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عنقود التمايز ١٤: عامل تكهن جديد في مرضى سرطان الدم اللمفاوي المزمن

أطروحة

مقدمة إلى مجلس كلية الطب في جامعة بابل كجزء من متطلبات نيل

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١٤٤٤هـ

٢٠٢٢م

الخلاصة :

الخلفية :

يعتبر مرض ابيضاض الدم اللمفاوي المزمن مرض عضال ويمثل سرطان الدم الاكثر شيوعا عند البالغين لدى الغرب حيث يتميز بتراكم تدريجي للخلايا اللمفاوية الناضجة في نخاع العظم والدم المحيطي وتميل هذه الخلايا الى ان تكون خالدة. وتتسبب في انماط سريرية مختلفة تتراوح بين المسار البطيء والتدهور السريع (المرض العدواني) . لذلك تركزت العديد من الجهود لإيجاد مؤشرات موثوقة يمكن ان تساعد في تنبؤ النتائج او شرح التباين السريري للمرض . برز عنقود التمايز ١٤ ليصبح عاملا جديدا كأحد هذه المؤشرات لكنه ما يزال يحتاج الى تقييم شامل لإمكانية اعتماده في تكهن المرض .

هدف الدراسة :

هدفت الدراسة الى تقييم مستوى عامل التمايز ١٤في مصل مرضى ابيضاض الدم اللمفاوي المزمن و دراسة ارتباط هذا المستوى مع مرحلة المريض السريرية وعوامل مختبرية وسريرية اخرى.

المرضى وطرق العمل :

شملت هذه الدراسة ٤٥ مريضا يعانون من ابيضاض الدم اللمفاوي المزمن ممن حضروا العيادات الخارجية في مستشفى بغداد التعليمي ومستشفى مرجان التعليمي من اكتوبر ٢٠٢١ الى ابريل ٢٠٢٢مع ٤٥ مشاركا بالغا سليما للمقارنة مع مجموعة المرضى وقد شخص جميع المرضى بناءا على الفحص السريري وصورة الدم وقياس التدفق الخلوي لكل منهم. وقسموا الى مرضى مصابين بابيضاض الدم اللمفاوي المزمن النموذجي (٩٥,٦%) ومرضى مصابين بابيضاض الدم اللمفاوي المزمن اللانموذجي (٤,٤%).وصنفوا الى ثلاث مجاميع حسب مرحلة المرض السريرية وفقا لمراحل نظام راي وبنت. تم جمع عينات الدم وتم اجراء فحص الدم مع صورة الدم وقياس مستوى عامل التمايز١٤ في مصل الدم بواسطة انزيم مرتبط مقايسة الممتز المناعي (ELISA) لمجموعة المرضى والمجموعة الضابطة.

النتائج : كانت اعمار المرضى تتراوح بين٤٠ سنة و٧٥ سنة ومعدل عمر ٥٩,٦٤سنة. غالبية المرضى كانت لجنس الذكور ٧١,١%. بينت الدراسة ان مستوى عامل التمايز ١٤ في مصل دم مرضى ابيضاض الدم اللمفاوي المزمن أعلى مما عليه في المجموعة الضابطة . وكان هناك ارتباطا

هاما لهذا المؤشر مع مراحل المرض المتقدمة حسب نظام راي وبنت وكذلك كان المستوى اعلى في المرضى الذين لديهم تضخم الطحال والكبد او مضاعفات خاصة نتيجة المرض ، واظهرت عدم وجود ارتباطا مع عمر او جنس المريض او صنف المرض (نموذجي او غير نموذجي) ونسب مكونات الدم والهيموغلوبين.

الاستنتاج : نستنتج من هذه الدراسة ان مرضى ابيضاض الدم اللمفاوي المزمن لديهم مستويات عالية لعامل التمايز ١٤ ويرتبط ارتباطا وثيقا بتقدم مراحل المرض ولذا فبالإمكان استخدامه كعامل تكهن للمرض وربما يمكن اعتماده كهدف للعلاج مستقبلا.

Repuplic of Iraq
Ministry of Higher Education and Scientific Research
University of Babylon
College of Medicine
Department of Pathology

CD14: A New Prognostic Factor in Patients with Chronic Lymphocytic Leukaemia

A thesis

Submitted to the Council of the College of Medicine and the committee of postgraduate studies of Babylon University in partial fulfillment of the requirement for the degree of Master in Pathology

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1444 AH

بِسم اللّهِ الرَّحمنِ الرَّحِيم

﴿ نَرۡفَعُ دَرَجَٰتٖ مَّن نَّشَآءُۗ وَفَوۡقَ كُلِّ ذِي عِلۡمٍ عَلِيمٞ ﴾

صَدَقَ اللّهُ العَظِيم

سورة يوسف الاية [٧٦]

Certification of Supervision

We certify that this thesis entitled " CD14: Anew prognostic factor in patients with chronic lymphocytic leukaemia " was prepared under our supervision at the **College of Medicine / Babylon University** as a partial requirement for the master degree in pathology.

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Examining committee certificate

We, the examiner committee, certify that we have read the thesis entitled **" CD14: Anew prognostic factor in patients with chronic lymphocytic leukaemia "** and have examined the student **(Sabreen Hameed Zghair)** in its contents, and that in our opinion it is accepted as a thesis for degree of Master in Science of Pathology.

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Dedication

To my large family:

My parents, *I could never have done anything in my life journey without your love, support and prays. Thank you for teaching me to trust Allah and believe in myself.*

My siblings, *the best gift my parents bring in my life, thanks for being my faithful friends. You give me the power to do my best.*

To my small family:

My husband, *thanks for your love, patience and friendship. Being always by my side makes everything possible.*

My sons *thank you for giving me the energy to continue every time I look into your eyes.*

Everything I do, and all the success I've got or will get is because of them and for them…

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Sabreen Hameed

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List of Abbreviations

Abbreviation	Key
μg	microgram
AIG	Autoimmune Granulocytopaenia
AIHA	Autoimmune Haemolytic Anaemia
ANOVA	Analysis Of Variance
BAK	Bcl-2 homologous Antagonist/killer
B-CLL	B cell Chronic Lymphocytic Leukaemia
Bcl-2	B Cell Lymphoma 2
BCR	B Cell Receptor
BP	Baise Pair
CBC	Complete Blood Count
CD	Cluster Of Differentiation
aCLL	Atypical ChronicLymphocytic Leukaemia
CLL-IPI	Chronic Lymphocytic Leukaemia International Prognostic Index
C-T	Cytosine to Thymine
DLBCL	Diffuse Large B-cell Lymphoma
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FL	Follicular Lymphoma
FMC7	Flinders Medical Center7
g/dL	Gram per deciliter
GPI	Glycoprotein inositol
Hb	Haemoglobin
HCL	Hairy cell leukaemia
HRP	Horseradish peroxidase
IFN	Interferon
IG	immunoglobulin
IGHV	Immunoglobulin heavy chain variable region
IL1	Interleukin 1
IRF3	Interferon regulatory factor 3
kDa	kilo dalton

LAP	Lymphadenopathy
LN	Lymph node
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Membrane associated protein kinase
MBL	Monoclonal B-cell lymphocytosis
mCD14	Membrane associated cluster of differentiation 14
MCL	Mantle cell lymphoma
MCL1	Myeloid cell leukemia-1
M- CLL	Mutated IGHV genes chronic lymphocytic leukaemia
MD	myeloid differentiation factor
mg/dL	Milligram per deciliter
Ml	Milliliter
MZL	Marginal zone lymphoma
NF-kB	Nuclear factor kappa B
OD	Optical density
PB	Peripheral blood
PLL	Prolymphocytic leukemia
sCD14	Soluble cluster Of differentiation14
sIg	Surface membrane immunoglobulins
SLL	Small lymphocytic lymphoma
SLVL	Splenic lymphoma with villous lymphocytes
SNP	Single nucleotide polymorphism
TLR	Tall like receptor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
U-CLL	Unmutated IGHV genes chronic lymphocytic leukaemia
WBC	White blood cell
WHO	World health organization
XIAP	X-linked inhibitor of apoptosis protein
ZAP70	Zeta chain associated protein kinase 70

Abstract

Background: Chronic lymphocytic leukaemia is presently an incurable disease representing the most prevalent adult leukaemia in western countries characterized by progressive accumulation of mature lymphocyte in bone marrow and peripheral blood that tend to be immortal cause heterogeneous clinical course, ranging from indolent course to aggressive disease. So, many efforts focus on finding reliable indicators that can help to predict the outcome or explain CLL clinical variability. Novel marker such as cluster of differentiation 14 (CD 14) has been emerged, But it need thorough assessment for its prognostic capacity in CLL.

Aims of the Study: The goal of the study was to assess the level of CD14 in CLL patients' serum and to evaluate the relation of CD14 with CLL stage and other clinical and laboratory haematological parameters and estimate its possible role as prognostic factor in CLL patients.

Subjects and Methods: This is a case control study included 45 CLL patients who were attending the outpatient clinic of haematology in Baghdad teaching hospital and Marjan teaching hospital from October 2021 to April 2022, together with 45 adult participants without CLL were involved for comparison with patients group. All patients involved were newly diagnosed as having CLL based on physical examination, immunophenotyping profile. According to their flowcytometry, divided into patients with typical CLL (95.6%) and patients with atypical CLL (4.4%). The patients staged according to Rai and Binet staging systems. Blood samples were collected from each

subject, and the following investigations were done: CBC, blood film examination and ELISA assay for serum CD14.

The results: The patients' ages ranged from 40 to 75 year with a mean age of (59.64 ± 8.01yr.). The majority of patients were males (N=32, 71.1%). The mean differences of CD14 concentration (mg/l) between study groups demonstrated significant higher level among patient group (1.77 ± 0.63) than control group (1.43 ± 0.43) with P value of 0.004. The level of serum CD 14 in atypical CLL (2.12 ± 0.44) have been found to be more than typical cases (1.75 ± 0.63) but the difference was statistically not significant (P=0.43).

There was no significant correlation of mean serum CD14 levels with patients gender (P=0.075) or age (P=0.148). CD14 level was higher in advanced stages with a mean of (2.11 ± 0.38, P=.022) in high risk / Rai stage and with a mean of (2.08 ± 0.39, P=0.035) in Binet stage C. There were significant differences between means of CD14 in patients with complication and those with organomegaly (P-value= 0.001and 0.015) respectively. There were no significant correlation between CD14 and hematological parameters although there was negative correlation with haemoglobin concentration and platelet count.

Conclusions: Patients with CLL had higher serum CD14 level than normal individuals and CD14 level was significantly associated with more advanced CLL stage. CD14 level was significantly higher in CLL patients with organomegaly and complicated cases. CD14 has no significant correlation with age, gender, lymphadenopathy, CLL subtype and hematological parameters. CD14 is considered a new prognostic factor in CLL was linked to clinical stage and disease progression. Hence, it can be used to predict CLL outcome and may be a future therapeutic target.

CHAPTER ONE

INTRODUCTION

1.1: Introduction

Chronic lymphocytic leukemia (CLL) is a lymphproliferative disorder recognized with accumulated small lymphocytes at lymph nodes, bone marrow, blood, liver, spleen, or often at other organs. These lymphocytes are marked with mature morphology and immature biology[1].

CLL is a prevalent leukaemia that affects adults in the Western world, accounting for about 30% of all leukaemias and approximately 10% of all haematological neoplasms[2]. There are 4.9 new cases per 100,000 per year in the UK and USA. The disease predominantly identified in older patients with a median age of 74 year at diagnosis[3].

CLL is a disorder of neoplastic B-cells, according to the World Health Organization (WHO) classification, whereas T-CLL is now known as T-cell prolymphocytic leukaemia[4].

CLL is caused mainly by defect in apoptosis mechanisms. A loss of apoptosis has been associated with prolonged survival of neoplastic B-cells expressing B-cell lymphoma2 (Bcl2). The Bcl2 protein family, which includes both pro and anti-apoptotic proteins, is a major regulator of the apoptotic cascade. Bcl2 was the first member of this family to be discovered and is still the best known[5]. Constitutive high-level expression of Bcl2 is a hallmark of CLL, assisting the malignant cells to resist apoptosis and resulting in accumulation of malignant B lymphocytes[6]. There is an inherited genetic susceptibility for CLL, with a six fold to nine fold increased risk for family members of patients with CLL[7].

The disease exhibit considerable heterogeneous clinical course, ranging from patients requiring treatment immediately after diagnosis to those not requiring treatment for many years; These two subsets are distinguished by

mutational status of the immunoglobulin heavy chain variable region gene (IGHV). Patients who have cells which express a non-mutated IGHV often have a more aggressive disease than those who have CLL cells that express a mutated IGHV[8]. Many patients are diagnosed after the incidental finding of a persistent lymphocytosis, whereas others have symptoms. Peripheral blood morphology and immunophenotyping are important diagnostic procedures. Prognosis can be determined using clinical staging, lymphocyte doubling time, and biomarkers. Because CLL has generally been thought to be incurable, the aims of therapy are to prolong survival and control symptoms[9].

CLL can be transformed to an aggressive lymphoma (Richter's Transformation) this occurs in 5% of CLL patient[10]. Despite considerable gains in CLL diagnosis, prognosis, and therapy over the last decades, little is known about how these advancements have influenced patient survival at the population level, particularly among newly diagnosed patients[11].

Atypical CLL (aCLL) a subtype of CLL characterized by larger cells, cells with abundant cytoplasm than the classical form and/or with the presence of cleaved cells. This subtype of CLL was associated to a particular cytogenetic finding (trisomy of chromosome 12) as well as to certain phenotypic findings (higher intensity of cell B markers, which are usually decreased in CLL such as brighter expression of sIg and positivity for FMC7) and a potentially lower Matutes score and a worse prognosis[12].

Cluster of differentiation 14 is one of several markers being studied for prognostic assessment in CLL patients. CD14 is a fascinating molecule with various functions and associations. It is now generally recognized that CD14 functions as a pattern recognition receptor for a wide range of ligands in innate immunity, including apoptotic cells, fungus, bacterial products and microbial cell walls[13].

CD 14 has been thought to play a broader role in cancer, atherosclerosis and metabolic disease regulation, in addition to its innate immunity function. CD14 protein exists in two forms: membrane molecule (mCD14) and a soluble form (sCD14)[14]. Although the precise function of the CD14 antigen in B-CLL is unclear, some studies revealed that CD14 expression in B-CLL represents a distinct prognostic factor and an important predictor of overall survival in patients with B-CLL[15].

1.2: Aims of the study

1. Assessment of serum CD14 level in CLL patients.

2. To evaluate the relationship between CD14 and CLL stage, as well as other clinical and laboratory haematological parameters and to estimate its possible role as a prognostic factor in CLL patients.

CHAPTER TWO

LITERATURE REVIEW

2.1: Chronic Lymphocytic Leukaemia (CLL)

2.1.1: Definition

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder of the B-lineage with a distinct morphology and immunophenotype. The cell of origin is now assumed to be an antigen experienced, activated B-cell, which in some patients is a postgerminal center B-cell that have undergone somatic hyper-mutation of IGHV gene, while in others the cells have developed response via a T-cell independent processes outside the germinal center and without hyper-mutation[16].

2.1.2: Epidemiology

CLL is the most common leukemia in the West that affects elderly people(64-70 year)[17]. According to the American Cancer Society's estimates for CLL in the United States for 2021 there were approximately 21,250 new cases of chronic lymphocytic leukemia and CLL responsible for around 4,320 fatalities, also CLL accounts for a quarter of all new leukemia cases. The chance of developing CLL in a person's lifetime is roughly 1 in 175 (0.57%)[18].

The incidence rates in men are nearly twice as high as in women. CLL is less common among people of African or Asian origin[19].

CLL account for 10% of all leukaemia diagnosed in Arab nations[20]. According to the most up-to-date issued Iraqi Cancer Boared Registry in 2019, leukemia was the fifth of top 10 most common cancers in Iraq accounting for 5.51% with 1977 new case / year. Unfortunately there is no registration for incidence of each type of leukemia[21].

In a local study in Karbala province in Iraq, CLL was the least common type of leukaemia which represented only 15.7% of all leukaemia types[22].

2.1.3: Etiology

The etiology is poorly understood, yet it is considered as an acquired disorder. Conversely there is a suggestion of genetic factors contribute to disease susceptibility; first-degree relatives of patients with CLL have an 8.5-fold increased risk of developing this disease, and the concordance of CLL is higher among monozygotic twins than among dizygotic twins[23].

Monoclonal B-cell lymphocytosis (MBL), which is more frequent in high-risk CLL families than the general population, may be an early genetic factor indicative of inherited predisposition, some studies suggest that geographic and racial variability in CLL incidence and prognosis reflect underlying differences in genetic risk factors[24].

Other studies has discovered a link between CLL and certain environmental conditions, such as working and living on a farm, hepatitis C virus seropositivity, and occupational history as a hairdresser. Amusingly, a moderate inverse association has been described with sun exposure, atopic conditions (asthma, hay fever, eczema), cigarette smoking[25]. Although radiation has been associated to an increased risk of other forms of leukemia, it has not been connected to an increased risk of CLL. Blood transfusions have not been identified as a risk factor[26].

2.1.4: Pathogenesis

CLL is distinguished by a typical failure in apoptosis, a physiological programmed cell death required for development regulation, homeostasis maintenance and tumorigenesis prevention. One of the hallmarks of cancer is

the ability to evade the apoptotic program, which is a key mechanism in clinical resistance to treatment. This is especially true for CLL [27].

The leukaemic cells in the blood are dormant but are unable to initiate their apoptotic program. This is due to a variety of factors, including impairment in the CLL cells' apoptotic machinery and an excess of survival signals from the microenvironment (complex mix of stromal cells, nurse-like cells, T-cells and macrophages), that generate chemokines and interleukins that activate survival pathways such as NF-kB. In CLL cells, these pathways are constantly active, resulting in the production and overexpression of critical antiapoptotic proteins like B-cell lymphoma-2 (BCL-2), myeloid cell leukemia-1 (MCL-1), Bcl-2 homologous antagonist/killer (BAK), and X-linked inhibitor of apoptosis protein (XIAP)[28].

Many studies have confirmed the fundamental role of B cell receptor (BCR) activation for CLL etiology, the BCR is consist of immunoglobulin (IG) molecules plus the CD79a/b subunits. There are two primary molecular subgroups: those with mutated IGHV genes (M-CLL) and those with unmutated IGHV genes (U-CLL). U-CLL derived from B cells that have not passed through the germinal center, while M-CLL derived from postgerminal center B cells[29].

The role of BCR-mediated signaling varies depending on IGHV mutation status: M-CLL cells are generally prone to anergy, whereas U-CLL cells are more prone towards cell growth and proliferation. Furthermore, anergic cells generally have a greater susceptibility to apoptosis unless anti-apoptotic proteins such as BCL2 are upregulated, as is the case in CLL cells [30]. Epigenetic studies found a third subtype with a profile made of cases with

moderate IGHV mutation. All three epigenetic subgroups differ in their use of IGHV genes, stereotypes, genomic aberrations and clinical outcome[31].

CLL is not a sudden event, it start and progress slowly, for this reason it is called chronic. Based on this idea it is suggested that most, monoclonal B cell lymphocytosis (MBL), a very indolent cell expansion defined by less than 5,000 monoclonal B cells in the peripheral blood, precedes most, if not all cases of CLL. MBL is found in about 5% of the elderly people and has a risk of developing into CLL of about 1% / year[32].

CLL is characterized by progressive immunological dysregulation in the cellular, humoral, and innate immune components, which is associated with an early increase in the absolute number of circulating T-cells, particularly immunosuppressive T-regulatory cells and myeloid-derived suppressor cells[33]. The leukemic B cells are responsible for initiating and perpetuating the immunological dysregulation seen in the disease by generating immunosuppressive cytokines such as transforming growth factor-β, down regulating critical surface molecules required for development of a functional immune system and release of exosomes. Aberrant functioning immune cell contribute to the pathogenesis of CLL through release of cytokines, chemokines and small extracellular vesicles that promote CLL growth and survival[34].

2.1.5: Diagnosis

2.1.5.1: Clinical Features

Over 80% of patients come at an early stage of disease, often with no symptoms. Lymphocytosis is frequently the only abnormality detected while investigating other unrelated medical conditions[35]. 10% of patients present

with B symptoms (unexplained fever, unintentional >10% body weight loss in the preceding 6 months, or drenching night sweats)[36]. Some patients may have palpable lymphadenopathy or organomegaly; bone marrow involvement can lead to anaemia and thrombocytopenia. A complication of CLL such as a high-grade disease transformation, an autoimmune disease may lead to its diagnosis[37]. Another category present with repeated infections which may be major and necessitate inpatient management.

High risk of infections could be attributed to many immunological defects mainly hypogammaglobulinemia, abnormalities in T-cells, neutrophil , monocyte and complement system[38]. Extra nodal and/or extra medullary presentations of CLL rare, with the skin and central nervous system being the most frequent sites of involvement[39].

2.1.5.2: Laboratory Findings

A- Complete Blood Count and Morphology

A complete and differential blood cell count (CBC) usually reveals absolute lymphocytosis of 5000 B lymphocytes/L that lasts at least three months. The leukemic cells seen in the blood smear are tiny, mature lymphocytes with a narrow cytoplasmic border and a compact nucleus devoid of apparent nucleoli and partially aggregated chromatin. Other distinguishing morphologic features of CLL include Gumprecht nuclear shadows or smudge cells observed as cell debris[40].

Large atypical cells, cleaved cells, or prolymphocytes, are often seen on the peripheral smear and may account for up to 55% of blood lymphocyte. If this percentage of prolymphocytes is exceeded, prolymphocytic leukaemia (B-cell PLL) is a more likely diagnosis[41]. A picture of anaemia (which may be due to

the infiltration of the bone marrow by the disease, autoimmunity, iron, folate or vitamin B12 deficiency particularly in the elderly or poorly nourished people) can be present. Likewise, thrombocytopenia is infrequent at diagnosis[42].

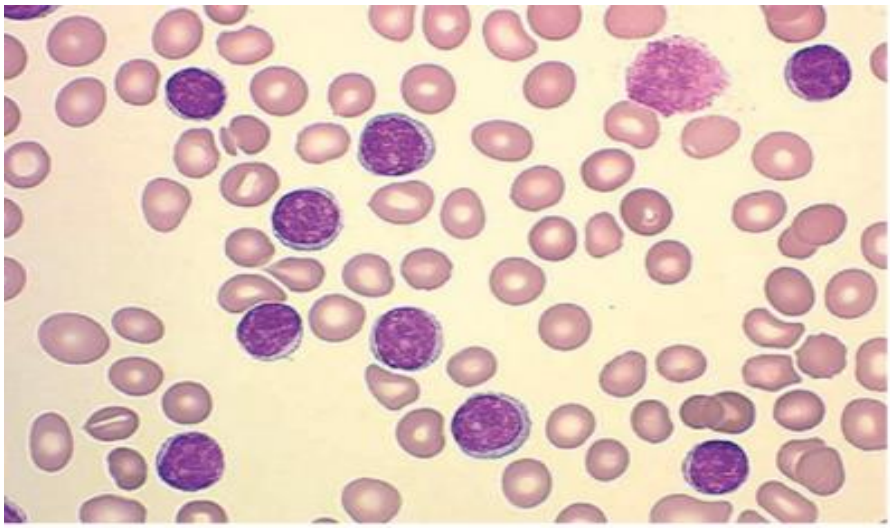

Figure 2.1: peripheral smear from a patient with CLL[42]

B- Immunophenotyping

The immunophenotyping analysis has an essential role in the definitive diagnosis of CLL. CLL cells exhibit a specific immunophenotypic feature on flowcytometry, such as CD5+, CD19+, CD23+, weak surface membrane immunoglobulins (sIg), and negative or dim expression of CD79b and FMC7[43]. Scoring system was defined for diagnosis of CLL depending on immunophenotyping profile which composed of five parameters that include CD5, CD22, CD23, and FMC7. Thus, a score of 4-5 indicates typical CLL and a score of 3 suggest atypical CLL whereas 0 - 2 excludes CLL[44].

Table 2.1: Scoring system for diagnosis of (CLL)[44]

Marker	point	
	1	0
CD5	Positive	Negative
CD23	Positive	Negative
FMC7	Negative	Positive
SIG	Low	Medium/high
CD22/CD7	Low/negative	Medium/high

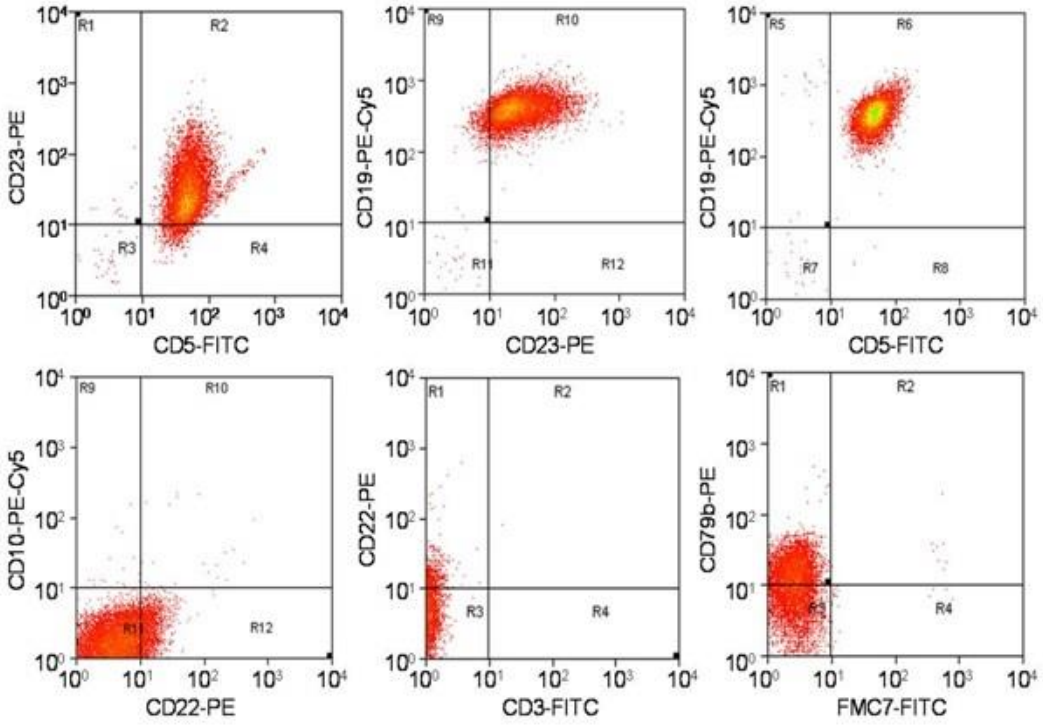

Figure 2.2: Flowcytometric profile of PB sample of CLL patient[44]

C- Pathology Features

- **Bone marrow biopsy:** Bone marrow aspiration and biopsy are not advised for routine diagnosis. It is indicated when there is diagnostic doubt or clinical suspicion of lymphomatous transformation, and it is particularly beneficial in detecting the source of pre-treatment

cytopenias and prolonged post-treatment cytopenias[45]. The presence of CLL cells in the bone marrow smear should be distinguished, with a percentage of more than 30%. The infiltration can have a nodular, interstitial, or diffuse growth pattern in a bone marrow biopsy[46].

- **Lymph nodes biopsy:** LN biopsy is not essential for the diagnosis of CLL, but is indicated when there is diagnostic uncertainty or clinical suspicion of lymphomatous transformation into Richter syndrome[47]. Now a positron emission tomography (PET) scan can detect CLL transformation to RS with high sensitivity, specificity and negative predictive value[48].

2.1.5.3: Criteria for CLL Diagnosis

According to the International Workshop on Chronic Lymphocytic Leukaemia (iwCLL) the following criteria are used for CLL diagnosis:[4]

- The presence in the peripheral blood of $\geq 5x10^9$/L B lymphocyte situated for at least 3 month. The clonality of these lymphocytes needs to be confirmed by demonstrating immunoglobulin light chain restriction using flow-cytometry.

- The leukaemic cells found in the PB smear are characterized by mature appearance, small lymphocytes with a narrow border of cytoplasm and a dense nucleus losing discernible nucleoli and having aggregated chromatin. Larger, atypical lymphocytes or prolymphocytes may be found but must not be more than 55%.

CLL cells coexist the B cell surface antigens CD19 and CD20 together with CD5, CD23, CD43 and CD200. When compared to normal lymphocytes, the

levels of surface CD20, sIg and CD79b are often low. Each clone of leukaemic cells expresses either the kappa or lambda Ig light chains, or neither of the two[49].

2.1.6: Differential Diagnosis

The main entities that should be included in the differential diagnosis for CLL are[50]:

- Mantle cell lymphoma (MCL) in leukemic phase.

- Prolymphocytic leukemia (B-PLL).

- Hairy cell leukemia (HCL)

- Follicular lymphoma in leukemic phase (FL)

- Marginal zone lymphoma (MZL), in particular splenic lymphoma with villous lymphocytes (SLVL).

Immunophenotyping is aid in differential diagnosis. Biopsy of affected lymph nodes and bone marrow , as well as genetic and molecular analysis can be beneficial in difficult cases[51].

2.1.7: Staging

In 1970, two clinical staging systems (Rai and Binet) were established and are still commonly used. They are easy to use in clinical practice, rely soley on clinical data, and consider lymph node , spleen and liver involvement, as well as the existence of cytopenias (anemia and thrombocytopenia)[52,53]. In recent times, the modified Rai[54] and Binet clinical staging systems are routinely applied to divide the patients into different prognostic groups.

The survival of CLL patients is varied according to the staging of the illness as for patients with Rai 0 or Binet A, it was with average of >10 years, for CLL patients with stage I and II or B, the average survival reached >8 years, while for CLL patients with stage III and IV or C, the average survival reached to about 6.5 years[55].

Clinical staging is still used in routine clinical practice and it can reliably predict the outcomes of the patients at the time of initial diagnosis[56].

Table 2.2: staging systems for CLL[44]

Stage		Definition
Binet- system		
A		**Hb ≥ 10 *g/dl* platelets ≥100× 10^9/L <3 involved lymphoid *sites*[a]**
B		**Hb ≥10 *g/dl* Platelets ≥100× /10^9/L ≥3 involved lymphoid sites[a]**
C		**Hb <10 *g/dl* platelets <100× 10^9/L (Not immune in nature)**
Modified Rai staging system		
Low risk	0	**Lymphocytosis> 5x10^9/L**
Intermediate risk	I II	**Lymphocytosis and lymphadenopathy Lymphocytosis and hepatomegaly and/or splenomegaly with/without lymphadenopathy**
High risk	III IV	**Lymphocytosis and Hb <11 g/dl with/without lymphadenopathy/organomegaly Lymphocytosis and platelets <100× 10^9/l with/without lymphadenopathy/organomegaly**

Hb, haemoglobin ; a Binet's system considers five possible sites of involvement: cervical, axillary, inguinal lymphadenopathy (either uni or bilateral), spleen and liver.

2.1.8: Prognosis

Rai and Binet staging systems have limited effectiveness in individuals with no disease-related symptoms at an early clinical stage. As a result, new

prognostic keys are being explored[57]. CLL has a highly variable natural history, with survival varying from months to decades after diagnosis. Some patients require little or no therapeutic intervention and have a normal life expectancy, while others require multiple courses of treatment and eventually die from the disease and its complications. Generally, the disease is still considered incurable, but it is essential to appreciate that, patients can live for decades after therapy intiation [58].

Table 2.3: Commonly Used Prognostic Markers in CLL including those used in CLL-IPI[59]:

Prognostic Marker	Better Prognosis	Worse Prognosis
Sex	Female	Male
Age	<65 y	>65 y
Stage: Rai Binet	 0 A	 I-IV B and C
Lymphocyte count	$<12 \times 10^9$/L	$\geq 12 \times 10^9$/L
Lymphocyte doubling time	>12 mo.	<12 mo.
Number of "smudge cells"	≥30%	<30%
β2-Microglobulin level	<3.5 mg/L	>3.5 mg/L
Flow cytometry: B-cell count	$<11 \times 10^9$/L	$\geq 11 \times 10^9$/L
CD38	<20% cells positive	≥20% cells positive
ZAP-70	<20% cells positive	≥20% cells positive
FISH/TP53 mutations	Del 13q	Del 17p and/or TP53 mutation
IGHV mutation status	Mutated	Un-mutated

2.1.9: Complications

2.1.9.1: Autoimmunity

Patients with CLL have a 5–10% risk of developing autoimmune concequences, which usually result in cytopaenia. These autoimmune cytopaenias can arise at any stage of the disease and don't have prognostic significance by their own. Autoimmune haemolytic anaemia (AIHA) is the most prevalent autoimmune complication, followed by immune thrombocytopaenia and pure red blood cell aplasia and very rarely, autoimmune granulocytopaenia (AIG).

By contrast, non-haematological autoimmune complications of CLL such as acquired angioedema, glomerulonephritis and paraneoplastic pemphigus are rare; The first is a result of a secreted product of the tumor, but the last two seem to be a product of the disordered immune system, and may be triggered by treatment with purine analogues suggesting a mechanism involving regulatory T cells[60].

2.1.9.2: Infections

Infectious complications continue to be a major cause of morbidity and mortality in patients with chronic lymphocytic leukaemia (CLL). The pathogenesis of infections in these patients is multi- factorial, related to inherent immune defects and therapy-related immunosuppression[61]. In fact, they account for the predominant cause of death in most cases. Infectious mortality estimated to be between 30 and 50%. The organisms causing infections to CLL patients are changing from common bacterial organisms to

less common opportunistic pathogens such as Pneumocystis, Listeria, mycobacteria, herpes viruses, and Candida[62]

Table 2.4: Pathogenesis of infection in patients with CLL[62]

▪ **CLL RELATED RISK FACTORS**
Hypo-gammaglobulinemia
Cell mediated immunity
Neutropenia, neutrophil function and the complement system
Duration of disease
Advanced disease stage
▪ **THERAPY RELATED RISK FACTORS**
Steroid-induced immune dysfunction
Purine analogs, alemtuzumab induced T-cell defects
Neutropenia

2.1.9.3: Second Neoplasia

CLL patients have an increased risk of other malignancies. This may be due to surveillance bias, treatment or immunosuppression[63]. The relative risk has been assessed to be two times higher than in the overall population. Melanoma, lung carcinoma, lymphoma, Kaposi sarcoma, central nervous system and gastrointestinal cancers are the most frequently observed. Skin cancers other than melanoma can also be seen, but at the same rate of the general population[42].

2.1.9.4: Disease Transformation:

4% to 20% of previously treated CLL patients progress to high-grade lymphoma. Richter transformation is defined as a transformation of CLL into aggressive lymphoma, most often diffuse large B-cell lymphoma (DLBCL). Traditional chemotherapy used to treat de novo diffuse large B-cell lymphoma

has a low response rate in these individuals, and they have a comparable or shorter overall survival rate[64]. This event occurs in the natural history of CLL. The majority of cases are clonally related to the original CLL clone, while a minority develop from an unrelated clone[65].

2.1.10: Treatment

It is very difficult to cure CLL and so the approach to therapy is generally conservative, aiming for symptom control rather than a normal blood count. Indeed, chemotherapy given too early in the disease can shorten rather than prolong life expectancy. Another important fact is that many patients never need treatment[66].

Based on their physical condition, comorbidities, and predicted life expectancy, there are three groups of elderly patients can be identified. First, physically well patients with no or minor comorbidities that reduce their life expectancy should be treated with standard therapies. Second, patients with relevant comorbidities that affect life expectancy who should have modified disease- control therapy. Third, patients with a multiple and/or severe comorbidities that significantly reduce life expectency should receive the best supportive care possible[67].

Different therapeutic goals should be followed for these three patient groups. The goal for the first group is to achieve long-term remission and prolong survival, whereas the second and third groups should seek disease control and symptom palliation[68]. Treatment should be started only if there is a clear indication based on iwCLL criteria[69].

The following are the indications for treatment:

- Evidence of marrow failure identified by the development or worsening of anemia and/or thrombocytopenia.
- Massive (at least 6 cm below the left costal edge) or progressive or symptomatic splenomegally.
- A massive lymph node (i.e at least 10 cm in longest diameter) or a progressing or symptomatic lymphadenopathy.
- Progressive lymphocytosis with a 50% increase within two months or a lymphocyte doubling time < 6 months.
- Autoimmune complications including anaemia or thrombocytopenia poorly responsive to corticosteroids or other traditional therapy.
- Significant disease related symptoms (constitutional symptoms) like fatigue, night sweating, weight loss and fever.

2.2: Cluster of Differentiation 14 (CD14)

2.2.1: CD14 Definition

CD14 is a cell surface glycoprotein expressed mainly in innate immune response cells such as monocytes, macrophages, neutrophils, and B cells[70]. CD14 was first discovered as a monocytes marker to signal intracellular processes in response to bacterial encounters. Then, CD14 was identified to be a tall like receptor -co-receptor for detecting pathogen-associated molecular patterns. On the other hand, CD14 has been revealed as a multifunctional receptor and its effect on a verity of diseases have been further investigated[71].

2.2.2: CD14 Structure and Gene Location

CD14 is a single-copy gene mapped to 5q31.1. Consisting of ≈3900 base pair (BP) organized in 2 exons and encodes a protein of 375 amino acids [72]. The crystal structure of CD14 reveals that it is a dimer with the two subunits together forming a horseshoe-shaped configuration similar to the structure of TLR ectodomain. Each subunit has a hydrophobic pocket at its amino terminus which is the main component of the LPS-binding site in CD14. The binding sites of CD14 for different TLR ligands appear to overlap, as LPS can compete with DNA and peptidoglycan; dsDNA can partially compet with polyI:C and lipoteichoic acid can compete with peptidoglycan for CD14 binding[73].

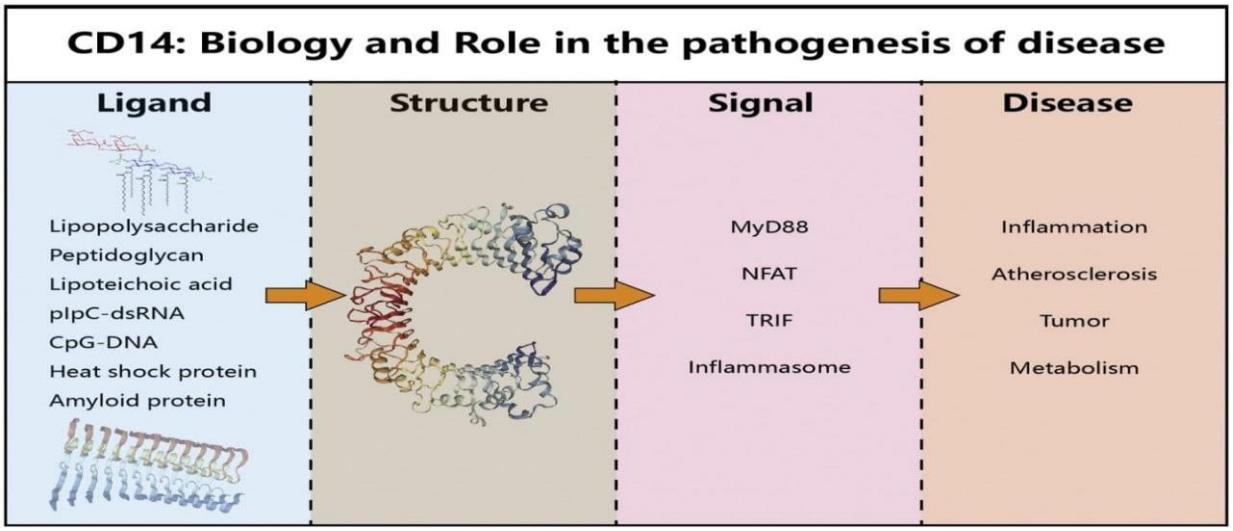

Figure 2.3: CD14 ligand, structure, signals and disease related[14]

2.2.3: CD14 Polymorphism

The human CD14 gene exhibits sequence variations, the underlying argument in favor a single nucleotide polymorphism (SNP) in the promoter region that occurred in CD14 at locus -159 & -260 from the transcription start

point, preceding a Cytosine to Thymine (C-T) transition. these two polymorphisms may affect the function of CD14, which may play an important role in disease development[74].

2.2.4: CD14 Types and Tissue Distribution

There are two forms of CD14[75][76]:

The first one is a 55kDa glycoprotein fixed to the membrane **(mCD14)** by a glycosylphosphatidylinositol (GPI) tail (anchor), that is highly expressed on monocytes, macrophages, and neutrophils but has a lesser surface expression observed on a range of other hematopoietic and stromal cells.

The second form is free, soluble CD14 **(sCD14),** that lake an anchor with a molecular weight of 48–56 kDa**,** exists in plasma, serum, cerebrospinal, and other body fluids. sCD14 either appears after shedding of mCD14 (by at least three different mechanisms that include bypassing of Glycoprotein inositol(GPI) addition, cleavage of the GPI anchor by phospholipase D, and direct proteolytic cleavage from the cell surface) or is directly secreted from intracellular vesicles.

2.2.5: CD14 Signals and Functions

CD14 appears to act like a coreceptor for LPS. It transports LPS to the TLR4/myeloid differentiation factor-2(MD-complex) and activating downstream signaling pathways including the MAPK, NF-kB, and IFN regulatory factor 3 (IRF3) pathways; leading to the generation of pro-inflammatory cytokines and type I IFNs[77]. Current studies reported that CD14 is involved in the activation of TLR2, TLR3, TLR7, and TLR9 since CD14-deficient macrophages and dendritic cells exhibit diminished inflammatory responses to these TLRs specific ligands[73].

sCD14 facilitate LPS-induced inflammatory response in endothelial and epithelial cells that do not express the membrane form of CD14. These results suggest that CD14 is a critical pattern-recognition receptor in the innate immunity against a broad spectrum of ligands[78]. Recently, its identified that CD14 may has a role is in clearance of apoptotic cells by phagocytic function[79].

2.2.6: Role of CD14 in CLL

The survival of CLL cells is linked to NFκB constitutive activity that plays a major role in disease development and evolution. Monocytes aid CLL cell survival by secreting soluble CD14, which activates NFκB in these cells, while CLL cells actively alter their microenvironment by promoting accessory monocytes to secret CD14[13]. The CD14 molecule is expressed on the surface of monocytes and macrophages and functions as an LPS receptor, coupled to LPS-binding protein, in mediating LPS-induced tumor necrosis factor (TNF) production.This glycoprotein with anti-apoptotic and pro-proliferative functions harbor high-risk chromosome abnormalities and advanced disease, However, the significance of the soluble version of CD14 is still being contested[80].

Numerous studies have shown that monocytes may be categorized into three distinct subsets based on the differential expression of CD14 and CD16 markers.The majority of monocytes in healthy conditions are classified as classical monocytes, which have the $CD14^{++}CD16^{-}$ phenotype. These classical monocytes play largely phagocytic and antitumor roles. Importantly, this monocyte subset gives rise to so called M1 macrophages that, in some contrast to M2 macrophages, exert numerous potent antitumor effects. The other

monocytes subgroups are nonclassical $CD14^{+}CD16^{++}$ and intermediate $CD14^{++}CD16^{+}$ monocytes[81], These two monocyte subsets were significantly expanded in patients with a variety of inflammatory and/or malignant diseases. Recently, according to some studies, the non-classical monocytes can secrete significantly higher TNF-α level. Notably, TNF-α promotes the proliferation of leukemic B cells and plays an important role in the progression of B-CLL[82]. Remarkably, larger numbers of non-classical monocytes have recently been found in patients with CLL. The presence of this finding was shown to be more prevalent in patients who have unfavorable genetic abnormalities[83]. Indeed, CLL patients' circulating monocytes have recently been proven to play a crucial role in the survival of leukaemic cells. Furthermore, the monocytes from patients with CLL have been demonstrated to develop in vitro into big, adherent cells able to protect leukaemic cells from both spontaneous and drug-induced death. However, it is unclear whether increased numbers of non-classical or classical monocyte that is associated with a poor prognosis. To now, it's also unknown if immune chemotherapy can reduce the number of these proinflammatory monocyte subtypes[84].

CHAPTER THREE

MATERIALS AND METHODS

3.1: Materials

3.1.1: Chemicals

The chemicals and materials used throughout this study were listed in table (3.1).

Table 3.1: Chemicals and materials used throughout the study and their origin.

Chemical/material	Manufacturer
Human cluster of differentiation 14, CD14 ELISA kit	BT LAB Bioassay technology laboratory (Shanghai, China)
Leishman stain	SYRBIO (SAR)
Oil	SYRBIO (SAR)
deionized water	Baghdad/ Iraq

3.1.2: Instruments

All instrument used throughout this study were listed in table (3.2).

Table 3.2: Instrument used throughout the study and their origin

Instrument/Equipment	Manufacturer
Deep Freeze	Naïve (Turkey)
Hematology Analyzer	BOUL MEDICAL AB (Sweden)
ELISA (chromate 4300)	Awareness technology (USA)
Incubator	Memmert (Germany);
Centrifuge REF 2300	Hettich(Germeny)
Calibrated micropipettes	Slammed(Germany)
Light microscope	Olympus (Japan)
EDTA tube (5 ml)	Wafi medical laboratories (Iraq)
Blood collection plain tube	SAIL BRAND (China)

Eppendorff tube (1.5 ml)	Eppendorf (Germany)
Slides	SAIL BRAND (China)
Gloves	Broche(Malaysia)
Plastic syringes; 5ml	Meheco (China)
Rack	Meheco (China)
Timer	Junghans, Germany
Disposable pipette tips	Eppendorf (China)
Filter paper	China

3.1.3: Study Subjects

This study was conducted on a total of 90 Iraqi adult from two Iraqi centers: Baghdad Teaching Hospital and Marjan Teaching Hospital. We studied forty five patients who attended the outpatient clinic of haematology in these hospitals during the period from October 2021 to April 2022. They were newly diagnosed as having Chronic Lymphocytic leukemia (CLL). Another forty five adult participants without CLL were included and served as a normal control for comparison with the patients study group; all control members had normal CBC measured at the time of blood sampling.

The informed consent was acquired from both patients and control groups in the presence of witnesses from the medical staff.

3.1.3.1: Patients

Forty five adult patients newly diagnosed with chronic lymphocytic leukaemia based on physical examination by a specialist, morphological assessment of peripheral blood films, as well as flowcytometric immunophenotyping profile were included in this study. According to their immunophenotyping profile, there were 95.6% of patient with typical CLL

(N=43) and 4.4% with atypical CLL (N=2). The patient group included 32 male (71.1%) and 13 female (28.9%) their age range from 40 to 75 year.

Criteria for selection of patients in this study were:

- Patients were randomly selected regarding age and sex.
- Those patients were newly diagnosed by consultant physician and specialized haematopathologist according to diagnostic criteria of CLL including immunophenotyping by flowcytometry (patients with Matutes score 4 and 5 were selected and regarding those with score 3 other markers were used as CD200 and 43)

For reliable assessment of CD14, alcoholic patients and those who have severe trauma, secondary malignancy, tuberculosis, chronic liver disease, AIDS and infection at time of blood sampling were excluded from this study. Also, Patients with small lymphocytic lymphoma, monoclonal B cell lymphocytosis, Richter's syndrome and B-cell pro-lymphocytic leukemia were not included in this study.

All patients have been physically examined and investigated for complete blood count, blood film and all having flowcytometry reports taken from their data or hospital archives where they were done and the information was obtained using a questionnaire form.

3.1.3.2: Control

Forty five healthy subjects were taken as a control group. They were all adults who were most closely matched for the age and sex of patients. For all

controls, clinical history were taken, physical examination and complete blood count (CBC) were done.

3.2: Methods

3.2.1: Study Design

This is a case-control study. It included 90 individuals, 45 patients and 45age and sex-matched healthy controls. The patients divided into patients with typical CLL and those with atypical subtype and all staged according to Modified Rai Staging System into three groups: low risk (stage 0), moderate risk (stage I-II) and high risk (stage III-IV) and according to Binet Staging System into another three groups including A, B and C. Soluble CD14 level was measured by ELISA assay for both patient and control groups.

3.2.2: Collection of Blood Sample

Before blood sampling, all participants were informed about the study objectives and their consents were obtained. Blood samples were drawn from patients and control individuals by venepuncture using a disposable syringe. Four milliliter of blood was collected from each patient and control. Tow ml of blood were taken and dispensed in ethylene diamine tetra acetic acid (EDTA) tubes and sent to the laboratory for complete blood count and blood film evaluation. Another 2 ml dispensed in plain tubes and left for around twenty minutes to clot at room temperature. After that, it was centrifuged for 20 min. at 2000 RPM to obtain serum (the supernatant without sediment). The serum was divided into (500µl) aliquots in Eppendorff tubes. These tubes were labeled and stored in the deep freezer (-40ºC) until the time of testing CD14.

3.2.3: Biochemical Tests

3.2.3.1: sCD14 ELISA Assays

3.2.3.1.1: Principle

The kit used in our study was an Enzyme Linked Immunosorbent Assay (ELISA) that had 96-well plate. Human CD14 antibody has been pre-coated on the plate. CD14 which is present in the sample, is added to the well and binds to antibodies coated on them. The biotinylated Human CD14 Antibody is then added to the sample and binds to CD14. After that Streptavidin-HRP is next added which binds to the Biotinylated CD14 antibody. During the washing step after incubation, unbound Streptavidin-HRP is rinsed away. Then, substrate solution is added, and the color develops in accordance to the amount of Human CD14. The reaction is stopped by adding an acidic stop solution, and lastly the absorbance is measured at 450 nm.

3.2.3.1.2: Standard Preparation

At the beginning, all reagents brought to room temperature before use. Then, we reconstituted the 120µl of the standard (9.6mg/L) with 120 µl of standard diluent to generate a 4.8mg/L standard stock solution. We allowed the standard to sit for 15 mins with gentle agitation prior to making dilutions. After that, we prepared duplicate standard points by serially diluting the standard stock solution (4.8mg/L) 1:2 with standard diluent to produce 2.4mg/L, 1.2mg/L, 0.6mg/L and 0.3mg/L solutions.

3.2.3.1.3: Preparation of Wash Buffer

We diluted 20ml of Wash Buffer Concentrate 25x into distilled water to yield 500 ml of 1x Wash buffer.

3.2.3.1.4: Assay Procedure

The assay was performed following the instructions in the kit's booklet, which are outlined in the steps bellow:

1. We prepared all reagents, standard solutions and samples as instructed. Brought all reagents to room temperature before use. The assay is performed at room temperature.
2. We determined the number of strips required for the assay and inserted the strips in the frames for use. We stored the unused strips at 2-8°C.
3. We added 50µl standard to standard well. We didn't add biotinylated antibody to standard well because the standard solution contains biotinylated antibody.
4. We added 40µl sample to sample wells and added 10µl anti-CD14 antibody to sample wells, then we added 50µl streptavidin-HRP to sample wells and standard wells (not blank control well). We mixed well and cover the plate with a sealer and incubated for 60 minutes at 37°C.
5. We removed the sealer and wash the the plate 5 times with wash buffer. We soaked wells with 300µl wash buffer for a minimum of 30 seconds for each wash then we blotted the plate onto paper towels for dryness.
6. We put 50µl of substrate solution A into each well, followed by 50µl of substrate solution B to each well. We incubated plate covered with a new clean sealer at 37°C in the dark for 10 minutes.
7. We added 50µl of stop solution in each well. The color changed from blue into yellow rapidly.
8. after 10 minute of adding the stop solution, we assessed the optical density (OD value) of each well using a microplate reader set to 450 nm.

3.2.3.1.5: Standard curve

The standard curve generated by plotting the average OD for each standard on the vertical (Y) axis against the concentration on horizontal (X) axis, then a best fit curve through the points on a graph was drown. These calculations performed with computer-based curve fitting software program (Microsoft Excel 2010) and the line determined by regression analysis.

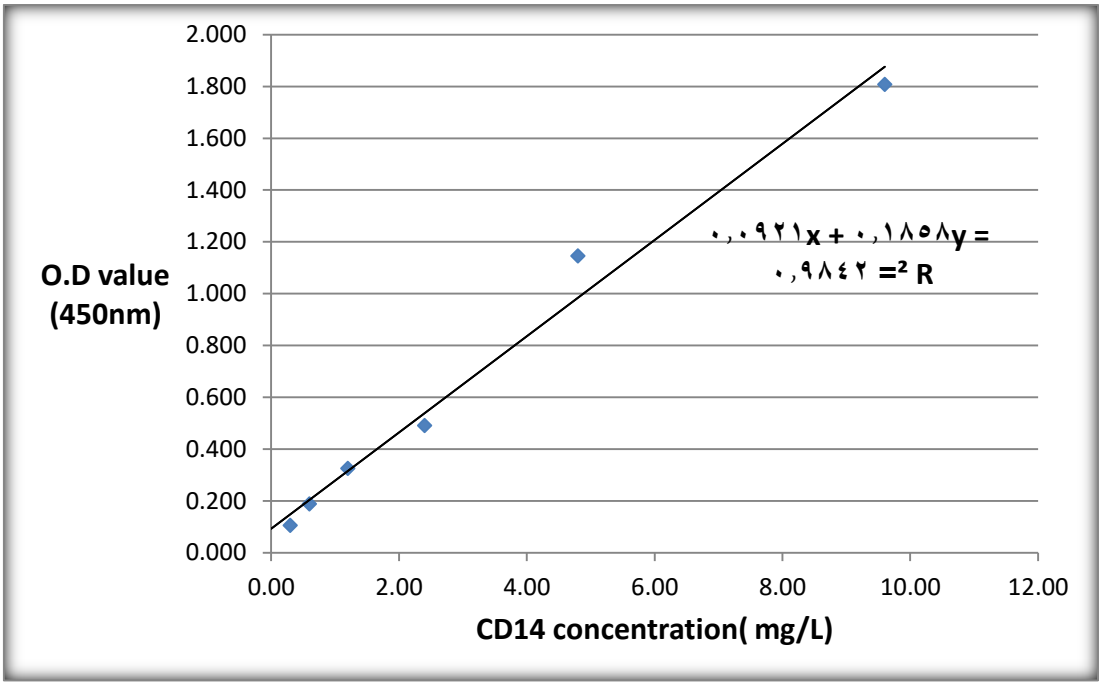

Fig 3.1: CD14 standard curve

3.2.4: Hematological Studies

3.2.4.1: Automated Blood Count

Blood samples were drawn and placed in EDTA tubes before being sent to the laboratory for a CBC using a Haematolog Auto Analyzer (5 parts differential). The blood is mixed inside the tube (without vigorous shaking) and then transferred into the analyzer, which assesses the number and types of different blood cells. The Auto analyzer system obtains a standard volume of

blood using narrow tubing and provides measured data using a mix of procedures:

- Photometric measurement of light absorbance is used to detect Hemoglobin content that measures the amount of hemoglobin in the entire sample as well as within each red cell.
- Volumetric impedance is used to determine cellular concentration and volume distributions of WBC, RBC and Platelets.
- Optical light scattering and diffraction measurement are utilized to evaluate leukocyte differential parameters (lymphocyte, monocyte, neutrophil, eosinophil, and basophil percentages)

3.2.4.2: Blood Film Preparation:

Blood film made on clean glass slide by using EDTA anti-coagulated blood sample. The slide measured 75 x 25mm and approximately 1mm thick. A small drop of blood is placed in the center line of a slide about 1 cm from one end (the frosted end). Then the drop spreaded by a second slide used as spreader placed in front of the drop at an angle of about 30 degree to make a thin layer of blood over the slide. After spreading, the slide labelled and allowed to dry in the air and then stained with Leishman stain and examined.

3.2.4.3: Blood Film Examination

Blood film examined systematically, starting with macroscopic observation of the stained film to assess whether the spreading technique was satisfactory and whether there are any abnormal particles present, then progresses from low power to high power microscopic examination.

Microscopic examination of a blood film aims to determine the morphological features of blood cellular elements. The results obtained are reliable when the smear was well made and stained.

3.3: Data Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 27. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Student t-test was used to compare means between two groups. ANOVA test was used to compare means between three groups or more. Correlation coefficient was used to assess the relationship between two continuous variables. Pearson chi-square was used to find the association between categorical variables. A p-value of ≤ 0.05 was considered as significant.

CHAPTER FOUR

THE RESULTS

4.1: Age and Gender Distribution in Study Groups

The Mean age of patients ± SD was (59.64 ± 8.01) years with older patient was 75 years and younger patient was 40 years. Majority of patients were males (N=32, 71.1% as shown in table (4.1)

Table 4.1: The Distribution of patients with chronic lymphocytic leukemia according to age and gender (N=45)

Study variables		
	mean	range
Age (years)	(59.64 ± 8.01)	(40-75)
Gender	No.	%
Male	32	71.1%
Female	13	28.9%
Total	45	100.0%

4.2: Staging of CLL in Patients Group

4.2.1: Staging According to Modified Rai Staging System

By applying the Modified Rai Staging System[54], the studied patients were categorized into the following:

1. 7 (15.6%) patients were in low risk.
2. 23 (51.1%) patients were in moderate risk.
3. 15 (33.3%) patients were in high risk, as shown in figure (4.1).

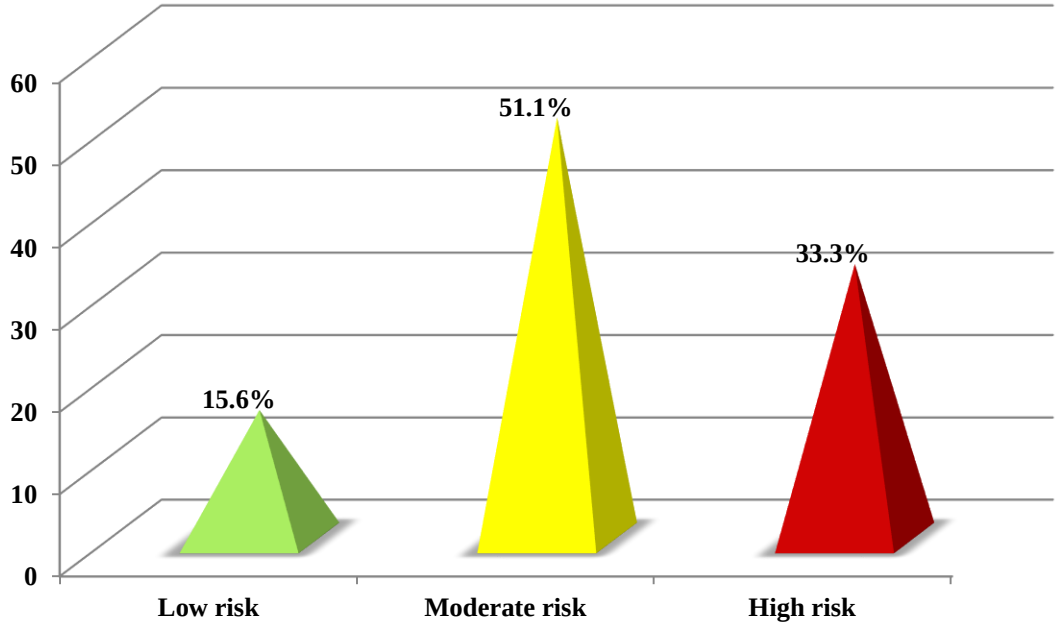

Figure 4.1: Distribution of patients with chronic lymphocytic leukemia (CLL) according to Modified Rai stage (N=45)

4.2.2: Staging According to Binet Staging System

The distribution according to Binet Staging System[52] including (stage A, B and C) was as following:

1. 12 (26.7) patients were in stage A.
2. 19 (42.2) patients were in stage B.
3. 14 (31.1) patients were in stage C, as shown in figure (4.2).

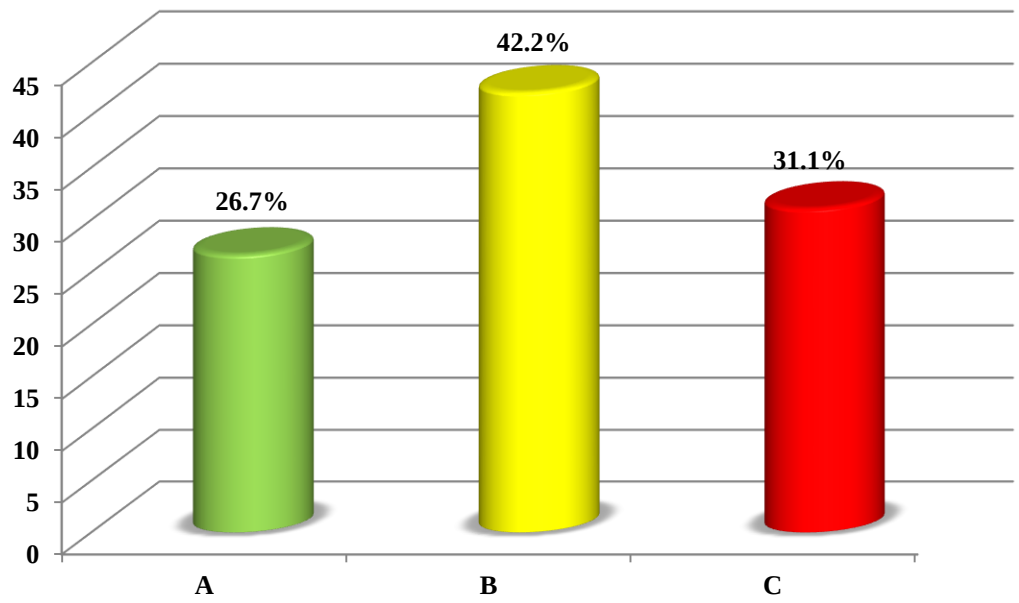

Figure 4.2: Distribution of patients with chronic lymphocytic leukemia according to Binet stage (N=45)

4.3: Hematological Parameters in Study Groups

The comparison in haematological parameters including (platelet count ($\times 10^9$/L), Hb level (g/dl), absolute lymphocyte count($\times 10^9$/L) and total WBC count ($\times 10^9$/L)) according to study groups (patients with CLL and control group) revealed a statistically significant differences between the study groups in terms of platelet count, Hb level, absolute lymphocyte count and total WBC count (P-value <0.001). The normal individuals in control group showed a significant higher means of platelet count and Hb level and lower means of absolute lymphocyte count and total WBC count (Table 4.2). There were Thrombocytopenia in 13.3 % of patients with CLL (figure 4.3) and anemia in 26.7% of patients(most of them had normochromic anemia due to bone marrow infiltration and anemia of chronic disease with lesser patient had hypochromic anemia) (figure 4.4).

Table 4.2: The mean differences of haematological parameters in study groups

haematological variable	Study group	N	Mean ± SD	t-test	P-value
Platelet count (×10^9/L),	CLL	45	169.79 ± 73.03	**-5.496**	**<0.001***
	Control group	45	245.44 ± 56.48		
Hb level (g/dl)	CLL	45	11.76 ± 2.46	**-5.212**	**<0.001***
	Control group	45	14.03 ± 1.57		
Absolute lymphocyte count(×10^9/L)	CLL	45	54.72 ± 51.34	**6.859**	**<0.001***
	Control group	45	2.21 ± 0.82		
Total WBC count (×10^9/L)	CLL	45	68.99 ± 64.52	**6.401**	**<0.001***
	Control group	45	7.40 ± 1.74		

*P ≤ 0.05 was significant.

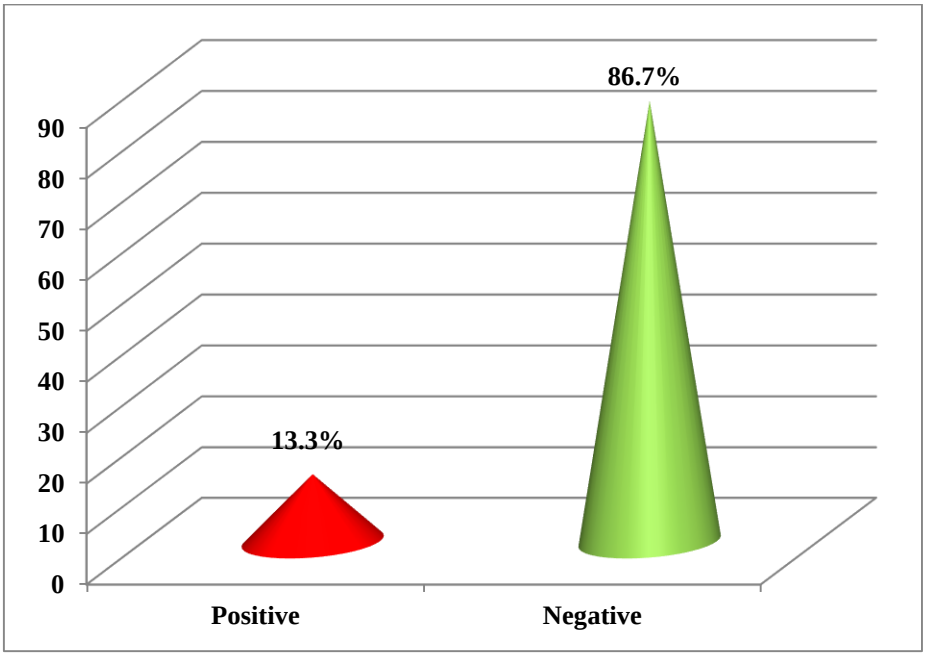

Figure 4.3: The Distribution of patients with chronic lymphocytic leukemia according to thrombocytopenia (N=45)

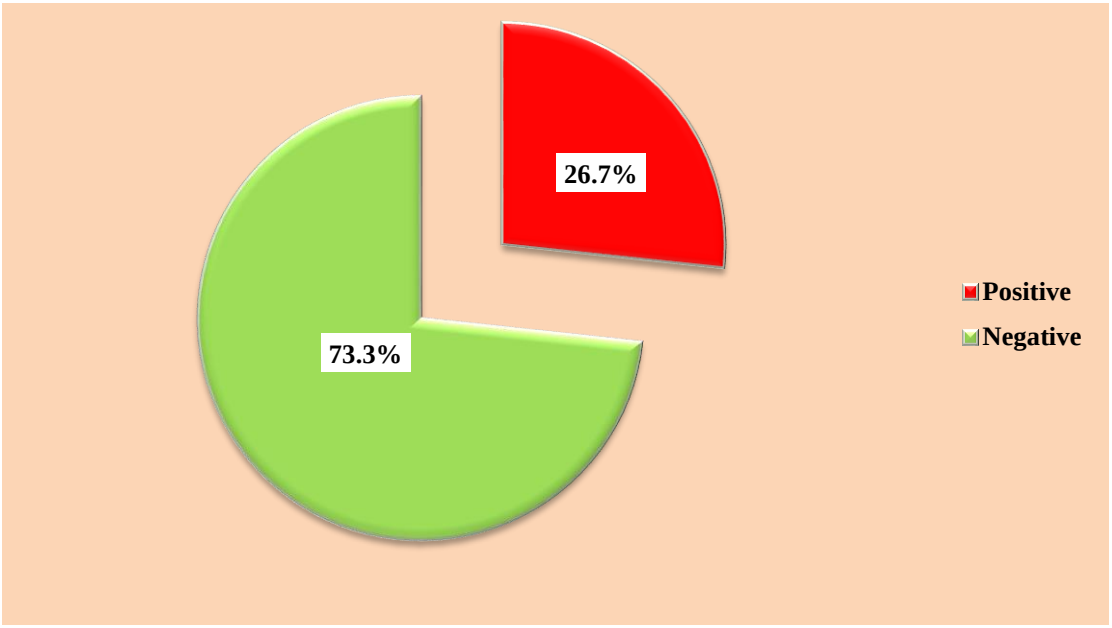

Figure 4.4: The Distribution of patients with chronic lymphocytic leukemia according to anemia (N=45)

4.4: Clinical Parameters in Patients Group

The statistical analysis of clinical variables that demonstrated in patient group including (lymphadenopathy, organomegaly and complications) showed lymphadenopathy presented in 86.7% of patients, organomegaly presented in 66.7% of patients while complications presented in 8.9% of patients. The complications included (hemolytic anemia and red cell aplasia) as in table (4.3)

Table 4.3: The Distribution of patients with CLL according to clinical parameters (N=45)

clinical parameters	No.	%
Complications		
Yes	4	8.9%
No	41	91.1%
Total	45	100.0%
Lymphadenopathy		
Yes	39	86.7%
No	6	13.3%
Total	45	100.0%
Organomegaly		
Yes	30	66.7%
No	15	33.3%
Total	45	100.0%

4.5: Patients Distribution According to CLL Subtypes

Most patients were having typical CLL according to flowcytometric analysis as shown in table (4.4).

Table 4.4: The Distribution of patients according to CLL subtypes (N=45)

Study variables	No.	%
CLL subtype		
Typical	43	95.6%
A typical	2	4.4%
Total	45	100.0%

4.6: CD14

4.6.1: Comparison between Study Groups

The mean differences of CD14 concentration (mg/l) according to ELISA technique demonstrate significant higher level among patient group compared to the control group, as shown in figure (4.5).

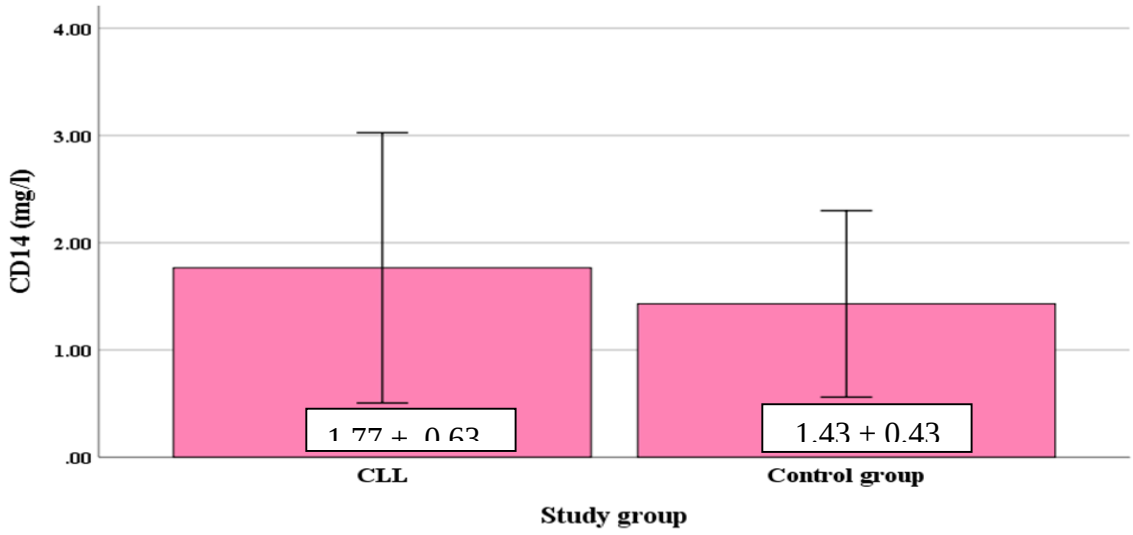

Figure 4.5: The mean differences of CD14 (mg/l) in patients and control group (N=90, P=0.004*)

4.6.2: CD14 Distribution with Gender and Age in patients group

4.6.2.1: CD14 Distribution with Gender

The mean of CD14 among female was (2.03 ± 0.75) which is slightly higher than that of male (1.66 ± 0.55) with no significant differences between these two groups (P-value =0.075) as shown in table (4.5)

Table 4.5: The mean differences of CD14 (mg/l) according to gender in patients group (N=45)

Investigated variable	Gender	N	Mean ± SD	t-test	P-value
CD 14 (mg/l)	Female	13	2.03 ± 0.75	1.826	0.075
	Male	32	1.66 ± 0.55		

*P ≤ 0.05 was significant.

4.6.2.2: CD14 Distribution with Age

No statistically significant correlation was established between mean of CD14 (mg/l) and patient ages as shown in table (4.6).

Table 4.6: The mean differences of CD14 (mg/l) according to age in patients group (N=45)

Study variables	Mean ± SD	r	P-value
CD 14 (mg/l)	**1.77 ± 0.63**	**0.219**	**0.148**
Age (years)	**59.64 ± 8.01**		

*P ≤ 0.05 was significant.

4.6.3: Comparison with CLL Stage

4.6.3.1 Comparison with Modified Rai Stages

There were statistically significant differences between means of CD14 level (mg/l) according to Modified Rai stage and increased level with stage progression and higher level associated with high risk stage (III-IV) as in table (4.7) and figure (4.6) (the actual difference was between high and low risk and between moderate and high risk).

Table 4.7-A: The mean differences of CD 14 (mg/l) according to Modified Rai stage

Study variables	Modified Rai stage	No.	Mean ± SD	F	P-value
CD 14 (mg/l)	Low risk	7	1.41 ± 0.32	**4.166**	**0.022***
	Moderate risk	23	1.65 ± 0.73		
	High risk	15	2.11 ± 0.38		

*P value ≤ 0.05 was significant

Table 4.7-B: Multiple comparisons (LSD)

Study variables	Modified Rai stage		P-value
CD 14 (mg/l)	Low risk	Moderate risk	0.355
		High risk	**0.014***
	Moderate risk	High risk	**0.025***

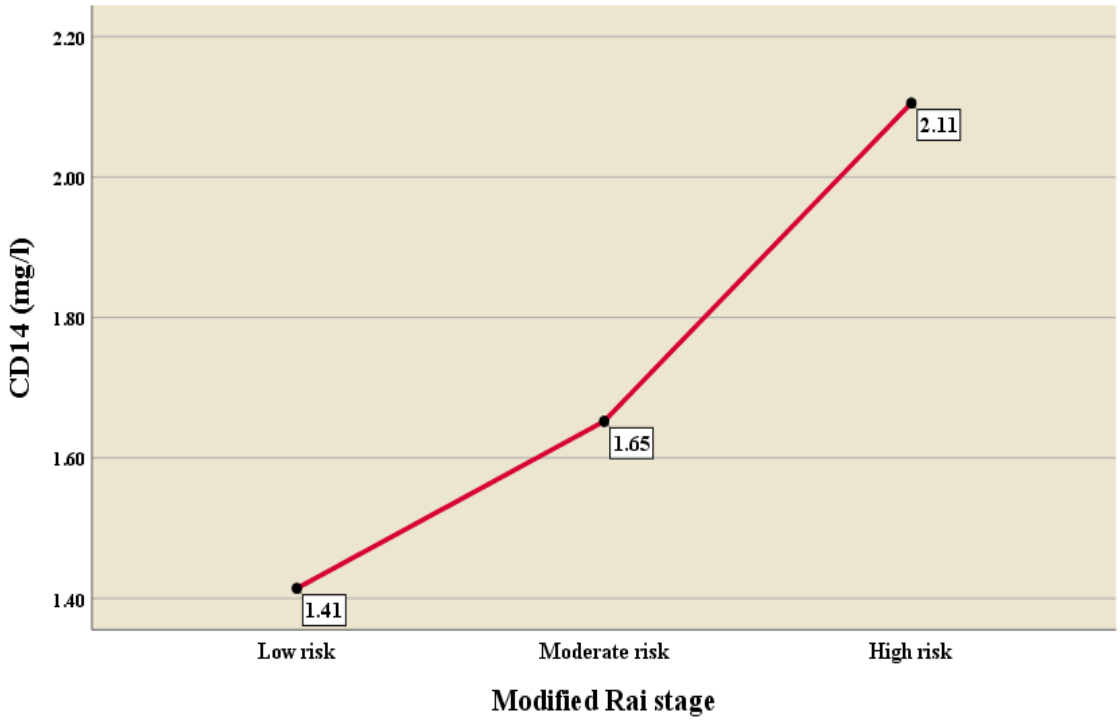

Figure 4.6: The mean differences of CD14 (mg/l) according to modified Rai stage (N=45, P=0.022*)

4.6.3.2: Comparison with Binet Stages

A statistically significant differences between means of CD14 (mg/l) according to Binet stage were marked with higher level associated with stage C where the mean was 2.08 ± 0.39 (P-value = 0.035*) as shown in table (4.8) and figure (4.7) (the actual difference was between stage A and C).

Table 4.8-A: The mean differences of CD 14 (mg/l) according to Binet stage

Study variables	Binet stage	N0.	Mean ± SD	F	P-value
CD 14 (mg/l)	A	12	1.46 ± 0.28	**3.629**	**0.035***
	B	19	1.73 ± 0.81		
	C	14	2.08 ± 0.39		

*P value ≤ 0.05 was significant

Table 4.8-B: Multiple comparisons (LSD)

Study variables	Binet stage		P-value
CD 14 (mg/l)	A	B	0.222
		C	**0.011***
	B	C	0.1

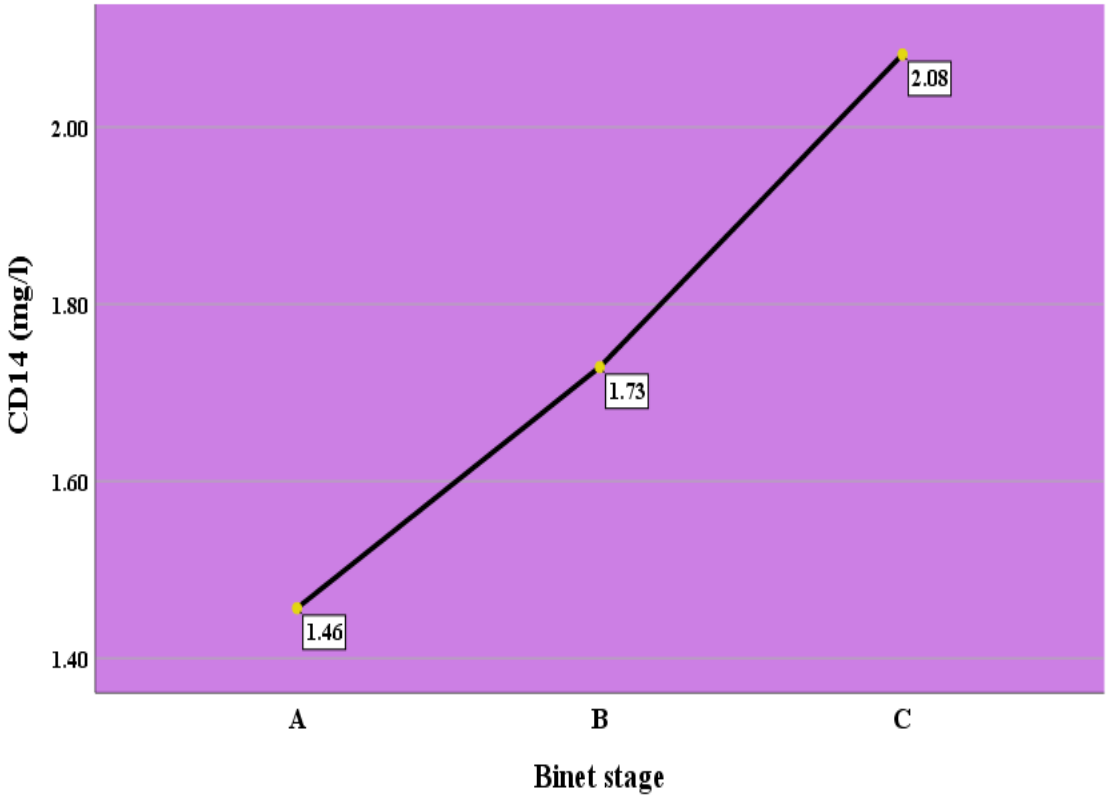

Figure 4.7: The mean differences of CD14 (mg/l) according to Binet stage (N=45, P=0.035*)

4.6.4: Correlation with CLL Subtype

There were no significant differences between means of CD14 (mg/l) according to subtypes, as shown in table (4.9).

Table 4.9: The mean differences of CD14 (mg/l) according to CLL subtype (N=45)

Study variable	Study group	N	Mean ± SD	t-test	P-value
CD14 (mg/l)	Typical CLL	43	1.75 ± 0.63	-0.798	0.43
	Atypical CLL	2	2.12 ± 0.44		

*P ≤0.05 was significant.

4.6.5: Correlation with Hematological Parameters in patients group

The comparison between CD 14 (mg/l) and haematological variables platelet count ($\times 10^9$/L), Hb level (g/dl), absolute lymphocyte count ($\times 10^9$/L) and total WBC count ($\times 10^9$/L)) among patients with CLL revealed that there was no significant correlation between CD 14 (mg/l) and total WBC and absolute lymphocyte count, but there was a negative correlation between Hb level and platelet count with CD14 concentrations as the concentration increased with lower Hb level and platelet count, though this was not statistically significant as in table (4.10).

Table 4.10: The correlation of CD14 (mg/l) and haematological parameters among patients with CLL (N=45)

Study marker	Study variables	Mean ± SD	R	P-value
CD 14 (mg/l) (1.77 ± 0.63)	Platelet count ($\times 10^9$/L)	169.79 ± 73.03	-0.134	0.38
	Hb level (g/dl)	11.76 ± 2.46	-0.259	0.086
	Absolute lymphocyte count($\times 10^9$/L)	54.72 ± 51.34	0.185	0.223
	Total WBC count ($\times 10^9$/L)	68.99 ± 64.52	0.218	0.151

*P ≤ 0.05 was significant

4.6.6: Correlation with Clinical Parameters in patients group

The mean differences in CD14 (mg/l) according to clinical parameters (complication, lymphadenopathy and organomegaly) showed significant differences between means of CD14 (mg/l) and complication, and organomegaly (P = 0.001and0.015) respectively, but there is no significant differences between means of CD14 (mg/l) and lymphadenopathy (P-value=0.178) (table 4.11).

Table 4.11: The mean differences of CD14 (mg/l) according to clinical variables in patients group (N=45)

clinical variable	Complication	N	Mean ± SD	t-test	P-value
CD 14 (mg/l)	Yes	4	2.75 ± 1.04	**3.743**	**0.001***
	No	41	1.66 ± 0.49		
clinical variable	**LAP**	**N**	**Mean ± SD**	**t-test**	**P-value**
CD 14 (mg/l)	Yes	39	1.81 ± 0.65	1.369	0.178
	No	6	1.44 ± 0.34		
Study variable	**Organomegaly**	**N**	**Mean ± SD**	**t-test**	**P-value**
CD 14 (mg/l)	Yes	30	1.92 ± 0.69	**2.528**	**0.015***
	No	15	1.44 ± 0.27		

*P ≤ 0.05 was significant.

CHAPTER FIVE

DISCUSSION

DISCUSSION

Chronic lymphocytic leukemia (CLL) is a low-grade B –lineage lymphoid malignancy with variable clinical course[85].

Chronic lymphocytic leukemia, which accounts for around a quarter of all new leukemia cases, primarily affects older persons, with males having a slightly higher risk than females[86].

CD14 is a glycosylphosphatidylinositol (GPI) anchored receptor known to act as a co-receptor for many TLRs, both at the cell surface and in the endosomal compartment. CD14 can be expressed by cells of both hematopoietic and non-hematopoietic origin as a cell membrane or secreted protein[87].

Our study aimed to discover the possible role of this protein in chronic lymphocytic leukemia and its possibility to be a prognostic factor by comparing its level with patient and control group in addition to estimate its relation with patients stage and some haematological and clinical parameters.

5.1: Clinicopathological Assessment

5.1.1: Age Distribution

This study found the mean age of patients was 59.64 years (table 4:1) which is comparable to that reported by other Iraqi workers where the mean age in their studies were 57.18 and 59.24 respectively[88][89]. While it was lower than that reported in western countries[90][91]. These differences may be related to the effect of geographical, environmental factors and population structure between Iraq and Western countries that made life expectancy lower among Iraqi population.

5.1.2: Gender Distribution

The incidence of registered cases of CLL in our study which included 32 (71.1%) male and 13(28.9%) female (table 4:1) was higher in male; where the male: female ratio was 2.4:1. Some local research in Baghdad and Erbil found the same result with M:F ratio of 2.5:1 and 2.7:1 respectively[92][93]. While it was higher than that found in other local study where the ratio was 1.7:1[89] and western study[94] where the ratio was 1.4:1. The real cause of male predominance in CLL disorder is unknown[95], But it may be related to some oncogenic influences like dietary habitudes and cigarette smoking or even hormonal abnormalities.

These differences in multiple studies may be related to variation in population structures and different sample size.

5.1.3: Staging System Distribution

According to Modified Rai Staging System, most patients were in moderate (51.1%) and high risk (33.3%) stages (84.4% of total 45 cases) while the lowest percent in low risk stage (15.6%) (Figure 4.1). Other Iraqi studies accomplished by Aladdin S. Naji[96] and Kawa Hassan[97] found the majority of patients had either advanced or intermediate stage 45(91%), 105(86.7%) of the total cases respectively and also low risk stage were the lowest . The same was found when applying Binet staging system (Figure 4.2) where higher number of patients in stage B (42.2%) and C (31.3%) similar to Haider Al-Dahery result, where he found 55.6% of patient with stage B and 44.4% with stage C[98].

Our study revealed a higher incidence of advanced stages than in western countries as they found more than 50% of cases diagnosed at an early stage of

the disease[99], the cause of this disparity could be linked to ignorance and poor primary health care with no routine checkups as well as Iraq's high poverty rate, which forces people to see a doctor only when they have severe symptoms, all of which delay disease identification at an early stage

5.1.4: Haematological and Clinical Assessment

In the assessment of haematological parameters (Table 4.2), the mean total white blood cells (WBC) count of patients (68.99 ± 64.52) was markedly higher than the mean of control group (7.40 ±1.74). As diagnostic criteria for CLL all patients had a lymphocytosis with a mean of absolute lymphocyte count in peripheral blood (54.72±51.3) compared to (2.21±0.82) for control group, which observed in all previous researches[98][90][94][100][101].

The mean platelet count (169.79±73.03) and Hb level (11.76 ± 2.46 mg/dl) in patients group were lower than that of control group where mean platelet and Hb level were (245.44±56.48) and (14.03 ±1.57 mg/dl) respectively and these results found to be similar to that found by other studies[89][98]

Anemia was noticed in 12 patients (26.7%) (Figure 4.4) and thrombocytopenia in 6 patients (13.3%) (Figure 4.3). Those parameters (anaemia and thrombocytopenia) representing a determinant for high risk stage in Binet and modified Rai staging Systems[53][54].

Regarding clinical parameters, LAP was the most common finding present in 86.7% of patient (Table 4.3) and this go with other study where the LAP was the commonest presentation [88] .

5.1.5: CLL Subtype Distribution

The immunophenotyping assessment of CLL patients revealed the majority of patients 43(96.6%) were of 4-5 IPT score / typical CLL while only 2(4.4%) had a score of 3 / atypical CLL (table 4.4). This result goes with worldwide publications regarding B-CLL immunophenotyping where typical CLL is the commonest subtype [102][103],[104],[105].

5.2: CD14 marker

5.2.1: Level of CD14 in CLL Patients and Control Group

In this study, we used quantitative ELISA kit to assess the level of serum CD14 in patient and control groups. We discovered that soluble CD14 level was significantly higher in patient group (1.77 ± 0.63) compared to control group(1.43 ± 0.43) with p-value of 0.004 (Figure 4.5). This result was in agreement with other workers in many previous studies, some of them reported higher mean of sCD14 in CLL patients[5][13][80][106] and the other revealed increased frequency of cellular CD14 expression on B-CLL cells[107].

5.2.2: Correlation sCD14 Level with Gender and Age

In the present study, the level of sCD14 concentration was greater in females than males (Table 4.5), however, the difference is not significant. In addition there was no significant correlation between sCD14 level and the age of patients (Table 4.6). Similar result was reported by other studies[5][106].

5.2.3: Correlation of sCD14 Level with Stage of Disease

In this study, sCD14 level significantly associated with advance stage of disease as the level was markedly higher in advanced stages including high risk group (Modified Rai Stage III-IV) (Table 4.7 and Figure 4.6) and Binet stage C (Table 4.8 and Figure 4.7) that coincident with the result of other studies[5][13][80][106]. There is a study discovered a direct link between CD14 expression and advanced clinical stages which are associated with shorter overall survival[108]. This strong relation reflects the importance of CD14 as prognostic factor in CLL.

5.2.4: Correlation of sCD14 Level with CLL Subtype

There was no significant correlation with CD14 and CLL subtype, although the level was higher in atypical than typical cases (table 4.9). The result may be due to few no. of patients had atypical CLL involved in this study. This finding is unique and didn't take in consideration in previous studies.

5.2.5: Correlation of sCD14 Level with Haematological and Clinical Parameters

Regarding haematological parameters (table 4.10), We found a negative correlation of CD14 with haemoglobin level and platelet count as the concentration increased with decreased haemoglobin level and platelet count although it was not significant (The non-significant association may be due to few number of patient with low hemoglobin and platelet count in our study and the negative correlation can be explained by higher concentration of CD14 in advanced CLL stage that associated with lower haemoglobin level

and platelet count). There were no significant correlation with total WBC and absolute lymphocyte counts. In the assessment of CD14 relation to clinical parameters in this study, there was a significant correlation of CD14 to organomegaly (P=0.015), but there was no considerable association with lymphadenopathy (Table 4.11). There was only one study showed that, there was no significant relation between the level of CD14 and clinical, laboratory and haematological parameters[5]. The other study discovered a significant negative correlation of CD14 to haemoglobin level[106]. These differences may be related to sample number, different technique used, work environment and the way of patient selection.

5.2.6: Correlation of sCD14 Level with Specific Complications

Some patient (N=4, 8.9%) in this study showed specific complication (Auto immune hemolytic anemia /three patients and red cell aplasia /one patient) and statistical analysis showed significant high CD14 concentration among these complicated cases (table 4.11). This finding is unique and didn't take in consideration in previous studies.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1: Conclusions

From the current study we can conclude the following:

1. Patients with CLL had higher serum CD14 level than normal individuals.
2. CD14 level is significantly associated with more advanced CLL stage.
3. CD14 level has a negative correlation with haemoglobin concentration and platelet count.
4. CD14 has no significant correlation with age, gender, lymphadenopathy, total WBC count, absolute lymphocyte count and CLL subtype.
5. CD14 is considered a new prognostic factor in CLL is linked to clinical stage and disease progression. Hence it can be used to predict CLL outcome and may be a future therapeutic target.

6.2: Recommendations

1. A prospective cohort study with larger sample size and follow up of patients with assessment of marker level during the course of disease (pretreatment, during treatment and post treatment) to assess the relation of CD14 in patient outcome, complications and treatment responsiveness.

2. Studying the correlation of CD14 with other known poor prognostic factors in CLL such as CD38, IgHV gene mutational status and chromosomal aberration.

3. Studying the association of CD14 in other lymphoproliferative disease.

4. Educating people through health programs on the importance of routine checkups for early diagnosis and avoiding problems associated with advanced disease .

CHAPTER SEVEN

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APPENDIX

Appendix

I. The forma of the patients used in this study:

Flow No.			Code No.			Tel No.	
Name:							
Age:							
Gender:			Male			Female	
Occupation:							
Address:							
Past medical history:							
History of alcohol intake:							
History of trauma:							
Presenting symptoms:							
Lymphadenopathy:		cervical		axillary		inguinal	
Organomegaly:		hepatomegaly		splenomegaly			
Complications:							
Viral status:							
CBC	Total WBC	Absolute lymphocyte count	RBC	Hb	MCV	MCH	platelet
Blood film:							
Retic % if done:							
Coombs test if done:							
Diagnosis by:		Flowcytometry		Bone marrow			
Stage:	Rai		binet		Modified rai		
IPTscore:							
sCD 14 level:							