مالك يوم الدين ۝ إياك نعبد وإياك
نستعين ۝ اهدنا الصراط المستقيم ۝ صراط الذين أنعمت عليهم غير
المغضوب عليهم ولا الضالين ۝

Student declaration

I the undersigned, Ph.D. student declare that this thesis entitled "**Quantitative Analysis of Kallikrein-Related Peptidases *KLK6, KLK10*, and *KLK14* Gene Expression in Childhood Acute Lymphoblastic Leukemia**" is my original effort and has never been performed in any other university and that all resources of materials have been duly acknowledged.

Shwan Majid Ahmad

MSc in Chemistry with Medicinal Chemistry- University of Glasgow

Date: 01/06/2022

Supervisors' certification

We certify that this thesis entitled "**Quantitative Analysis of Kallikrein-Related Peptidases *KLK6*, *KLK10*, and *KLK14* Gene Expression in Childhood Acute Lymphoblastic Leukemia**" accomplished by (**Shwan Majid Ahmad**), was created under our direction at the Department of Biochemistry, College of Medicine, University of Sulaimani in partial completion of the criteria for the degree of **Doctor of Philosophy** (**Ph.D.**) in **Clinical Chemistry**.

Assistant Professor
Dr. Basima Sadiq Ahmed
Ph.D. in Clinical Biochemistry
Department of Biochemistry and Clinical Chemistry
College of Pharmacy
University of Sulaimani

Assistant Professor
Dr. Karzan Ghafur Khidhir
Ph.D. in Molecular Biology and Genetics
Department of Biology
College of Science
University of Sulaimani

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Assistant Professor
Dr. Shirwan Hamasalih Omer
MSc, Ph.D. in Clinical Neurophysiology
Dean of the College of Medicine

Date: 24 /07/2022

Examination committee certification

We certify that we have read the thesis entitled "**Quantitative Analysis of Kallikrein-Related Peptidases *KLK6, KLK10,* and *KLK14* Gene Expression in Childhood Acute Lymphoblastic Leukemia**", prepared by (**Shwan Majid Ahmad**), and as members of the examining committee discussed its content with the student, it meets the standards of a thesis in partial fulfillment for the degree of **Doctor of Philosophy (Ph.D.)** in **Clinical Chemistry**.

Dr. Salar Adnan Ahmed
Professor of Clinical Biochemistry
College of Medicine- Hawler Medical University
(Chairman)

Dr. Karim Jalal Karim
Assist. Prof. of Genetics and Molecular Biology
Faculty of Science and Health- Koya University
(Member)

Dr. Ali Ibrahim Mohammed
Assist. Prof. of Hematopathology
College of Medicine-University of Sulaimani
(Member)

Dr. Sadat Abdulla Aziz
Assist. Prof. of Cell Molecular Physiology
College of Veterinary Medicine- University of Sulaimani
(Member)

Dr. Namir Ghanim Al-Tawil
Professor of Community Medicine
College of Medicine- Hawler Medical University
(Member)

Approved by the Council of the College of Medicine.

Assistant Professor
Dr. Shirwan Hamasalih Omer
MSc, Ph.D. in Clinical Neurophysiology
Dean of the College of Medicine
Date: /09/2022

Dedication

I ask Allah Almighty to accept this study with His forgiveness for mistakes I have ever made

Dedicate to;

- The Prophet of Allah; Muhammad (Peace and Blessings of Allah be upon him)
- My Father; to whom I ask Allah to rest his soul in Paradise
- My Merciful Mother, the Sun in my Life
- My Lovely Sisters, the Flowers in the same Garden
- To every candle that burns itself to enlighten the way for others

Acknowledgments

In the Name of Allah, Most Gracious, Most Merciful

Praise be to **Allah**, the Almighty, for helping me with His Kindness and Mercy to accomplish this study.

I would like to express my gratitude and admiration for my supervisors: Assistant Professor **Dr. Basima Sadiq Ahmed** and Assistant Professor **Dr. Karzan Ghafur Khidir**, for their valuable encouragement and efforts in supervising this thesis.

All respect and thanks to the **Deanery** of **College of Medicine**, the **Chairman** of the **Biochemistry Department**, and all my esteemed **teachers**, **colleagues**, and the **staff** working at the college for their support through my postgraduate study.

My deepest acknowledgement and thanks to the head, the doctors, the nurses, and all the staff working at **Hiwa Cancer Hospital**, especially to the staff at Pediatric Department, Biochemistry Laboratory, Hematology Laboratory, Flowcytometry Laboratory, and Molecular Biology Laboratory; great gratitudes to **Dr. Lana Anwar Tahir**, **Dr. Dana Ahmed Abdullah**, **Dr. Muhammed Abdulwahab**, **Mr. Nawzad Omer Ahmad**, **Dr. Sozan**, **Mr. Alan**, **Dr. Kani**, **Dr. Zardasht**, **Dr. Dashti**, **Dr. Ali**, **Mr. Bryar**, **Mr. Salah**, **Mr. Omed**, **Mr. Hersh**, **Mr. Thamir**, and **Mr. Rizgar** for their cooperation and assistance in gathering cases, samples, and data, also allowed me to conduct the qPCR assays at the Molecular Biology Laboratory.

My endless thanks to all the pediatric patients and healthy volunteers who were generous to participate in this study.

My heartfelt appreciation to the head and staff of **Kurdistan Institution for Strategic Studies** for their kindness and continuous support in where the core of this research project has been performed; my special thanks to **Mr. Bahez Osman**.

My deepest thanks would go to my honorable **family**, **my dear mother, and my lovely sisters** for their patience and supports throughout the years of my studies.

The last but not least, I would like to thank all whose names I have not mentioned for their assistance in performing this study.

Shwan Majid Ahmad

Abstract

Background: Acute lymphoblastic leukemia (ALL) is the most widely diagnosed pediatric cancer. It is caused by the rapid proliferation of immature lymphoid in the blood, bone marrow, and other tissues. In a five-year survival rate, more than 80% of pediatric patients are cured. Despite high survival rates, sensitive and specific molecular biomarkers are required for cancer diagnosis and prognosis, assignment of better risk stratification, and therapeutic outcomes for childhood ALL. Human kallikrein related-peptidases (KLKs) are a family of fifteen unique released serine proteases that characterize the human genome's longest uninterrupted cluster located on chromosome 19. KLKs are present in a range of tissues and bodily fluids, where they influence normal physiological function. The aberrant expression of various kallikrein-related peptidases has been related to diverse diseases and cancers. The potential of kallikrein-related peptidases in clinical oncology has been recognized. A very well-known member of the KLK family with clinical relevance is the prostate-specific antigen (PSA/KLK3) screening test. However, molecular analysis of other members of the KLK family in hematological malignancies is new, and investigation into the involvement of KLKs in cancer is currently ongoing.

Objectives: This research aimed to determine mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* as potential biomarkers for diagnosis and/or prognosis of childhood ALL, and to assess the effect of chemotherapy on their expression profile after one month and three months of receiving chemotherapy.

Materials and Methods: This investigation was a prospective, analytical, observational, and case-control study. The participants were children aged 1 to 15 years, including 23 patients who were admitted to the Pediatric Department in Hiwa Cancer Hospital, Sulaimaniyah, Kurdistan Region of Iraq, and they were newly diagnosed with ALL. Also, healthy pediatric volunteers (n=12) were selected as the control group. Blood samples were collected from leukemic patients at three different times: at diagnosis with ALL, following one month, and three months of receiving chemotherapy. Total RNA was extracted from blood samples, followed by cDNA

synthesis, then mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* were analyzed using quantitative real-time PCR (qRT-PCR).

Results: mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* in blood samples from pediatric ALL patients were significantly downregulated compared to healthy blood donors (p=0.002, p=0.0001, and p=0.0007 respectively). *KLK6*, *KLK10*, and *KLK14* mRNA expression levels were significantly downregulated in ALL patients after one month of receiving chemotherapy compared to their levels in normal blood samples (p=0.0292, p<0.0001, and p<0.0001 respectively). The genes' expression was also significantly downregulated in ALL patients who received three months of chemotherapy compared to their levels in normal blood samples (p=0.0038, p=0.0175, and p<0.0001 respectively). ROC curve analysis revealed the significant diagnostic value of the *KLK6*, *KLK10*, and *KLK14* expression to discriminate ALL patients from normal counterparts (AUC=0.822, 95% CI=0.6735-0.9713, SE=0.076, p=0.0029), (AUC=0.886, 95% CI=0.7720-1.000, SE=0.0582, p=0.0004), and (AUC=0.851, 95% CI=0.7087-0.9931, SE=0.0726, p=0.0012) respectively. Univariate logistic regression analysis demonstrated that the three genes could be used as prognostic biomarkers for ALL (OR=0.2289, 95% CI=0.0557-0.9399, p=0.0115), (OR=0.0228, 95% CI=0.0008851-0.2299, p <0.0001), and (OR=0.0716, 95% CI= 0.003912-0.4610, p=0.0002) respectively. In ALL patients who received one-month chemotherapy, *KLK10* and *KLK14* mRNA expression levels were downregulated compared to newly diagnosed patients (p=0.4413 and p=0.0039 respectively), whereas *KLK6* mRNA expression was upregulated (p=0.4413). In ALL patients, *KLK6* and *KLK14* mRNA expression were downregulated after three months of chemotherapy compared to their level in the patients upon diagnosis (p=0.6794 and p=0.1336 respectively), while *KLK10* mRNA was upregulated (p=0.0602).

Conclusions: The present study revealed that *KLK6*, *KLK10*, and *KLK14* mRNA expression is significantly downregulated in pediatric ALL patients compared to the control group, implying that it would have diagnostic relevance. Thus, *KLK6*, *KLK10*, and *KLK14* expressions at the mRNA level could be used as molecular biomarkers in the diagnosis and prognosis of ALL.

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Abbreviation	Stand for
A	Absorbance
ACTB	Beta-actin gene
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AUC	Area Under the Curve
BFM	Berlin-Frankfurt-Munich
BLAST	Basic Local Alignment Search Tool
BM	Bone Marrow
BMI	Body Mass Index
bp	Base pair
C	Cytosine
°C	Celsius, also called degree centigrade
CA 125	Cancer Antigen/ Carbohydrate Antigen 125
CAG	Cancer-Associated Gene
CBC	Complete Blood Count
CCG	Children's Cancer Group
CD	Cluster Designations
cDNA	Complementary DNA
CI	Confidence Interval
CLL	Chronic Lymphocytic Leukemia
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
Ct	Cycle threshold
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic Acid
EGIL	European Group for the Immunological Characterization of Leukemias
FISH	Fluorescence In Situ Hybridization
EMSP1	Enamel Matrix Serine Protease 1
FAB	French-American-British
G	Guanine
g	Gram
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HGNC	Human Gene Nomenclature Committee
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic Stem Cell Transplantation
ISIS	Daesh

IU	International units
KLK	Kallikrein Related-Peptidases
L	Liter
L_{1-3}	Lymphoblasts morphological categories 1-3
LDH	Lactate Dehydrogenase
LOE	Level Of Evidence
M	Molarity
Min	Minute
miRNA	MicroRNA
ml	Milliliter
MPO	Myeloperoxidase
MRD	Minimal Residual Disease
mRNA	Messenger Ribonucleic Acid
N	Number
NA	Not Available
NACB	National Academy of Clinical Biochemistry
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
ng	Nanogram
NTC	No Template Control
OR	Odds Ratio
p	Probability
PAR	Protease Activated Receptor
PAS	Periodic Acid Schiff
PCR	Polymerase Chain Reaction
POG	Pediatric Oncology Group
PSA	Prostate-Specific Antigen
qPCR	Quantitative Polymerase Chain Reaction or Real-time PCR
RBC	Red Blood Cell
ROC	Receiver Operating Characteristic
rpm	Round per minute
RQU	Relative Quantification Unit
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse transcription-polymerase chain reaction
SBB	Sudan Black B
SE	Standard Error of the Mean
Sec	Second
Sg	Seminogelin
SIGLEC	Sialic Acid-binding Ig-like Lectin
SNP	Single Nucleotide Polymorphisms
T_a	Annealing Temperature
TAP	Testicular Acid Phosphatase gene

Taq	Thermus Aquaticus gene
TBE	Tris/Borate/EDTA buffer solution
TLP	Traumatic Lumbar Puncture
T_m	Melting Temperature
UKALL	United Kingdom Acute Lymphoblastic Leukaemia
UV	Ultraviolet
WBC	White Blood Cell
WHO	World Health Organization
β-ME	β-Mercaptoethanol
µl	Microlitter

CHAPTER ONE

Introduction

Chapter One: Introduction

1.1 Background of the study

Acute lymphoblastic leukemia (ALL) is the most universally diagnosed pediatric malignancy (1). A standard-based clinical and laboratory analyses are performed to understand clinical-pathological aspects of the disease and approach to their diagnosis, treatment, and outline prognostic factors (2). The prevalence of the use of conventional molecular biomarkers for diagnosis, prognosis, and prediction of ALL in adults is four times surpassing compared to children (3).

There are also significant gaps in our understanding of various molecular features of kallikrein functions. Human kallikrein related-peptidases (KLKs) consist of a single family of fifteen homologous secreted serine proteases. They are significant in regulating normal physiological functions and their dysregulation is associated with the progression of diverse diseases and malignancies (4).

Tumor biomarkers are biomolecules that can be used to detect the presence of cancer, and/or provide information in patient management. They play a substantial role in the diagnosis of malignancies, prognosis, and prediction of treatment strategies (5). Cancer biomarkers could be nucleic acids, proteins, cells, metabolites, or processes such as proliferation, angiogenesis, or apoptosis (6).

Several kallikreins have been proposed as putative cancer biomarkers in clinical oncology as relevant diagnostic, prognostic, and monitoring molecular biomarkers in a range of human malignancies. The most well-known example is the worldwide use of KLK3 or (Prostate-Specific Antigen, PSA) in the diagnosis and monitoring of prostate cancer (7). The shortage of data on KLKs in hematological malignancies provided the impetus for the current work. This study put forth the hypothesis that there could be an association between kallikrein-related peptidases and leukemia. The goal of the current study was to investigate the possibility of using mRNA gene expression levels of *KLK6*, *KLK10*, and *KLK14* as diagnostic and/ or prognostic biomarkers for acute lymphoblastic leukemia, and to assess the effect of chemotherapy on their expression profile after one month and three months of receiving chemotherapy.

1.2Aim and objectives of the study

To address the hypothesis of the current study, the following objectives were formulated:

- Perform quantitative measurement of mRNA gene expression levels of the kallikrein-related peptidases: *KLK6*, *KLK10*, and *KLK14* in newly diagnosed childhood ALL patients and healthy control blood donors using the real-time qPCR.

- Correlate the mRNA expression levels of the *KLKs* in newly diagnosed childhood ALL patients and the continuous variables (age, lymphocyte count, WBC, and serum LDH concentration).
- Assess the potential diagnostic values of the *KLKs'* mRNA expression.
- Investigate the prognostic ability of the *KLKs'* mRNA expression.
- Evaluate the *KLKs'* mRNA expression levels of childhood ALL patients after one month and three months of receiving chemotherapy.

CHAPTER TWO

Literature Review

Chapter Two: Literature Review

2.1 Acute Lymphoblastic Leukemia (ALL)

Leukemias are defined as a group of malignant disorders that occur due to abnormal and uncontrolled proliferation of leukocytes. Leukemias affect the specialized hemopoietic tissue of the bone marrow and could affect peripheral blood, lymphoid tissues, and spleen. Depending on the period the disease would progress, leukemias are classified into acute or chronic (8). Mature cells in bone marrow stem cells are generated through various steps of self-renewal, maturation, and progressive differentiation. Immature, young, or primitive blast cells will then form mature peripheral blood cells (9). Acute leukemias are caused by an abnormal proliferation of the primitive cells and chronic leukemias are due to malignancy of the mature and maturing cells.

Acute lymphoblastic leukemia (ALL) is a cancer of lymphoblasts, which is composed of either B or T lineages. Approximately 85% of the ALL cases are precursor B-cell ALLs, which typically manifest as pediatric acute leukemias. Precursor T-cell ALLs are less common and tend to present more frequently in adults (10). ALL is the commonest pediatric cancer. The childhood ALL cure rate is more than 80% at five-year survival rates (11). However, less progress has been achieved in the treatment of adults ALL.

2.1.1 Epidemiology of ALL

ALL is the most common childhood malignancy. It represents 23% of pediatric cancer cases. In the United States, 80% of diagnosed ALL cases are in children (12). Infant ALL represents 2% to 3% of pediatric cases. The overall incidence rate in both the United States and the Nordic countries is 3.9 per 100,000/year in people younger than 15 years (13). ALL incidence rates in developed countries peak between the age of 2 to 5 years. The incidence of the disease is almost three times as frequent in whites as compared to blacks. It is slightly more common in males than in females; the male-to-female ratio is about 1.2 to 1.0 (14). With the exception, the frequency is slightly higher in female infants. Exposure to depleted uranium, as well as massive use of chemical weapons by the former Iraqi regime and recently by ISIS against Iraqi population, has been connected to a rise in leukemia rates in several Iraqi cities (15). According to a local study, the leukemia incidence rate per 100,000 pediatric age population was 3.57 in boys, and 2.97 in girls (16).

2.1.2 Etiology and risk factors of ALL

It is considered that the etiology of acute leukemia is idiopathic. Nevertheless, some factors have been examined as possible risk factors for the disease including genetic, environmental, and infectious risk factors (8, 13, 17-21). The genetic aspects include genetic abnormalities such as chromosome aneuploidy and translocations. The environmental aspects include in utero

exposure to ionizing radiation, maternal frequent exposure to pesticides, exposure to benzene, and drugs such as alkylating agents. The infection aspects include maternal infection during pregnancy which has been linked to an increased risk of childhood leukemia.

2.1.3 Classifications of ALL

2.1.3.1 FAB morphologic classification of ALL

The French-American-British (FAB) morphologic classification was first produced in 1976 (22). This classification relied on the morphological and cytochemical microscopic appearance of leukemic cells. Three morphological categories of lymphoblasts in ALL were defined namely L_1, L_2, and L_3 established on their size, cytoplasm, nucleus, chromatin, basophilia, and vacuolation. Generally, 85% of pediatric ALL diagnosed cases have L_1 morphology, 14% have L_2, and about 1% have L_3. The FAB classification is presented in **(Figure 2.1)** and **(Table 2.1)** (23, 24).

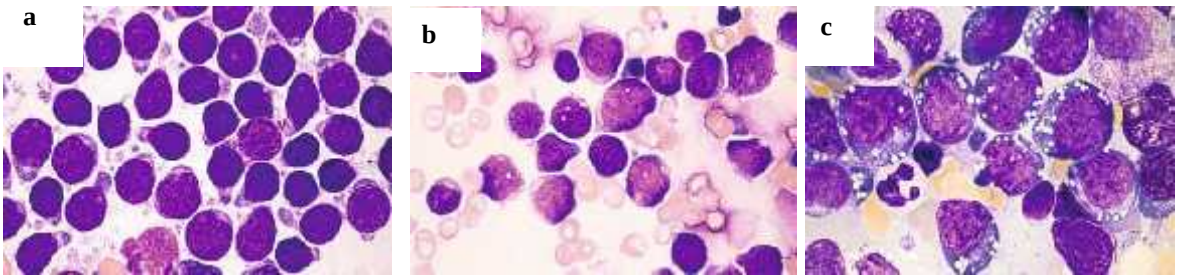

Figure 2.1. FAB morphologic appearance of blast cells in ALL.

This figure demonstrates FAB morphological classifications; [a]ALL-L_1: small and homogenous blasts, [b]ALL-L_2: lymphoblasts of varying size, and [c]ALL-L_3: prominent cytoplasmic vacuoles.

Table 2.1. FAB morphologic classification of blast cells in ALL.

Characteristics	L_1 morphology	L_2 morphology	L_3 morphology
Cell size	Small in size, uniform	Large, irregular size	Large, regular size
Nuclear shape	Round and regular nucleus	Irregular nucleus outline	Round nucleus
Nuclear chromatin	Homogeneous	Heterogeneous, condensed	Finely granular stippled
Nucleoli	Barely visible	Prominent	Prominent
Cytoplasmic amount	Scanty	Abundant	Abundant
Cytoplasmic basophilia	Intensity is slight to moderate	Variable	Very deep
Cytoplasmic vacuolation	Variable	Variable	Prominent

Special stains were used to identify the exact FAB group such as Periodic Acid Schiff (PAS), Sudan Black B (SBB), and Myeloperoxidase (MPO). Blasts in ALL cases cytochemically have negative results in both MPO and SBB reactions and usually positive results in PAS reactions (25-27). It is difficult to distinguish between B- and T-lineage ALL using morphological criteria, and much more difficult to identify B-lineage lymphoblasts from normal B-lineage lymphoid progenitors. (23).

2.1.3.2 Immunophenotype and cytochemical classification of ALL

Immunological markers are found on the surface, in the nuclei, and in the cytoplasm of leukemic cells. The surface markers can be detected using monoclonal antibodies conjugating with different fluorochromes. These cell surface markers are called cluster designations which are expressed as the CD.

Flow cytometry immunophenotyping is performed for the diagnosis and prognosis of ALL which is an accurate technique for assigning a cell line to one of the main cell lineages (28, 29). The European Group for the Immunological Characterization of Leukemias (EGIL) proposed a guideline for the immunological characterization of acute leukemias based on the idea that surface and cytoplasmic antigens may be present and/or absent during lymphocyte maturation. Then, the cell line and degree of differentiation of the leukemic process can be detected according to antigen expression (30). Most childhood ALL cases are developed as a result of the monoclonal proliferation of B-cell precursors (80%), mature B-cell (5%), and T-cell ALL (15%) (31). According to the bone marrow differentiation steps of normal B-cell progenitors, B-lineage ALL was classified into Pro-B, common Pre-B, Pre-B, and B-cell ALL (29, 32, 33). T-lineage ALL was categorized using differentiation antigens that corresponded to normal thymocyte differentiation levels, into Pre-T, T-Intermediate, and Mature or Medullar ALL (29, 33).

2.1.3.3 Cytogenetic and molecular classification of ALL

The World Health Organization (WHO) categorized hematological neoplasms in 2001, revised them in 2008, and then in 2016 (34, 35). The WHO produced a standard based on clinical and laboratory data derived from different methods including analysis of cell morphology, cytochemistry, immunophenotyping, cytogenetic and molecular genetics to understand clinical-

pathological aspects of diseases and approaches to their diagnosis, treatment, and outline prognostic factors (2, 35).

Genetic abnormalities in B-cell ALL is significantly related to the occurrence of specific chromosomal rearrangements involving hyperdiploidy (51-65 chromosomes) (36), hypodiploidy (≤44 chromosomes) (37), rearrangement of mixed-lineage leukemia (*MLL*) at 11q23 (38), t(9;22)(q34;q11)/*BCR-ABL1* (37), and t(12;21)(p13;q22)/*TEL-AML (ETV6-RUNX1)* (38).

Genetic deregulations in T-cell ALL are mostly related to the abnormal expression of normal transcription factor proteins. This is frequently a consequence of chromosomal translocations that commonly involve the 14q11 juxtaposing promotor and enhancer constituents of T-cell receptor genes (39). Frequent genomic features of T-ALL include *TAL1* deregulation, *LMO2* deregulation, *NOTCH1* mutations, and *MLL* rearrangements (37).

2.1.4 Clinical features of ALL

The onset of the signs and symptoms of childhood acute leukemias are mostly present within few weeks (40). The highly important clinical manifestations are the following:

2.1.4.1 Bone marrow failure

The bone marrow (BM) of children with ALL is extensively infiltrated by blasts. The presentation includes the symptoms of fatigue, paleness, fever, bleeding, bone pain, dyspnoea, angina, dizziness, weight loss, malaise, repeated infections, petechiae, and bruising. Anemia, neutropenia, and thrombocytopenia are often present in their CBC findings (9).

2.1.4.2 Neoplastic infiltration involvement

Lymphadenopathy, hepatomegaly, and splenomegaly are frequent and commonly painless (10). Enlargement of the kidneys can be seen in 30-50% of pediatric patients without having therapeutic and prognostic implications (9). Also, in rare cases, enlargement of the pancreas has been reported as a result of leukemic cells infiltrating the pancreas (41).

2.1.4.3 Central nervous system (CNS) manifestation

Meningeal spread may present with headaches, vomiting, and nerve palsies. CNS leukemia can be detected by morphological examinations of cerebrospinal fluid (CSF). Approximately 3-5% of ALL childhood patients show signs of CNS leukemia at initial diagnosis and 30-40% of patients at relapse (42).

2.1.4.4 Skeletal manifestation

Infiltration of bone marrow may appear as bone pain that markedly affects the long bones. This presents with a limp or refusal to walk. Bone tenderness is

mostly observed. At ALL diagnosis up to 25% of pediatric patients have radiographic abnormality osteopenia, and fractures are observed in 10% (43).

2.1.4.5 Genital system involvement

Painless testicular scrotum enlargement can be an indication of testicular leukemia or hydrocele caused by lymphatic blockage (24). The involvement of the uterus and cervix with ALL is extremely uncommon, and ovarian involvement is even rarer (44).

2.1.4.6 Gastrointestinal tract manifestation

Gastrointestinal manifestations in childhood ALL is present in one-fourth of patients at autopsy and usually during relapse. Patients with leukemic infiltrates are frequently asymptomatic or have non-specific and vague complaints. The most common symptoms are probably abdominal pain, diarrhea, nausea, vomiting, or gastrointestinal bleeding (45).

2.1.4.7 Mediastinal manifestation

Patients with precursor T-cell ALL frequently affect the thymus and appear with a mediastinal mass with or without associated pleural effusions. This results in respiratory distress and other signs of superior vena cava syndrome (46). About one-half of children with T-cell ALL have mediastinal masses (24).

2.1.4.8 Biochemical abnormalities

Frequent breakdowns of leukemic cells cause hyperuricemia (47). Hyperkalemia is often associated with massive cell lysis (48). Hyperphosphatemia with secondary hypocalcemia is often observed at diagnosis (49). High serum levels of lactate dehydrogenase (LDH) are detected at ALL diagnosis and relapse due to rapid cell turnover, and it is reported to be normalized during remission (24).

2.1.5 Diagnosis of ALL

The initial step in diagnosing childhood ALL begins with patient history and physical examination. After evaluation of the chief complaint and symptoms, several diagnostic tests and clinical procedures are ordered to approach the diagnosis of ALL, as formulated below:

- Complete Blood Count (CBC) with differential and peripheral blood smears are quick and inexpensive investigations to evaluate the cellular constituents and examine abnormalities (50).
- Bone marrow aspiration is performed for a definitive diagnosis of acute leukemia. It is usually operated under general anesthetic in children. Bone marrow aspirate assesses the cellularity, morphological, immunological, cytogenic, and enumeration of blast cells and mature cells. During treatment, BM aspiration is also performed to establish a response to treatment by the determination of minimal residual disease (MRD) (51). The standard criteria to confirm ALL diagnoses requires a minimum of 20% blast cells (48).

- Bone marrow trephine biopsy is performed when BM aspiration is not possible in a case with compact bone marrow and a ‘dry tap’ due to marrow fibrosis, infarction, or necrosis. It is also used as a diagnostic test to show the degree of disruption in bone marrow integrity as a result of leukemia development (52, 53).
- Cytochemical special stains are used to identify cell lineages (lymphoid, myeloid), such as Periodic Acid Schiff (PAS), Sudan Black B (SBB), and Myeloperoxidase (MPO) (54).
- Histochemistry and flow cytometry immunophenotyping studies are performed for BM aspirates and peripheral blood samples using a group of monoclonal antibodies to assign acute leukemia lineage (55).
- Cytogenetic and molecular studies are performed to identify specific genetic abnormalities using karyotyping, qPCR, FISH, or SNP array analysis (56).
- A lumber puncher test is performed for CSF cytologic analysis. Depending on CSF results, CNS involvement in childhood ALL (42) is classified as follows:

  CNS-1: less than 5 WBC/mm^3, and no blasts detected in the CSF.

  CNS-2: less than 5 WBC/mm^3, and blasts detected in the CSF.

  CNS-3: more than 5 WBC/mm^3, and blasts detected in the CSF or cranial nerve involvement or presence of cerebral mass. CSF with more than 10 RBC/mm^3 with or without blasts, is defined as a traumatic lumbar puncture (TLP). The following formula is used to define the presence of CNS leukemia:

if the ratio of CSF WBC/CSF RBC is greater than Blood WBC/Blood RBC then, CNS involvement in ALL is present (48).

- Chemistry assays are performed to detect liver and kidney problems produced by leukemic cell dispersal or the adverse effects of chemotherapy treatments.
- Ultrasound sonography and radiography are performed to check the presence of mediastinal mass, cardiomegaly, and hepatosplenomegaly (57).
- Electrocardiography (ECG) and echocardiogram are performed to check cardiac function (58).
- Tissue Human Leukocyte Antigen (HLA) typing is performed when a donor stem cell transplant is a part of ALL treatment (59).
- Cytomegalovirus antibody titer, hepatitis virus screen, and HIV screen are performed to detect infections (60, 61).
- Coagulation tests are performed to check blood is clotting properly (62).

2.1.6 Prognostic factors and risk stratification of childhood ALL

Several clinical and laboratory-based features were identified to have prognostic value in response to therapy (63). Patient age at diagnosis, initial WBC count, gender, immunophenotype, genetic alterations, and early responsiveness to induction therapy has been identified as highly important factors to assess prognosis and define risk group in childhood ALL **(Table 2.2)**. Several criteria have been defined to classify risk stratifications by some cooperative groups. For example, a set of risk criteria was constructed in 1993 by the Pediatric Oncology

Table 2.2. Prognostic factors in pediatric ALL.

Prognostic factors	Favorable	Unfavorable	Reference
Age (year)	1 to 9	<1 or ≥10	(64)
WBC count ($\times 10^9$/L)	<50	>50	(65)
Gender	Female	Male	(14)
Ethnicity	White	Black	(66)
Immunophenotype	B-cell lineage	T-cell lineage	(67)
Genotype	Hyperdiploidy>50 ; *ETV6-RUNX1* t(12;21)(TEL-AML1) Trisomies 4,10,17	Hypodiploidy<44 ; t(9;22)*BCR-ABL1* t(4;11)*MLL-AF4* t(17;19) *TCF3-HLF*	(68)
CNS involvement	CNS1	CNS3	(69)
Testicular Enlargement	Absent	Present	(70)
Time to remission	<14 days	>28 days	(65)
MRD at end of induction	<0.01%	≥0.01%	(71)

Group (POG) and Children's Cancer Group (CCG) (72). Their criteria defined standard risk as (a WBC count less than 50,000/µl and patients aged 1 to <10 years), and high-risk (all other ALL patients including T-ALL regardless of WBC count or age). The National Cancer Institute (NCI) risk group classification, defined standard-risk as (WBC count less than 50,000/µl and age less than 10 years) and high-risk (WBC count more than 50,000/µl and/or age 10 years or older) (73). Pediatric ALL cases most often are classified into standard-, medium-

, and high-risk stratify groups (9). Based on known prognostic factors, pediatric oncologists stratified patients into various risk groups. Lower-risk group, a favorable outcome, can be treated less intensively to reduce late side effects of treatment toxicities. Higher-risk group, unfavorable outcome, targeted with more aggressive and diverse types of therapies (74).

2.1.7 Treatment of ALL

Childhood ALL treatment protocols generally involve three main phases: induction, consolidation, and maintenance; including CNS prophylaxis therapy and intensive supportive care. The protocols include an intensive combination of chemotherapy regimens and it may be supplemented with hematopoietic stem cell transplantation and/or radiation therapy. These treatments take two to three years based on early therapy outcomes, the intensity of the current protocol, and the analysis of prognostic factors. Cure rates are more than 80% in pediatric patients at five-year survival rates (11, 75, 76).

2.1.7.1 Induction therapy

Remission induction is the first phase of chemotherapy, in which nearly all leukemic cells in the bone marrow are rapidly eradicated to reduce tumor burden and restore normal hematopoiesis. Patients in complete remission are defined as the bone marrow of normal cellularity, with less than 5% of lymphoblasts present, and CBC count within the normal range (77). The backbone of the induction regimen typically includes vincristine, asparaginase, and a glucocorticoid. For the

high-risk group, an additional fourth drug, anthracycline is added to the dose regimen to reduce bone marrow relapse (75, 78). On the day 29th of induction, ALL patients are tested for minimal residual disease (MRD). Based on the results of the MRD test and cytogenetic risk group, post-induction treatment is then introduced (79, 80).

2.1.7.2 Consolidation therapy

After the achievement of complete remission, the second phase of treatment for ALL patients is consolidation or intensification therapy. This additional therapy is to eradicate any remaining drug-resistant leukemic cells, which lowers the chance of relapse and survival improved. The patients receive the same drug schema used in the induction phase in combination with different drugs depending on the risk group assignment, the day 29 MRD result, the absolute neutrophil count, the platelets count, and their body mass index (BMI). The duration of the treatment and combination of the drugs considerably vary among patient populations (65). The most widely used consolidation schema is the Berlin-Frankfurt-Munich (BFM) protocol. The treatment of standard-risk patients in the consolidation phase includes the administration of cyclophosphamide, cytarabine, 6-mercaptopurine, and methotrexate. The treatment of high-risk patients receives additional regimens of asparaginase and vincristine (57, 81). Delayed intensification is introduced to all patients or

patients with higher risk. It improves the outcome and reduces the risk of relapse (82, 83).

2.1.7.3 Maintenance therapy

Maintenance or continuation therapy is performed in low intensity to prevent relapse and to further reduce nondetectable residual leukemic cells after consolidation therapy. The patients receive daily oral 6-mercaptopurine, weekly methotrexate, periodically corticosteroids, and vincristine for two to three years. The effectiveness of this phase of treatment is determined by the metabolism of 6-mercaptopurine to 6-thioguanine (57, 84). The metabolites of 6-mercaptopurine are quantified. The pediatric patients on continuation therapy are carefully monitored to address the related chemotherapy toxicity and compliance issues (85, 86).

2.1.7.4 CNS-directed therapy

At the time of diagnosis, leukemic CNS involvement is infrequent; it is seen in 3-7% of the patients and more than half of the cases in the absence of CNS-directed therapy. CNS provides a pharmacological sanctuary site for the leukemic cells, which are undetected at diagnosis and systemic chemotherapy cannot readily access them because of the blood-brain barrier. Specific CNS prophylaxis is introduced early in the protocols, to eradicate clinically CNS disease at the time of diagnosis and to avoid the risk of CNS relapse (87). CNS-directed therapies usually involve high-dose systemic chemotherapy, intrathecal

chemotherapy (87, 88), and cranial irradiation in a small subgroup of children with overt CNS (89). The suggested radiation dose is highly dependent on the systemic chemotherapeutic intensity. Treatment with cranial irradiation of 18 Gray is typically warranted in patients with CNS-3 at diagnosis. Even in the absence of cranial irradiation, intensified CNS-directed chemotherapy is related to attentional dysfunction in survivors of childhood ALL (90). CNS-directed chemotherapy carries the chance of secondary CNS neoplasms, seizures, encephalopathy, and neurocognitive toxicities that probably cause continual impairments in intelligence, memory, processing speed, attention, and administrative functions (91, 92).

2.1.7.5 Supportive care

Enhanced intensive supportive care is crucially important for ALL patients and it contributes to achieving complete drug dose, reducing chemotherapy-related toxicities, and improving survival rates (93). Supportive care mostly involves infection control (94, 95), management of tumor lysis syndrome (96-98), management of thrombosis (99, 100), management of thrombocytopenia and anemia (101-104), and hematopoietic stem cell transplantation (HSCT) therapy (105-107).

2.2 Human kallikrein-related peptidases

The human tissue kallikrein and kallikrein-related peptidases (KLKs) consist of a single family of fifteen homologous, highly conserved, secreted trypsin- or chymotrypsin-like serine proteases (108). Kallikrein was first isolated in high concentrations from pancreatic extracts by Werle and colleagues in the 1930s, and the term originated from the Greek word (*kallikreas*) which means the pancreas (109). Tissue and plasma kallikreins are two distinct types of kallikreins that have different molecular weights, gene structure, substrate specificity, immunological features, and the type of kinin produced from kininogens (110). Kallikreins are present in diverse tissues and bodily fluids acting as enzymes cleaving peptide bonds (111). Tissue KLKs have a wide spectrum of important roles in normal and pathophysiological processes including kinin formation, skin desquamation, blood pressure control, semen liquefaction, tissue remodeling, electrolyte balance, and prohormone processing (112). Plasma kallikrein is a glycoprotein encoded by the *KLKB1* a single gene mapped on human chromosome 4q34-35. It is only expressed by the liver cells, then it is secreted into the blood system (113, 114). The gene consists of 15 exons and encodes an inactive enzyme (114). The enzyme is then activated by the coagulation factor XII. High molecular weight kininogen is cleaved by plasma kallikrein, releasing the bioactive peptide bradykinin. (115, 116). Plasma kallikrein works as a mediator of inflammatory reactions, blood clotting, fibrinolysis, blood pressure, and bradykinin secretion (110, 115, 117).

2.2.1 Gene family of kallikrein-related peptidases

Human kallikrein-related peptidases present the largest uninterrupted cluster of serine proteases in the human genome. They are coded for by a family of fifteen functional genes clustered contiguously on chromosome 19 located at q13.3-13.4 (118-120). The human *KLK* locus is attached centromerically by the testicular acid phosphatase gene (*ACPT*), and telemetrically by a cancer-associated gene (*CAG*), and *Siglec-9* belongs to the SIGLEC (Sialic acid-binding Ig-like lectin) family (121, 122). A genetic map of the human *KLK* locus is illustrated in **(Figure 2.2)**.

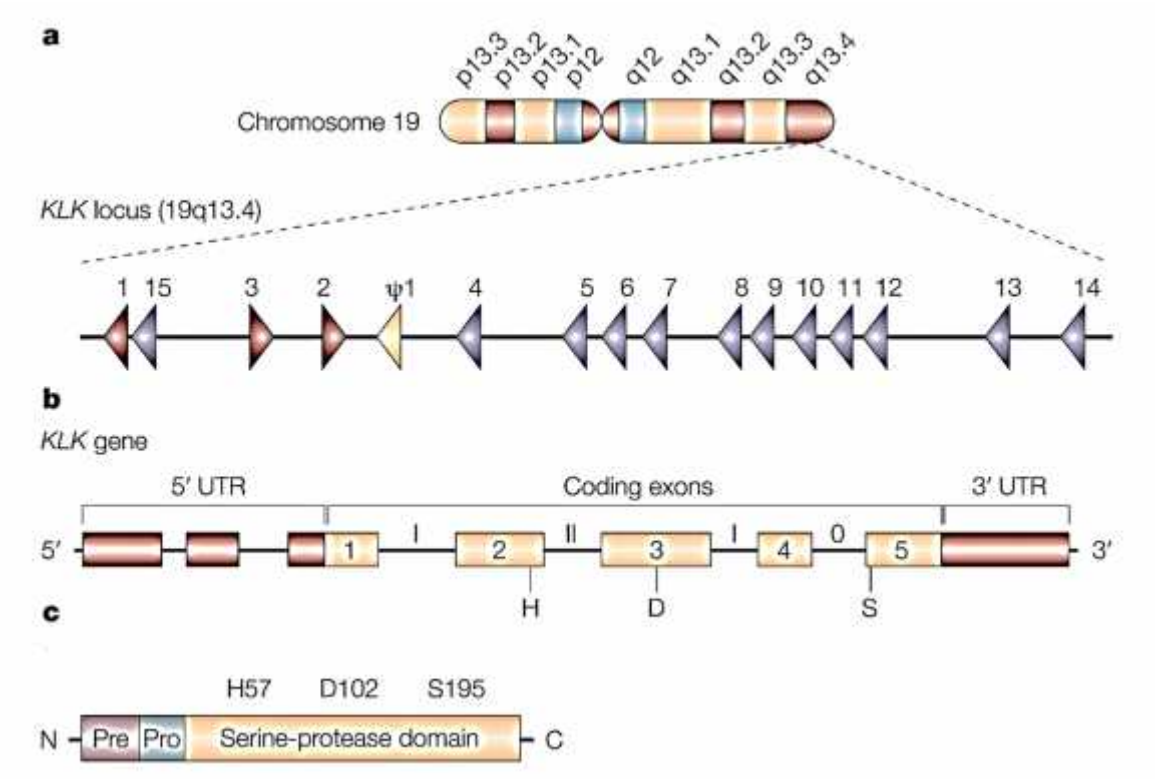

Figure 2.2. The human kallikrein locus, gene, and protein features.

Schematic representation of (**a**) The human *KLK* gene cluster found at chromosome 19q13.4 and the corresponding (**b**) gene, and (**c**) protein structure. This figure is adapted from (123).

The classical kallikreins are *KLK1* (tissue kallikrein), *KLK2* (glandular kallikrein), and *KLK3* (PSA), which were the first three genes discovered in the human kallikrein locus (124). *KLK4* to *KLK15* represent the rest of the kallikrein gene family, which was identified in the late 1990s. (125). Proteases KLK2-15 are formally named kallikrein-related peptidases, which are without confirmed kininogenase activity (119). The members of the human kallikrein gene family are compared below (120, 125):

- All genes of the family colocalize to the same chromosomal regions (19q13.3-13.4) in a linear arrangement.
- Serine proteases with a conserved catalytic triad (histidine, aspartic acid, and serine) are encoded by all genes.
- Although the family's genes all have five coding exons, certain members have one or more 5´ untranslated exons.
- The coding exons are close in size or identical.
- All members of the gene family have entirely conserved intron phases.
- At the DNA and amino acid levels, all of the family's genes exhibit strong sequence homologies (40-80 percent).
- Most KLKs in the family are controlled by steroid hormones including androgen, estrogen, and progestin in the body tissues (126, 127).
- Epigenetic-related processes including DNA methylation, histone modification, and miRNA-mediated modulation of mRNA levels have been

demonstrated to be involved in the transcriptional and post-transcriptional regulation of KLKs (128-130).

- Furthermore, it has been established that single nucleotide polymorphisms (SNP) affect the KLKs' mRNA and protein expressions or could adjust the proteolytic activity of the resulting KLK proteases (131).

2.2.2 Protein structure of kallikrein-related peptidases

Kallikrein-related peptidases are a subclass of secreted serine endoproteases within the S1 family of clan SA (132). KLKs are translated as a single chain of prepro-enzymes with varying lengths that share approximately 40% protein identity (133). The prepro-KLKs are proteolytically fragmented upon production from the secretory pathway at the amino-terminal signal peptide (134). Once secreted, the pro-KLKs are still inactive, and further processed via cleavage of the N-terminal propeptide by other KLKs or proteases to become active extracellular peptidases (133, 135). Activated KLKs function to cleave bonds within polypeptide chains with three conserved catalytic residues; always occurring at the position of His^{57}, Asp^{102}, and Ser^{195} (standard chymotrypsin numbering) that span the active site, histidine near the end of the second coding exon, aspartic acid in the middle of the third coding exon, and serine at the beginning of the fifth coding-exon **(Figure 2.2)** (136). All KLK proteins consist of ten fully conserved cysteine residues forming five disulfide bonds (136). An additional pair of cysteine residues occur in KLK4-12 and KLK15 which is

unique for kallikreins compared with other S1A peptidases. Kallikrein substrate specificity is determined by residue 189, which is placed at the base of the substrate-binding pocket (137). The substrate-binding pocket's amino acid could be aspartate, exhibiting trypsin-like specificity cleaving after arginine or lysine residues as occurring in all of KLK 1, 2, 4, 5, 6, 8, 10, 12, and 13 (138). KLK15 possesses trypsin-like specificity, except with Glu^{189} (139). For tyrosine, leucine, and phenylalanine residues, both KLK3 and KLK7 possess chymotrypsin-like specificity. They are with Ser^{189} and Asn^{189} residues, respectively (140). KLK9 possesses chymotrypsin-like specificity which has a Gly^{189} residue that is likely responsible for the inability of KLK9 to hydrolyze the pro-KLK sequences (139). KLK 11 and KLK 14 have both trypsin-like and chymotrypsin-like activities. Hydrolysis of the peptide bond is initiated when the hydroxyl oxygen atom of the catalytic Ser^{195} attacks the carbonyl of the substrate peptide bond utilizing His^{57} as a general base (141). All kallikreins include a highly conserved Gly^{193}, except for KLK10, which contains serine instead. (142). During hydrolysis, Gly^{193} is involved in stabilizing oxyanion intermediate of the internal peptide bond (143). It appears that KLK10 lacks proteolytic activity against traditional substrates due to the absence of Gly^{193} in its structure (139, 144). The proteolytic activity of KLKs is controlled by means of proenzyme activation, inactivation through internal fragmentation, and/or complex development with endogenous plasma and tissue inhibitors (145).

2.2.3 (Patho)physiological expressions and clinical relevance of kallikrein-related peptidases

KLKs have diverse expression profiles and are found as bioactive components in various tissues and biological fluids that are crucial in the regulation of basic physiological functioning **(Figure 2.3)**. The aberrant expression of a variety of kallikrein-related peptidases has been linked to a range of diseases and cancers. KLKs play a crucially important role as biomarkers, and PSA screening test is the best example of the clinical utilities of the family.

KLK1 is mostly produced in the pancreas, salivary gland, and kidney (146). By cleavage of low molecular weight kininogen, KLK1 releases kinin. This involves blood pressure regulation, pain induction, smooth muscle contraction, electrolyte balance, vascular permeability, neutrophil chemotaxis, and inflammation (147, 148). Besides, KLK1 involves in releasing nitric oxide, reducing oxidative stress, processing growth factors, and peptide bonds (147, 148). It was found that KLK1 was correlated with gastrointestinal stromal tumor invasion (149), and coronary artery disease (150).

Both KLK2 and KLK3 are extremely expressed in the prostate and seminal plasma (151). They have a role in seminal clot liquefaction and spermatozoa release through hydrolysis of seminogelin Sg-I and Sg-II, and the primary structural gel-forming proteins in human semen, fibronectin, is generated by seminal vesicles essential for sperm motility (152, 153). KLK3, commonly

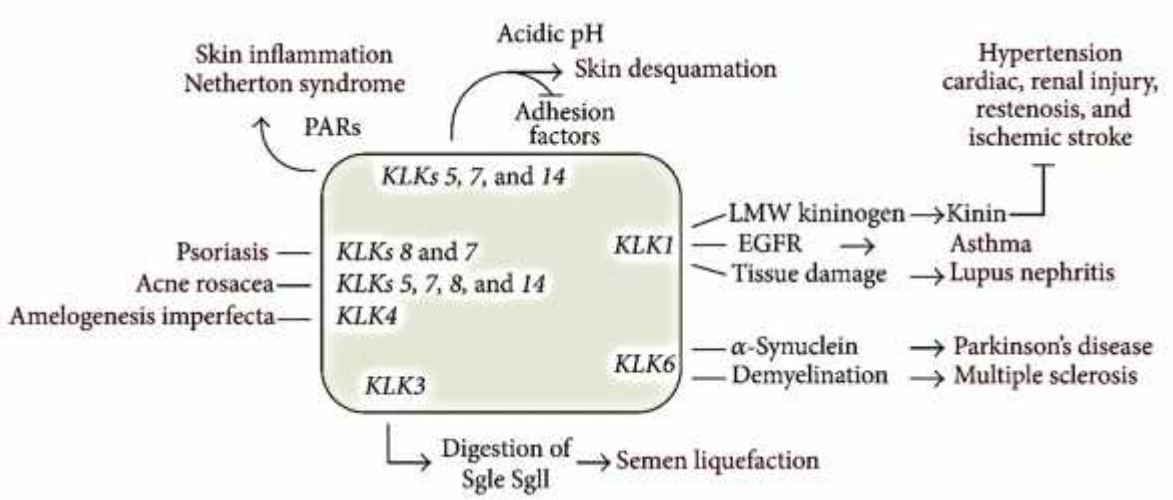

Figure 2.3. The functions of kallikreins.

Schematic representation of some functions of KLKs: They involve several normal physiological and pathological conditions. This figure is adapted from (154).

known as the prostate-specific antigen (PSA), along with KLK2 is controlled by androgens and is predominantly expressed in prostate and breast malignancies. They are biomarkers for both cancers' diagnosis, prognosis, and monitoring (5, 7, 124, 155-158).

KLK4 was originally designated as enamel matrix serine protease 1(EMSP1) and was shown a role in cleaving enamelin (159). KLK4 involves amelogenesis, namely the formation of tooth enamel to achieve a high degree of mineralization (160). KLK4 is expressed in many tissues and predominately in the prostate. KLK4 is overexpressed in prostate cancer and ovarian cancer tissues (161, 162). KLK4 overexpression was also observed in colorectal cancer and its mRNA expression in colorectal adenocarcinoma was reported to be clinically

relevant as a poor prognostic indicator for unfavorable disease-free survival (163).

KLK5 expression is abundant in diverse human tissues, its mRNA is highly expressed in skin, breast, testis, ovary, salivary gland, and esophagus (145). KLK5 is a major proteinase along with KLK7, and KLK14 involving skin desquamation by degrading desmosomal adhesion proteins in the outermost layer of the skin (164-167). Dysregulation of KLK5 expression was found in ovarian, breast, prostate, and testicular carcinomas (168-171). It was reported that KLK5 is a potential tumor biomarker in endocrine-related malignancies correlated with poor prognosis in ovarian and uterine cervical malignancies (172). Differential expression of KLK5 is also found in hormone-independent cancers such as lung and bladder carcinomas (168).

KLK6 is highly produced in healthy tissues of the CNS, kidney, pancreas, endometrium, mammary, and prostate (173). *KLK6* mRNA expression is differentially regulated and contributed as an unfavorable prognostic tumor marker depending on the cancer grade. It is downregulated in breast cancer (174), upregulated in colorectal adenocarcinoma (175), and endometrial carcinoma (176). In 2016, *KLK5-9* transcripts were analyzed in distinct cancerous human tissues including leukemia via nested reverse transcription PCR (177). High serum levels of KLK6 protein were observed in ovarian cancer (178, 179), uterine serous papillary carcinoma (180, 181), psoriasis (182), Alzheimer's disease

(183), and multiple sclerosis (184), and KLK6 was found to contribute in their pathogenesis.

KLK10 is expressed in the skin, tonsils, brain, pancreas, esophagus, and sex organs (185). KLK10 expression is hormone-regulated and tissue-specific (186, 187). KLK10 is aberrantly expressed in pancreatic cancer and hormone-dependent malignancies. *KLK10* represents a potential tumor suppressor gene, and its expression is downregulated in ALL, breast, prostate, and testicular malignancies (188-192). KLK10 possesses as a biomarker in the diagnosis and prognosis of pancreatic, colorectal, and ovarian cancers (193-195).

KLK14 is expressed in the CNS, skin, breast, prostate, testis, bone marrow, lymph node, colon, skeletal muscle, and lung (196-198). It is regulated by steroid hormones (199, 200). KLK14 has a role in the control of activation and/or inactivation of some kallikreins including KLK1, KLK3, KLK5, and KLK11 (201, 202). KLK14 also involves skin desquamation, seminal clot liquefaction, cancer growth, invasion, and angiogenesis (164, 201-204). This gene has been examined as a potential tumor marker; downregulated at the mRNA level in breast, prostate, and testicular malignancies (197). Also, its upregulation at the mRNA level can be considered a poor prognostic biomarker for patients with colorectal cancer; have discriminatory power between colorectal cancer and adenoma patients (205). **Table 2.3** demonstrates the evidence of deregulation of *KLK6*, *KLK10*, and *KLK14* in several cancers and diseases (154).

Table 2.3. Deregulation of *KLK6*, *KLK10*, and *KLK14* in cancers and diseases.

Disease	Kallikrein	Factor	Observation
Alzheimer's disease	*KLK6* (CSF), *KLK10* *KLK6* (brain, blood)	Increased expression Decreased expression	
Aneurism	*KLK6*	Decreased expression	Suggestion of unfavorable prognosis
Multiple sclerosis	*KLK6*	Increased expression	Advanced disease
Dementia with Lewy bodies	*KLK6*	Decreased expression	Suggestion of diagnostic marker
Psoriasis	*KLK6, KLK10*	Increased expression	Severity of skin lesions
Parkinson's disease	*KLK6*	Increased expression	Disease-associated marker
Breast cancer	*KLK10, KLK14* *KLK6* *KLK10*	Increased expression Increased expression Promoter methylation	Potential diagnostic biomarkers Suggestion of favorable prognosis
Colorectal cancer	*KLK6, KLK10*	Increased expression	Suggestion of unfavorable prognosis
Gastric cancer	*KLK6, KLK10*	Increased expression	Suggestion of unfavorable prognosis
Head and neck cancer	*KLK10* *KLK6, KLK10*	Promoter methylation Increased expression	Suggestion of unfavorable prognosis
Lung cancer	*KLK10* *KLK6* *KLK14*	Promoter methylation Increased expression Increased expression	 Suggestion of unfavorable Prognosis Diagnostic marker
Melanoma	*KLK6*	Increased expression	
Ovarian cancer	*KLK6* *KLK10, KLK14* *KLK10*	Increased expression Increased expression SNP	Advanced stage Suggestion of favorable prognosis
Prostate cancer	*KLK10* *KLK14*	SNP SNP	Suggestion of unfavorable prognosis
This table is adapted from (154).			

CHAPTER THREE

Materials and Methods

Chapter Three: Materials and Methods

3.1 Laboratory equipment, kits, and reagents

All instruments and tools used in this study are listed in **(Appendix A)**. The kits and reagents used in this study are listed in **(Appendix B)**.

3.2 Study design and ethical consideration

This study was a prospective, analytical, observational, and case-control study. The population involved in this study was male or female children aged 1-15 years. The participants involved in the study were admitted to the Pediatric Department in Hiwa Cancer Hospital in Sulaimani Governorate in Kurdistan/ Iraq and they were newly diagnosed with acute lymphoblastic leukemia (ALL). Also, healthy pediatric volunteers were selected as control individuals. The sample collection and molecular biology work, including the patients' follow-up started in June 2018 and was completed in January 2020.

The research proposal was approved by the Research Ethics Committee of the University of Sulaimani's College of Medicine (approval number 55 on September 17th, 2017), the Directorate of Health in Sulaimani Governorate, and the Scientific Committee at Hiwa Hospital **(Appendix C)**. The research and practical work of the present study was conducted at Kurdistan Institution for Strategic Studies, Molecular Biology Laboratory in Hiwa Cancer Hospital, and Bakhshin Hospital.

3.3 Enrollment and consent

A total of twenty-three newly diagnosed ALL patients and twelve healthy pediatric volunteers were qualified for the inclusion criteria of the current study.

The guardian of all of the recruited individuals was requested to complete an informed consent form in writing voluntarily before they were engaged in the research. The purpose, procedure, and benefits of the study were also explained to the participants and their families **(Appendix D)**. All participants have gone through a standardized interview process and all demographic information was collected on a form specially designed for the study, including sex, age, height, weight, and medical history and they received more information about the study protocol **(Appendix E)**.

3.4 Inclusion and exclusion criteria

Inclusion criteria for the patients:

- Newly diagnosed ALL patients and admitted to Pediatric Department in Hiwa Cancer Hospital. Clinical diagnosis of ALL cases was established by bone marrow examination and cell Immunophenotyping.
- Male or female pediatric participants.
- Accept to sign an informed written consent form, willing to participate in and comply with the study.

Exclusion criteria for the patients:

- A known malignancy and/or a hematological disorder other than ALL.

Inclusion criteria for the control group:

- Male or female healthy pediatric volunteers. They were examined by specialist physicians, and their status was confirmed clinically by laboratory examination results.
- Accept to sign an informed written consent form, willing to participate in and comply with the study.

Exclusion criteria for the control group:

- A known malignancy and/or a hematological disorder.

3.5 Sample collection

Two milliliters of venous blood were obtained from each patient's cubital vein using disposable syringes. The drawn blood was collected in a lavender tube, with potassium EDTA content. The samples were transported to Kurdistan Institution for Strategic Studies in a cool box. They were instantly used for molecular biology work. Blood samples were collected from leukemic patients at three different times: when diagnosed with ALL, after one month of receiving chemotherapy, and after three months of receiving chemotherapy. Diagnosis of childhood ALL cases was established by bone marrow examination and cell immunophenotyping. ALL patients were treated according to UKALL Interim Guidelines (206).

3.6 RNA extraction

The RNA was extracted using a Prime Prep™ Blood RNA Extraction Kit (GeNet Bio, Daejeon, South Korea) according to the manufacturer's instructions. This kit extracts total cellular RNA from whole blood. Contaminants and enzyme inhibitors such as heparin and hemoglobin were entirely removed. The wash buffers were made following the user's guide before commencing the experiment, by the addition of 20 ml and 44 ml of absolute ethanol to Buffer BRW1 and Buffer BRW2, respectively. A 200 µl of whole blood with 1 ml of Buffer BRR in a 1.5 ml eppendorf microcentrifuge tube was mixed. The tube was incubated on ice for 15 min until the cloudy mixture became translucent which indicates lysis of erythrocytes. The tube was vortexed twice during incubation. The tube was centrifuged at 3500 rpm for 15 min at 4°C. After centrifugation leukocytes formed a pellet and the supernatant was smoothly removed using micropipette. A 400 µl of Buffer BRR was added to the cell pellet and resuspended by pulse vortexing. The tube was centrifuged at 3500 rpm for 10 min at 4°C and the supernatant was completely removed using a micropipette.

Then, 350 µl of Buffer BRL and 3.5 µl of β-mercaptoethanol (β-ME, 14.2 M) were added to the pelleted leukocytes and mixed by vortexing for 15 sec. The lysate was transferred into Spin Column 1 (blue O-ring) placed in a 2 ml collection tube, and centrifuged for 2 min at 14000 rpm. After centrifugation, the flow-through was carefully transferred onto a new 1.5 ml eppendorf tube. After

that, 350 µl of 70% ethanol was added to the homogenized lysate. The tube was centrifuged at 3,500 rpm for 20 sec at 20°C.

Up to 700 µl of the mixture was transferred to Spin Column 2 and collected in a 2 ml tube, centrifuged at 14000 rpm for 1 min at 20°C and the pass-through was discarded. Spin Column 2 was reinserted onto the same collection tube. To wash the membrane, 700 µl of Buffer BRW1 was introduced to Spin Column 2 and centrifuged at 14000 rpm for 1 min at 20°C. The Spin Column 2 was carefully reinserted onto a clean collection tube. For another wash of the membrane, 500 µl of Buffer BRW2 was added to Spin Column 2, centrifuged at14000 rpm for 1 min at 20°C. The pass-through was removed and the Spin Column 2 was reinstalled in the same collecting tube. For further washing, this step was repeated twice. It was then centrifuged at 14000 rpm for 2 min at 20°C to remove residual wash, so during RNA elution, the membrane of the spin column dried, and no ethanol was carried over.

To elute the RNA, a 1.5 ml nuclease-free collection tube was inserted into Spin Column 2. In Spin Column 2, 40 µl of Buffer BRE was introduced to the membrane's center and left at room temperature for 2 min. At 20°C, it was centrifuged for 2 minutes at 14000 rpm. The purified RNA samples were then aliquoted and stored at (-20°C) for immediate use, and for long-term storage, it was kept at (-70°C).

3.7 Checking RNA integrity

The quantity, quality, and integrity of extracted RNA were evaluated. The concentration of RNA from all the samples was measured using Eppendorf Biophotometer, following the operating manual. A clean, dry, new cuvette was chosen to measure blank (zero absorbance) using 50 µl nuclease-free water. Then, the RNA sample's concentration was measured in µg/ml using a 5 µl RNA sample and 45 µl nuclease-free water. For gene expression analysis, only RNA samples with an absorbance ratio A260/280 greater than 1.8 and an A260/230 ratio of nearly 2 were selected (207). The integrity and quality of the RNA samples were examined on 1.5% agarose gel electrophoresis visualized by 1.0 % ethidium bromide. Two ribosomal RNA bands appeared, one of 18S rRNA and the other of 28S rRNA. The band of 28S rRNA had higher intensity than 18S rRNA (208). This gave a clue about the integrity of the RNA samples, and those with significant degradation were discarded (209).

3.8 cDNA synthesis

The RNA samples were reverse transcribed into a first-strand complementary DNA (cDNA) before PCR amplification, using 2X SuPrimeScript RT Premix (SR-3000) kit, following the manufacturer’s instructions **(Table 3.1)**. Also, a “no template control” NTC tube was prepared per experiment, which excludes the RNA template; instead 9 µl DEPC-treated water was included in the total.

Table 3.1. Reverse-transcription reaction components.

Reaction Components	Volume
SuPrimeScript RT Premix (2X)	10 µl
Oligo(dT)$_{18}$ primer (100 pmol/ µl)	1 µl
DEPC-treated H_2O	1 µl
Total RNA	8 µl
Total Reaction Volume	20 µl

Oligo(dT)$_{18}$ Primer is an 18-mer single-stranded oligonucleotide with 5' and 3' hydroxyl tails produced for use as a primer for cDNA synthesis.
The dNTP (Deoxynucleotide) mix is a pre-mixed solution that contains four nucleotides: dATP, dCTP, dGTP, and dTTP.
RNase-free DEPC-treated H_2O (diethyl pyrocarbonate-treated water) is used to minimize the risk of RNA degradation by RNases.

The thermal cycler instrument (Applied Biosystems, USA) was programmed as follows: stage 1; heating at 50°C for 60 min, stage 2; heating at 70°C for 10 min, then holding at 4°C **(Appendix F)**. Following this, the tubes were inserted into the thermal cycler and the cycling program started to perform cDNA synthesis. The cDNA samples were then aliquoted and kept at (-20°C) for immediate use and it was stored at (-70°C) for long-term storage.

3.9 Primers

In the present study, the used oligonucleotide primers of *KLK6* (210), *KLK10* (193), *KLK14* (211), *GAPDH* (212), and *ACTB* (213) **(Table 3.2)** were obtained from published articles. The primers were synthesized by (Macrogen, Seoul, South Korea).

Table 3.2. Primers used in PCR amplifications.

Primer Name	NCBI Reference Sequence	Primer sequence forward/reverse (5′--3′)	Product Size bp	Type of PCR
KLK6 ***(homo sapiens)***	**NM_001319949.1**	**Forward: GAAGCATAACCTTCGGCAAA** **Reverse: GGGAAATCACCATCTGCTGT**	**237**	Quantitative real-time PCR
KLK10 ***(homo sapiens)***	**NM_001077500.1**	**Forward: TCTACCCTGGCGTGGTCACC** **Reverse: GCAGAGCCACAGGGGTAAACAC**	**148**	Quantitative real-time PCR
KLK14 ***(homo sapiens)***	**NM_022046.4**	**Forward: GGTCATCACTGCTGCTCACT** **Reverse: GTGGGTCCGGGAGTTGTAGTT**	**142**	Quantitative real-time PCR
GAPDH ***(homo sapiens)*** ***"housekeeping"***	**NM_001289745.2**	**Forward: ATGGGGAAGGTGAAGGTCG** **Reverse: GGGTCATTGATGGCAACAATATC**	**107**	Quantitative real-time PCR
ACTB ***(homo sapiens)*** ***"housekeeping"***	**NM_001101**	**Forward: ATCTGGCACCACACCTTCTACAATGAGCTGCG** **Reverse: CTCATACTCCTGCTTGCTGATCCACATCTGC**	**837**	Conventional PCR

3.10 Conventional RT-PCR

To validate the process of reverse transcription of RNA and the quality of cDNA, conventional RT-PCR was carried out using *ACTB* primers and OnePCR™ Ultra (PCR Master Mix) kit (GeneDireX, Inc., US) according to **(Table 3.3)**. A "NTC" tube was prepared per experiment.

Table 3.3. Conventional RT-PCR reaction components.

Reaction Components	Volume	Final concentration
OnePCR™ Ultra Premix (2X)	10 µl	1X
Forward primer (10 pmol/ µl)	1 µl	0.5 µM
Reverse primer (10 pmol/ µl)	1 µl	0.5 µM
DEPC-treated D.W.	5 µl	
cDNA Template	3 µl	
Total Reaction Volume	20 µl	

The thermal cycler (Applied Biosystems) was programmed based on the cycling conditions **(Appendix G)**: stage 1; initial denaturation, heating at 94°C for 5 minutes, stage 2; amplification stage, heating at 94°C for 1 minute (denaturation step), then heating at 56°C for 1 minute (annealing step), next heating at 72°C for 1 minute (extension step), stage 2 was repeated for 35 cycles, after that stage 3; final extension for 10 minutes then holds on 4°C. The tubes were inserted into the thermal cycler and the cycling program started to perform PCR. The PCR products were stored at (-20°C). The target sequence's amplification was affirmed using 1.5% agarose gel electrophoresis.

3.11 Real-time PCR (qPCR)

The real-time quantitative PCR (qPCR) was performed using the Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to **(Table 3.4)**. A "NTC" tube was prepared per experiment. The Rotor-Gene Q was programmed following the cycling conditions in **(Table 3.5)**.

Table 3.4. Real-time PCR reaction setup.

Reaction Components	Volume/reaction	Final concentration
Rotor-Gene SYBR Green PCR Master Mix (2X)	10 µl	1X
Forward primer (10 pmol/ µl)	2 µl	1 µM
Reverse primer (10 pmol/ µl)	2 µl	1 µM
RNase-free water	4.5 µl	
cDNA Template	1.5 µl	≤100 ng/reaction
Total Reaction Volume	20 µl	

Table 3.5. Real-time PCR cycling conditions.

Step	Time	Temperature	Additional comments
PCR initial activation step	5 min	95°C	Activation of HotStarTaq *Plus* DNA Polymerase by this heating step
Two-step cycling- 40 times the following two steps are repeated			
Denaturation	5 sec	95°C	
Combined annealing/ extension	10 sec	60°C*	Fluorescence data collection is performed

*According to manufacturer's guidelines; this temperature should be used for QuantiTect Primer Assays and all primer sets with a T_m well below 60°C.

Furthermore, to verify the reaction specificity of target gene amplification, the Rotor-Gene Q was programmed to conduct a melting curve analysis for the qPCR products **(Appendix H)**. To ensure data reliability, all qPCR reactions were

carried out twice. The PCR products were stored at (-20°C). The samples were measured and the cycle threshold (Ct values) for each PCR cycle was established by detecting fluorescence. The relative quantification method was used for the qPCR analysis. The gene expression at the mRNA level was calculated using the ($2^{-\Delta\Delta Ct}$) comparative CT method (214).

3.12 Agarose gel electrophoresis

The RNA samples and the PCR products were visualized by agarose gel electrophoresis (215, 216). Briefly, 1.5 g of agarose was added to 100 ml 1X TBE (Tris/Borate/EDTA) buffer solution. The mixture was boiled in a microwave with sporadic swirling until completely dissolved. The melted agarose was cooled at room temperature to about 55°C, then 10 µL of ethidium bromide was added. The gel was placed into the tray containing the comb in its position, and it was left at room temperature for at least 20 minutes to solidify. The comb was removed gently, and the gel with the tray was placed in the electrophoresis tank containing 1X TBE buffer.

RNA samples were mixed with 6X loading dye (0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 30% Glycerol) in a ratio of 1:3 (2 µl of the dye and 6 µl of the RNA), and they were loaded into individual gel wells. Conventional RT-PCR products had a red color as the kit contains a loading dye, so 8 µl of the PCR product was directly loaded into the individual gel wells. An appropriate DNA ladder was loaded into a gel well to determine the specific PCR product size.

The gels were run at 60 Volts and 100 Volts for 50 min, for RNA samples and PCR products, respectively. The electrophoresis bands in the gel were visualized by a UV transilluminator with a gel documentation system and photographed.

3.13 Statistical analysis

Since the distributions of *KLK6*, *KLK10,* and *KLK14* mRNA expression levels in ALL patients and normal controls were not Gaussian, therefore an appropriate non-parametric test was used to assess the differences between the groups. The Kruskal-Wallis test was used to compare the *KLKs'* mRNA expression of all study groups namely the normal controls, the newly diagnosed ALL patients before starting chemotherapy, the patients after one month of chemotherapy, and the patients after three months of receiving chemotherapy. The Mann-Whiney *U*-test was used to compare the *KLKs'* mRNA expression of the normal controls and the ALL patients at the three conditions: on diagnosis, after one month of chemotherapy, and after three months of receiving chemotherapy. The Wilcoxon Signed-Rank test was used to compare the *KLK's* mRNA expression in ALL patients before and after chemotherapy.

Relationships between mRNA expression levels of the *KLKs* on diagnosis and the continuous variables involved in this study were evaluated by Spearman's correlation analysis (r_s).

Receiver operating characteristic (ROC) analysis was used to investigate the potential diagnostic relevance of the *KLKs'* mRNA expression by plotting sensitivity against (1-specificity). The Hanley and McNeil method was used to estimate the area under the curve (AUC) (217).

Logistic regression analysis was conducted using the mRNA expressions of the *KLKs* on diagnosis as a continuous variable, for the prediction of the presence of ALL.

GraphPad Prism 8 software was used to analyze the data. In all statistical tests, a probability value less than 0.050 was defined as the level of statistical significance: (* $p< 0.05$, ** $p<0.01$, *** $p< 0.001$, and **** $p< 0.0001$). All probabilities were two-tailed.

CHAPTER FOUR

Results

Chapter Four: Results

4.1 Recruitment and deposition

A total of twenty-three newly diagnosed ALL patients, and twelve normal controls were qualified to involve in this prospective, analytical, and case-control study. Real-time qPCR relative quantification of mRNA expression of *KLK6*, *KLK10*, and *KLK14* was performed for nineteen ALL patients and all of the normal controls. The quantification of mRNA expression of the three KLKs in all the leukemic patients was carried out at three different times: when diagnosed with ALL, after one month of receiving chemotherapy, and after three months of chemotherapy.

4.2 Demographic and clinical characteristics of the study cohort

The results of this study indicated that ALL patients' age ranged from 1 to 15 years with a mean of 6.61 years (±0.95 SE), and a median of 5.0 years. The age of the controls ranged from 2-13 years with a mean of 7.17 years (±1.15 SE), and a median of 5.5 years. Males were dominated with 69.57% and 58.33% in the patients and controls, respectively. The two groups' age and sex were not significantly different with (p=0.72) and (p=0.71), respectively. The demographic and clinical variables of the cohort are shown in **(Table 4.1)**.

Table 4.1. Demographic and clinical variables of the study cohort.

Groups / Variables	ALL Patients (n=23)	Controls (n=12)	Statistical test t/ Fisher's exact test	*p*-value[e]
Age (year; Mean ±SE)	6.61±0.95	7.17±1.15	t=0.3572	0.7233
Sex (N, %)				
Male	16 (69.57%)	7 (58.33%)	Fisher's exact test	0.7091
Female	7 (30.43%)	5 (41.67%)		
WBC[a] ($x10^6$ /ml; Mean± SE)	10.96±2.829	7.543±0.6986	t=0.8591	0.3965
Lymphocytes[b] ($x10^6$ /ml; Mean± SE)	7.278±2.007	3.303±0.4621	t=1.411	0.1677
Serum LDH[c] (IU/L; Mean± SE)	1285±270.1	NA[d]	-	-
ALL sub-type (N, %)				
B-ALL	18 (78.26%)	NA[d]	-	-
T-ALL	5 (21.74%)			

These data are for the newly diagnosed ALL patients before starting chemotherapy and the control group; Reference ranges: [a] White Blood Cells (3.5-10.0) $x10^6$ /ml; [b] Lymphocytes count (0.5-5.0) $x10^6$ /ml; [c] Lactate Dehydrogenase (240-480) IU/L; [d] NA: Not Available; [e] *p* was calculated by (t) unpaired t-test or Fisher's exact test.

The mean total WBC for the patients and controls were 10.96 and 7.543 X 10^6/ml, respectively, and they were not significantly different (p=0.3965). The mean of lymphocytes for the patients and controls were 7.28 and 3.3 X 10^6/ml, respectively, and they were not significantly different (p=0.1677). The patients' serum LDH concentration was highly increased. The diagnosis of ALL sub-type in the patients was 78.26% B-ALL and 21.74% T-ALL. The patients aged younger than 8 years were more frequent. The distribution of age groups among ALL sub-types and controls is demonstrated in **(Figure 4.1)**.

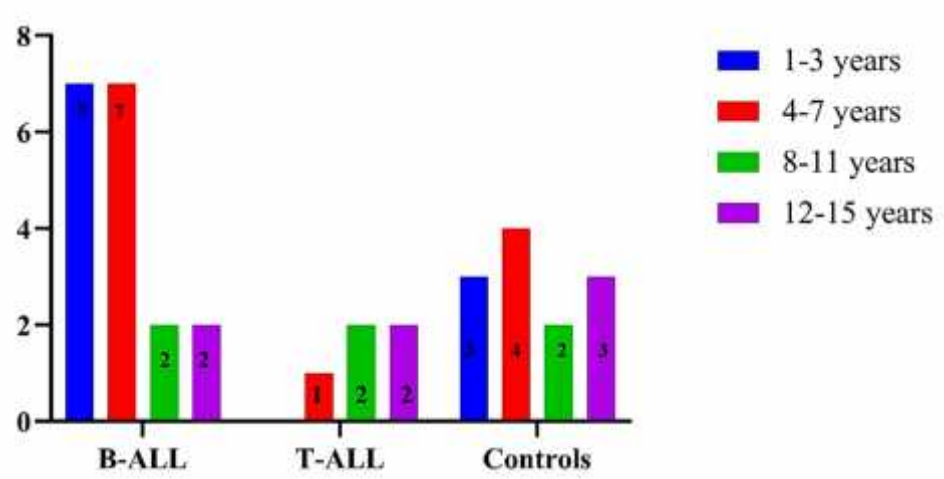

Figure 4.1. Distribution of age groups among ALL subtypes and controls.

4.3 Total RNA quantity and quality assessment

The concentration and purity of extracted total RNA for each sample were measured by Eppendorf Biophotometer at 260 and 280 nm. The integrity and quality of the RNA samples were also evaluated by agarose gel electrophoresis stained with ethidium bromide. Two sharp bands appeared, one of 18S rRNA and the other of 28S rRNA (ribosomal RNA). The band of 28S rRNA was more intense than 18S rRNA. This was used to evaluate the quality of total RNA samples **(Figure 4.2)**.

4.4 cDNA quality assessment

The quality of cDNA samples was evaluated using conventional RT-PCR using *ACTB* primers. Expression of the *ACTB* gene was an indication that cDNA samples would be suitable to be used for performing real-time qPCR. The PCR

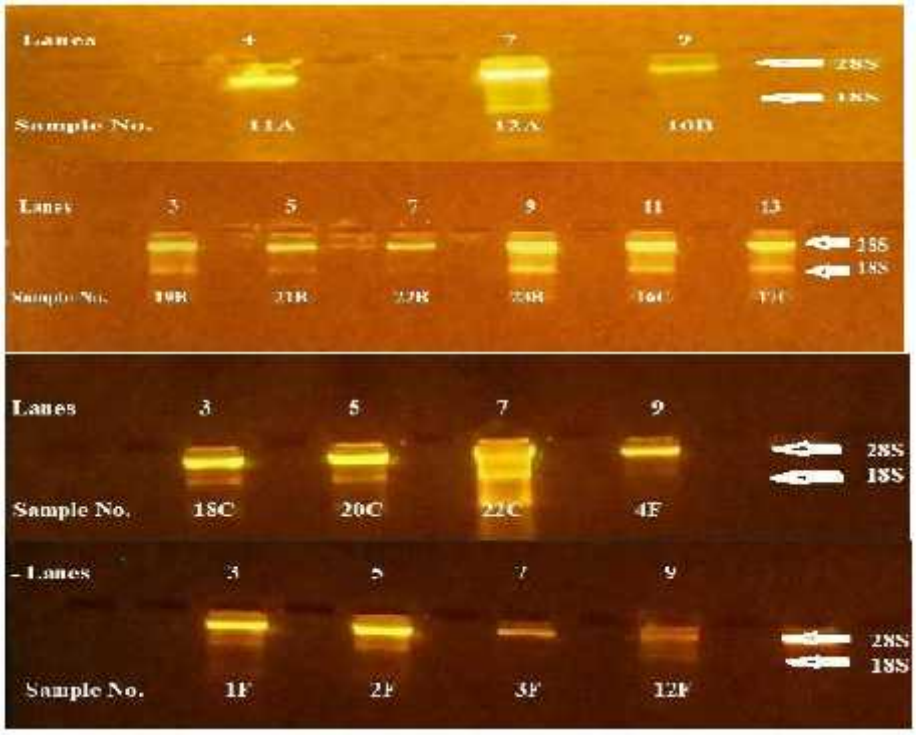

Figure 4.2. Agarose gel electrophoresis for total RNA samples.

This figure illustrates agarose gel electrophoresis for total RNA of selected samples: A; ALL patients on disease diagnosis, B; ALL patients after one month of receiving chemotherapy, C; ALL patients after three months of receiving chemotherapy, F; normal controls. Each lane represents RNA from its corresponding sample number; the RNA samples were loaded into a 1.5% TBE agarose gel and detected with ethidium bromide staining.

products were assessed by agarose gel electrophoresis. The primers of *ACTB* were successful in amplifying the 837 bp amplicon **(Figure 4.3)**. In the negative controls, amplification of no bands was an indicator of free genomic DNA contamination.

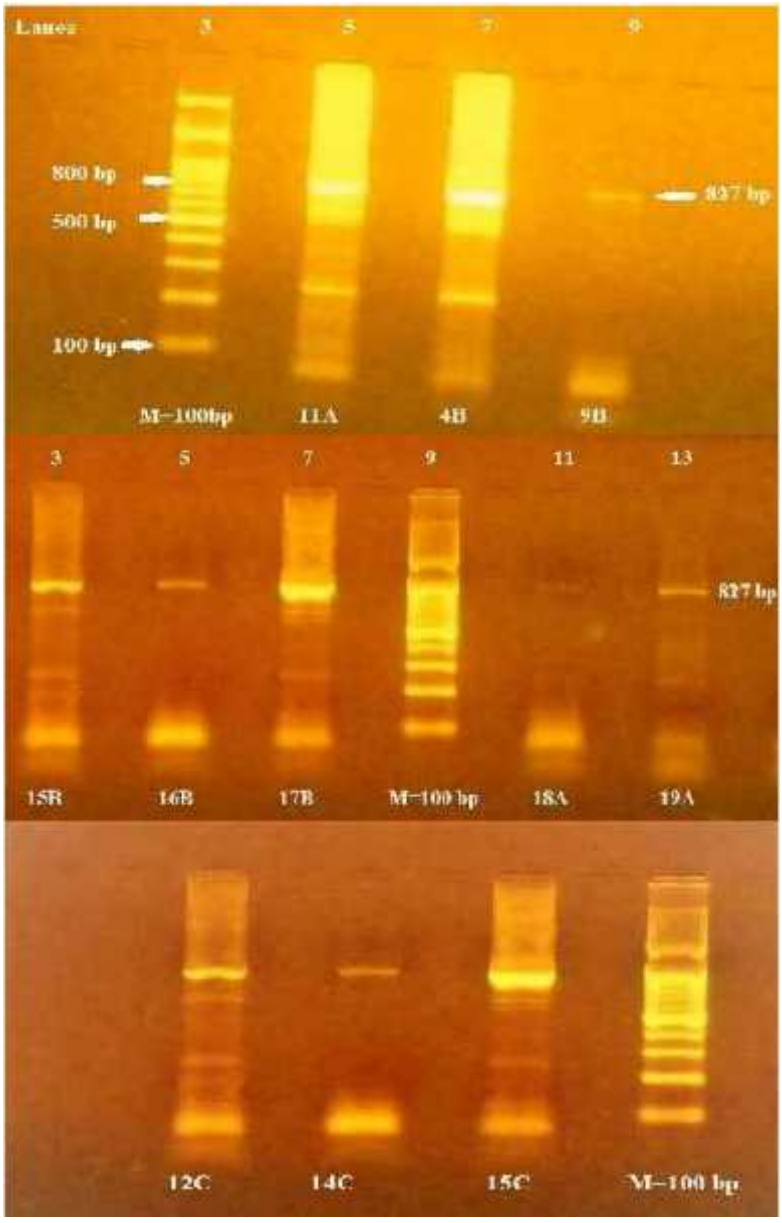

Figure 4.3. Agarose gel electrophoresis of *ACTB* PCR products.

This figure illustrates agarose gel electrophoresis of *ACTB* conventional RT-PCR products for selected cDNA samples: A; ALL patients on disease diagnosis, B; ALL patients after one month of receiving chemotherapy, C; ALL patients after three months of receiving chemotherapy. M: 100bp DNA ladder. The PCR product samples were loaded into a 1.5% TBE agarose gel, expected *ACTB* amplicon size is 837 bp.

4.5 Quantitative analysis of the *KLK*s' mRNA expression

A quantitative real-time PCR assay was developed to measure the relative expression of genes of interest *KLK6*, *KLK10*, and *KLK14* at their mRNA level.

4.5.1 Expression of *KLK6* mRNA in the cohort

KLK6 mRNA expression levels of all the studied groups were compared relative to each other using the Kruskal-Wallis test (p=0.0159; **Figure 4.4**).

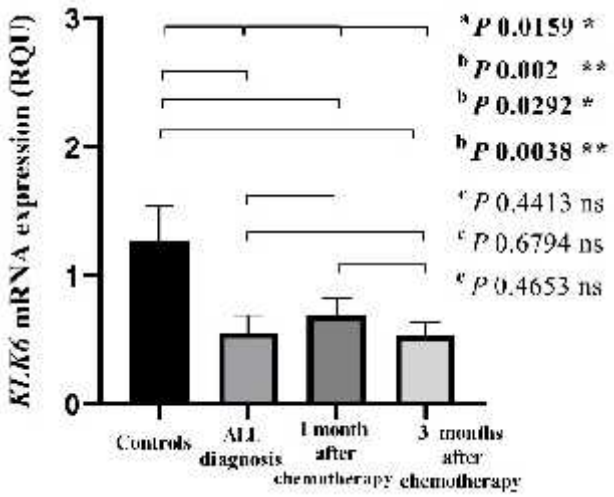

Figure 4.4. Bar-graph of *KLK6* mRNA expression in the cohort.

This graph demonstrates the distribution of *KLK6* mRNA expression in each of the normal controls, the newly diagnosed ALL patients before starting chemotherapy, the patients after one month of chemotherapy, and the patients after three months of receiving chemotherapy. *P*-values were calculated using the Kruskal-Wallis test (a) to compare the *KLK6* mRNA expression of all study groups, the Mann-Whitney *U* test (b) to compare the *KLK6* mRNA expression of the normal controls and ALL patients at the three conditions: on disease diagnosis, after one month of chemotherapy, and after three months of chemotherapy, and the Wilcoxon Signed-Rank test (c) to compare the *KLK6* mRNA expression in the ALL patients before and after treatments. *KLK6* mRNA expression in the normal controls was significantly higher than in ALL patients at the three conditions (p=0.002; p=0.0292 and p=0.0038 respectively by the Mann-Whitney *U* test).

KLK6 mRNA expression levels in newly diagnosed ALL patients were significantly decreased compared to their levels in normal blood samples (p=0.002; **Figure 4.4**). The expression level of *KLK6* mRNA in ALL patients after one month of chemotherapy and after three months of receiving chemotherapy were significantly decreased compared to their levels in normal blood samples (p=0.0292 and p=0.0038 respectively; **Figure 4.4**).

The expression level of *KLK6* mRNA in ALL patients after one month of chemotherapy was increased compared to their level in the patients on disease diagnosis (p=0.4413; **Figure 4.4**). While the *KLK6* mRNA expression level in ALL patients after three months of chemotherapy was slightly decreased compared to their level in the patients on disease diagnosis (p=0.6794; **Figure 4.4**).

4.5.2 Expression of *KLK10* mRNA in the cohort

KLK10 mRNA expression levels of all the studied groups were compared relative to each other by the Kruskal-Wallis test (p<0.0001; **Figure 4.5**).

KLK10 mRNA expression levels in newly diagnosed ALL patients were significantly decreased compared to their levels in normal blood samples (p=0.0001; **Figure 4.5**). The expression level of *KLK10* mRNA in ALL patients after one month and after three months of chemotherapy was significantly

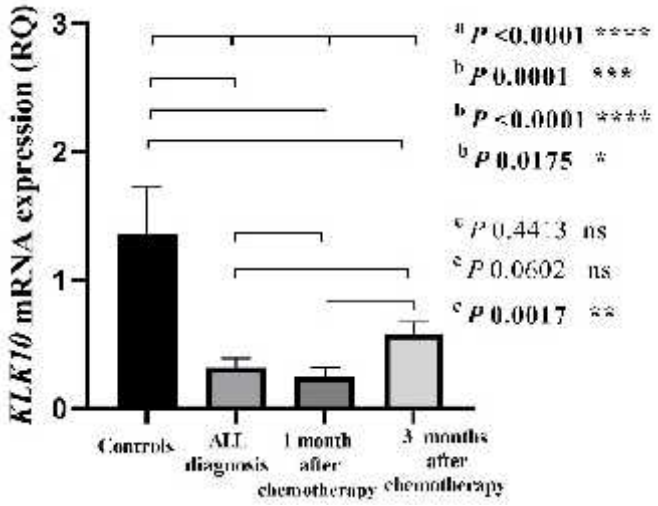

Figure 4.5. Bar-graph of *KLK10* mRNA expression in the cohort.

This graph demonstrates the distribution of *KLK10* mRNA expression in each of the normal controls, the newly diagnosed ALL patients before starting chemotherapy, the patients after one month of chemotherapy, and the patients after three months of receiving chemotherapy. *P*-values were calculated using the Kruskal-Wallis test (a) to compare the *KLK10* mRNA expression of all study groups, the Mann-Whitney *U* test (b) to compare the *KLK10* mRNA expression of the normal controls and ALL patients at the three conditions: on disease diagnosis, after one month of chemotherapy, and after three months of chemotherapy, and the Wilcoxon Signed-Rank test (c) to compare the *KLK10* mRNA expression in the ALL patients before and after treatments. *KLK10* mRNA expression in the normal controls was significantly higher than in ALL patients at the three conditions (p=0.0001; p<0.0001 and p=0.0175 respectively by the Mann-Whitney *U* test).

decreased compared to their levels in normal blood samples (p<0.0001 and p=0.0175 respectively; **Figure 4.5**). The expression level of *KLK10* mRNA in ALL patients after one month of chemotherapy was decreased compared to their level in the patients on disease diagnosis (p=0.4413; **Figure 4.5**). While the *KLK10* mRNA expression level in ALL patients after three months of chemotherapy was increased compared to their level in the patients on disease diagnosis (p=0.0602; **Figure 4.5**).

4.5.3 Expression of *KLK14* mRNA in the cohort

KLK14 mRNA expression levels of all the studied groups were compared relative to each other using the Kruskal-Wallis test (p<0.0001; **Figure 4.6**).

KLK14 mRNA expression levels in newly diagnosed ALL patients were significantly decreased compared to their levels in normal blood samples (p=0.0007; **Figure 4.6**). The expression level of *KLK14* mRNA in ALL patients after one month of chemotherapy and three months of chemotherapy were

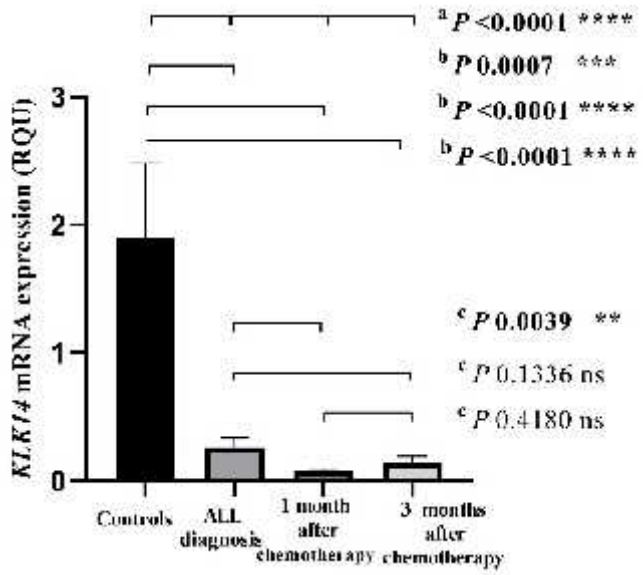

Figure 4.6. Bar-graph of *KLK14* mRNA expression in the cohort.

This graph demonstrates the distribution of *KLK14* mRNA expression in each of the normal controls, the newly diagnosed ALL patients before starting chemotherapy, the patients after one month of chemotherapy, and the patients after three months of receiving chemotherapy. *P*-values were calculated using the Kruskal-Wallis test (a) to compare the *KLK14* mRNA expression of all study groups, the Mann-Whitney *U* test (b) to compare the *KLK14* mRNA expression of the normal controls and ALL patients at the three conditions: on disease diagnosis, after one month of chemotherapy, and after three months of chemotherapy, and the Wilcoxon Signed-Rank test (c) to compare the *KLK14* mRNA expression in the ALL patients before and after treatments. *KLK14* mRNA expression in the normal controls was significantly higher than in ALL patients at the three conditions (p=0.0007; p<0.0001 and p<0.0001 respectively by the Mann-Whitney *U* test).

significantly decreased compared to their levels in normal blood samples (p<0.0001 and p<0.0001 respectively; **Figure 4.6**).

The *KLK14* mRNA expression level in ALL patients after one month and three months of chemotherapy were decreased compared to their level in the patients on disease diagnosis (p=0.0039 and p=0.1336 respectively; **Figure 4.6**).

4.6 Descriptive statistics of the *KLKs'* mRNA expression in the cohort

The data from **Table 4.2**, **Table 4.3**, and **Table 4.4** demonstrate the descriptive statistics of mRNA expression levels of *KLK6*, *KLK10*, and *KLK14*, respectively. The tables describe the mean, range, and percentile of the mRNA expressions of the *KLKs* in all the groups in the cohort.

Table 4.2. Descriptive statistics of *KLK6* mRNA expression.

Variables	Mean± SE[b]	Range	Percentile		
			25th	Median	75th
***KLK6* mRNA in normal controls (RQU[a]; n=12)**	1.264±0.2780	0.3157-3.909	0.5967	1.159	1.610
***KLK6* mRNA in newly diagnosed ALL patients (RQU[a]; n=19)**	0.5554±0.1351	0.01506-2.44	0.2343	0.3135	0.7055
***KLK6* mRNA in ALL patients after one month of chemotherapy (RQU[a]; n=19)**	0.6862±0.1403	0.03033-2.440	0.1337	0.6402	1.062
***KLK6* mRNA in ALL patients after three months of chemotherapy (RQU[a]; n=19)**	0.5260±0.1080	0.02622-1.749	0.1903	0.3092	0.6492

[a] Relative Quantification Unit; [b] Standard Error of the mean

Table 4.3. Descriptive statistics of *KLK10* mRNA expression.

Variables	Mean± SE[b]	Range	Percentile		
			25th	Median	75th
***KLK10* mRNA in normal controls (RQU[a]; n=12)**	1.362±0.3585	0.2934-4.629	0.5126	0.9609	1.688
***KLK10* mRNA in newly diagnosed ALL patients (RQU[a]; n=19)**	0.316±0.071	0.0202-1.134	0.0884	0.2089	0.5038
***KLK10* mRNA in ALL patients after one month of chemotherapy (RQU[a]; n=19)**	0.2375±0.0799	0.0056-1.496	0.02337	0.08367	0.3016
***KLK10* mRNA in ALL patients after three months of chemotherapy (RQU[a]; n=19)**	0.5793±0.0927	0.06125-1.538	0.2484	0.5288	0.7224

[a] Relative Quantification Unit; [b] Standard Error of the mean

Table 4.4. Descriptive statistics of *KLK14* mRNA expression.

Variables	Mean± SE[b]	Range	Percentile		
			25th	Median	75th
***KLK14* mRNA in normal controls (RQU[a]; n=12)**	1.906 ±0.5761	0.091-6.197	0.3935	1.155	3.455
***KLK14* mRNA in newly diagnosed ALL patients (RQU[a]; n=19)**	0.2623±0.07146	0.0208-1.358	0.0572	0.1733	0.3873
***KLK14* mRNA in ALL patients after one month of chemotherapy (RQU[a]; n=19)**	0.0782 ±0.0152	0.00937-0.2119	0.01953	0.05153	0.1341
***KLK14* mRNA in ALL patients after three months of chemotherapy (RQU[a]; n=19)**	0.1457±0.04312	0.00676-0.5402	0.01700	0.04612	0.2701

[a] Relative Quantification Unit; [b] Standard Error of the mean

4.7 Correlations between the continuous variables in ALL patients

The data from **Table 4.5**, **Table 4.6**, and **Table 4.7** demonstrate the findings of Spearman's correlation coefficient analysis of *KLK6*, *KLK10*, and *KLK14* expressions at the mRNA levels and the continuous variables in newly diagnosed ALL patients. The mRNA expression levels were not significantly correlated with patient age, lymphocyte count, WBC, and serum LDH concentration. Except for *KLK14* mRNA level was negatively correlated with WBC (r_s=-0.6, p=0.007). This investigation also observed a positive relationship between WBC and ALL patients' lymphocyte count (r_s=0.83, p <0.0001), and the patient's age and serum LDH level (r_s=0.71, p <0.001).

Table 4.5. Correlations between *KLK6* mRNA expression and the continuous variables in newly diagnosed ALL patients.

Variables	*KLK6* mRNA (n=19)	Age (n=23)	Lymphocytes (n=23)	WBC[b] (n=23)	LDH[c] (n=19)
***KLK6* mRNA**					
r_s[a]		0.16	-0.17	-0.17	-0.31
95% CI		-0.3338 to 0.5796	-0.5863 to 0.3248	-0.5892 to 0.3208	-0.7042 to 0.2390
p-Value		0.52	0.5	0.49	0.25
Age					
r_s[a]	0.16		-0.48	-0.26	0.71
95% CI	-0.3338 to 0.5796		-0.7516 to -0.07408	-0.6148 to 0.1837	0.3705 to 0.8849
p-Value	0.52		**0.02 ***	0.23	**0.001 ****
Lymphocytes					
r_s[a]	-0.17	-0.48		0.83	-0.5
95% CI	-0.5863 to 0.3248	-0.7516 to -0.07408		0.6279 to 0.9275	-0.7830 to -0.04422
p-Value	0.5	**0.02 ***		**<0.0001 ****	**0.03 ***
WBC[b]					
r_s[a]	-0.17	-0.26	0.83		-0.24
95% CI	-0.5892 to 0.3208	-0.6148 to 0.1837	0.6279 to 0.9275		-0.6345 to 0.2543
p-Value	0.49	0.23	**<0.0001 ****		0.32
LDH[c]					
r_s[a]	-0.31	0.71	-0.5	-0.24	
95% CI	-0.7042 to 0.2390	0.3705 to 0.8849	-0.7830 to -0.04422	-0.6345 to 0.2543	
p-Value	0.25	**0.001 ****	**0.03 ***	0.32	

[a] Spearman's Correlation Coefficient; [b] White Blood Cells; [c] Lactate Dehydrogenase

Table 4.6. Correlations between *KLK10* mRNA expression and the continuous variables in newly diagnosed ALL patients.

Variables	*KLK10* mRNA (n=19)	Age (n=23)	Lymphocytes (n=23)	WBC[b] (n=23)	LDH[c] (n=19)
***KLK10* mRNA**					
r_s[a]		-0.09	-0.10	-0.31	-0.14
95% CI		-0.5339 to 0.3913	-0.5409 to 0.3830	-0.6806 to 0.1768	-0.6056 to 0.3948
p-Value		0.71	0.68	0.19	0.6
Age					
r_s[a]	-0.09		-0.48	-0.26	0.71
95% CI	-0.5339 to 0.3913		-0.7516 to -0.07408	-0.6148 to 0.1837	0.3705 to 0.8849
p-Value	0.71		**0.02 ***	0.23	**0.001 ****
Lymphocytes					
r_s[a]	-0.10	-0.48		0.83	-0.50
95% CI	-0.5409 to 0.3830	-0.7516 to -0.07408		0.6279 to 0.9275	-0.7830 to -0.04422
p-Value	0.68	**0.02 ***		**<0.0001 ****	**0.03 ***
WBC[b]	-0.31	-0.26	0.83		-0.24
r_s[a]	-0.6806 to 0.1768	-0.6148 to 0.1837	0.6279 to 0.9275		-0.6345 to 0.2543
95% CI	0.19	0.23	**<0.0001 ****		0.32
p-Value					
LDH[c]	-0.14	0.71	-0.50	-0.24	
r_s[a]	-0.6056 to 0.3948	0.3705 to 0.8849	-0.7830 to -0.04422	-0.6345 to 0.2543	
95% CI	0.6	**0.001 ****	**0.03 ***	0.32	
p-Value					

[a] Spearman's Correlation Coefficient; [b] White Blood Cells; [c] Lactate Dehydrogenase

Table 4.7. Correlations between *KLK14* mRNA expression and the continuous variables in newly diagnosed ALL patients.

Variables	*KLK14* mRNA (n=19)	Age (n=23)	Lymphocytes (n=23)	WBC[b] (n=23)	LDH[c] (n=19)
***KLK14* mRNA**					
r_s[a]		0.06	-0.33	-0.60	-0.31
95% CI		-0.4202 to 0.5087	-0.6877 to 0.1640	-0.8314 to -0.1816	-0.7091 to 0.2297
p-Value		0.82	0.17	**0.007 ****	0.23
Age					
r_s[a]	0.06		-0.48	-0.26	0.71
95% CI	-0.4202 to 0.5087		-0.7516 to -0.07408	-0.6148 to 0.1837	0.3705 to 0.8849
p-Value	0.82		**0.02 ***	0.23	**0.001 ****
Lymphocytes					
r_s[a]	-0.33	-0.48		0.83	-0.50
95% CI	-0.6877 to 0.1640	-0.7516 to -0.07408		0.6279 to 0.9275	-0.7830 to -0.04422
p-Value	0.17	**0.02 ***		**<0.0001 ******	**0.03 ***
WBC[b]	-0.60	-0.26	0.83		-0.24
r_s[a]	-0.8314 to -0.1816	-0.6148 to 0.1837	0.6279 to 0.9275		-0.6345 to 0.2543
95% CI	**0.007 ****	0.23	**<0.0001 ******		0.32
p-Value	-0.31	0.71	-0.50	-0.24	
LDH[c]	-0.7091 to 0.2297	0.3705 to 0.8849	-0.7830 to -0.04422	-0.6345 to 0.2543	
r_s[a]	0.23	**0.001 ****	**0.03 ***	0.32	
95% CI					
p-Value					

[a] Spearman's Correlation Coefficient; [b] White Blood Cells; [c] Lactate Dehydrogenase

4.8 ROC curves for sensitivity and specificity of the *KLKs*

Besides quantification of mRNA expression of *KLK6*, *KLK10*, and *KLK14*; the diagnostic accuracy of the genes in ALL was evaluated by receiver operating characteristic (ROC) analysis. The area under the ROC curve (AUC) was achieved from the plotting of sensitivity versus (1-specificity), and the optimal diagnostic cutoff point was revealed.

4.8.1 Diagnostic evaluation of *KLK6* mRNA expression in ALL

The ROC curve in **Figure 4.7** illustrated that *KLK6* mRNA expression could very efficiently discriminate ALL from normal counterparts (AUC =0.822, 95% CI=0.6735-0.9713, SE=0.076, *p*=0.0029). The ROC curve analysis revealed that 0.7614 RQU is the best diagnostic cutoff value. The sensitivity of this cutoff value was 84.21%, whereas the method's specificity was 75.0%.

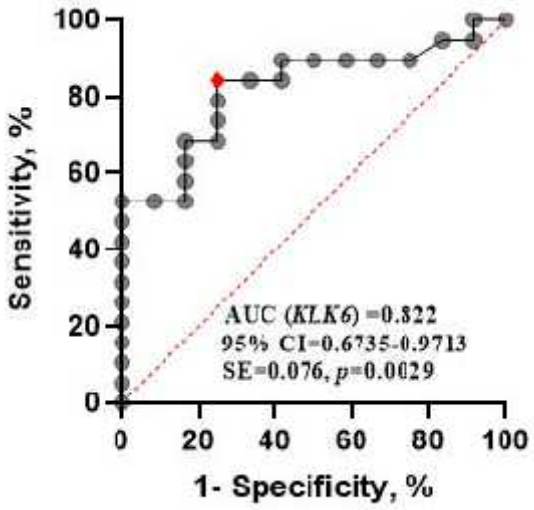

Figure 4.7. ROC curve for *KLK6* mRNA expression.

Receiver operating characteristic (ROC) analysis for quantified *KLK6* mRNA expression. It reveals that *KLK6* can be applied to diagnose ALL and discriminate it from normal controls; AUC, Area Under Curve.

4.8.2 Diagnostic evaluation of *KLK10* mRNA expression in ALL

The ROC curve in **Figure 4.8** illustrated that *KLK10* mRNA expression could very efficiently discriminate ALL from normal counterparts (AUC =0.886, 95% CI=0.7720-1.000, SE=0.0582, *p*=0.0004). The ROC curve analysis revealed that 0.5399 RQU is the best diagnostic cutoff value. The sensitivity of this cutoff value was 89.47%, whereas the method's specificity was 75.0%.

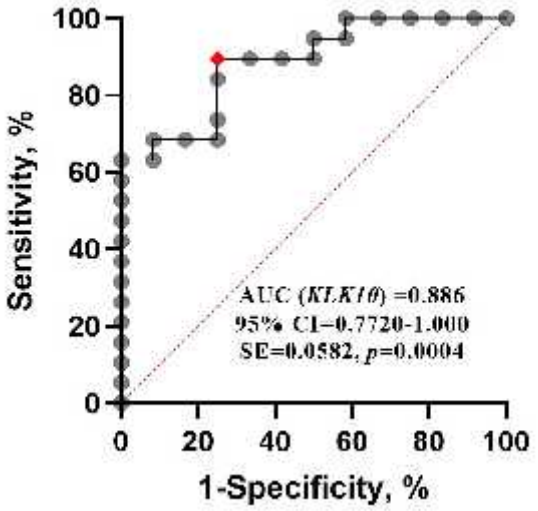

Figure 4.8. ROC curve for *KLK10* mRNA expression.

Receiver operating characteristic (ROC) analysis for quantified *KLK10* mRNA expression. It reveals that *KLK10* can be applied to diagnose ALL and discriminate it from normal controls; AUC, Area Under Curve.

4.8.3 Diagnostic evaluation of *KLK14* mRNA expression in ALL

The ROC curve in **Figure 4.9** illustrated that *KLK14* mRNA expression could very efficiently discriminate ALL from normal counterparts (AUC =0.851, 95% CI=0.7087-0.9931, SE=0.0726, *p*=0.0012). The ROC curve analysis

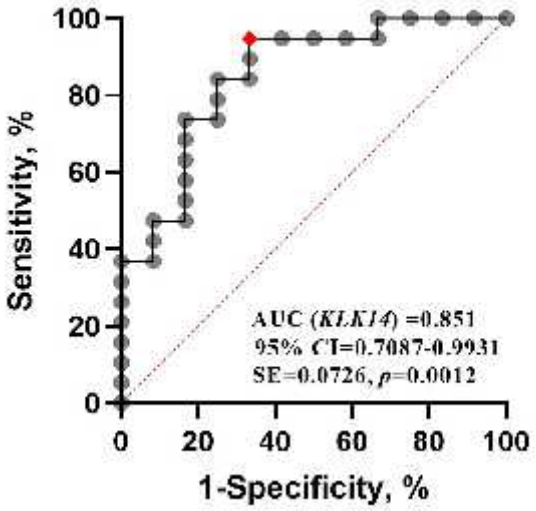

Figure 4.9. ROC curve for *KLK14* mRNA expression.

Receiver operating characteristic (ROC) analysis for quantified *KLK14* mRNA expression. It reveals that *KLK14* can be applied to diagnose ALL and discriminate it from normal controls; AUC, Area Under Curve.

revealed that 0.5402 RQU is the best diagnostic cutoff value. The sensitivity of this cutoff value was 94.74%, whereas the method's specificity was 66.67%.

4.9 Logistic regression analysis for the *KLKs'* mRNA expression

To further investigate the discriminatory significance of *KLK6, KLK10*, and *KLK14* mRNA expression, their quantified expression was used as a continuous variable to construct a univariate logistic regression model to predict the presence of ALL.

4.9.1 Logistic regression model for *KLK6* mRNA expression

Univariate logistic regression analysis demonstrated that patients with reduced *KLK6* mRNA expression levels establish a significant prognostic marker for ALL (crude odds ratio [OR]=0.2289, 95% CI=0.04428-0.7544, p=0.0115).

4.9.2 Logistic regression model for *KLK10* mRNA expression

Univariate logistic regression analysis demonstrated that patients with reduced *KLK10* mRNA expression levels establish a significant prognostic marker for ALL (crude odds ratio [OR]=0.0228, 95% CI= 0.0008851-0.2299, p<0.0001).

4.9.3 Logistic regression model for *KLK14* mRNA expression

Univariate logistic regression analysis demonstrated that patients with reduced *KLK14* mRNA expression levels establish a significant prognostic marker for ALL (crude odds ratio [OR]=0.0716, 95% CI=0.003912-0.4610, p=0.0002).

CHAPTER FIVE

Discussion

Chapter Five: Discussion

Biomarkers are biomolecules detected in body fluids or tissues that may indicate normal or pathological activity (6). Biomarkers are classified into many categories based on their use and molecular changes. Tumor biomarkers indicate the presence of cancer, which could be nucleic acids, proteins, cells, metabolites, or processes such as proliferation, angiogenesis, or apoptosis. They could serve as diagnostic, prognostic, and/or monitoring biomarkers (218). Acute lymphoblastic leukemia (ALL) is the most common hematological malignancy diagnosed in children. In ALL, precursor lymphoblasts are obstructed at an early stage of differentiation, proliferate quickly, and displace normal bone marrow hematopoietic cells (57). Despite the favorable survival rates of childhood ALL, it is essential to have sensitive and specific molecular biomarkers for the diagnosis and prognosis of the disease; assign better risk classification, and consequently better clinical results. Accumulative evidence robustly demonstrated that kallikrein-related peptidases have a wide interest in clinical oncology (219). They serve as diagnostic and/or prognostic biomarkers in diverse human cancers such as prostate (7, 220), breast (221, 222), ovarian (223, 224), lung (225), colorectal (175, 193), and gastric cancer (226). Nevertheless, the research into the effects of KLKs on cancer is currently ongoing, and the investigation of other members of the KLK group in hematological malignancies has not been tested.

5.1 Kallikrein-related peptidases in hematological malignancies

Human kallikrein-related peptidases designate a group of fifteen functional genes of serine proteases on chromosome 19q13.3-13.4. Few papers have been published investigating kallikreins in hematological malignancies: in 2004, Roman-Gomez, J. *et al* for the first time found that *KLK10* expression was strongly reduced at mRNA level in precursor B-cell ALL and 69% of samples diagnosed with ALL. Moreover, the study found loss of expression in *KLK10* due to hypermethylation in ALL cell lines compared to normal cell lines and proposed it as a factor for an unfavorable prognosis in childhood ALL (188). Down expression of *KLK10* in ALL has been reported in other studies (123, 154, 227, 228). In 2014, Kashuba demonstrated that chronic lymphocytic leukemic cells express constituents for the kinin-kallikrein system signaling pathway. In the study kininogen, an important protein of the kinin-kallikrein system was overexpressed and associated with CLL prognosis (229). In 2015, for the first time, *KLKB1* expression was investigated by Adamopoulos *et al* who discovered a significant increase in *KLKB1* mRNA expression in CLL patients and very efficiently distinguished from healthy blood donors (230). In 2016, *KLK5-9* transcripts were analyzed in distinct cancerous human tissues including leukemia via nested reverse transcription PCR (177). In 2016, for the first time, *KLK14* expression was examined by Kontos *et al* found a strong overexpression of

KLK14 mRNA in CLL patients than in the normal population and related to poor prognosis in CLL (231).

Treatment of pediatric ALL patients has shown vast progress over the past decades, increasing considerations of remission rates and prognosis of the patients. Nevertheless, some patients regardless of presenting favorable clinical characteristics may be overtreated or suffered from unpleasant outcomes (66, 232). The discovery of new prognostic biomarkers is essential to predict the patients' outcomes and monitor their response to therapy.

Thus, the present study aimed to quantify mRNA expression levels of the kallikrein-related peptidases: *KLK6*, *KLK10*, and *KLK14* in newly diagnosed childhood ALL patients and healthy control blood donors utilizing an accurate and sensitive real-time qPCR predicated on SYBR Green chemistry. Besides, to assess their potential diagnostic and/or prognostic biomarker suitability for acute lymphoblastic leukemia. In addition, to evaluate alterations in mRNA expression levels of *KLK6, KLK10,* and *KLK14* in childhood ALL patients who received one month and then three months of chemotherapy.

5.2 Evaluation of *KLK6* mRNA expression as a potential biomarker in pediatric ALL

In this study, the differential diagnostic usefulness of the *KLK6* mRNA expression was investigated using qPCR. This study found that *KLK6* mRNA expression levels were considerably reduced in ALL patients compared to normal blood samples (p=0.002; Figure 4.4; Table 4.2). The differential diagnostic value was demonstrated via ROC curve analysis (AUC =0.822, 95% CI=0.6735-0.9713, SE=0.076, p=0.0029; Figure 4.7). The ROC curve results illustrated that *KLK6* mRNA expression could very efficiently discriminate ALL from normal counterparts, the optimal diagnostic cutoff value was revealed to be 0.7614 RQU. Using this cutoff value, the method's sensitivity was 84.21%, and its specificity was 75.0%. This establishes that *KLK6* mRNA expression could serve as a diagnostic biomarker for ALL. Univariate logistic regression analysis confirmed that patients with reduced *KLK6* mRNA expression are more possible to suffer from ALL ([OR]=0.2289, 95% CI=0.04428-0.7544, p=0.0115). This establishes that a reduction in *KLK6* mRNA expression could be a prognostic biomarker for ALL. This investigation also found that *KLK6* mRNA expression levels were significantly downregulated in ALL patients after one month and after three months of chemotherapy compared to their levels in normal blood samples (p=0.0292 and p=0.0038 respectively; Figure 4.4, Table 4.2). The expression level of *KLK6* mRNA in ALL patients after one month of chemotherapy was overregulated compared to their level in the patients on disease diagnosis

(p=0.4413; Figure 4.4, Table 4.2). Whereas the *KLK6* mRNA expression level in ALL patients after three months of chemotherapy was slightly downregulated compared to their level in the patients on disease diagnosis (p=0.6794; Figure 4.4, Table 4.2).

KLK6 mRNA expression is differentially regulated and contributed to unfavorable prognostic tumor biomarkers, for instance, downregulation of *KLK6* mRNA expression was detected in breast cancer, and contributed as a prognostic tumor marker depending on the cancer grade (174, 233, 234). KLK6 protein has a function in the invasion and metastasis of malignant tumors (235). *KLK6* mRNA and protein overexpression was found as potential prognostic indicators of gastric malignancy (226, 236). *KLK6* mRNA and protein are expressed in ovarian cancer. This protein overexpression was found as a diagnostic and prognostic biomarker of ovarian cancer (237, 238). *KLK6* mRNA and protein overexpression was shown as significant screening and prognostic biomarkers in colorectal adenocarcinoma (175, 239, 240). KLK6 protein is overexpressed and considered a poor prognostic biomarker in non-small cell lung cancer (225). In 2016, *KLK5-9* transcripts were analyzed in distinct cancerous human tissues including leukemia via nested reverse transcription PCR (177).

KLK6 is a serine protease, that involved a proteolytic cascade. CNS is rich with KLK6 cleaving the amyloid precursor protein in perineuronal net places and the extracellular matrix and it is a potential element of Alzheimer’s disease

pathogenesis (183). KLK6 protein expression is associated with immune cell survival by a molecular mechanism through stimulation of protease activated receptor-1 (PAR-1) in neurons (241, 242). PAR-1 was suggested to be significant for a variety of immunological responses that depend on homeostasis maintenance and immune clearance apoptosis (242).

Protease activated receptor-1 (PAR-1) is a transmembrane G-coupled prototypic receptor that is stimulated by thrombin and other serine proteases including kallikreins (KLK5, KLK6, and KLK14) is being correlated with various physiological and pathological processes (243, 244). PAR-1 plays potential roles in promoting tumor cell proliferation, infiltration, and metastasis, stimulated by tumor-derived serine proteases and matrix metalloproteinases (245, 246). PAR-1 is upregulated in a variety of human cancers including leukemia, colon, prostate, breast, and ovarian cancer (247). PAR-1 is drastically overexpressed in aggressive acute leukemia subtypes and influences blast cells to egress from bone marrow to peripheral blood (248). PAR-1 expression denotes an unfavorable prognostic biomarker at diagnosis of childhood ALL (249). PAR-1 is involved in leukemogenesis as well as it has an ultimate role in the eradication of primitive leukemia stem cells in AML (250).

It was examined that active KLKs produced by tumors and inflamed tissues can have hormonal features and that their proteolytic ability is regulated by proteinase inhibitors that can be found in cancer-related fluids (251). The

potential role of KLKs in hormone-processing was suggested; it was reported that KLK5-8, and KLK10-14, are released in the human pituitary gland, located with the growth hormone, and KLK5, KLK6, and KLK14 potentially contribute to this hormone's proteolytic degradation into functional fragments (252).

5.3 Evaluation of *KLK10* mRNA expression as a potential biomarker in pediatric ALL

In this study, the differential diagnostic usefulness of the *KLK10* mRNA expression was investigated using qPCR. This study found that *KLK10* mRNA expression levels were considerably reduced in ALL patients compared to normal blood samples (p=0.0001; Figure 4.5; Table 4.3). The differential diagnostic value was demonstrated via ROC curve analysis (AUC =0.886, 95% CI=0.7720-1.000, SE=0.0582, p=0.0004; Figure 4.8). The ROC curve results illustrated that *KLK10* mRNA expression could very efficiently discriminate ALL from normal counterparts, the optimal diagnostic cutoff value was revealed to be 0.5399 RQU. Using this cutoff value, the method's sensitivity was 89.47%, and its specificity was 75.0%. This establishes that *KLK10* mRNA expression could serve as a diagnostic biomarker for ALL. Univariate logistic regression analysis confirmed that patients with reduced *KLK10* mRNA expression are more possible to suffer from ALL ([OR]=0.0228, 95% CI=0.0008851-0.2299, p<0.0001). This establishes that a reduction in *KLK10* mRNA expression could be a prognostic biomarker for ALL. This investigation also found that *KLK10* mRNA expression

levels were significantly downregulated in ALL patients after one month and after three months of chemotherapy compared to their levels in normal blood samples ($p<0.0001$ and $p=0.0175$ respectively; Figure 4.5, Table 4.3). The expression level of *KLK10* mRNA in ALL patients after one month of chemotherapy was slightly downregulated compared to their level in the patients on disease diagnosis ($p=0.4413$; Figure 4.5, Table 4.3). Whereas the *KLK10* mRNA expression level in ALL patients after three months of chemotherapy was overregulated compared to their level in the patients on disease diagnosis ($p=0.0602$; Figure 4.5, Table 4.3).

In 1996, the *KLK10* gene was characterized as a possible tumor-suppressor gene; and its down expression in breast cancer cell lines was observed (253). *KLK10* mRNA expression was analyzed in breast tissues by in situ hybridization; found further supportive results of the decrease of expression in breast malignant tissues compared to normal samples (189). This down-expression was proposed due to *KLK10* exon-3 methylation (254). *KLK10* is associated with four CpG islands, the largest one is located on exon 3 of the gene (255). These studies linked CpG island hypermethylation and down expression of KLK10 at both mRNA and protein levels (256). *KLK10* transcriptional silencing is associated with hypermethylation of CpG islands within promoter or gene coding regions. (254). *KLK10* inhibits carcinogenesis and is considered a tumor suppressor gene. It was examined that hypermethylation of *KLK10* CpG island plays a crucially

significant role in tumor-specific loss and down expressions of KLK10 mRNA and protein in ALL, breast, and prostate cancers (130, 188, 254, 257, 258). In early breast cancer patients, methylation of the gene's third exon possessed a prognostic value (258). Reduction in *KLK10* mRNA expression in precursor B-cell ALL was proposed as a factor for an unfavorable prognosis in ALL (188). Previous studies also found that the *KLK10* gene is over-expressed by steroid hormones via binding to their receptors which act as a binding molecule to activate or suppress transcription (186). KLK10 protein overexpression is observed in ovarian tumors that correlates to its elevation in the serum and possesses a significant value as tissue and serological markers for diagnosis, prognosis, and monitoring of ovarian cancer (195, 224, 259-261). It was reported that *KLK10* overexpression could serve as a diagnostic and prognostic biomarker for pancreatic and colorectal cancers (193, 194, 262-264). It was observed that overexpression of *KLK10* mRNA is an independent biomarker for predicting poor prognosis in gastric cancer, whereas urinary KLK10 protein could also play a non-invasive marker to predict inoperable and incurable gastric cancer (265, 266).

5.4 Evaluation of *KLK14* mRNA expression as a potential biomarker in pediatric ALL

In this study, the differential diagnostic usefulness of the *KLK14* mRNA expression was investigated via qPCR. This study found that *KLK14* mRNA expression levels were considerably reduced in ALL patients compared to normal blood samples (p=0.0007; Figure 4.6; Table 4.4). The differential diagnostic value was demonstrated via ROC curve analysis (AUC =0.851, 95% CI=0.7087-0.9931, SE=0.0726, p=0.0012; Figure 4.9). The ROC curve results illustrated that *KLK14* mRNA expression could very efficiently discriminate ALL from normal counterparts, the optimal diagnostic cutoff value was revealed to be 0.5402 RQU. Using this cutoff value, the method's sensitivity was 94.74%, and its specificity was 66.67%. This establishes that *KLK14* mRNA expression could serve as a diagnostic biomarker for ALL. Univariate logistic regression analysis confirmed that patients with reduced *KLK14* mRNA expression are more possible to suffer from ALL ([OR]=0.0716, 95% CI=0.003912-0.4610, p=0.0002). This establishes that a reduction in *KLK14* mRNA expression could be a prognostic biomarker for ALL. This investigation also found that *KLK14* mRNA expression levels were significantly downregulated in ALL patients after one month and three months of chemotherapy compared to their levels in normal blood samples (p<0.0001 and p<0.0001 respectively; Figure 4.6, Table 4.4). The expression level of *KLK14* mRNA in ALL patients after one month of chemotherapy was significantly downregulated compared to their level in the patients on disease diagnosis

(p=0.0039; Figure 4.6, Table 4.4). The *KLK14* mRNA expression level in ALL patients after three months of chemotherapy was downregulated compared to their level in the patients on disease diagnosis (p=0.1336; Figure 4.6, Table 4.4).

KLK14 gene is positioned at 19q13.4 within the human kallikrein locus. KLK14 protein is a serine protease with trypsin-like substrate specificity (198). *KLK14* differential expression was reported as potential diagnostic and/or prognostic biomarkers in ovarian, breast, testicular, prostate, and colorectal malignancies. It was reported that KLK14 plays a marker of a promising outcome of ovarian cancer (196). Elevation of its mRNA level has been implicated with lower cancer stage, reduced tumor grade, optimal residual tumor size, better cancer-free survival, and overall survival (108). The National Academy of Clinical Biochemistry (NACB) Laboratory Medicine Practice has designated KLK14 as a serum marker with clinical utility in ovarian cancer differential diagnosis, prognostic prediction, and tumor monitoring with the level of evidence (LOE) of IV and/or V (267, 268).

It was reported that the downregulation of *KLK14* gene expression is a potential biomarker linked with a poor prognosis for breast cancer (196, 222, 269). It was also assessed that the gene plays a possible independent diagnostic marker in breast tumor biopsies (270). Furthermore, it was found that *KLK14* expression could serve as a predicting biomarker for chemotherapy response in breast cancer (211). Downregulation of *KLK14* gene expression in testicular germ

cell tumors was observed (192, 271). Quantification of KLK14 mRNA and protein levels in the cancerous and non-cancerous prostate tissues was found to be downregulated by androgen receptor signaling and associated with aggressiveness of the tumor suggesting an unfavorable prognosis of the disease (272-275). Colonic cancers produce KLK14 protein, which activates protease-activated receptor-2 (PAR-2) by signaling human colon cancer cells (276). PAR-2 is a G protein-coupled receptor stimulated by intramolecular binding of a tethered ligand which is liberated by the proteases, primarily of the serine protease group including trypsin and kallikreins (KLK2/4/5/6/14) (277). PAR-2 works as a cell surface sensor for various extracellular and cell surface-associated proteases (278). It was observed that overexpression of KLK14 protein in cytosolic extracts from colorectal cancer tissues was significantly correlated with patients' overall survival, and it was established as a significant prognostic biomarker in staging and grading of the disease (279). Also, its upregulation at the mRNA level can be considered a marker of unfavorable prognosis for patients with colorectal cancer; have discriminatory power between colorectal cancer and adenoma patients (205). Upregulation of *KLK14* mRNA in CLL patients was observed to be associated with an unfavorable prognosis of the disease (231).

5.5 Assessment of the correlations between the continuous variables in ALL patients

The outcomes of the Spearman's correlation coefficient analysis of the mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* and the continuous variables in newly diagnosed ALL patients revealed that there was no significant correlation between the mRNA expression levels and the patient's age, lymphocyte count, WBC, and serum LDH concentration. Except for *KLK14* mRNA level was negatively correlated with WBC (r_s=-0.6, p=0.007). The present study found a positive relationship between WBC and ALL patients' lymphocyte count (r_s=0.83, p <0.0001). Patients with ALL frequently have an increase in lymphocytes (280). Also, there was a positive correlation between the patient's age and serum LDH level (r_s=0.71, p <0.001). This is in line with past research on adults (281).

5.6 Limitation and strength of the study

The present investigation has certain limitations as well as some strengths. A small number of individuals qualified for the inclusion criteria of the study leads to a decrease in the sample size of the study. Some of the pediatric individuals refused to participate in the study because venipuncture is an invasive procedure for children. The study could not continue to follow up the ALL patients to examine the mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* after receiving the latter phases of chemotherapy because the duration of the

present study was limited. A large percentage of samples could not be examined due to a lack of resources and the high cost of qPCR analyses in our country.

This study was successful to identify between ALL and the control group as demonstrated by ROC curve analysis, which showed that *KLK6*, *KLK10*, and *KLK14* mRNA expression levels have a favorable diagnostic utility in ALL. In addition, according to univariate logistic regression analysis patients with downregulated *KLK6*, *KLK10*, and *KLK14* mRNA expression are more likely to develop ALL. This establishes that reduction in *KLK6*, *KLK10*, and *KLK14* mRNA expression could be considerable prognostic biomarkers for ALL. This is the first analysis to investigate *KLK6*, *KLK10*, and *KLK14* mRNA expression in pediatric ALL patient samples after induction and three months of receiving chemotherapy. Changes in the *KLK6*, *KLK10*, and *KLK14* expression profiles after one month and three months of receiving chemotherapy could suggest that the patients were responding to treatment, and the *KLK6*, *KLK10*, and *KLK14* expression profiles could have an impact on disease outcome and could be targeted therapeutically.

5.7 Conclusions

The results of the present study conclude the followings:

1. The mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* in ALL patients were significantly reduced compared to normal blood samples.

2. The mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* revealed significant diagnostic value to distinguish ALL patients from normal counterparts.

3. The reduction in mRNA expression levels of *KLK6*, *KLK10*, and *KLK14* was found to be significant prognostic molecular biomarkers for ALL.

4. The mRNA expression of the genes of interest in newly diagnosed childhood ALL patients was not significantly proportional to patient age, lymphocyte count, WBC, and serum LDH concentration. Except for *KLK14* mRNA level significantly had a negative correlation with WBC.

5. Expression of *KLK6*, *KLK10*, and *KLK14* at mRNA levels was significantly downregulated in ALL patients after one month and three months of receiving chemotherapy compared to their levels in normal blood samples.

6. Thus, this study suggests that *KLK6*, *KLK10*, and *KLK14* mRNA expression levels could be used as molecular biomarkers in the diagnosis and prognosis of ALL. In addition, the expression profile of the *KLKs* could be utilized to predict how well ALL patients respond to chemotherapy.

5.8 Recommendations

The followings are the suggested additional future research based on the findings of this study:

1. Further study on a larger scale of ALL patients and normal blood donors are required to see whether quantification of mRNA expressions of *KLK6*, *KLK10*, and *KLK14* in peripheral blood samples could be utilized in clinical practice to diagnose and predict the presence of ALL.

2. A longer time-schedule follow-up of the patients is required for a more clinical evaluation of the *KLK6*, *KLK10*, and *KLK14* mRNA expression for ALL patients' prognosis and response to chemotherapy.

3. Since mRNA expressions of *KLK6* and *KLK14* in ALL have not been examined before, no prognostic cutoff values were suggested. It is required to categorize their mRNA expression levels and investigate their prognostic significance in ALL.

4. Since levels of mRNA expression of *KLK6*, *KLK10*, and *KLK14* in ALL pediatric patients have been studied for the first time after induction therapy and three months of chemotherapy, further investigations are required to see whether the expression profile of the genes of interest could have the potential to influence disease outcomes and to be therapeutically targeted.

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Appendices

Appendices

Appendix A. Equipment used in the study.

Equipment	Company	Origin
Alcohol Pad	Sugama	China
Aluminum Foil	Sanita	Lebanon
Analytical Balance	Kern Sohn	Germany
Biological Safety Cabinet & Clean Benches	Thermo Scientific	USA
Biophotometer	Eppendorf	Germany
Blood Transportation Box	Krew	India
Conical Flasks	Pyrex	Germany
Deep Freezer (-20 °C)	GFL	Germany
Deep Freezer (-70 °C)	GFL	Germany
Deionizer Nanopure	Thermo Scientific	USA
Digital Camera	Canon	Japan
Disposable Syringe	Set Medical	Germany
EDTA Tube	VACUTEST	Italy
Electronic Stopwatch	Optima	Japan
Electrophoresis Gel Tank	Analytik Jena	Germany
Electrophoresis Power Supply	Analytik Jena	Germany
Eppendorf Tubes	Accumax	India
Gel Documentation System	Cleaver	UK
Graduated Cylinder	Isolab	Germany
Hot Plate Magnetic Stirrer	Roth	Germany
Ice Maker	Scotsman	USA
Microcentrifuge	VWR	Germany
Microwave	Sharp	UK
Multicentrifuge	Heraeus	Germany
Nitrile Gloves	Avedo safety	Greece
Oven	Binder	Germany
Paint Markers	Staedtler	Germany
Parafilm Tape	Sigma Aldrich	USA
PCR Tubes	Accumax	India
pH-Meter	Knick	Germany
Precision Micropipettes	Eppendorf	Germany
Printer	Canon	Japan
Racks	Eppendorf	Germany
Refregirated Centrifuge 5417R	Eppendorf	Germany
Refrigerator (4.0 °C)	Haier	China
Rotor Gene Q Cycler	Qiagen	Germany
Spatula Spoon	Usbeck	Germany
Sterile Cotton	LanYuhan	China

Thermal Cycler	Applied Biosystems	Singapore
Thermometer	Memmert	Germany
Thermomixer Compact	Eppendorf	Germany
Tips	Accumax	India
Tourniquet	Indiamart	India
UV Sterilisation Cabinet	Cleaver	UK
UV Transilluminator	Cleaver	UK
Vertical Autoclave	Indiamart	India
Vortex Mixer	Stuart	UK
Water Bath	Memmert	Germany

Appendix B. Kits and reagents used in the study.

Kits and Reagents	Company	Origin
2X SuPrimeScript RT Premix (SR-3000) Kit	GeNet Bio	South Korea
Absolute Ethanol	Merck	Germany
Agarose	Scharlau	Spain
Agarose Gel Loading Dye	GeNet Bio	South Korea
Boric Acid (MW=61.83 g/mol)	Scharlau	Spain
Deionized Sterile Distilled Water	Bioneer	South Korea
DNA Ladder	GeNet Bio	South Korea
Ethidium Bromide	Promega	USA
Nuclease-Free Water	Ambion	USA
OnePCR™ Ultra (PCR Master Mix) Kit	GeneDireX	USA
Prime PrepTM Blood RNA Extraction Kit	GeNet Bio	South Korea
Primers	Macrogen	South Korea
Rotor-Gene SYBR Green PCR Kit	Qiagen	Germany
EDTA Disodium Salt Dihydrate (MW=372.24 g/mol)	Promega	USA
Tris Base (MW=121.14 g/mol)	Promega	USA
Water, DEPC Treated	GeNet Bio	South Korea
β-Mercaptoethanol	AppliChem	Germany

Appendix C. Ethical committee approval.

The Ethical committee of the College of Medicine should fill this field.

Ethical committee of College of Medicine has met to assess the plan and suggestions of the postgraduate and scientific committees regarding the research project of:

PhD student: **Shwan Majid Ahmad**

Title of the project: **Human kallikrein-related peptidase associated with leukemia**

In the field of **Biochemistry** in the specification of **Clinical Biochemistry and Molecular Biology** under supervision of:

Supervisor: Assistant professor, **Dr Basima Sadiq Ahmed Jaff-** PhD in Clinical Biochemistry, University of Sulaimani- College of Pharmacy- Department of Clinical Biochemistry

Co-Supervisor: Lecturer, **Dr Karzan Ghafur Khidhir-** PhD & Post Doctorate in Molecular Genetic- University of Sulaimani- College of Science- Biology Department

As a result, the committee has decided to approve the PhD project.

Members of the ethical committee of College of Medicine:

No: 55

Date: 17-9-2017

Ass.Prof. Dr. Bakhtiar Mohamed Mahmoud
Head of Committee

Ass.Prof. Dr. Saeed A.Latteef A.Kareem
Member

Ass.Prof. Dr. Farhad M. Abdulkarim Barzinji
Member

Ass.Prof. Dr.Tavga Ahmed Aziz
Member

Ass.Prof. Dr.Mokamad Rasheed Ameen
Member

Ass.Prof. Dr.Sardar Rashid Hama Salih
Member

Dr. Anwar Aboubaker Kareem
Member

Dr. Dyary Hiewa Othman
Member

Dr. Fattah Hama Raheem Fattah
Member

UNIVERSITY OF SULAIMANI
COLLEGE OF MEDICINE
ETHIC COMMITTEE

Appendix D. Consent form.

Dear Sir/ Madam

I am (Shwan Majid Ahmad), PhD student in Clinical Chemistry- College of Medicine- University of Sulaimani, my research protocol is titled: Human Kallikrein-related Peptidase associated with Leukemia.

To perfonn this Ph.D. study I need to collect blood samples from pediatric age individuals: patients diagnosed with leukemia before and after treatment, and healthy control individuals. The project has been approved by each of the Scientific and Ethical Committee at the College of Medicine, The Directorate of Health in Sulaimani Governorate, and the Hiwa Cancer Hospital Scientific Committee.

I would like to ask for your agreement on the participation of your child in this study. His/her name and data will be kept confidential…. Kind Regards

Agreement and Participation:

I have read this consent form and voluntarily I agree and sign to participate our child (……) in the study.

Name: Signature:

Relation to the child: Date:

Mobile Number:

Study Code:

The researcher: Shwan Majid Ahmad

Appendix E. Questionnaire.

Date of interview:

Study code: Name:

Date of Birth: Age:

Sex: Blood group:

Ethinicity: Residence address:

Occupation: Mobile Number:

Any chronic disease:

Any hematological disease:

Family history of cancer/hematological diseases:

Below to be filled for the patients at Hiwa Cancer Hospital who enrolled in the study

Patient ID: Treating doctor:

Date of admission: Date of diagnosis:

Diagnosis type: Diagnosis sub-type:

Date of the blood collections for this study

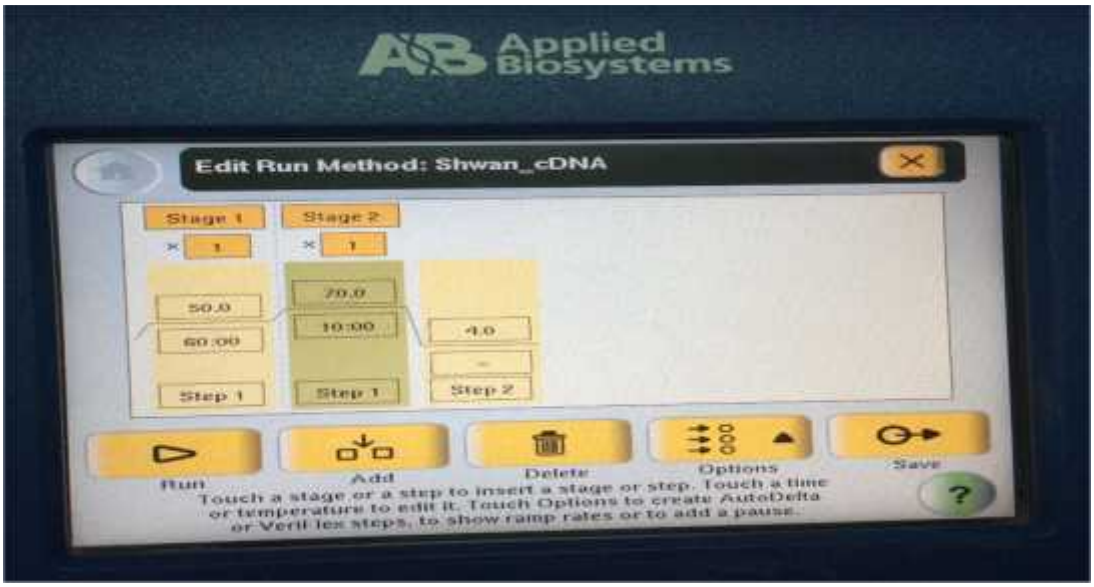
Date of the first sample:

Date of the second sample:

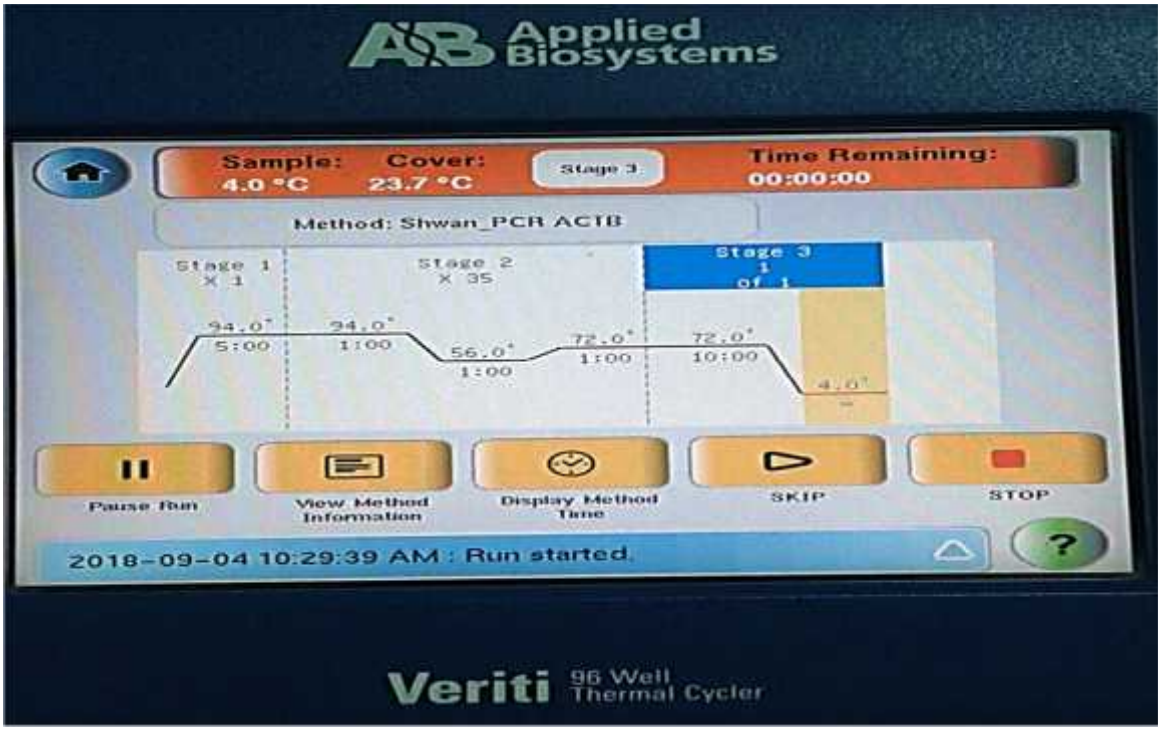
Date of the third sample:

This data is kept confidential and it is only used for research purposes…. Kind regards…. Shwan Majid Ahmad

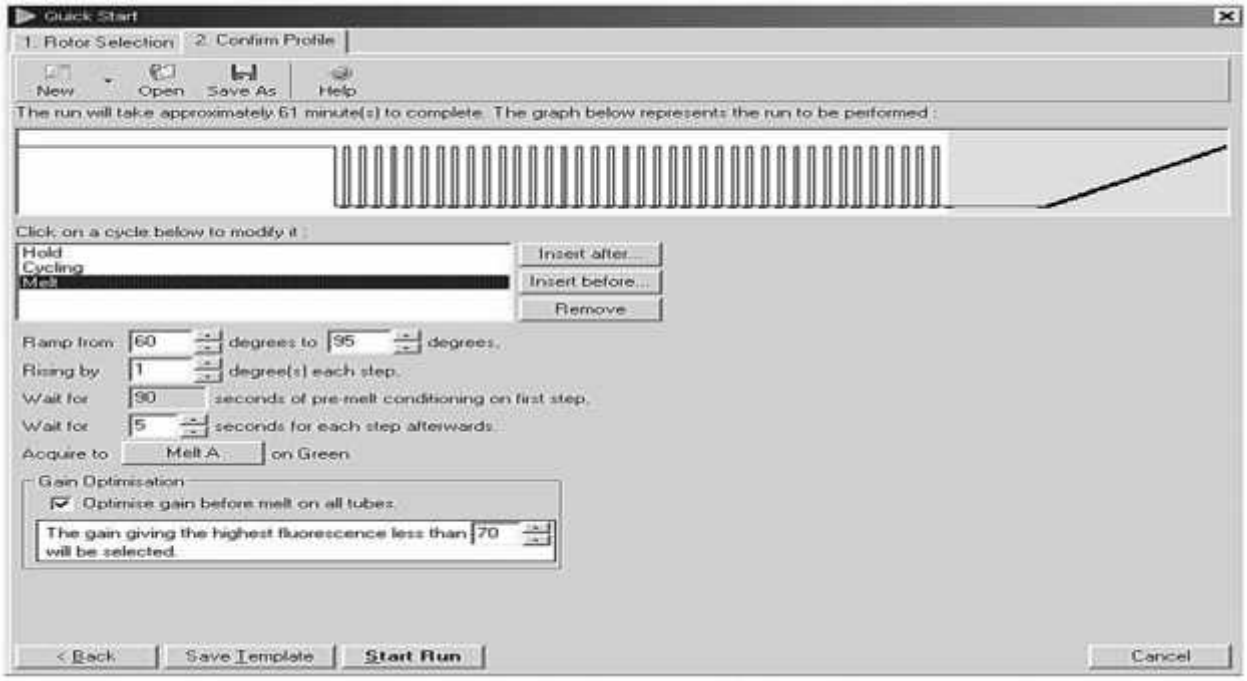
Appendix F. cDNA synthesis cycling conditions.



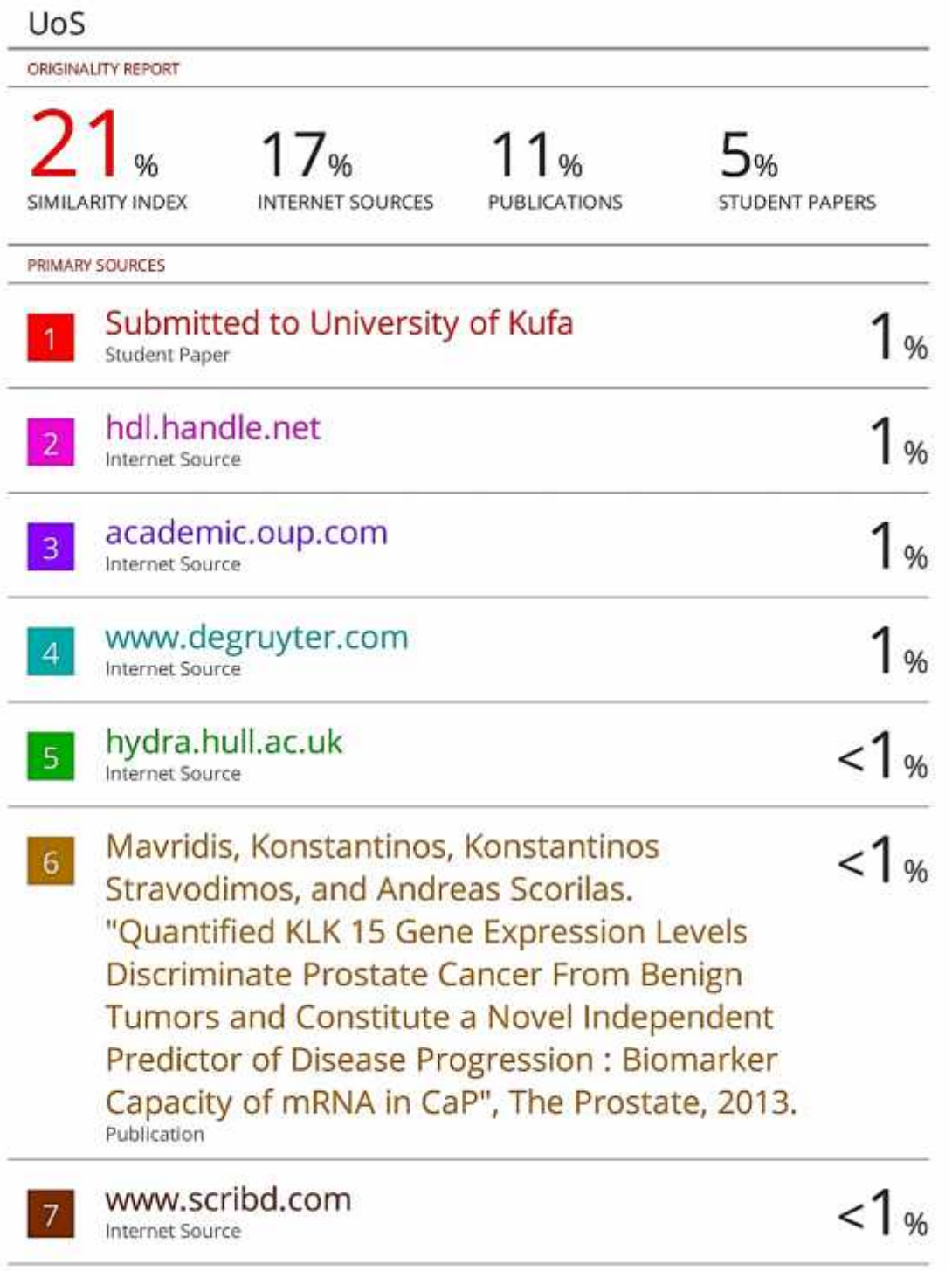
Appendix G. Conventional RT-PCR cycling conditions.



Appendix H. Melting curve analysis.



Appendix I. Originality report.



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Appendix J. Publications relating to the present project

PeerJ

2021-2022
Impact Factor = 3.061

Prospective quantitative gene expression analysis of kallikrein-related peptidase *KLK10* as a diagnostic biomarker for childhood acute lymphoblastic leukemia

Shwan Majid Ahmad[1], Basima Sadq Ahmed[2], Karzan Ghafur Khidhir[3] and Heshu Sulaiman Rahman[4]

[1] Department of Biochemistry, College of Medicine, University of Sulaimani, Sulaimaniyah, Iraq
[2] Department of Biochemistry & Clinical Chemistry, College of Pharmacy, University of Sulaimani, Sulaimaniyah, Iraq
[3] Department of Biology, College of Science, University of Sulaimani, Sulaimaniyah, Iraq
[4] Department of Physiology, College of Medicine, University of Sulaimani, Sulaimaniyah, Iraq

ABSTRACT

Background. The most common malignancy in children is acute lymphoblastic leukemia (ALL). This study aimed to explore *KLK10* mRNA expression as a potential diagnostic biomarker for ALL in children and to examine the effect of chemotherapy on *KLK10* mRNA expression following the induction and after three months of receiving chemotherapy.

Methods. In this prospective study, total RNA was extracted from blood samples of 23 pediatric ALL patients on diagnosis, after one month and three months of receiving chemotherapy. Healthy pediatric volunteers ($n = 12$) were selected as control individuals. After cDNA synthesis, *KLK10* mRNA gene expression levels were quantified using quantitative real-time PCR (qRT-PCR).

Results. *KLK10* mRNA expression levels were significantly decreased in leukemic cells compared to their levels in cells of normal blood samples ($p = 0.0001$). *KLK10* expression levels in ALL patients after one month and three months of receiving chemotherapy decreased compared to normal blood samples ($p < 0.0001$ and $p = 0.0175$ respectively). The expression level of *KLK10* mRNA in ALL patients after one month of chemotherapy was decreased compared to their level on diagnosis ($p = 0.4413$). *KLK10* mRNA expression levels in ALL patients after three months of chemotherapy were increased compared to their level on diagnosis ($p = 0.0602$). The ROC curve illustrated that *KLK10* mRNA expression could very efficiently discriminate ALL patients from normal counterparts (AUC=0.886, 95% CI [0.7720–1.000], SE = 0.0582, $p = 0.0004$).

Conclusion. *KLK10* mRNA expression could serve as a potential diagnostic molecular biomarker for ALL in children.

Submitted 21 January 2022
Accepted 3 May 2022
Published 31 May 2022

Corresponding author
Shwan Majid Ahmad,
shwan.majid@univsul.edu.iq,
shwanchemistry83@yahoo.com

Academic editor
Vladimir Uversky

Additional Information and Declarations can be found on page 11

DOI 10.7717/peerj.13489

Subjects Biochemistry, Molecular Biology, Oncology, Pediatrics, Medical Genetics
Keywords Acute lymphoblastic leukemia, Chemotherapy, Diagnostic biomarker, Kallikrein-related peptidase, *KLK10*, Prospective study, Quantitative real-time PCR

How to cite this article Ahmad SM, Ahmed BS, Khidhir KG, Rahman HS. 2022. Prospective quantitative gene expression analysis of kallikrein-related peptidase *KLK10* as a diagnostic biomarker for childhood acute lymphoblastic leukemia. *PeerJ* 10:e13489
http://doi.org/10.7717/peerj.13489

الخلاصه

(AUC=0.851, 95% CI=0.7087-0.9931, SE=0.0726, p=0.0012) **على التوالى. أظهر تحليل الانحدار اللوجستي أحادي المتغير، أن الجينات الثلاثة يمكن أن يكون مؤشراً موثوقًا للتنبؤ ب** ALL

(OR=0.2289, 95% CI=0.0557-0.9399, p=0.0115) **و**

(OR=0.0228, 95% CI=0.0008851-0.2299, p <0.0001) **و**

(OR=0.0716, 95% CI= 0.003912-0.4610, p=0.0002) **على التوالى. في المرضى** ALL **الذين تلقوا العلاج الكيميائي لمدة شهر واحد، إنخفض مستويات التعبير** mRNA **من** *KLK10* **و** *KLK14* **مقارنة بالمرضى الذين تم تشخيصهم حديثًا (**p=0.04413 **و** p=0.0039 **على التوالى)، بينما زاد التعبير** *KLK6* mRNA (*p*=0.04413). **في المرضى** ALL**، بينما إنخفض التعبير** mRNA **من** *KLK6* **و** *KLK14* **بعد ثلاثة أشهر من العلاج مقارنة بمستواهم في المرضى عند التشخيص (**p=0.6794 **و** p=0.1336 **على التوالى)، بينما زاد التعبير** *KLK10* mRNA (*p*=0.0602).

الاستنتاجات: كشفت هذه الدراسة أنخفاض مستويات التعبير mRNA **لكل من** *KLK6* **و** *KLK10* **و** *KLK14* **بشكل كبير للأطفال المرضى** ALL **مقارنة بالأصحاء (مجموعة التحكم)، بأنها ذات علاقة بالتشخيص. لذا يمكن استخدام مستويات التعبير** mRNA **من** *KLK6* **و** *KLK10* **و** *KLK14* **كمؤشر بيولوجي جزيئي في تشخيص و تنبؤ المرضى** ALL.

الخلفية: سرطان ابيياض الدم الليمفاوي الحاد (ALL) هو أكثر أنواع السرطانات إنتشارا عند الأطفال بسبب التكاثر السريع خلايا لمفاوية الغير الناضجة في الدم ونخاع العظم والأنسجة الأخرى. فى معدل البقاء على قيد الحياة لمدة خمس سنوات يتم علاج أكثر من 80٪ من الأطفال المصابين. بالرغم من هذه النسبة العالية أن المؤشرات الحيوية الجزيئية مطلوبة للتنبؤ بالسرطان و تحديد المخاطرالمرضى والنتائج العلاجية. أن KLKs كاليكرين ذات الصلة بيبتديز هي مجموعة مكونة من خمسة عشر سيرين بروتينيز فريدة، تتميز بأنها أطول عنقود غير منقطع جينوم الأنسان تقع على الكروموسوم 19 و توجد في الأنسجة وسوائل الجسم، و تؤثر على الوظائف الفسيولوجية. أن تعبير الغير الطبيعى لهذه KLKs يرتبطً بالأمراض و السرطانات و درست تأثيراتها فى علم الأورام السريري، حيث و'جدت علاقة وثيقة بينها و بين أنتيجين الخاص بالبروستات (PSA/KLK3)، و أن التحليل الجزيئي لأعضاء اخرى من مجموعة KLKs لأورام الدم الخبيثة موضوع جديد والتحقق من إسهام KLKs في السرطانات مستمرة لحد الأن.

الأهداف: يهدف البحث الى تحديد مستويات التعبير mRNA ل *KLK6* و *KLK10* و *KLK14* كمؤشر بايولوجي فعال للتشخيص و التكهن لسرطان الدم الليمفاوي عند الأطفال، وتقييم تأثير العلاج الكيميائي على ملف تعبيرهم بعد شهر وثلاثة أشهر من العلاج الكيميائي.

المواد و الطرق: هذا البحث دراسة تنبؤيه، تحليلية، مبنية على الملاحظة، و تحكيم الحالات المرضية و الغير المرضية. تمت هذه الدراسة على أطفال تتراوح أعمارهم بين (1 – 15) سنة، 23 طفلاً مصابا بالمرض ALL حديثًا وافدين في مستشفى هيوا للسرطان فى مدينة السليمانية. و تم اختيار 12 متطوعا كأطفال أصحاء كمجموعة تحكم. تم جمع عينات الدم من مرضى ALL في ثلاث فترات مختلفة: عند التشخيص بـ ALL، بعد شهر وثلاثة أشهر من العلاج الكيميائي. تم عزل أجمالي الحمض النووي الريبيRNA من عينات الدم ، و تحضير cDNA، و من ثم تحليل مستويات تعبير mRNA من *KLK6* و *KLK10* و *KLK14* باستخدام PCR الكمي في الوقت الحقيقي (qRT- PCR).

النتائج: إنخفض مستويات تعبير ل mRNA من *KLK6* و *KLK10* و *KLK14* بشكل كبير في عينات الدم المأخوذة للأطفال المرضى ALL مقارنة بدم الأصحاء، و تم أيجاد علاقة احصائية مهمة (p=0.002 وp=0.0001 و p=0.0007 على التوالى). إنخفضت مستويات تعبير mRNA من *KLK6* و *KLK10* و *KLK14* بشكل كبير في المرضى الذين تلقوا العلاج الكيميائي لمدة شهر مقارنة بمستوياتهم في عينات الدم الأطفال الأصحاء،و ايجاد علاقة احصائية مهمة (p=0.0292 وp<0.0001 و p<0.0001 على التوالى). كما إنخفض تعبيرات الجينات بشكل كبير في المرضى ALL الذين تلقوا العلاج لمدة ثلاثة أشهر مقارنة بمستوياتهم في عينات الدم للأطفال الأصحاء و تم ايجاد علاقة احصائية مهمة (p=0.0038 وp=0.0175 وp<0.0001 على التوالى). كشف تحليل منحنى ROC عن القيمة التشخيصية الهامة لتعبير *KLK6* و *KLK10* و *KLK14* للتمييز بين المرضى ALL و الأصحاء

(AUC=0.822, 95% CI=0.6735-0.9713, SE=0.076, p=0.0029) و

(AUC=0.886, 95% CI=0.7720-1.000, SE=0.0582, p=0.0004) و

التحليل الكمي للتعبير الجيني للكاليكرين ذات الصلة ببيبتديز *KLK6* و *KLK10* و *KLK14* في سرطان الدم الليمفاوي الحاد عند الأطفال

اطروحة مقدمة الى مجلس كلية الطب فى جامعة السليمانية كجزء من متطلبات نيل شهادة الدكتوراه في الكيمياء السريرية

من قبل

شـوان ماجد احمد محمود

بأشراف

الأستاذ المساعد

د.باسمه صادق احمد جاف

الأستاذ المساعد

د.كارزان غفور خضر

1443 هجرى 2022 ميلادى 2722 كوردى

یەك بە دوای یەك). **هەروەها ئاستی دەربڕینی بۆهێڵەکان لە نەخۆشانی** ALL **کە بۆ ماوەی سێ مانگ چارەسەری کیمیاویان وەرگرتوە نزمترە بە بەراورد بە نمونەی خوێنی ساغ، نرخی ئاماریان کاریگەرە** (p=0.0038 **و** p<0.0001 **و** p=0.0175 **یەك بە دوای یەك**). **شیکاری چەماوەی** ROC **گرنگی توانای بەهای دەستنیشانکردنی ئاستی دەربڕینی** mRNA**ی** *KLK6* **و** *KLK10* **و** *KLK14* **پیشاندا لە جیاکردنەوەی توشبوانی** ALL **لەگەڵ گروپی کۆنترۆڵ**

(AUC=0.822, 95% CI=0.6735-0.9713, SE=0.076, p=0.0029) **و**

(AUC=0.886, 95% CI=0.7720-1.000, SE=0.0582, p=0.0004) **و**

(AUC=0.851, 95% CI=0.7087-0.9931, SE=0.0726, p=0.0012) **یەك بە دوای یەك. شیکاری پاشۆچونی لۆجستی یەك گۆڕاو ئەوەی پیشاندا کەمبونەوەی ئاستی هەر سێ بۆهێڵەکە لەوانەیە وەك زیندەنیشاندەرێکی پێشبینیکەر بەکاربێن بۆ دۆخی نەخۆشانی** ALL (OR=0.2289, 95% CI=0.0557-0.9399, p=0.0115) **و**

(OR=0.0228, 95% CI=0.0008851-0.2299, p <0.0001) **و**

(OR=0.0716, 95% CI= 0.003912-0.4610, p=0.0002) **یەك بە دوای یەك. لە نەخۆشانی** ALL **کە ماوەی یەك مانگ چارەسەری کیمیاویان وەرگرتبوو ئاستی دەربڕینی** mRNA**ی** *KLK10* **و** *KLK14* **کەمی کرد بە بەراورد بە نەخۆشانی تازە دەستنیشانکراو** (p=0.04413 **و** p=0.0039 **یەك بە دوای یەك**)، **لە کاتێکدا ئاستی دەربڕینی** mRNA**ی** *KLK6* **زیادی کرد** (p=0.04413). **لە نەخۆشانی** ALL **کە ماوەی سێ مانگ چارەسەری کیمیاویان وەرگرتبوو ئاستی دەربڕینی** mRNA**ی** *KLK6* **و** *KLK14* **کەمی کرد بە بەراورد بە نەخۆشانی تازە دەستنیشانکراو** (p=0.6794 **و** p=0.1336 **یەك بە دوای یەك**)، **لە کاتێکدا ئاستی دەربڕینی** mRNA**ی** *KLK10* **زیادی کرد** (p=0.0602).

دەرئەنجامەکان: ئەم تویژینەوەیە ئەوەی دەرخست ئاستی دەربڕینی mRNA**ی** *KLK6* **و** *KLK10* **و** *KLK14* **لە منداڵانی نەخۆشی** ALL **بە ڕێژەیەکی بەرچاو کەمدەکا بە بەراورد بە منداڵانی گروپی کۆنترۆڵ، ئەمەش ئەوەی نیشاندا ئەم بۆهێڵانە گرنگی دەستنیشانکردنیان هەیە. بۆیە ئاستی دەربڕینی** mRNA**ی** *KLK6* **و** *KLK10* **و** *KLK14* **دەتواندرێت بەکاربهێنرێت وەك زیندەنیشاندەرێکی گەردی بۆماوەیی لە دەستنیشانکردن و پێشبینیکردنی** ALL.

پاشخان: شێرپه نجه‌ی کتوپڕی خرۆکه‌ی سپی خوێن (ALL) به‌ربلاوترین شێرپه نجه ده‌ستنیشانکراوه‌کانه له منداڵان. به‌هۆی له‌ڕاده‌به‌ده‌ر زیادبونی لیمفه‌خانه پێنه‌گه‌یشتوکان له خوێن و مۆخی ئێسك و شانه‌کانی تر. له ڕێژه‌ی پێنج ساڵ مانه‌وه له ژیاندا، زیاتر له 80٪ نه‌خۆشانی منداڵان چاره‌سه‌ر ده‌کرێن. سه‌ره‌ڕای به‌رزی ئه‌م ڕێژه‌یه، زینده‌نیشانده‌ری بۆماوه‌یی گه‌ردی هه‌ستیار و تاییه‌ت پێویستن بۆ ده‌ستنیشانکردن و پێشبینیکردنی شێرپه نجه وه باشتر دیاریکردنی ئاستی مه‌ترسی و ده‌رئه نجامه‌کانی چاره‌سه‌ر. کالیکرینی په‌یوه‌ندیدار به پێپتیده‌یز KLKs پێکهاتوه له کۆمه‌ڵه‌یه‌کی پانزه ئه‌ندامی سه‌ربه‌خۆی ده‌ردراوی پرۆتیه‌یزی سێرین، به‌وه ناسراون دڕێژترین کۆمه‌ڵه‌ی نه‌پچڕاوی جینۆمی مرۆڤن ده‌که‌ونه سه‌ر کرۆمۆسۆمی ژماره 19. KLKs له زۆرێك له شانه و شله‌کانی له‌شدا هه‌ن، کاریگه‌رییان هه‌یه له‌سه‌ر چالاکی و فه‌رمانه فسیۆلۆجیه‌کان. ده‌ربڕینی نائاسایی ئه‌م کالیکرینانه په‌یوه‌ندیان هه‌یه به زۆرێك له نه‌خۆشی و شێرپه نجه. به‌کارهێنانی کالیکرینه‌کان له کلینکی شێرپه نجه‌زانی دا ناسراون. ئه‌نتیجینی تاییه‌ت به پرۆستات (PSA/KLK3) له گرنگترین کالیکرینه‌کانن به‌کاردێت له پشکنینی کلینکی. له‌گه‌ڵ ئه‌وه‌شدا، شیکاری بۆماوه‌یی گه‌ردی ئه‌ندامه‌کانی تری خێزانی KLKs له شێرپه نجه‌ی خوێندا نوێیه، وه لێکۆڵینه‌وه له په‌یوه‌ندی KLKs به شێرپه نجه‌وه به‌رده‌وامه.

ئاماڕنجه‌کان: ئه‌م توێژینه‌وه‌یه ئاماڕنجی دیاریکردنی ئاستی ده‌ربڕینی mRNA ی *KLK6* و *KLK10* و *KLK14* وه‌ك زینده‌نیشانده‌رێکی گه‌ردی بۆماوه‌یی له ده‌ستنیشانکردن و پێشبینیکردنی شێرپه نجه‌ی کتوپڕی خرۆکه‌ی سپی خوێن له منداڵان، هه‌روه‌ها هه‌ڵسه‌نگاندنی کاریگه‌ری چاره‌سه‌ری کیمیاوی له پڕۆفایلی ده‌ربڕینه‌کانیان دوای یه‌ك مانگ وه سێ مانگ له وه‌رگرتنی چاره‌سه‌ری کیمیاوی.

که‌ره‌سته و ڕێگاکانی توێژینه‌وه: ئه‌م توێژینه‌وه‌یه لێکۆڵینه‌وه‌یه‌کی داهاتویی چاوه‌ڕێکردنه وه شیکاری چاودێریکردنه له که‌سی نه‌خۆش و ساغ. به‌ژداربوانی ئه‌م توێژینه‌وه‌یه منداڵانی ته‌مه‌ن 1 بۆ 15 ساڵان بوون، 23 نه‌خۆشی تازه ده‌ستنیشانکراوی ALL له به‌شی منداڵان له نه‌خۆشخانه‌ی هیوا له شاری سلێمانی، هه‌روه‌ها 12 منداڵی ساغ وه‌ك گروپی کۆنترۆڵ به‌ژداربون. نمونه‌ی خوێن له توشبوان به ALL وه‌رگیران له سێ کاتی جیا دا: له کاتی ده‌ستنیشانکردنی نه‌خۆشیه‌که‌یان، هه‌روه‌ها دوای یه‌ك مانگ وه سێ مانگ له وه‌رگرتنی چاره‌سه‌ری کیمیاوی. ترشه‌ناوکی ڕایبۆز RNA جیاکرایه‌وه له نمونه‌ی خوێنه‌کان وه cDNA لێ دروستکرا، پاشان شیکاری ئاستی ده‌ربڕینی mRNAی *KLK6* و *KLK10* و *KLK14* بۆ کرا به به‌کارهێنانی quantitative real-time PCR (qRT- PCR).

ئه نجامه‌کان: ئاستی ده‌ربڕینی mRNAی *KLK6* و *KLK10* و *KLK14* له نمونه‌ی خوێنی منداڵی نه‌خۆشی ALL نزمتره به به‌راورد به نمونه‌ی خوێنبه‌خشی ساغ به‌شێوه‌یه‌کی به‌رچاو (p=0.002 وp=0.0001 وp=0.0007) یه‌ك به یه‌ك دوای یه‌ك). ئاستی ده‌ربڕینی mRNAی *KLK6* و *KLK10* و *KLK14* له نه‌خۆشانی ALL که بۆ ماوه‌ی یه‌ك مانگ چاره‌سه‌ری کیمیاویان وه‌رگرتوه نزمتره به به‌راورد به نمونه‌ی خوێنی ساغ، به‌های ئاماریان کاریگه‌ره (p=0.0292) و

شـیکاری چەندێتی دەربڕینە بۆهێڵیەکانی کـالیکرینی پەیوەندیدار بە پێپتیدەیز *KLK6* و *KLK10* و *KLK14* لە شێرپە نجەی کتوپڕی خرۆکەی سپی خوێن لە منداڵان

نامـەیەکە پێشکەشکراوە بە ئە نجومەنی کۆلێجی پزیشکی زانکۆی سلێمانی وەک بەشێک لە پێداویستیەکانی بەدەستهێنانی بڕوانامەی دکتۆرا لە کلینکەڵ کمستری

لەلایەن

شـوان ماجد احمد محمود

بەسەرپەرشتی

پرۆفیسۆری یاریدەدەر
د.باسمە صادق احمد جاف

پرۆفیسۆری یاریدەدەر
د.کارزان غفور خضر

1443 کۆچی　　2022 زاینی　　2722 کوردی