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Alteration of Biochemical Immunological and Molecular Markers of Chronic Lymphocytic leukemia in Sample of Iraqi Patients

A Dissertation

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بسم الله الرحمن الرحيم

وَٱللَّهُ أَخۡرَجَكُم مِّنۢ بُطُونِ أُمَّهَٰتِكُمۡ لَا تَعۡلَمُونَ شَيۡـٔٗا وَجَعَلَ لَكُمُ ٱلسَّمۡعَ وَٱلۡأَبۡصَٰرَ وَٱلۡأَفۡـِٔدَةَ ۙ لَعَلَّكُمۡ تَشۡكُرُونَ ٧٨

صدق الله العظيم

سورة النحل: (78)

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Haider

Summary

Chronic lymphocytic leukaemia (CLL) is a monoclonal malignancy characterized by an accumulation of small and mature looking B lymphocytes in the blood, bone marrow and other tissues. The B-lymphocytes developed in a wrong way lead to an immune disorder, and are typically characterized by expression of some of CD markers as (CD5,CD38 and ZAP-70) determined by immunophenotyping. The aim of this study was to investigate the extent to which immunological, biochemical, and molecular parameters are altered in CLL patients, and the potential of applying these alterations as biomarkers for CLL in Iraqi patients.

This study was conducted on randomly selected 55 CLL patients referred to the National Center for Blood Diseases, AL-Mustansiyria University for evaluation and treatment during the period extending from Oct. 2013 to Sept. 2015. CLL patients included 18 newly diagnosed untreated patients (12 males and 6 females), with a mean age of 55±12 yr and age range of(40-80)yr. According to the Binet staging system for CLL, 10 of these patients were in stage B and 8 in stage C. Besides, this study includes 37 already diagnosed CLL patients who are currently receiving treatment. They included 28 males and 9 females, with a mean age of 57±9 yr and on age range of 45-71 yr. Twenty three 23 patients of this group were in stage B and 14 patients in stage C. Nineteen apparently healthy subjects were also involved in this study. They included 9 males and 10 females, with a mean age of 61 ± 17 yr. and an age range of 30-90 yr.

A Complete peripheral blood picture examination in the untreated newly diagnosed CLL patients revealed absolute lymphocytosis, mild anemia, decreased platelet count and elevated ESR level as compared to controls. A Follow-up analysis of the data obtained from those patients in response to

combination therapy (rituximab, fludaribine, cyclophosphamide(RFC)) for 1-8 months (median 4.5 months) normalized WBC, and Lymphocytes counts and anemia, but failed to correct the lowered platelet counts or the elevated ESR values.

The significant biochemical changes observed in the untreated CLL patients were those of slight elevations of albumin and uric acid levels, which were not corrected by RFC therapy. Nevertheless, these values remained within the reference values. Serum levels of the major antioxidant, glutathione (GSH) showed a significant decline and serum levels of malondialdehyde (MDA) were significantly elevated in untreated CLL patients. RFC therapy failed to normalize these values, suggesting the presence of sustained increased endogenous oxidative stress in CLL patients.

Furthermore, five out of the (18) (27.8%) newly diagnosed CLL patients were diabetic, and showed hyperglycemia and elevated HbA1c levels.

The immunological profile of the newly diagnosed CLL patients revealed the presence of increased expression of CD5, CD 38, and ZAP-70. Similarly, the levels of IL-6 and IL-10 were elevated in the serum of untreated CLL patients. RFC therapy managed to normalize only the expression of ZAP-70.

Real Time-PCR results showed the presence of mutated in immunoglobulin heavy-chain variable region (IgVH) in 3 out of 8 untreated and 4 out of 8 treated CLL patients, while healthy controls did not show any mutation in IgVH.

Another 37 old CLL patients who were previously diagnosed and received therapy for a range of 4 months to 8.5 years (median 21.5 months) showed comparable results to those obtained after a median 4.5 months of RFC therapy. Furthermore, 11 out of 37 (29.7%) old treated CLL patients were diabetic and showed hyperglycemia and elevated

HbA1c levels compared to euglycemic old treated patients and control subjects.

In conclusion, CLL patients of the present study showed various immunological, biochemical, and molecular changes. The interesting finding that 16out of 55 CLL patients (i.e. 29%) had hyperglycemia and elevated HbA1c levels deserves further studies to assess its clinical significance and whether or not it may be utilized as a potential risk factor for the incidence of CLL in Iraqi patients.

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List of Abbreviations:

AITL	Angioimmunoblastic lymphoma
AML	acute myeloblastic leukemia
AMPK	Activated monophosphate kinase
BL	Burkitt Lymphoma
classical HD,	classical Hodgkin’s disease
CLL	Chronic Lymphocytic Leukemia
CML	chronic myelogenous leukemia
CD	Cluster of differentiation
DCs	dendritic cells
DM	diabetics mellitus
DLBCL	Diffuse large B-cell lymphoma
dNTP	Deoxynucleotide tri phosphate
FL	Folicullar lymphoma
HL	Hodgkin's lymphoma
IFN-γ	Interferon – gamma
IL-	Interleukin
IgVH	immunoglobulin heavy-chain variable region
LMW	low molecular weight
MALT,	mucosa- associated lymphoid tissue
MCL	Mantle cell lymphoma

List of Abbreviations:

MF	mycosis fungoides
NCBI	National Center for Biotechnology Information
NK	Natural Killer
NHL	Non - Hodgkin's lymphoma
NF-κB	Nuclear factor- kappa B
NLPHD,	nodular lymphocytepredominant Hodgkin’s disease
OS	Oxidative stress
PB	Peripheral blood
PTCL	Peripheral T-cell lymphoma
PLL,	prolymphocytic leukaemia
RAPD	Random amplified polymorphic DNA
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcriptase polymerase chain reaction
SLL	Small Lymphocytic Leukemia
Tregs	Regulatory T cells
TMB	TeteraMethylBenzidine
TNF-α	Tumor Necrosis Factor –alpha

1. INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world accounting for 40% of all leukemia characterized by the clonal proliferation and accumulation of B-lymphocytes. It affects mainly elderly patients as the median age of diagnosis is about 72 years and the male to female ratio is 2 : 1. So far, Rai and Binet staging systems are used for predicting CLL patients' outcome. The symptoms and signs of the disease arise from a clonal excess of B- cells caused mainly by defects that prevent programmed cell death (apoptosis). Diagnosis in hematopathology depends mainly on cytomorphology and histologic features with the application of immunophenotypic analysis by flow cytometry (FCM) and immunohistochemistry (Cherie, 2004; Jurisic, et al., 2008). The overlapping morphological and immunophenotypic features of mature B-cell can be solved by flowcytometric or immunohistochemical immunophenotypic techniques. Flow cytometric Immunophenotyping has become a necessary diagnostic tool in the last 15 years (Poeta, et al., 2001; Abdel-Ghafar, et al., 2012). In addition, flow cytometry can be used to identify expression of targets for potential antibody-directed therapy and provide some prognostic information (Fiona, 2008).

Which have been established but mainly concern symptomatic CLL patients, are lymphocyte CD38 expression and CD5, presence of ZAP-70, immunoglobulin heavy (IGHV) gene mutation status, and cytogenetic profile (Rassenti, et al., 2004; Krober, et al., 2006). In symptomatic patients the presence of unmutated Ig heavy chain variable region, the presence of ZAP-70, and CD-38 expression predict worse clinical outcome (Kyrtsonis, et al., 2013).

Interleukin 2 (IL-2) and its receptors have been shown to play a central role in the mechanism controlling the growth of neoplastic B cells (Malavasi, et al., 2011). On the other hand, the leukemic cells in B-CLL can themselves express and secrete some cytokines including proinflammatory cytokines, such as TNFa (Jablonska, et al., 2005; Schulz, et al., 2011), and (IL-6) and IL-10 receptors (DiLillo, et al., 2010). IL-6 is of special interest in B-CLL, because this cytokine acts as a B-cell stimulatory factor. The production of IL- 6 *in vitro* varies significantly among patients with different stages of CLL. (Gorgun, et al., 2009).

Cytokines such as tumor necrosis factor (TNF)- α, interleukin IL-2, IL-4, IL-6, IL-8, IL-10 and interferon (IFN)-α have been proposed to play a role in the activation, growth and apoptosis of leukemic B-cells. (Burger, et al., 2005).

This genetic diversity of cancer cells is essential for progression of the disease (Calin, et al., 2005). On the other hand, accumulation of reactive oxygen species (ROS) from antioxidant deficiency, mitochondrial dysfunction, inflammation, phagocytosis (myeloperoxidase activity), exogenous stress (exogenous oxidants, redox cycling agents, UV irradiation, chemicals, endotoxins, and hyperoxia) result in a state of redox imbalance known as oxidative stress. ROS alter biological macromolecules (DNA, carbohydrates, proteins, and lipids). which contributes to genomic instability. Previous analyses have demonstrated an impairment in the antioxidant defense system and an enhancement in the damaged DNA base 8-oxo-2-deoxyguanosine (8-oxo-dG) in both the preleukemic state of monoclonal B-cell lymphocytosis and CLL(Oltra, et al., 2001; Collado, et al., 2012).

Immune cell functions are linked to ROS production and antioxidant defense. Therefore, antioxidant deficiency can be one cause of immune function suppression, affecting both innate T-cell-mediated immune response and adaptive antibody response. Tumor lysis syndrome was observed in some CLL patients (Hileman, et al., 2001). Tumor lysis syndrome is characterized by a series of metabolic disorders induced by rapid tumor cell death and release of toxic cellular contents into circulation. It is defined by abnormal elevation in serum uric acid, potassium, phosphate and lactate dehydrogenase (LDH), leading to serious complications such as neurological abnormalities, kidney damage, cardiovascular events, and potentially death (Tiu, et al., 2007; Tosi, et al., 2008; Blum, et al., 2011). Tumor lysis syndrome is typically rare in CLL (Cheson, 2009; Gertz, 2010).

1.1 Objectives of the study:

a) Assessment of hematological and biochemical profile in CLL patients and healthy subjects.
b) Assessment of lymphocytes immunophenotyping (CD5, CD38, and ZAP-70) using flow cytometry.
c) Estimation of serum proinflammatory cytokines (IL-6/IL-10).
d) Assessment 0f antioxidant defense mechanisms in serum of CLL patients and healthy subjects such as glutathione (GSH), and malondialdehyde (MDA).
e) Determination of IGHV mutational status using Rt-PCR and DNA sequencing.
f) Assessment of glycemic evaluation in CLL patients.

1.2 Literature Review

1.2.1 Definition of Chronic Lymphoid Leukemia (CLL)

Chronic lymphocytic leukemia is a disease in which mature lymphocytes become cancerous and gradually replace normal cells as functionally incompetent lymphocytes in the blood, bone marrow, liver, spleen and lymph nodes as a result of prolonged lifespan with impairment of normal apoptosis in CLL due to over expression of the Bcl-2 gene product, which is an anti-apoptotic protein. (Poeta, et al., 2001; Mir, et al ; 2013). CLL is the most common type of leukemia in North America and Europe. It is rare in Japan and Southeast Asia, which indicates that heredity plays some role in its development (Wierda, et al., 2007). CLL is characterized by the progressive accumulation of mature, monoclonal CD5+ CD19+ CD23+ B lymphocytes in the peripheral blood, lymph nodes, spleen and bone marrow (Chiorazzi, et al., 2005). CLL cells can present with absence of mutations in the immunoglobulin variable region genes (IgVH) and can over-express zeta-chain TCR-associated protein kinase 70 kD (ZAP-70) which both are correlated with the aggressiveness of the disease (Burger, et al., 2009). The common chromosome abnormalities with CLL patients are deletion of 13q14, trisomy 12, deletions at 11q22-q23, NOTCH1 and structural abnormality of 17p involving the p53 gene (Dohner, et al : 2000; Pospisilova, et al: 2012).

1.2.3 Etiology and Pathogenesis

One study noted an increase in CLL in some rural communities, suggesting that an environmental agent(s) associated with farming plays a role (Zent, et al, 2008). However, other studies found that the incidence of CLL apparently was not associated with exposure to

pesticides, sunlight, ionizing radiation, or known carcinogens (Byrd, et al.,2014). Also, a few studies noted an increase in CLL among persons chronically exposed to electromagnetic fields. Some studies found a relatively high prevalence of infection with type C hepatitis virus (HCV) in patients with CLL compared with that of the general population, suggesting a possible pathogenic role. Women noted a nonsignificant trend toward reduced risk of this leukemia with increasing parity, prompting speculation that pregnancy lowers the risk for CLL. However, hormones have not been demonstrated to play any role in the development of this disease (Lichtman, et al., 2007).

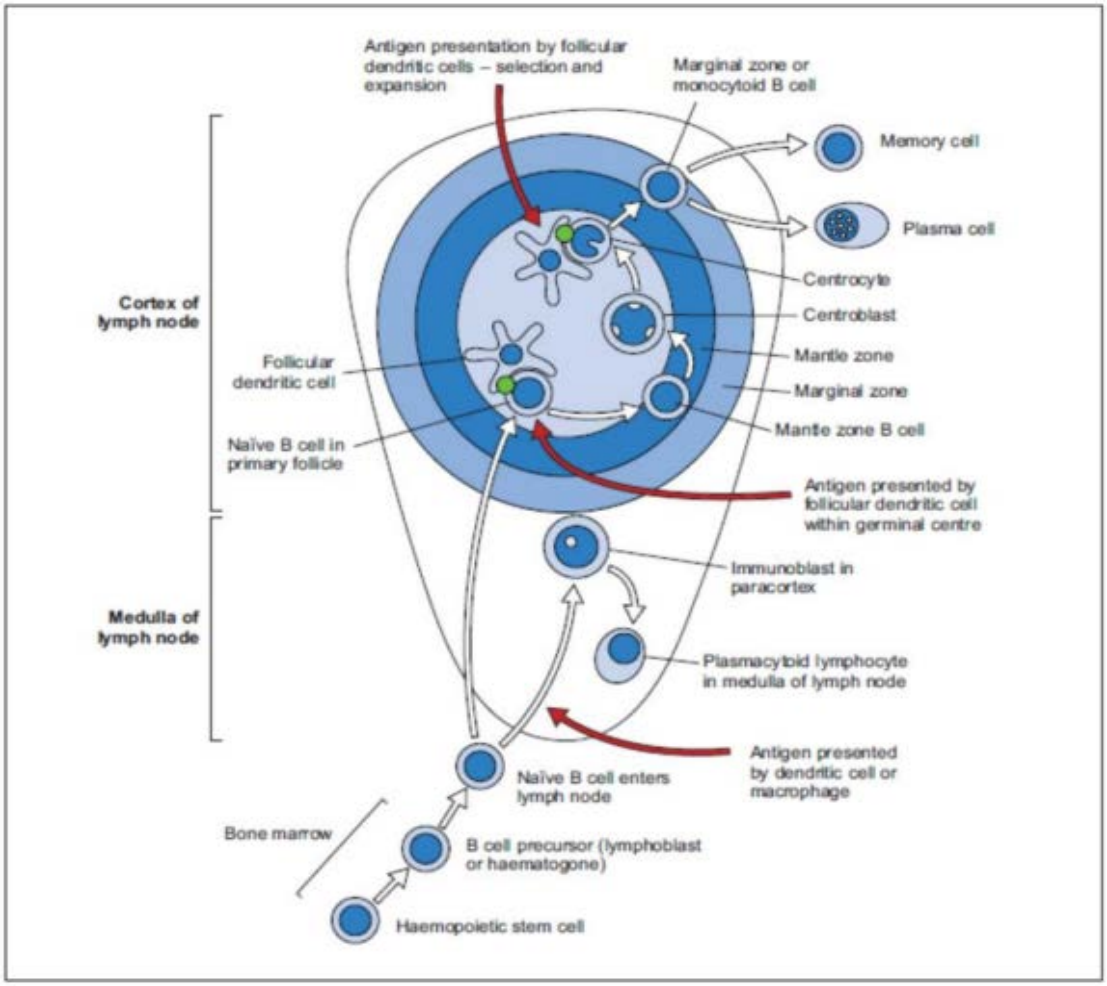
Recent data suggest that familial CLL may more commonly show somatic hypermutation of the immunoglobulin heavy-chain variable region, suggesting a more indolent disease course. Monoclonal B-cell lymphocytosis (MBL) has been identified recently as a likely precursor to CLL (Brown, et al; 2008).

1.2.4 Pathophysiology of malignant B-cell CLL

Basically tumorigenesis is a multi-step pathway but the earliest events in lymphoid neoplasia are difficult to recognize, although histological progression is a well-recognized feature of many lymphoid neoplasms. No“benign neoplasms” in the lymphoid system had been recognized, and this is a fact that may be related to the propensity of lymphoid cells to circulate or home, and not remain confined to a single anatomic site (Muller. et al., 2001).

These naive B cells undergo clonal expansion in germinal centers (GCs) which are found in the cortex of lymph nodes (Küppers, 2004; Swerdlow, et al., 2008). In the lymph node GC, the immunoglobulin genes are further modified by somatic hypermutation, a process by

which cells undergo rapid mutations and class- switch recombination (Jaffe, 2009; Swerdlow, et al., 2008).



Figure(1.1) The normal development of a B lymphocyte (Estella, et al., 2007).

A diagrammatic representation of the journey of the normal B-cell from the bone marrow to the lymph node crossing peripheral blood and its evolution is shown in (figure 1.1) demonstrating a haemopoietic stem cell in the bone marrow which gives rise to a B-cell precursor and then to a naive B cell which migrates either to secondary lymphoid tissues such as a lymph node primary follicle or medulla. If the B cell is presented with antigen by a dendritic cell or macrophage, further development occurs. A naive (IgM- or IgD-expressing) B cell in the primary follicle responds to antigen by class switching and migration

to the mantle zone (Dooley, et al., 2006). The mantle zone B cell then migrates back into the germinal centre and transforms to a centroblast and then a centrocyte within what is now a secondary follicle containing a germinal centre (Merbl, et al., 2007). These germinal centre cells undergo somatic hypermutation before migrating to the marginal zone and then the blood stream. Postgerminal centre B cells become memory cells in blood or tissues or plasma cells in tissues. Tumors corresponding to almost all stages of B-cell development have been found in humans. Most lymphoid tumors have gene rearrangements characteristic of the cell type from which they arose (Swerdlow, et al., 2008).

Consequently, all mature B-cell lymphomas have rearranged immunoglobulin genes and usually express (surface or cytoplasmic) immunoglobulins. They represent the malignant tranformations of cells that were able to recognize antigen via their B-cell receptors (Swerdlow, et al., 2008).

Somatic hypermutations of the immunoglobulin genes occur in the germinal center, lymphomas carrying mutated IGHV genes are regarded as germinal center or post- germinal center neoplasms whereas lymphomas without IGHV mutations represent the pre-germinal center neoplasms. Likewise, IGH class switching is a typical germinal center process so that lymphomas that express IgG are considered to stem from B cells that have already passed the germinal center (Kantor, et al., 1997).

The assumed relationships between normal B-cell differentiation and B lineage neoplasms is demonstrated in figure (1.2) showing the malignant B-cells neoplasms with their normal counter-part B-cells during their evolution.

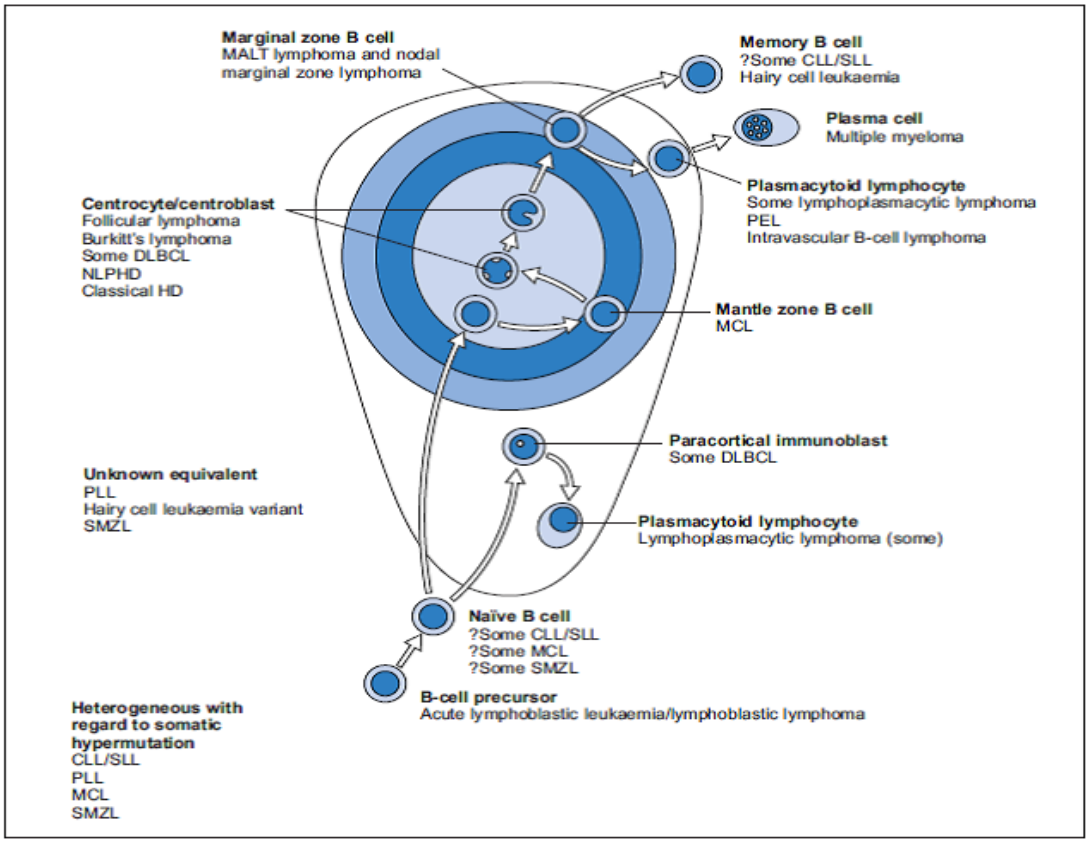


Figure (1.2) The assumed putative relationships between normal B-cell differentiation and B-lineage neoplasms (Estella, et al., 2007).

The specificity of cytogenetic aberrations nowadays is a key feature in mantle cell lymphoma, Burkitt lymphoma and follicular lymphoma (Chaganti, et al., 2004). Chromosomal abnormalities, along with immunophenotypic data, have helped define and categorize CLL (Chaganti, et al., 2004). Immunophenotypic data are frequently included to improve the diagnostic specificity (Jaffe, 2009). Nevertheless, currently immunophenotypic is the defining diagnostic future in CLL only (Jaffe, 2009).

1.2.5 Staging systems for CLL

The two widely accepted systems are those of Rai (1975) and Binet (1981).

Rai's staging system takes the view that CLL cells accumulate first in the blood and bone marrow, then in lymph nodes and spleen, finally leading to bone marrow failure. The chances of a patient surviving will depend largely on the stage at which he or she presents to the physician ' s attention. This is used more often in the United States.

Binet system: this is used more widely in Europe (Catovsky, et. al., 2011).

Binet staging system: This divides CLL into 3 stages (Binet,1981):

1- Binet stage A: Fewer than 3 areas of lymphoid tissue are enlarged, with no anemia or thrombocytopenia.
2- Binet stage B: 3 or more areas of lymphoid tissue are enlarged, with no anemia or thrombocytopenia.
3- Binet stage C: Anemia and/or thrombocytopenia are present.

1.2.6 Complications of CLL

1.2.6.1 Autoimmunity

Autoimmune complications are well recognized in CLL, occurring in 10% to 25% of patients at some time during their disease course. Autoimmunity in CLL predominantly targets blood constituents, and autoimmune hemolytic anemia is the most common form of autoimmune disorder. Auto-antibodies in CLL are polyclonal and differ in specificity and isotype from the immunoglobulins secreted directly by CLL cells (Dearden, 2008).

1.2.6.2 Infectious complications

Infections continue to be one of the major causes of morbidity and mortality in patients with CLL. Organisms causing infections to CLL patients are changing from common bacterial organisms to less common opportunistic pathogens such as Pneumocystis, Listeria, mycobacteria, herpesviruses, and Candida (Wadhwa, 2006)

1.2.7 Biomarkers for CLL

Biomarkers for CLL range from general markers that are measured in serum or blood, to protein markers detectable using flow cytometry. Biomarkers can aid in prognostication and therapy selection (Hallek, et al., 2008). Two of the most reliable molecular prognostic markers, both of which are offered in routine diagnostics, are the immunoglobulin heavy chain variable (*IGHV*) gene mutational status and fluorescence *in situ* hybridization (FISH) detection of prognostically relevant genomic aberrations (e.g. 11q−, 13q−, +12 and 17p−). In addition to these markers, a myriad of additional biomarkers have been postulated as potential prognosticators in CLL, on the protein (e.g. CD38, ZAP70, TCL1), the RNA (e.g. *LPL, CLLU1*, micro-RNAs) and the genomic (e.g. *TP53, NOTCH1*, *SF3B1* and *BIRC3* mutations) level (Bain, 2010).

1.3 Flow cytometry immunophenotyping (FCI)

Flow cytometry immunophenotyping (FCI) evaluates individual cells in suspension for the presence and absence of specific antigens (phenotype) (Fiona, 2008). The currently used FCI are new multicolor (detecting many CD markers inside and on the surface of the same single malignant cell) and multiparametric (combining studying many

physical characters of cell size and granularity alongside the multiple CD marker). Flow cytometry remains an indispensable tool for the diagnosis, classification, staging, and monitoring of hematologic neoplasms (Jurisic, et al., 2008). Flow cytometry analysis requires the identification of the cells population of interest by gating, a process by which events (cells) are electronically selected based on predefined criteria. Gating may be based on light scatter and/or fluorescence (i.e. antigen expression such as CD45) (Pettitt, et al., 2012). Interpretation of antigen expression by these gated events is generally performed manually, operator-guided and interactive, and, as such, is highly subjective (Pettitt, et al., 2012). Flow cytometric immunophenotyping is a useful tool in diagnostic hematopathology. Various types of specimens are suitable for Flow cytometric immunophenotyping which include peripheral blood, bone marrow, aspirates, and core biopsies, and all types of blood fluids (Jurisic, et al., 2008).

Flow cytometry immunophenotyping studies can recognize CLL which is similar in immunophenotype to normal mature lymphoid cells (surface immunoglobulin on mature B cells) and lack of antigenic features of immaturity, such as expression of CD34 or weak intensity staining for CD45 (Matutes, 2000). Furthermore, FCI can be used to identify expression of targets for potential antibody-directed therapy and provide some additional prognostic information such as CD38 and ZAP-70 expression in CLL (Zent, 2010).

1.4 Alteration in the immune system in CLL:

CLL is not a static disease that results simply from accumulation of long-lived lymphocytes but is rather likely to be a dynamic integrative process composed of cells that proliferate and die albeit at reduced levels compared to normal cells (Chiorazzi and Ferrarini, 2006).

The microenvironment is necessary and/or plays a pivotal role in maintaining the enhanced survival of CLL cells *in vivo*. Human bone marrow stromal cells (BMSC) have been demonstrated to support the survival of CLL cells when both cell types were co-cultured *in vitro* (Panayiotidis, et al., 1996; Lagneaux, et al., 1998; Kay, et al., 2007). Further investigation has suggested that CLL B-cells need to have intimate contact with BMSC in bone marrow (Lagneaux, et al 1998), T cells in lymph nodes (Kater, et al 2004), and nurse-like cells (NLC) in lymphatic tissues (Burger, et al 2000, Tsukada, et al 2002) to maintain survival. Among the adverse prognostic factors for CLL patients, accumulation of mature monoclonal CD5, CD38 (Deaglio, et al 2003, Deaglio, et al 2007), ZAP-70 expression (Chen, et al 2002), and the immunoglobulin variable heavy chain region (IGHV) mutational status (Lanham et al., 2003).

1.4.1 CD-5

It is a 57 kD glycoprotein which is encoded by a gene situated on the long arm of chromosome 11(Wojciech, 2010; Ortolani, 2011). CD5 has a role as a regulator of cell death and as a receptor for pathogen associated molecular patterns. In addition to its function as an inhibitory receptor it is implicated in the proliferative response of activated T cells and in T-cell helper function (Soldevila, et al., 2011). CD-5 is expressed on most mature T lymphocytes, but not on all of them. CD-5 can be considered a T-associated antigen, but not a T-specific molecule (Simons, et al,. 2013). It is expressed on the membrane of a subset of peripheral blood B lymphocytes, ranging between 17% and 25% of all B lymphocytes (Sandes, et al., 2013). CD5 is expressed either by "typical" or "atypical" B-CLL its increased expression is correlated with deletion of the long arm of chromosome

13, also it is expressed on element of B-CLL in plasmacytoid transformation (Wojciech, 2010).

1.4.2 CD-38

CD38 is a 46 kD glycoprotein encoded by a gene situated on chromosome 4. CD38 is a multifunctional ectoenzyme (cyclic ADP ribosylhydrolase) widely expressed in hematopoietic cells. It plays a role in the regulation of cell activation and proliferation regulating the intracytoplasmic concentration of calcium, but it also behaves as a receptor, modulating cell to cell and cell and co-operating in transmembrane signal transmission. CD38 is expressed on different cellular types, particularly on 50% of adult bone marrow B cells and germinal center B cells. CD38 is also expressed at high levels on plasma cells. It is expressed in committed hematopoietic stem cells and other hematopoietic precursors during early differentiation and activation (Ortolani, et al., 2011; Sandes, et al., 2013).

14% to 56% cases of B-cell chronic lymphocytic leukemia (B-CLL) c, CD38 is expressed in at least 30% of the cells. CD38 is not generally expressed in hairy cell leukemia (HCL); however, a report exists in which in a less than 50% of cases, the antigen is expressed on at least 20% of the cells (Ortolani, et al., 2011).

In B-CLL, evaluation of CD38 antigen expression is exceedingly important, as it represents a bad prognostic indicator (Damle, 1999) and correlates with the presence of unfavorable indicators including the presence of trisomy 12 , deletion of the long arm of chromosome 11 and deletion of the short arm of chromosome 17 (Athanasiadou, 2006).

1.4.3 70-kDa zeta-associated protein (ZAP-70)

The zeta-chain associated protein of 70 kD (ZAP-70), an intracellular tyrosine kinase which play an important role in T-cell receptor signaling, natural killer cell activation, and early B-cell development (Schweighoffer , et al., 2003). ZAP-70 protein is not expressed in most normal mature B-cells, but is expressed in various B- and T-cell lymphomas (Wiestner, et al., 2003; Admirand, et al., 2004). ZAP-70 protein expression measured by flow cytometry was associated with unmutated immunoglobulin heavy chain variable region (IGHV) genes (Kipps, 2000; Chen, et al., 2002), a prognostic factor in CLL that participates in early B-cell differentiation (Schroeder, et al., 1994; Crespo, et al., 2003). Other studies have found that ZAP-70 is associated with enhanced signaling by the cell-surface immunoglobulin receptor in CLL B cells (Crespo, et al., 2003; Chen, et al., 2005). In a larger series of patients, Rassenti et. al., (2004) have shown that an increased expression of ZAP-70 by CLL is a more significant predictor of need for treatment than the presence of unmutated IgVH gene. Moreover, the expression of ZAP-70 appears to be constant over time.

1.4.4 Cytokines in CLL

Cytokines are low molecular weight (less than 80 kD) glycoproteins produced by a number of cell types, predominantly leukocytes that regulate immunity, inflammation and hematopoiesis. They are produced from various sources during the effector phases of immune responses and regulate a number of physiological and pathological functions including innate immunity, acquired immunity and inflammatory responses (Ghosh, 2012) .

The source of cytokines in CLL appears to be polyclonal B cells rather than the leukemic cells. (Lee, et al., 1998). In regard to survival,

some investigators (Kitabayash, et al., 1995) suggested that IL-6 and IL-10 prevents apoptotic death of CLL cells (Aderkaet, 1993; Reittie, et. al., 1996).

1.4.4.1 Interleukin-6 (IL-6)

IL-6 is a protein of 26 kD, with 183 amino acid forming the mature protein; its coding gene located on chromosome no. 7. Interleukin-6 is a pleiotropic cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, monocytes, normal hematopoietic cells, and lymphocytes. IL-6 is an IL-1 inducible and represents the best marker of biologically active IL-1 (Van, 1990; Fayad, et al., 2001).

IL-6 is of special interest in B-CLL, because this cytokine acts as a B-cell stimulatory factor (BSF-II), mediates B-cell differentiation and can stimulate the growth of B-cell lymphoid malignancies such as myeloma (Aderka, 1993). In contrast, it has been proven that IL-6 is also able to inhibit TNF-a induced proliferation of B-cells from CLL patients (Van, et al., 1993).

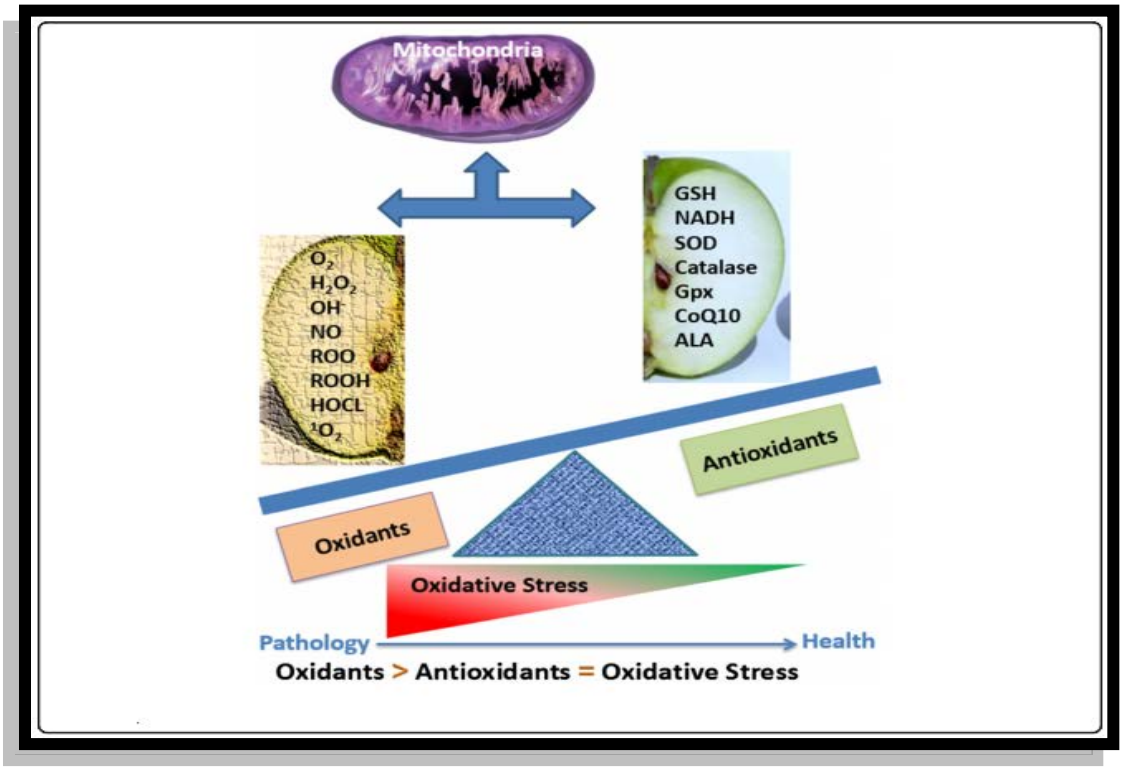
1.4.4.2 Interleukin-10

IL-10 is a protein of 35 kD. It is produced by several T-cell subpopulations, such as Th2, NK cells, and a variety of cell types, including macrophages, dendritic cells and B cells (Banchereau et al., 2012). IL-10 production has strong immunosuppressive effects via inhibition of Th1 type cytokines, including interferon-gamma and interleukin-2. Secretion of IL-10 from Treg cells, macrophages and other leukocytes followed by subsequent binding to IL-10 receptors on macrophages and dendritic cells has been linked to a reduced antigen presentation and an increased T-cell anergy (Shalev *et al.,* 2011). Additionally, IL-10 has been demonstrated to be effective in decreasing the levels of some pro-inflammatory cytokines (IL-2, IL-6,

IL-1β, IL-12, GM-CSF TNF-α and IFN-γ) (Tang, et al., 2011) . IL-10 prevents apoptotic death of CLL cells, whereas others suggest that it enhances them (Flckiger, et al., 1994).

1.5 Oxidative Stress (OS) in CLL

Oxidative stress (OS) results when there is an imbalance between the generation of oxygen-free radicals or reactive oxygen species (ROS) and response from the antioxidant defense systems figure (1-3) (Finkel and Holbrook, 2000). Free radicals are molecules or molecular fragments that contain one or more unpaired electrons which make them highly reactive (Jones, 2008).



Figurer (1.3) Types of oxidants and antioxidants which imbalance will lead to Oxidative Stress (Finkel and Holbrook, 2000).

ROS are endogenous and exogenous factors generated by the mitochondria, (Valko, et al., 2006) that alter biological macromolecules (DNA , carbohydrates, proteins, and lipids) (Kumar, et al., 2008). Moreover, production of ROS, reactive oxygen intermediate (ROI) and reactive nitrogen intermediate (RNI) are part of human body's physiological processes (Nathan, 2003). OS additionally attenuates immune responses by leading to dysfunctions and even apoptosis of NK- and T-cells, suggesting a role in tumor immune-escape. ROS-related lesions that do not cause cell death can stimulate the development of cancer , Alzheimer's disease, Huntington's disease, and multiple sclerosis as well as atherosclerosis, heart failure, and myocardial infarction (Poschke, et al., 2011). The interaction between growing cancer cells and the host immune response also generate OS (Renschler, 2004; Weinberg, 2009)**.** Production of ROS, reactive oxygen intermediate (ROI) and reactive nitrogen intermediate (RNI) are part of human body's physiological processes (Nathan, 2003). The Chronic OS results from little oxidative damage which accumulates during the life cycle of the cell and subsequently disrupts essential cellular functions and triggers many cancers. OS has been recognized to play a role in CLL development and treatment response (Renschler, 2004; Weinberg, 2009).

Oxidative stress contributes to genomic instability in CLL, but its relationship with the acquisition of specific chromosomal abnormalities is unknown (Haferlach, et al., 2010; Rossi, et al., 2013). CLL cells might contribute to the cancer-associated oxidative stress (Ristow and Zarse, 2010; Kumar, et al., 2014) and malignant lymphocytes from CLL patients have been demonstrated to produce abundantly superoxide anions (Finkel, 2000). This metabolic condition is regularly seen in cancer patients and results from accumulating

reactive oxygen species (Valko, et al., 2007). The Chronic OS results from little oxidative damage which accumulates during the life cycle of the cell and subsequently disrupts essential cellular functions and triggers many cancers. OS has been recognized to play a role in CLL development and treatment response (Renschler, 2004; Weinberg, 2009). ROS-related damages in lymphocytes from patients with monoclonal B-lymphocytosis and CLL reported increased levels of oxidatively modified DNA and lipids in the sera of untreated CLL patients due to increased oxidative phosphorylation in CLL cells **(**Jitschin, et al., 2014.**)**. Many chemotherapeutic drugs have been shown to exert their biologic activity through induction of OS in affected cells (Udensi and Tchounwou, 2014).

1.5.1 Alteration in tissue antioxidant defense mechanism

Under normal circumstances the effect of reactive species is balanced by the antioxidant action as enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, monoamine oxidase (MAO) and non-enzymatic action as glutathione (GSH), α-tocopherol, cysteine, thioredoxin,and vitamins which are acts as ROS scavengers (Halliwell, 1999; Jones, 2008). Essentially cellular functions, such as gene expression, are influenced by the balance between OS and antioxidant conditions. Antioxidant defenses are extremely important as they represent the direct removal of free radicals (pro-oxidants), providing maximal protection for biological sites (Arrigo, 1999).

1.5.1.1 Glutathione (GSH)

GSH is considered to be one of the main antioxidant agents preventing damage to important cellular components caused by ROS

(Pompella et al., 2003). GSH is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine (which is attached by normal peptide linkage to a glycine). Thiol groups are reducing agents. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form, can be reduced back by glutathione reductase, using NADPH as an electron donor (Landry, 2013 ; Narciso, et al., 2013). The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity (Pompella, et al., 2003).

Patients who suffer from cancer have a significantly decreased amount of GSH in the blood and tumor, given a complete response to the treatment (Peklak-Scott, 2005; Lyakhovich, et al., 2006). Lymphocytes from acute lymphocytic leukemia and B-cell CLL patients contain decreased levels of total GSH activities; however, GSH protein expression is increased in serum from leukemia compared with that from healthy control subjects (Nishiura, et al., 1992).

1.5.1.2 lipid peroxidation

Whenever a free radical interacts with another molecule, secondary radicals may be generated that can then produce yet more radical species. The classical example of such a chain reaction is lipid peroxidation, and the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralized by chain breaking antioxidant (Zwart, et al., 1999). These antioxidants scavenger radicals in membranes and lipoprotein particles are crucial in preventing lipid peroxidation (Esterbauer, et al., 1991). As an indicator of lipid peroxidation, malondialdehyde (MDA) is made

up by free radicals during tissue damage when (ROS) degrade polyunsaturated lipids, and used in the measurement of oxidative stress (Halliwell, 2007).

A recent study evaluating ROS-related damages in lymphocytes from patients with monoclonal B-lymphocytosis and CLL reported increased levels of oxidatively modified DNA and lipids in the sera of untreated CLL patients due to increased oxidative phosphorylation in CLL cells (Jitschin, et. al., 2014).

1.6 Biochemical changes in CLL

The clinical course and biology of CLL is heterogeneous; some patients die within months of diagnosis, whereas others live longer than 20 years (Rozman, 1995). The Rai and Binet staging systems help identify patients who have more advanced disease and require treatment (Rai, et al., 1975). Because most patients present with early- or intermediate-stage disease, additional markers are needed to stratify patients who are at increased risk of disease progression with concomitant decreased survival. Although renal involvement in advanced haematological malignancies is common, glomerulonephritis associated with lymphoproliferative disorders is rare, and the related pathogenetic mechanisms are still poorly understood (Da'as, et al., 2001). CLL is more commonly associated with membranoproliferative glomerulonephritis and membranous nephropathy (Lien and Lai, 2011). Tumor lysis syndrome is typically rare in CLL (Cheson, 2009; Gertz, 2010), but its occurrence with advanced disease stage, characterized by bulky tumor burden, elevated white blood cell count and β2-microglobulin, and reduced albumin level (Tosi, et al., 2008; Blum, 2011). With the increased development of targeted therapies,

Tumor lysis syndrome is becoming more prevalent and is now observed more commonly in diseases that were previously characterized as low risk for Tumor lysis syndrome, such as CLL (Cheson, 2009; Gertz, 2010).

1.6.1 Liver function in CLL

The liver may be involved in systemic diseases that mainly affect other organs. Patients with CLL often show mild to moderate liver enlargement and extensive lymphocytic infiltration in the portal tracts, with functional impairment of the liver in late stages (Schwartz and Shamsuddin 1981; Wilputte, et al., 2003). Even in the absence of liver metastasis, renal cancer causes hepatomegaly and abnormal liver function test results. Following tumor resection, however, these liver abnormalities return to normal, suggesting that the previously observed abnormalities were caused by a hepatotoxic hormone secreted from the tumor (Guevara , 2007).

1.6.2 Renal function in CLL

Renal infiltration with leukaemic cells is a common finding in patients suffering with chronic lymphocytic leukaemia **.** CLL is characterized by a progressive accumulation of monoclonal incompetent lymphocytes and is similar to peripheral B-cell neoplasm small lymphocytic lymphoma (Yogo, et al., 2009). Acute renal failure in CLL patients can be associated with acute tubular necrosis, uric acid nephropathy, light chain nephropathy, obstructive nephropathy, amyloidosis, hypercalcemia, glomerulonephritis, and cryoglobulinemia (Boudville, et al., 2001). Though a large proportion of patients with CLL will have significant renal infiltration (60–90%), rarely does this result in renal impairment and the infiltrate characteristically spares the

tubules (Barcos, et al., 1987; Blum, et al. 2011). Although the mechanism of renal failure in CLL is not clear it may be associated with intrarenal obstruction and ischemia which occurs secondary to the compression of the tubular lumen with CLL cells (Junglee, 2012). Renal involvement by CLL cells could be nodular or diffuse and may cause fibrosis in the same areas. Kidney size can be either increased or normal and proteinuria has been generally mild (Hewamana, et al., 2009).

1.7 Genetics of CLL

Development of leukemia is affected by a number of factors including environment and genetic background (DeVita, et al., 2009). CLL is a common hematological malignancy in Western countries and it is very rare in Asian countries (Rai and Keating , 2000). Furthermore, Asians including Japanese immigrants to USA continue to have a low incidence of CLL (Gale, et al.,2000; Pan, et al., 2002). Cytogenetic analyses of CLL identified chromosomal abnormalities including del11q23 affecting the ATM gene, tri12, del 13q14, and del17p13 affecting TP53 gene (Stilgenbauer, et al., 1998). In addition, gains and losses in Xp11.2-p21 and Xq21- qter (Summersgill,et al., 2002). Molecular studies on CLL identified three genes: IgVH, CD38 and ZAP-70 that correlate with CLL prognosis (Rosenwald, et al., 2001; Del Principe, et al., 2006). A CLL-specific microRNA signature was also identified, suggesting that microRNA deletion could be involved in CLL, and it is relatively intact with fewer aberrations than other types of leukemia (Calin, et al., 2005).

1.7.1 IGHV.

(IGHV) somatic hypermutation of the B cell receptor (BCR) variable region is a physiological event during antigen driven maturation of B cells in secondary lymphoid tissue. It can be measured by comparing the clonal (IGHV) sequence to known germline sequences in the clinical laboratory. CLL clones that utilize an unmutated IGHV have decreased time from diagnosis to first treatment and poorer survival than those who express mutated IGHV genes (Hamblin, et al., 1999; Damle, et al., 1999; Tobin, et al., 2003). Moreover, the IGHV1encoded (IgH) expressed in CLL commonly have stereotypic motifs in the third complementarity determining region, resulting from the rearrangement and restricted reading-frame-use of certain IGHD and IGHJ gene segments (Mauro, et al., 2000; Robak, 2007; Tsimberidou, et al., 2009). The prevalent use of unmutated IGHV1 and nonstochastic pairing with light chains suggests that plays a role in the development and/or progression of CLL (Rai, et al., 1975; Binet, et al., 1981).

1.7.2 Molecular investigation by Polymerase chain reaction (PCR)

The polymerase chain reaction is an *in vitro* technique which allows the amplification of a single or a few copies of a piece of DNA, and rapidly produce thousands to millions of copies of a particular DNA sequence in a matter of only few hours. The Requirements of PCR reaction are Template, Deoxy nucleotide triphosphate (dNTPS), Primers, PCR buffer and DNA polymerase, Magnesium concentration (Pherson and MØller, 2001).

In Reverse Transcription polymerase chain reaction (RT-PCR) an RNA strand is first reverse transcribed into its DNA complement using the enzyme reverse transcriptase, and the resulting cDNA is amplified

using traditional or real-time PCR (Hamblin, 2000; Bustin, 2002). RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript. (Troutt et al., 1992).

1.8 Treatment of CLL

The clinical course of CLL is highly diverse; some patients have indolent disease, never needing treatment, whereas others have aggressive disease, requiring treatment at initial presentation. The aim of treatment in CLL is to improve symptoms and put the disease into a remission, this requires measures to reduce the number of abnormal CLL cells in the body (Pettitt, et al., 2012). Prognostic factors, including clinical and laboratory features, have been correlated with clinical outcomes (Lobetti, et al., 2013; Cuthill, et al., 2013).

The most comman protocol used in treatment of CLL is immune chemotherapy consumed as a combination of Rituximab, Fludarabine and Cyclophosphamide (RFC). Fludarabine is the purine analogue. Cyclophosphamide is an alkylating agent. The rationale for using it in combination with fludarabine is based on the ability of fludarabine to inhibit the excision repair of DNA interstrand cross-links induced by cyclophosphamide and therefore to potentiate its cytotoxic activity in CLL cells (Yamauchi, et al., 2001). Rituximab also has the ability to down-regulate the expression of the antiapoptotic protein Bcl-2, thereby enhancing the sensitivity of CLL cells to fludarabine-induced apoptosis (Wierda, et al., 2007). R-FC is the treatment of choice for most fit people with CLL because it has been shown to result in improved survival. Chlorambucil works well in some people, especially people with a milder form of the disease, while bendamustine as an initial treatment for people with stage B or C

disease who cannot have fludarabine. (Hallek, et al., 2008; Catovsky, et al., 2011; Pettitt, et al., 2012), Pentostatin has the potential advantage of being less myelosuppressive than fludarabine (Robak, et al., 2000). Rituximab combined with bendamustine (R-B), might be a useful treatment in people who are less fit and who relapse early after being treated with chlorambucil or in fitter people who cannot have R-FC because of kidney problems (Pettitt, et al., 2012).

1.9 Remission and Minimal Residual Disease

Recent therapies for CLL, such as monoclonal antibodies and stem-cell transplantation, reduce CLL cells to a much lower level than previously possible. Modern techniques, such as four-color flow cytometry for the unique combination of B-CLL immunophenotypic antigens or allele-specific polymerase chain reaction for the immunoglobulin-gene rearrangement, can detect as few as a single CLL cell in 100,000 normal cells (Jurisic, et al., 2008). The ability to detect extremely low levels of CLL and to eradicate CLL to below this level allows us to address whether the lack of improved survival for purine analogs compared with alkylating agents is primarily due to lack of efficacy. The most appropriate patients in whom to address the correlation of minimal residual disease (MRD) negative remissions and survival are those with the worst prognosis (Vassilakopoulos et al. 2001). Although recent advances in our understanding of the biology of CLL, such as the adverse impact of p53 dysfunction, 17p deletions or ataxia-teleangiectasia gene mutations, germ-line immunoglobulin genes, or ZAP-70 expression, allow the identification of patients at diagnosis with a poor prognosis (Moreton and Kennedy, et al., 2005).

Chapter Two

Subjects, Materials and Methods

Chapter Two
Subjects, Materials and Methods

2.1 Subjects:

This study was conducted on 55 adult Iraqi patients referred to the National Center of Hematology, AL-Mustansiyria university for evaluation and treatment during the period extending from Oct. 2013 to Sept. 2015. They were diagnosed as having Chronic Lymphocytic leukemia (CLL) based on physical examination by a specialist, morphological assessment of peripheral blood films and bone marrow smear by aspirate (BSA) examination (in difficult cases), as well as flow cytometric immunophenotypic profile.

CLL patients included 18 newly diagnosed, untreated patients (12 males and 6 females), with a mean age of 55±12 yrs. and an age range of 40-80 yrs. According to the Binet staging system for CLL, 10 patients were in stage B and 8 in stage C. Another 37 previously diagnosed, and treated CLL patients (28 males and 9 females), with a mean age of 57±9 yrs. and an age range of 45-71 yr. Twenty three patients of this group were in stage B and 14 patients in stage C.

Five patients (2 in stage C and 3 in stage B) of the newly diagnosed untreated (5/18) group and eleven patients (7 in stage C and 4 in stage B) of the treated (11/37) group had type II diabetes mellitus with an oral hyperglycemic medication. Collectively, 16 out of the 55 CLL patients (i.e. 29.1%) reported a history of type II diabetes mellitus.

Nineteen healthy subjects were also involved in this study. They included 9 males and 10 females, with a mean age of 61±17yr. and an age range of 30-90 yrs.

Two males patient was excluded from the study. They were only patients with stage A among all patients of the present study.

The treatment given to CLL patients consist of the following combination therapy Rituximab, Fludarabine and Cyclophosphamide (RFC), Rituximab was given at 375 mg/m^2 on day one (or over 2 days), Fludarabine given at 30 mg/m^2 on day one to day three, , and Cyclophosphamide at 300 mg/m^2 on day one to day five (Hallek *et al*., 2008; Catovsky *et al*., 2011; Pettitt *et al*., 2012).

2.1.1 Research protocol: (Case-Control Study)

The effects of RFC combination therapy on the various studies parameters were followed up in the newly diagnosed untreated patients. Peripheral blood samples were obtained from these patients during their second visit to the center for evaluation of their clinical response to the initial therapy. The follow-up period range between 1-8 months, with a median of 4.5 months (mean ±SD; 4.78±2.36) months. Only 14 patients of the untreated newly diagnosed patients (14/18) managed to revisit the center.

Unlike the newly diagnosed CLL patents, the other already treated 37 CLL patients lack a pretreatment values for the current studied parameters. Instead, those patients received treatment for a period range between 4 months to 8.5 yrs. with a median of 21.5 months (mean±SD; 28.16±22.41) months. Accordingly, the obtained data served to reflect the effect of RFC therapy on the various study parameters after a median duration of 21.5 months.

Collectively, the present research protocol encompass two groups of CLL patients. First, 18 newly diagnosed patients with pre-treatment values, and followed up for a median of 4.5 months RFC therapy. Second, 37 already treated CLL patients with a median of 21.5 months of therapy; as illustrated in figure (2.1).

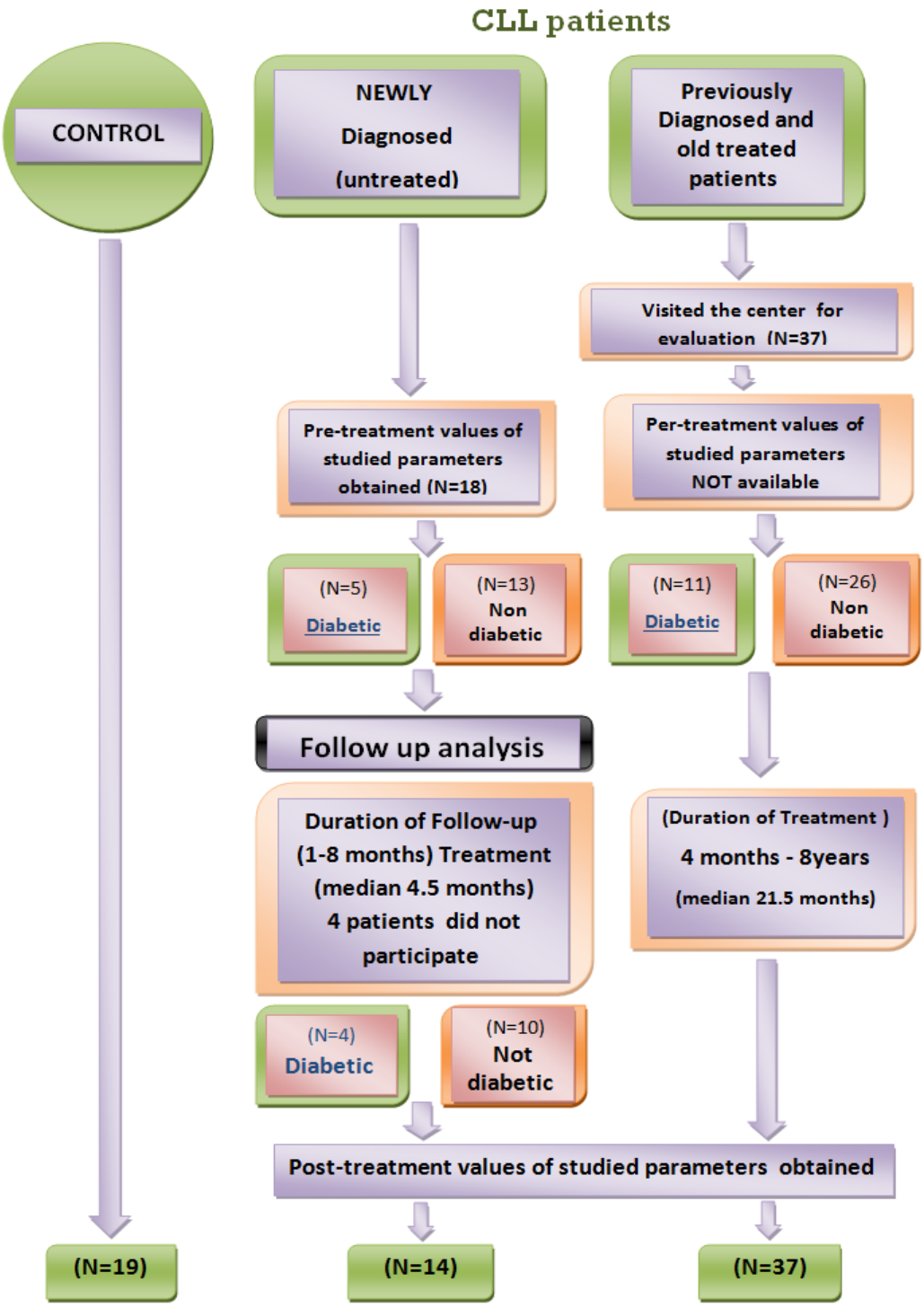


Figure (2.1) Research protocol (Case-Control Study).

2.1.2 Studied Parameters in the present protocol:

1- Complete Blood Picture: (conducted by the National center for blood diseases)

2- Biochemical Profile: (FBS/HbA1c, lipid profile/cholesterol, triglyceride, Blood urea, Creatinine, AST, ALT, Total bilirubin, Albumin , uric acid and Antioxidant status; Glutathione (GSH) and Malondialdehyde (MDA).

3- Immunological Profile:

A-Immunophenotyping: (CD5, CD38, and ZAP-70).

B- Proinflammatory Cytokines: (IL-6/IL-10).

4-Molecular Profile: (IGHV mutational status and DNA sequencing).

2.2. Materials

2.2.1: Apparatus

Apparatus used in this study are listed in table (2-1).

Table (2-1) Apparatus used and their manufactures

No.	Item	Company	Origin
1	Blood collection plain tubes	AFMH	England
2	Blood collection plastic can tubes	AFMH	China
3	Blood collection Pyrex test tubes	AFMH	=
4	Cooling centrifuge	Tomy Seiko company	Japan
5	Distillator	American	USA
6	EDTA containing tubes	AFMH	England
7	Eppendorff bench centrifuge	Netherland and Hinz Gbm 2000	Germany
8	Eppendorff tube	Eppendorf	=
9	Balance	Eppendorf	=
10	Flow cytometry	Apogee	England
11	Gel electrophoresis system	Thermo	USA
12	Gel Imaging System	BioRad	=

13	Incubator	Fisher	Germany
14	Magnetic stirrer with hot plate	Lassco	India
15	Microfuge	Eppendorf	Germany
16	Micropipette	Eppendorf	=
17	Microwave	Showinc	Thailand
18	NanoDrope Spectrometer	Thermo	Germany
19	Oven	Chilipison	U.K
20	Plastic syringes; 10ml	Meheco	China
21	Plastic wares	-	=
22	Rack	-	=
23	Refrigerator	Samsung	Korea
24	Spectrophotometer	Shimadzue	Japan
25	Tips	Eppendorf	China
26	Thermocycler	BioRad	USA
27	Ultra Violet Ray	Zhejiang	China
28	Vortex	Stuar Scientific	Germany
29	Volumetric cylinders	Volac	England
30	Volumetric Flasks	Volac	=
31	Water bath Memmert	Memmert	Germany
32	Vortex	Vortex 2 gene	USA
33	5- Part Differential Auto Analyzer Hematology	Convergence	Germany

2.2.2 Chemicals

Chemicals used in this study are listed in table (2-2).

Table (2-2) Chemicals materials used in the present study

NO.	Item	Company	Origin
1	Absolute Ethanol	Sigma	U.S.A
2	Agarose	Promega	=
3	Albumin Kit	GLOBE	Italy
4	alkaline phosphatase (ALP) Kit	GLOBE	=
5	Creatinine Kit	DIALAB	Austria
6	Ethidium bromide	Sigma	U.S.A
7	ELISA kit for quantitative determination of Interleukin 6(IL-6) in serum or plasma	R&D Systems	=
8	ELISA kit for quantitative determination of Interleukin 10(IL-10)	R&D Systems	=

	in serum or plasma		
9	Glucose Kit	DiaSys,	Germany
10	Normal Saline	ADWIC	U.S.A
11	Nuclease free water	Partec	Germany
12	Ficol-Histopacue	Sigma	U.S.A
13	FITC Mouse Anti_Human CD4	Partec	Germany
14	AST/AST – L Kit	DIALAB	Austria
15	ALT/ALT – L Kit	DIALAB	=
16	Human Glutathione (GSH) ELISA Kit (cat.CSB-E09495h)	Cusabio	U.S.A
17	Human Malondialchehyche (MDA) ELISA Kit (Cat.CSB-E08557h	Cusabio	=
18	Separation of mononuclear cells	Meltinyi biotec	=
19	PE Mouse Anti_Human CD8	Partec	Germany
20	Pro. Anti_Human ZAP- 70	Cusabio	U.S.A
21	Total Cholesterol Kit	DIALAB	Austria
22	Triglycerides Kit	TECO	U.S.A
23	Urea Kit	DIALAB	Austria
24	Uric acid Kit	DIALAB	=
25	6X Loading dye	Promega	U.S.A
26	10X TAE buffer	Promega	=

2.3 Methods

2.3.1 Blood samples collection:

Before blood sampling, all participants were informed about the study objectives and their consents were obtained.

Blood samples were obtained from patients and control subjects, in a fasting state, by venepuncture using a 10ml disposable syringe. Three ml of blood were obtained and dispensed in EDTA tubes and submitted to the lab. for evaluation of complete blood picture using (5- Part Differential Auto Analyzer Hematology) and measurement of HbA1c (D-10™ Dual Program Testing System). Another 3 ml were dispensed in plain tubes and left for around an hour to clot at room temperature. Then, it was centrifuged at 1000 rpm for 30 minutes to collect serum. The serum was divided into aliquots (250µl) in Eppendorff tubes and stored in the deep

freezer (-20ºC) until use for measuring glutathione (GSH), and malondialdehyde (MDA), cytokines (IL-6 and IL-10) and other biochemical profiles. Another 2ml were obtained in (EDTA) tubes for the lymphocytes separation. The last 2ml of the blood sample were obtained in (EDTA) tubes was placed in a cool box and transferred to private lab for determination of lymphocyte phenotyping (expressions of CD5, CD38, and ZAP-70) using flow cytometry (Dhot *et al*., 2003; Yaghmaie *et al*; 2008).

2.3.2 Measurement Hemoglobin,PCV, White Blood cell, Red Blood Cell, and Platelets.

Three ml of blood were obtained and dispensed in EDTA tubes which was submitted to the lab for complete blood count by (5- Part Differential Auto Analyzer Hematology).

- Principles of operation :

five-part differential Auto analyzer uses a combination of methods to provide measurement results.

⊕ Volumetric impedance is used to determine the cellular concentration and volume distributions of (WBC), (RBC), and (PLT).

⊕ Photometric measurement of light absorbance is used to determine hemoglobin concentration.

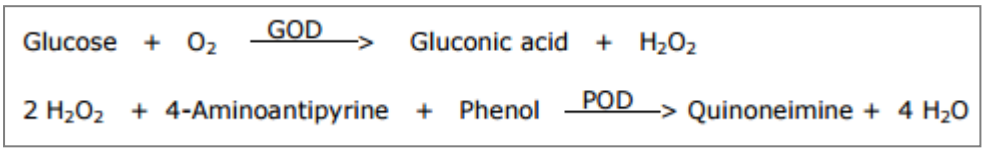
⊕ Optical measurement of light scattering and diffraction is used to determine five part leukocyte (LYM.%, MON.%, NEU.%, EOS.%, and BAS.%) differential parameters.

2.4 Measurement of biochemical profiles:

2.4.1 Determination of Fasting Blood Glucose (DiaSys, Germany)

2.4.1.1 Principle of the assay

Determination of glucose was done after enzymatic oxidation by glucose oxidase. The colorimetric indicator is quinoneimine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction) (Barham *et al.*, 1972).



GOD=glucose oxidase; PDO = hydrogen peroxide.

2.4.1.2 Blood glucose assay procedure:

a. An aliquot (10µl) of the serum was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 10 min. (sample)

b. An aliquot (10µl) of the standard was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 10 min (Standard)

c. An aliquot 1 ml of reagent R was added to the tubes and incubated at 37°C for 10 min (Blank).

d. The samples and standard were read at (500 nm) wavelength against the Blank. The color is stable for at least 1 hr.

2.4.1.3 Calculation of the results

The concentrations (mg/dl) of Glucose tests were calculated as in the sample as follows:

$$\text{Glucose [mg / dl]} = \frac{(A)sample\ x\ 100(standard\ conc.)}{(A)standard}$$

2.4.2 HbA1c Determination. (Bio-Rad D-10™ Dual)

2.4.2.1principle of operation

The Bio-Rad D-10™ Dual Program is based on chromatographic separation of the analytes by using ion-exchange high-performance liquid chromatography (HPLC).

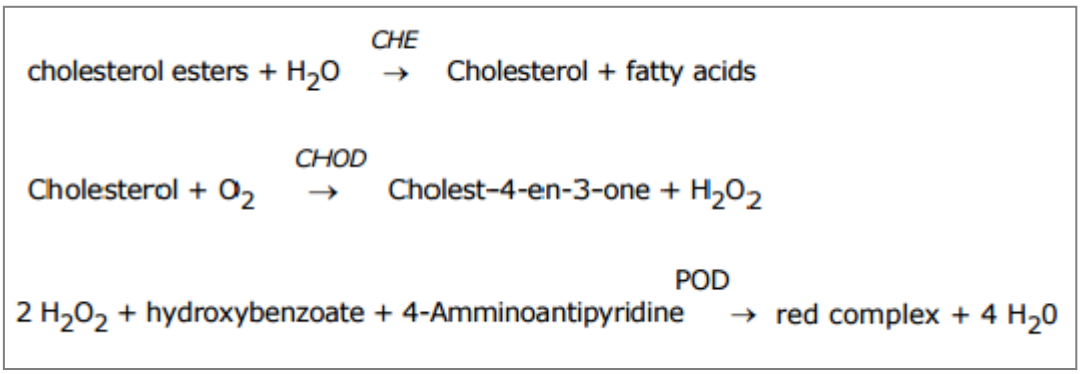
2.4.2.3 HbA1c assay procedure:

1- One ml of whole blood was added into EDTA tube for each patient and control.
2- The EDTA tube was left on the shaker for two min.
3- The sample tubes were loaded into the D-10 sample rack and placed in the D-10, The samples were automatically diluted on the D-10 and injected into the analytical cartridge.
4- The D-10 delivered a programmed buffer gradient of increasing ionic strength to the cartridge where the hemoglobins were separated based on their ionic interactions with the cartridge material.

2.4.3 Determination of Cholesterol Total (DIALAB, Austria)

2.4.3.1 Principle of the assay

The measurement is based on the following enzymatic reactions:



2.4.3.2 Cholesterol Total assay procedure:

a. An aliquot (10µl) of the serum was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 10 min. (sample)

b. An aliquot (10µl) of the standard was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 10 min (Standard)

c. An aliquot 1 ml of reagent R was added to the tubes and incubated at 37°C for 10 min (Blank).

d. The samples and standard were read at (510 nm) wavelength against the Blank. The color was stable for at least 1 hr.

2.4.3.3 Calculation of the results

The concentration (mg/dl) of cholesterol total tests were calculated in the samples as follows: (Bachorik, 1995)

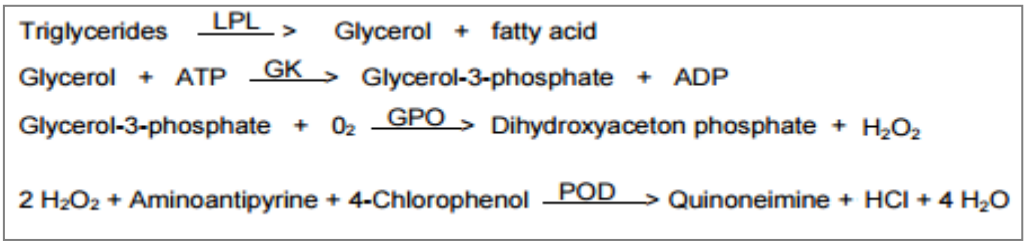
$$\text{Cholestero[mg/dL]} = \frac{\text{Sample}}{\text{Standard}} \times 200 \quad \text{Standard,Conc.}$$

.

2.4.4 Determination of Ttriglycerides (DIALAB, Austria)

2.4.4.1 Principle of the assay

The ddetermination of triglycerides was done after the enzymatic splitting with lipoprotein lipase. Indicator is quinoneimine generated from 4-aminoantipyrine and 4-chlorophenol by a hydrogen peroxide under the catalytic action of peroxidase.



LPL= lipoprotein lipase;

2.4.4.2 Triglycerides assay procedure:

a. An aliquot (10µl) of the serum was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 10 min. (sample)

b. An aliquot (10µl) of the standard was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 10 min (Standard)

c. An aliquot 1 ml of reagent R was added to the tubes and incubated at 37°C for 10 min (Blank).

d. The samples and standard, were read at (510 nm) wavelength against the Blank. The color is stable for at least 1 hr.

2.4.4.3 Calculation of the results

The concentration (mg/dl) of triglycerides tests were calculated in the samples as follow:

$$\text{Triglycerides (mg/dL)} = \frac{\text{Sample}}{\text{Standard}} \times 200 \quad \text{Standard,Conc.}$$

2.4.5 Determination of Urea (GLOBE Italy)

2.4.5.1 Principle of the assay

Urease hydrolyzes urea into ammonia and carbon dioxide.

Glutamate dehydrogenase catalyzes the reaction of ammonia with 2-ketoglutarate and oxidizes NADH into NAD+.

$$\text{Urea} + 2\,H_2O \xrightarrow{\text{Urease}} 2\,NH_4^+ + 2\,HCO_3^-$$

$$\text{2-Ketoglutarate} + NH_4^+ + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-Glutammate} + NAD^+ + H_2O$$

GLDH= glutamated dehydrogenase

2.4.5.2 Urea assay procedure:

a. An aliquot (10µl) of the serum was added to tubes containing (10µl) of Reagent A1, then it was mixed well and incubate for 5 minutes at 37 °C., then (200µl) of Reagent B was added and mixed well and incubated for 30 seconds at 37 °C, then A1 of sample was read. After precisely 60 seconds, the absorbance A2. (sample) was read.

b. An aliquot (10µl) of the standard was added to tubes containing (10µl) of Reagent A1, then it was mixed well and incubated for 5 minutes at 37 °C. Then (200µl) of Reagent B was added. Mixed well and incubated for 30 seconds at 37 °C then A1 of standard, after precisely 60 seconds read absorbance A2. (standard) was read.

c. An aliquot 1 ml of reagent A1 was added to the tubes and incubated at 37°C for 10 min (Blank).

d. The samples and standard were read at (340 nm) wavelength against the Blank.

2.4.5.3 Calculation of the results

The concentrations (mg/dl) of Urea tests were calculated in the samples as follows:

$$\Delta A = [(A_1 - A_2) \text{ sample or standard}] - [(A_1 - A_2) \text{ Blank}]$$

$$\text{Urea, mg/dl} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 50$$

2.4.6 Determination of Creatinine (DIALAB, Austria)

2.4.6.1 Principle of the assay

Serum and urine Creatinine reacts with Picric acid in alkaline solution yielding a yellow-orange coloured compound. The intensity of the colour is directly proportional to the Creatinine concentration present in the sample (REAGENT A:Sodium hydroxide and REAGENT B: Picric acid**).**

2.4.6.2 Creatinine assay procedure:

a. An aliquot (50µl) of the serum was added to tubes containing (1000µl) of Reagent 1, then it was mixed well and incubated for 5 minutes at 37 °C. Then (250µl) of Reagent 2 was added. Mixed well and incubated 1 min at 37 °C and A1 was read, incubated for exactly 2 min again. (sample)

b. An aliquot (50µl) of the standared was added to tubes containing (1000µl) of Reagent 1, then it was mixed well and incubate for 5 minutes at 37 °C. Then (250µl) of Reagent 2 was added and mixed well and incubated for 1 min at 37 °C and A1 was read. Incubated for exactly 2 min again.(standard)

c. An aliquot 1 ml of reagent R1 was added to the tubes and incubated at 37°C for 10 min (Blank).

d. The samples and standard were read at (490 nm) wavelength against the Blank.

2.4.6.3 Calculation of the results

The concentrations (mg/dl) of Creatinine tests were calculated in the samples as follows:

$$\Delta A = (A2 - A1)\ \text{sample or stdandard}$$

$$\text{Creatinine (mg/dL)} = \frac{\Delta A\ \text{Sample}}{\Delta A\ \text{Standard,}} \times 2\ \text{Standard,Conc.}$$

2.4.7 Determination of AST (Aspartate aminotransferase) (DIALAB, Austria)

2.4.7.1 Principle of the assay

NADH (Nicotinamide adenine dinucleotide) is oxidized to NAD+ , the resulting decrease in absorbance at 340 nm is directly proportional to the activity of AST in the sample.

$$\text{L-Aspartate} + \text{2-Oxoglutarate} \xrightarrow{\text{AST}} \text{L-Glutammate} + \text{Oxalacetate}$$

$$\text{Oxalacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-Malate} + \text{NAD}^+$$

2.4.7.2 AST assay procedure Bireagent procedure:

a. An aliquot (100µl) of the serum was added to tubes containing 1ml of Reagent A, then it was mixed well and incubated at 37°C for 5 min. (sample)

b. (100µl) of the Reagent B was added to tube sample after first incubation, then it was mixed well and incubated at 37°C. After 1 minute the absorbance (A) was read at 340 nm. The absorbance was read again 1, 2, 3 minutes thereafter

c. An aliquot 1 ml of Reagent A was added to the tubes and incubated at 37°C for 1 min (Blank).

d. The samples and standard were read at (334 nm) wavelength against the Blank.

2.4.7.3 Calculation of the results

The concentrations (mg/dl) of AST tests was calculated in the samples as follows:

Calculate ΔA/min , Factor (F)= 1905

AST (U/l) = ΔA/min x (F)

2.4.8 Determination of ALT (Alanine Aminotransferase) (DIALAB, Austria)

2.4.8.1 Principle of the assay

NADH is oxidized to NAD+ . The resulting decrease in absorbance at 340 nm is directly proportional to the activity of ALT in the sample.

$$\text{L-Alanine} + \text{2-Oxoglutarate} \xleftrightarrow{\text{ALT}} \text{L-Glutammate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xleftrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^+$$

2.4.8.2 ALT assay Bireagent procedure:

a. An aliquot (100µl) of the serum was added to tubes containing 1ml of Reagent A, then it was mixed well and incubated at 37°C for 5 min. (sample)

b. (100µl) of the Reagent B was added to tube sample after incubation, then it was mixed well and incubated at 37°C. After 1 minute the absorbance (A) read at 340 nm. The absorbance was read again 1, 2, 3 minutes thereafter

c. An aliquot 1 ml of Reagent A was added to the tubes and incubated at 37°C for 1 min (Blank).

d. The samples and standard were read at (340 nm) wavelength against the Blank.

2.4.8.3 Calculation of the results

The concentrations (mg/dl) of ALT tests were calculated in the samples as follows:

Calculate ΔA/min , Factor (F)= 1905

ALT (U/l) = ΔA/min x (F)

2.4.9 Determination of total bilirubin (DIALAB, Austria)

2.4.9.1 Principle of the assay

Bilirubin is formed from the heme portion of hemoglobin released by aged or damaged red blood cells. It is then converted in the liver to bilirubin monoglucuronide and bilirubin diglucuronide. Free bilirubin is not soluble in aqueous solution and requires solubilization dimethylsulfoxide and ethylene glycol used as asolvents for the total bilirubin assay. The absorbance of bilirubin at 555 nm is directly proportional to the bilirubin concentration in the sample.

2.4.9.2 Total bilirubin assay procedure:

a. An aliquot (100µl) of the serum was added to tubes containing (1000µl) of Reagent 1, then (10µl) of Reagent 2 was added and mixed well and incubated for 2 min at 37 °C and the absorbance. was read (sample).

b. An aliquot (100µl) of the Calibrator was added to tubes containing (1000µl) of Reagent 1, then (10µl) of Reagent 2 was read mixed well and incubate 2 min at 37 °C and read absorbance was read (standard)

c. An aliquot 1 ml of reagent R1 was added to the tubes and incubated at 37°C for 5 min (Blank).

d. The samples and Calibrator were read at (555 nm) wavelength against the Blank.

2.4.9.3 Calculation of the results

The concentrations (mg/dl) of total bilirubin tests were calculated in the samples as follows:

$$\text{Factor} = 12.9$$

$$\text{Total Bilirubin (mg/dL)} = \frac{\text{Sample}}{\text{Calibrator}} \times \text{Factor}$$

2.4.10 Determination of Albumin (GLOBE Italy)

2.4.10.1 Principle of the assay

Albumin binds with Bromo Cresol Green (BCG) at pH 4.2 causing a shift in absorbance of the yellow BCG dye. The blue-green colour formed is proportional to the concentration of albumin, when measured photometrically between 540–630 nm with maximum absorbance at 625 nm.

2.4.10.2 Serum Albumin assay procedure:

a. An aliquot (5µl) of the serum was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 5 min. (sample)

b. An aliquot (5µl) of the standard was added to tubes containing 1ml of Reagent R, then it was mixed well and incubated at 37°C for 5 min (Standard)

c. An aliquot 1 ml of reagent R was added to the tubes and incubated at 37°C for 5 min (Blank).

d. The samples and standard, were read at (546 nm) wavelength against the Blank. The color is stable for at least 60 minutes.

2.4.10.3 Calculation of the results

The concentration (g/dl) of Albumin tests was calculated in the samples as follows:

$$\text{Albumin (g/dl)} = \frac{\text{A sample}}{\text{A standard}} \times 4$$

2.4.11 Determination of uric acid (DIALAB, Austria)

2.4.11.1 Principle of the assay

Uric acid is oxidized by uricase into an allantoin with a production of hydrogen peroxide which, under the catalytic influence of peroxidase reacts with 4-aminofenazone and N-ethyl-N-(hydroxi-3-sulphopropil)-p-toluidine (ESPT)

$$\text{Uric Acid} + 2\,H_2O + O_2 \xrightarrow{\text{URICASE}} \text{Allantoine} + CO_2 + H_2O_2$$
$$\text{TBHBA} + \text{4-AAP} + H_2O_2 \xrightarrow{\text{POD}} \text{Chinonimine} + 2\,H_2O + HBr$$

2.4.11.2 uric acid assay procedure:

a. An aliquot (25µl) of the serum was added to tubes containing 1ml of Reagent $R^{\text{uric acid}}$, then it was mixed well and incubated at 37°C for 10 min. (sample)

b. An aliquot (15µl) of the standard was added to tubes containing 1ml of Reagent $R^{\text{uric acid}}$, then it was mixed well and incubated at 37°C for 10 min (Standard)

c. An aliquot 1 ml of reagent R was added to the tubes and incubated at 37°C for 10 min (Blank).

d. The samples and standard, were read at (550 nm) wavelength against the Blank. The color was stable for at least 30 minutes.

2.4.11.3 Calculation of the results

The concentrations (mg/dl) of uric acid tests were calculated in the samples as follows:

$$\text{Uric Acid (mg/dl)} = \frac{\text{Sample}}{\text{Stand}} \times 6 \text{ Standard Conc.}$$

2.5 Antioxidant enzymes

2.5.1 Glutathione (GSH) (Cusabio, U.S.A.)

2.5.1.1 Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for GSH has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any GSH present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for GSH is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of GSH bound in the initial step. The color development stops and the intensity of the color is measured.

2.5.1.2 Kit components for detection of glutathione (GSH):

Reagents	Quantity
Assay plate (12 x 8 coated Microwells)	1(96 wells)
Standard (Freeze dried)	2
Biotin-antibody (100 x concentrate)	1 x 120 µl
HRP-avidin (100 x concentrate)	1 x 120 µl
Biotin-antibody Diluent	1 x 10 ml
HRP-avidin Diluent	1 x 10 ml
Sample Diluent	1 x 20 ml
Wash Buffer (25 x concentrate)	1 x 20 ml
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

2.5.1.3 Preparation of Solutions

1. **Biotin-antibody (1x)** - the vial was centrifuged before opening. Biotin-antibody was diluted to a 100-fold dilution. A suggested 100-fold dilution is 10 µl of Biotin-antibody + 990 µl of Biotin-antibody Diluent.

2. **HRP-avidin (1x)** - the vial was centrifuged before opening. HRP-avidin was diluted to a 100-fold dilution. Which is 10 µl of HRP-avidin + 990 µl of HRP-avidin Diluent.

3. **Wash Buffer(1x)** - If crystals were formed in the concentrate, they were warmed up to room temperature and mixed gently until the crystals completely dissolved. A20 ml of Wash Buffer Concentrate (25 x) was diluted into distilled water to prepare 500 ml of Wash Buffer (1 x).

4. **Standard**

The standard was centrifuged at 6000-10000rpm for 30s.The Standard was reconstituted with 1.0 ml of Sample Diluent. This reconstitution produces a stock solution of 50 µg/ml. The standard was mixed to ensure a complete reconstitution and allow the standard to sit for a minimum of 15

minutes with gentle agitation prior to making dilutions. A 250 µl of Sample Diluent was pipetted into each tube. The stock solution was used to produce a 2-fold dilution series. Each tube was mixed thoroughly before the next transfer. The undiluted Standard served as the high standard (50µg/ml). The sample Diluent served as the zero standard (0 µg/ml).

2.5.1.4 GSH Assay Procedure

All reagents and samples were brought to room temperature before use. The sample was centrifuged again after thawing before the assay. All samples and standards were assayed in duplicate.

1. All reagents, working standards, and samples were prepared as directed in the previous sections.
2. The number of wells to be used was determined according to the Assay Layout Sheet and put any remaining wells and the desiccant were put back into the pouch, ziploc was sealed, stored unused wells at 4°C.
3. one hundred µl of standard and serum per well were added, covered with the adhesive strip provided, and incubated for 2 hours at 37°C. A plate lay out is provided to record standards and samples assayed.
4. The liquid of each well was removed without washing.
5. one hundred µl of Biotin-antibody (1x) was added to each well. covered with a new adhesive strip. Then incubated for 1 hour at 37°C.
6. Each well was aspirated and washed, repeating the process two times for a total of three washes. Washing was done by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, any remaining wash Buffer was removed by aspirating ordecanting. The plate was inverted and blotted it against clean paper towels.

7. one hundred µl of HRP-avidin (1x) was added to each well. The microtiter plate was covered with a new adhesive strip and incubated for 1 hour at 37°C.
8. The aspiration/wash was processed for five times as in step 6.
9. Ninety µl of DTMB Substrate was added to each well, incubated for 15-30 minutes at 37°C, and protected from light.
10. Fifty µl of Stop Solution was added to each well, and the plate was gently tapped to ensure a thorough mixing.
11. The optical density of each well was determined within 5 minutes, using a microplate reader set to 450 nm. If a wavelength correction is available, set to 540 nm. The readings at 540 nm was substracted from the readings at 450 nm. This subtraction will correct the optical imperfections in the plate.

2.5.1.5 Calculation of the result:

Using the professional soft "Curve Expert 1.3" to make a standard curve is recommended (figure 2-2). The duplicate readings wre averaged for each standard and the sample and the average zero standard optical density were subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and a best fit curve was drawn through the points on the graph. The data may be linearized by plotting the log of the GSH concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis.

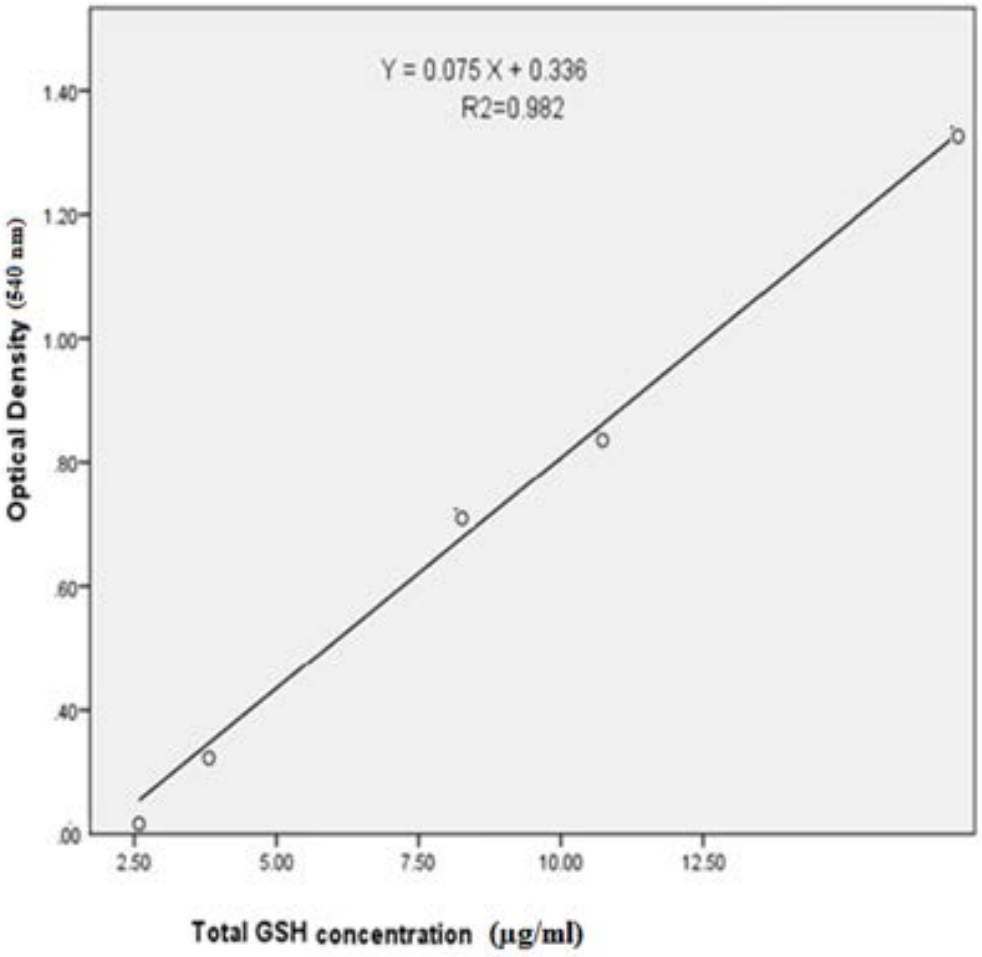


Figure (2.2) Standard curve of total glutathione (GSH)

2.5.2 Malondialdehyde (MDA) (Cusabio, U.S.A.)

2.5.2.1 Principle of the assay

The microtiter plate provided in this kit has been pre-coated with an antibody specific to MDA.

Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for MDA and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3'5, 5' tetramethyl-benzidine) substrate solution is added to each well.

Only those wells that contain MDA, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color.

The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of MDA in the samples is then determined by comparing the O.D. of the samples to the standard curve.

The standard curve concentrations used for the ELISA's were 20 nmol/ml, 10 nmol/ml, 5 nmol/ml, 2.5 nmol/ml, 1.25 nmol/ml, 0.625 nmol/ml,0. 312 nmol/ml.

2.5.2.2 Kit components for detection of Malondialchehyche (MDA)

Reagent	Quantity
Assay plate	1
Standard	2
Sample Diluent	1 x 20 ml
Biotin-antibody Diluent	1 x 10 ml
HRP-avidin Diluent	1 x 10 ml
Biotin-antibody	1 x 120µl
HRP-avidin	1 x 120µl
Wash Buffer	1 x 20 ml (25×concentrate)
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml

2.5.2.3 Preparation of Solutions

All reagents were brought to room temperature before use.

1. **Wash Buffer** The solution was warmed up to room temperature and mixed gently until the crystals were completely dissolved. 20 ml of Wash Buffer Concentrate was diluted into distilled water to prepare 500 ml of Wash Buffer.
2. **Standard** The Standard was reconstituted with 1.0 ml of Sample Diluent. This reconstitution produced a stock solution of 20000 pmol/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The Sample Diluent serves as the zero standard (0 pmol/ml).
3. **Biotin-antibody** The working concentration specified on the vial label was diluted using Biotin-antibody Diluent(1:100), respectively.
4. **HRP-avidin** The working concentration specified on the vial label using HRP-avidin was diluted Diluent(1:100), respectively.

2.5.2.4 MDA Assay Procedure:

All reagents were brought and samples to room temperature before use. All samples, standards, and controls were assayed in duplicate.

1. one hundred µl of Standard, Blank, or serum Sample were added per well, covered with the adhesive strip and incubated for 2 hours at 37° C.
2. The liquid was removed from each well without washing.
3. one hundred of Biotin-antibody working solution was added to each well and incubated for 1 hour at 37°C.
4. Each well was aspirated and washed three times for a total of three washes. The wash was done by filling each well with Wash Buffer (200µl) using a squirt bottle. Complete removal of liquid at each step is essential to good performance. After the last wash, any remaining Wash

Buffer was removed by aspirating or decanting. The plate was inverted and blotted it against clean paper towels.

5. 100µl of HRP-avidin working solution was added to each well. The microtiter plate was covered with a new adhesive strip, and incubated for 1 hour at 37°C.
6. The aspiration and washing was repeated three times as step 4.
7. 90µl of TMB Substrate was added to each well and incubated for 30 minutes at 37°C.
8. 50µl of Stop Solution was added to each well.
9. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

2.5.2.5 Calculation of the result:

The duplicate readings for each standard, control, and sample were averaged and subtracted from the average zero standard optical density. A standard curve was created by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the MDA concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. If samples were diluted, the concentration read from the standard curve was multiplied by the dilution factor. (figure 2-3)

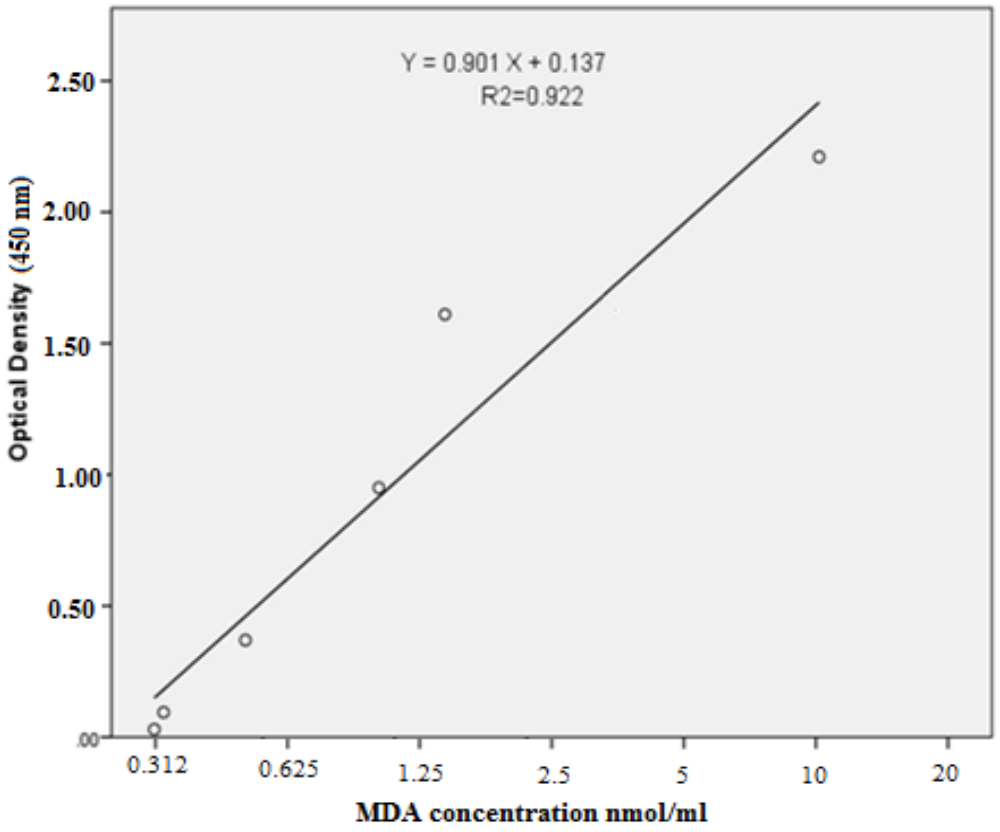


Figure (2.3) Standard curve of Malondialdehyde (MDA)

2.6 Immunophenotyping:

In this study, Immunophenotyping CD5, CD38 and Zap-70 expression were investigated by using fully equipped desktop four –color (Ibrahim *et al*., 2001) flow cytometry (FCM). CyFlow Cube features a modular optical concept. This allows using different lasers as light sources (www.partec.com/applications.html accessed at July,10,2013).

The CyFlow Cube allows easy optimization of the optics for any application by simple exchange of optical filters and mirrors.

Data acquisition, instrument control, and data analysis are controlled and performed by the CyView software.

-Antibody labeling (PARTEC. CyFlow®)

Antibody labelling was done by mixing 100 microlitre of whole blood with conjugated antibodies (10 microlitres) in a test tube, mixed thoroughly. Incubated for 15 minutes in the dark at room temperature.

-Leukocyte fixation

For leukocyte fixation, 100 microlitre of reagent A was added and mixed thoroughly and incubated for 10 min in the dark.

-Erythrocyte lysis

For erythrocyte lysis, 2.5ml of reagent B was added ,shaked gently and incubated for 20min in the dark. Then the sample was analysed on the flowcytometer. Some samples after fixation were stored at 2-8°C, protected from light, up to 24hr until analysis.

Flow cytometry data was analyzed in bivariateplots of two- or three-colour analyses with the application of electronic gates based on the scatter characteristic of cells. The measurement of the intensity of staining of cells by flow cytometry provide an absolute value for the light intensity. The measurement was performed by comparing cell fluorescence with an external standard by using different commercially available beads in kits, which usually comprised two tubes. One tube contains four types of beads with four different levels of fluorescence uptake: one very dim, one very bright and two intermediate; the other tube contains blank (non fluorescent beads) (Matutes *et al*., 2011).

The instrument set up was so that the fluorescence signal of the tube with the blank (unlabelled) beads is located in the region between 0 and 10^1 and four other peaks of fluorescence are seen along the axis of the relevant fluorochrome. The fluorescence voltage is established, and these settings maintained throughout the rest of the analysis of the unknown samples (Matutes *et al*., 2011).

The samples for a particular monoclonal Ab, run with the fluorescence settings obtained from beads stained with the corresponding monoclonal antibody, so that one fluorescence standard curve obtained for each monoclonal antibody. The data obtained from the flow cytometer and the standard curve, was automatically produced as shown in the Figure (2.4).The identification of cells was performed using forward scatter (FSC) versus side scatter (SSC) parameters . Antigen expression was considered to be positive when the percentage of positive cells was equal or greater than 20% (Bain *et al.*, 2010).

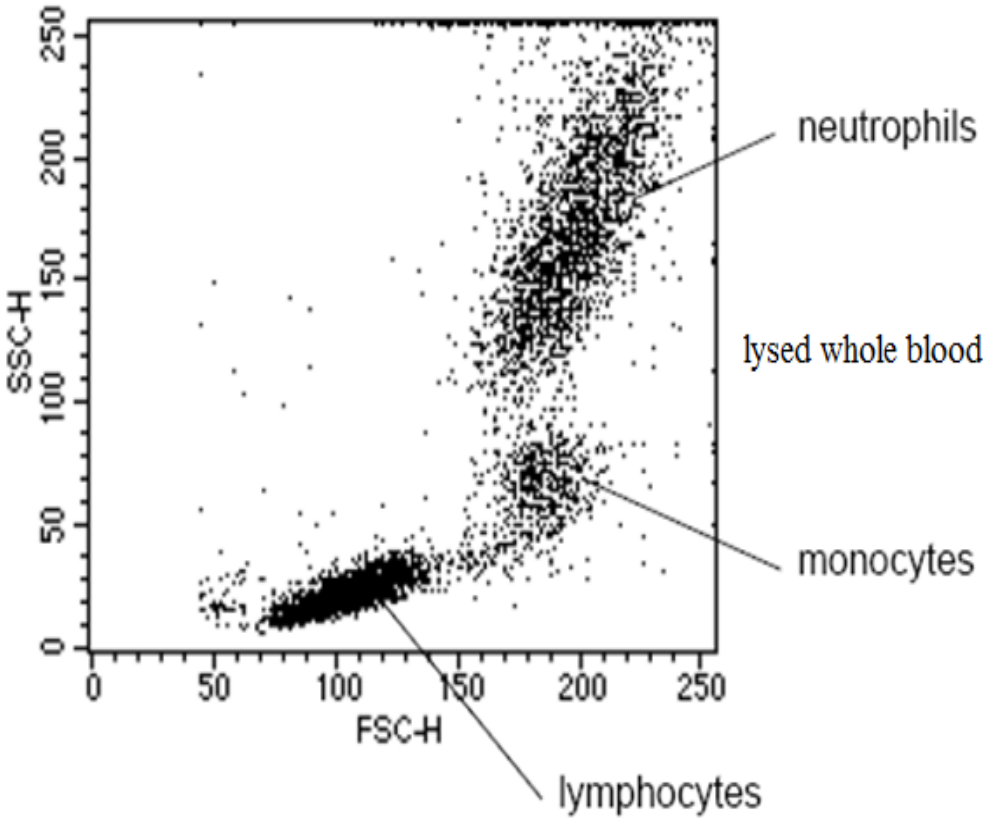


Figure (2.4) The forward and side scatter light signals

(leach *et al.*, 2013)

2.7 Interleukins

2.7.1 Interleukin 6 detection (R&D Systems USA)

2.7.1.1 Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.7.1.2 Kit components for detection Interleukin-6:

Kit component	Description
Interleukin 6 microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human IL-6.
IL-6 Standard	1.5 ng/vial of recombinant human IL-6 in a buffered protein base with preservatives; lyophilized.
IL-6 Conjugate	21 mL/vial of polyclonal antibody specific for human IL-6 conjugated to horseradish peroxidase with preservatives
Assay Diluent RD1W	11 mL/vial of a buffered protein base with preservatives.
Calibrator Diluent RD6F	21 mL/vial of animal serum with preservatives. For serum/plasma samples.
Calibrator Diluent RD5T	21 mL/vial of a buffered protein base

	with preservatives. For cell culture supernate samples.
Washer buffer concentrate	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. May turn yellow over time.
Color reagent A	12 mL/vial of stabilized hydrogen peroxide
Color reagent B	12 mL/vial of stabilized chromogen (tetramethylbenzidine).
Stop solution	6 mL/vial of 2 N sulfuric acid.

2.7.1.3 Preparation of Solutions

1. **Poly propylene tubes** were used in this procedure.

2. **Washing solution:** An aliquot (20 ml) of washing concentrate was diluted up to 500 ml with distilled water.

3. **Substrate solution:** Color Reagents A and B was mixed together in equal volumes within 15 minutes of use and protected from light. 200 µL of the resultant mixture is required per well.

4. **IL-6 standard**: The lyophilized IL-6 standard was reconstituted with 5.0 mL of Calibrator Diluent RD6F.This reconstitution produces a stock solution of 300 pg/mL. The standard was left at least 15 minutes with gentle agitation before making dilutions.

2.7.1.4 Assay Procedure (As manufacture's instruction)

Before carrying out the assay procedure, the kit was left at room temperature (22ºC) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

I- One hindered µl Assay Diluent RD1W was added to each well.

II- Serial concentrations (0, 3.13, 6.25, 12.5, 25 ,50 and 100) pg/mL of the standard were made using the assay diluent after reconstitution of standard vial with 1 ml of diluent water.

III- An aliquot (100 µl) of the standard or serum sample was added to the well. After that, the well was mixed and the plate was covered and incubated for 2 hours at room temperature.

IV- The wells were washed with four cycles of washing using the washing solution, with the aid of a microtiter plate washer.

V- 200 µL of IL-6 Conjugate was add to each well. Then, covered with a new adhesive strip and incubated for 2 hours at room temperature.

VI- The washing step was repeated (step iv).

VII- An aliquot (200 µl) of substrate reagent was added to each well, and the plate was covered and incubated in dark for 20 minutes at room temperature.

VIII- An aliquot (50 µl) of stop solution was added to each well, and the absorbance was read at a wave length of 570 nm using ELISA reader within 30 minutes after stopping reaction.

2.7.1.5 Calculations:

The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample Figure (2-5) using a curve fitting equation.

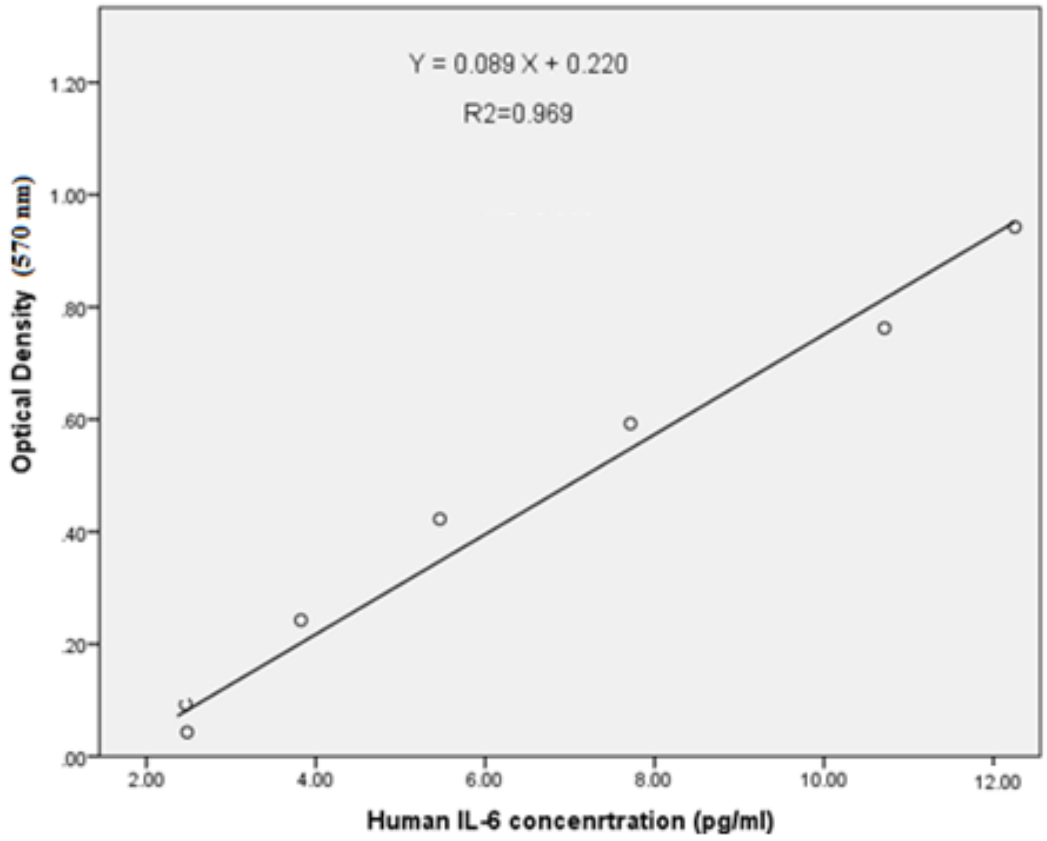


Figure (2-5) Standard curve of Interleukins-6.

2.7.2 Interleukin 10 detection (R&D Systems USA)

2.7.2.1 Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique (ELISA). A monoclonal antibody specific for Interleukin 10 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-10 present is bound by the immobilized antibody after washing away any unbound substances, an enzyme –linked monoclonal antibody specific for IL-10 is added the wells. Following a wash to remove any unbound antibody –enzyme reagent a substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.7.2.2 Kit components for detection of Interleukin-10:

Kit component	Description
Interleukin 10 microplate	96 well polystyrene microplate coated with a mouse monoclonal antibody against IL-10
IL-10 standard	5.0 ng /vial of recombinant human IL-10 in a buffered protein base with preservatives lyophilized
IL-10 conjugate	21 mL/vial of mouse monoclonal antibody against IL-10 conjugated to horseradish peroxidase with preservatives
Assay Diluent RD1W	11mL/vial of a buffered protein base with preservatives
Calibrator Diluent RD6P	21 mL/vial of animal serum with preservatives
Washer buffer concentrate	21 mL/vial of a 25-fold concentrated solution buffered surfactant with preservatives
Color reagent A	12 mL/vial of stabilized hydrogen peroxide
Color reagent B	12 mL/vial of stabilized chromogen (tertamethylbenzidine)
Stop solution	6 mL/vial of 2 N sulfuric acid

2.7.2.3 Preparation of Solutions

1. Poly propylene tubes were used in this procedure.

2. **Washing solution:** An aliquot (20 ml) of washing concentrate was diluted up to 500 ml with distilled water.

3. **Substrate solution:** Color reagent A and color reagent B were mixed together in equal volumes within 15 minutes of use. The mixture was protected from light.

4. **IL-10 standard**: The lyophilized IL-10 standard was reconstituted with 1.0 mL of distilled water .This reconstitution produces a stock solution of

5000 pg/mL. The standard was left at least 15 minutes with gentle agitation before making dilutions.

2.7.2.4 Assay Procedure

Before carrying out the assay procedure, the kit was left at room temperature (22ºC) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

I- Fifty (50 µl) Assay Diluent RD1W was added to each well.

II-Serial concentrations (0, 7.8, 15.6, 31,2, 62.5, 125, 250 and 500) pg/ml of the standard were made using the assay diluent after reconstitution of standard vial with 1 ml of diluent water.

III- An aliquot (200 µl) of the standard or serum sample was added to the well. After that, the well was mixed and the plate was covered and incubated for 120 minutes at room temperature.

IV- The wells were washed with four cycles of washing using the washing solution, with the aid of a microtiter plate washer.

V- An aliquot (200 µl) of conjugate solution was added to each well, and the plate was covered and incubated for 120 minutes at room temperature

VI- The washing step was repeated (step iv).

VII- An aliquot (200 µl) of substrate reagent was added to each well, and the plate was covered and incubated in dark for 30 minutes at room temperature.

VIII- An aliquot (50 µl) of stop solution was added to each well, and the absorbance was read at a wave length of 450-630 nm using ELISA reader within 30 minutes after stopping reaction.

2.7.2.5 Calculations:

The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample Figure (2-6), using a curve fitting equation.

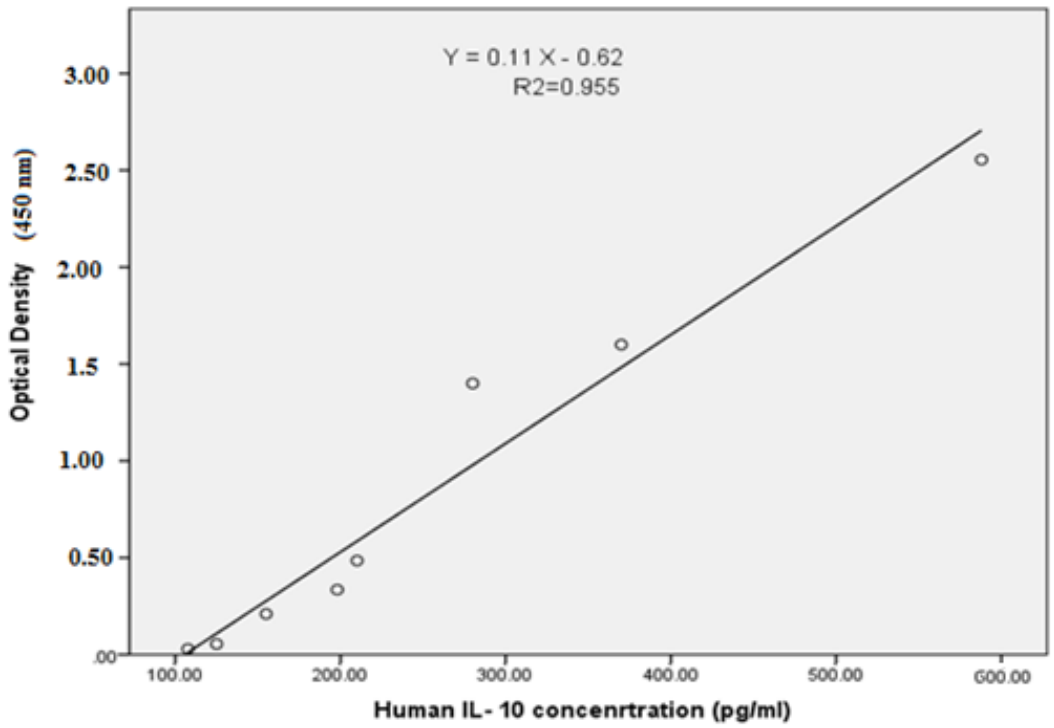


Figure.(2-6) Standard curve of IL-10.

2.8 Molecular study

2.8.1 Separation of mononuclear cells (MELTINYI BIOTEC)

Mononuclear cells (MNCs) were separated from blood sample by density gradient centrifugation according to the protocol described by Markowicz *et al*. (2006) , as shown in figure (2-7):

1. Fresh EDTA blood was diluted 1:1 in normal saline; mixed gently until it was homogenous.
2. The cell suspension was centrifuged in cooling centrifuge (at 4^{o} C ,2000 rpm for 10 min) on equal volume of Ficoll-Paque.

3. The upper layer was drawn off using a clean Pasteur pipette, the MNCs rich zone, then this zone was isolated and transferred into a new 10 ml tube and washed twice with normal saline through centrifugation at 2000 rpm for 8 min and 1000 rpm for 10 min, respectively. The final pellet was re-suspended with normal saline and was considered ready for isolation of DNA and determination of IGHV mutational status using RT-PCR and DNA sequencing.

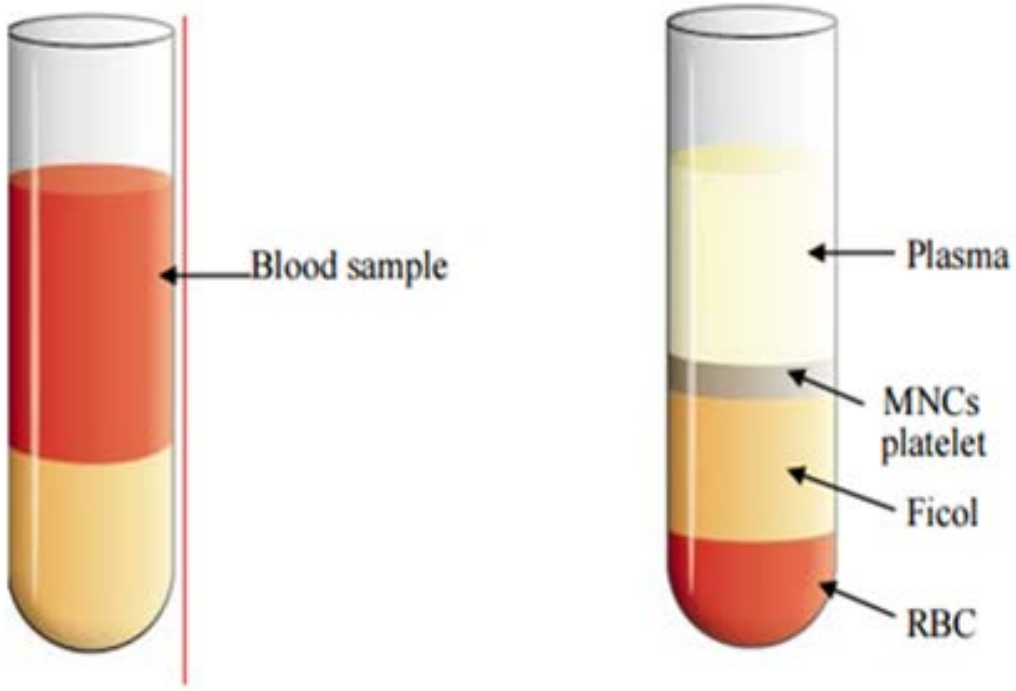


Figure (2-7) Isolation of mononuclear cells from blood sample (Meltinyi Biotec Kit - Ficoll-Paque).

2.8.2 Total RNA Extraction

Total RNA was isolated from lymphocytes(which were isolated in section 2.8.1) according to the protocol of SV total RNA isolation system protocol (Z3105) after modification which supplied by Promega USA.

The procedures followed were:

1. One hundred µl of lymphocytes were added to the RNA Lysis Buffer. 175µl of RNA Lysis Buffer were added to the cells. The cells were suspended and by pipetting.
2. About 350µl of RNA Dilution Buffer was added and mixed by inverting 3–4 times.
3. The tubes were placed in a water bath at 70°C for 3 minutes. Then centrifuged at 14000 rpm for 10 min. The cleared lysate was transfered to a sterile tube by pipetting.
4. Two hundred µl of 95% ethanol was added to the cleared lysate, and mixed by pipetting 3–4 times. The mixture was transfered to the Spin Column Assembly. Centrifuge at 12,000–14,000 × g for one minute.
5. Spin Basket was taken from the Spin Column Assembly, and discarded the liquid was discarded in the Collection Tube. The Spin Basket was then back into the Collection Tube. 600µl of RNA Wash Solution was added to the Spin Column Assembly, centrifuged at 12,000–14,000 × g for 1 minute.
6. The collection Tube was emptied as before and placed it in a rack. For each isolation to be performed, DNase incubation was prepared by mix combining 40µl Yellow Core Buffer, 5µl 0.09M $MnCl_2$ and 5µl of DNase I enzyme per sample in a sterile tube (in this order). Only the amount of DNase incubation mix required was prepared and pipetted carefully, and mixed by gentle pipetting; without vortex. The DNase I on ice was kept while it is thawed. 50µl of this freshly prepared DNase incubation mix was applied directly to the membrane inside the Spin Basket, and incubated for 15 minutes at 20–25°C.
7. After incubation, 200µl of DNase Stop Solution was added to the Spin Basket, and centrifuged at 12,000–14,000 × g for 1 minute.

8. Six hundred µl RNA Wash Solution (with ethanol added) was added and centrifuged at 12,000–14,000 ×g for 1 minute. 250µl RNA Wash Solution was added and centrifuged at high speed for 2 minutes.

9. For each sample, one capped 1.5ml Elution Tube were removed. The Spin Basket from the Collection Tube was transferred to the Elution Tube, and 100µl Nuclease-Free Water was added to the membrane. The Spin Basket Assemblies were placed in the centrifuge with the lids of the Elution Tubes facing out then centrifuged at 12,000–14,000 × g for 1 minute. The Spin Basket was removed and discarded. The Elution Tube containing the purified RNA was capped and stored at –70°C.

2.8.3 Determination of RNA Purity and concentration

This method is used to estimate the purity of the RNA by using a NanoDrope Spectrometer.

2.8.4 Partial IgVH Region Amplification and Sequencing

The partial IgVH Region was amplified using of specific primer pair using in RT-PCR reactions which provide a simple and rapid method for preparation of a specific region of cDNA for sequencing.

This part of research was done in ASCO Learning Center/Al-Harthia/Baghdad.

Primers

Lyophilized primers were dissolved in a nuclease free water to give a primary concentration of (100 µM) (as stock solution). For working solution, 10µl of stock was diluted with 90µl of nuclease free water to get (10 µM) as a final concentration. The sequence of these primer was explained in Table: (2-3).

Table (2-3): Primer Sequence used for GSTM1 deletion detections.

Primer	Sequence	size
VH FR2	5' TGGRTCCGMCAGSCYYCNGG '4	200
JH JHE	5' ACCTGAGGAGGACGGTGACC '4	

RT PCR (Marasca *et al*., 2005)

One Step Reverse Transcription PCR and specific primers were used as the following calculation and programming: Table(2-4) show Mixture A component.

Table(2-4): Mixture A component

Component	Volume (µl)
qPCR Mix	12.5
RT mix	0.5
VH FR2	1
JH JHE	1
Nuclease free water	8
DNA sample	2
Final volume	25

Table (2-5): PCR program for mixture A.

No	Steps	Temperature C°	Time	No. Of cycles
1	DNA synthesis	37	15min	
2	Initial Denaturation	95	5min	1 cycle
3	Denaturation	94	30sec	35cycles
	Annealing	60	30sec	
	Extension 1	72	30sec	
4	Final Extension	72	7min	1 cycle
5	Holding	4	-	1 cycle

2.8.5 Agarose Gel Electrophoresis (Sambrook *et al*., 1989)

After DNA extraction and PCR amplification, Agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA or amplification. PCR was completely dependable on the extracted DNA criteria.

Solutions (Ready to used)

-1 X TBE buffer.

-Loading dye.

-DNA ladder marker.

-Ethidium bromide (10mg / ml)

A-Preparation of agarose

1. one hundred ml of 1X TBEwas taken in a beaker.
2. One gm (for 1%) agarose was added to the buffer.
3. The solution was heated to boiling (using water bath) until all the gel particles were dissolved.
4. One µl of Ethidium Bromide (10mg/ml) was added to the agarose.
5. The agarose was stirred in order to get mixed and to avoid bubbles.
6. The solution was allowed to cool down at 50-60C°.

B- Casting of the horizontal agarose gel

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to gel at room temperature for 30 minutes. The comb was carefully removed and the gel was placed in the gel tray. The tray was filled with 1X TBE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

C- DNA loading

Two µl of loading buffer was applied to each 5µl DNA sample, and samples were added carefully to the individual wells. PCR products were loaded directly. Electrical power was turned on at 7v/cm for 1-2 h. DNA moved from Cathode to plus Anode poles. The Ethidium bromide stained bands in gel were visualized using Gel imaging system.

2.8.6 DNA Sequencing

PCR products were performed and sent for sequencing by Macrogen Company/Korea. Data were analysed using immunoglobulins and T cell receptor sequences (IgBLAST).

2.9 Statistical Analysis

The results were analyzed using the statistical analyzing system (SAS 2012).

Each value represents mean ± standard deviation (SD). Statistical analyses were performed using student´s t-test (for two group analysis). Multiple mean comparisons were made with the one–way analysis of variance (ANOVA) using a significant level of $P < 0.05$. Specific group differences were determined using Tukey´s test.

Chapter Three

Results and Discussions

3. Results and Discussion

3.1 Age, Gender and Binet stage distribution in CLL patients

3.1.1 Age, Gender and Binet stage:

The highest age incidence was between age groups (50-59) yrs. and (60-69) yrs., as approximately (34.5%) of the incidence of the disease occurred at each of those age groups Table (3-1).

Table (3-1): Age distribution of Iraqi CLL patients of the present study:

CLL Patients	Age group (yrs)					Total Number and %
	(40-49)	(50-59)	(60-69)	(70-79)	(80-89)	
Newly diagnosed	4	7	5	1	1	18 (32.7%)
Old treated	6	12	14	5	-	37 (67.3%)
Total	10	19	19	6	1	55 (100%)

The total CLL patients enrolled in the present study are composed of 40 males (72.27%) and 15 females (27.27%) with higher incidence in male than in female (ratio 2.6:1), as presented in Figure (3-1). Lamanna *et al*. (2011) reported that the incidence ratio of male to female (2.1:1) with the highest incidence occurs also frequently in middle age. Furthermore, Graig *et al*., (2010), and Freireich, (2010) reported that the incidence of CLL increases with age, and the median age at presentation is (65-70)yrs. They also reported that the male: female ratio is 2:1. Other study reported that CLL is a male-predominant disease, although the reason for this difference is not known (Jemal *et al*., 2010).

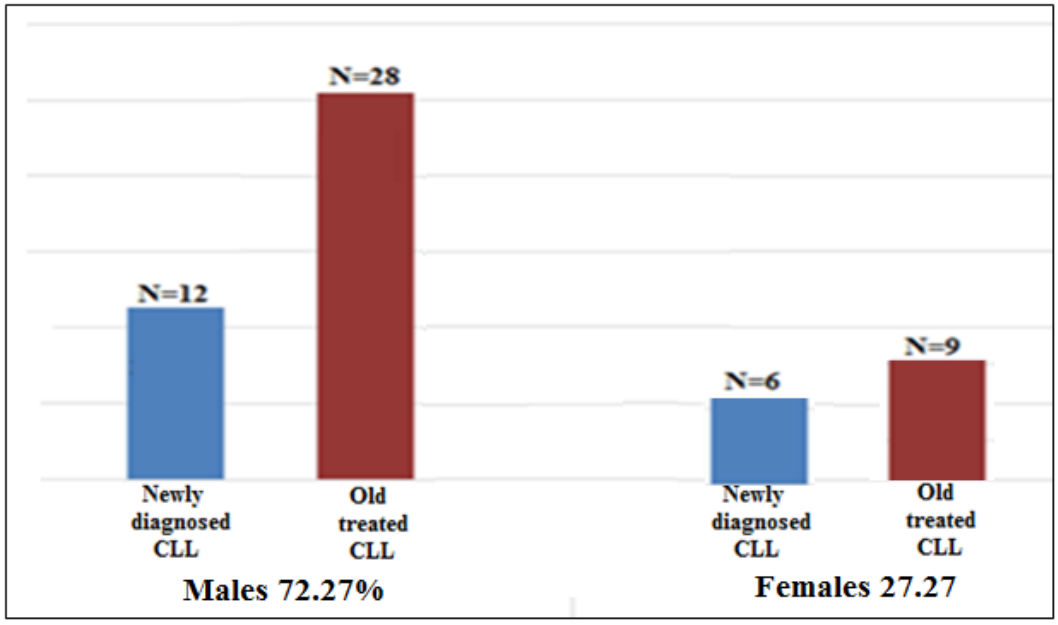


Figure (3-1) Gender distribution of Iraqi CLL patients of the current study.

The distribution of the patients according to Binet staging is presented in table (3.2) which showed that the highest percent of the patients in this study (60%) fell within Binet stage B of the CLL patients which is considered the intermediate or moderate risk stage in CLL patients, while the lowest percent of the patients (40%) fell within Binet stage C of the CLL patients which is considered the most advanced stage in CLL patients (Rawstron *et al*., 2002).

As table (3.2) showed, the predominance of stage B of CLL is also applied to both newly diagnosed as well as old treated patients.

Table (3.2) Distribution of Iraqi CLL patients according to Binet staging

CLL Patients	Binet staging		Total number and %
	B number and %	C number and %	
Newly diagnosed	10 (56%)	8 (44%)	18 (100%)
Old treated	23 (62%)	14 (38%)	37 (100%)
Total	33 (60%)	22 (40%)	55 (100%)

Clinical staging systems developed by Rai *et al* (1975) and Binet *et al*(1981) were able to predict long-term survival of patients with CLL, but have failed to predict specific disease course in patients with early-stage CLL. Advances in the identification and understanding of genomic and molecular markers are helping better predict disease progression and survival (Damle *et al*., 1999; Dohner *et al*., 2000).

3.2 General features:

3.2.1 General features in newly diagnosed CLL patients:

3.2.1.1 Hematological profile:

Complete peripheral blood picture examination in the untreated newly diagnosed CLL patients revealed leukocytosis (85.5±65.3x 10^9cells/L), manifested by absolute Lymphocytosis (48.99±26x 10^9cells/L). Six patients (6/18) showed WBC values range between (85-232 x10^9cells/L) and lymphocyte count range between (30-97x 10^9cells/L).

Other findings include mild anemia manifested by lowered RBC, Hb and PCV values, lower platelet count and increased ESR level as compared to controls. Seven patients (7/18) showed thrombocytopenia (range between 51-130x10^9cells/L).

The combination therapy administered to those patients was followed up for a median of 4.5 months (range 1-8 months). It managed to normalize

WBC and Lymphocytes counts, as well as anemia but failed to correct the lowered platelet counts as well as the increased ESR values (Table 3-3).

Table (3-3) Hematological profile in control and newly diagnosed (untreated and treated) Iraqi CLL patients: A follow up study.

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Treated (4.5months) (n=14)
WBCs (10^9/L)	6.7±1.39 [a]	85.6±65.38 [b]	17.61±31.36 [a]
Lymphocytes (10^9/L)	2.3±0.64 [a]	48.9±25.99 [b]	14.1±26.96 [a]
RBCs (10^{12}/L)	4.5±0.56 [a]	3.6±0.80 [b]	4.22±0.78 [ab]
Hb (g/dl)	12.9±1.40 [a]	11.5±1.68 [b]	12.0±1.21 [ab]
PCV (%)	40±5.50 [a]	36±4.39 [b]	37±4.86 [ab]
PLT (10^9/L)	320±69 [a]	168±71.31 [b]	142±88 [b]
ESR(mm/hr.)	15±9.31 [a]	42±15.74 [b]	31±8.13 [b]

Values are expressed as mean ± standard deviation (SD). Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

It is evident that there is a wide range of variability in each value (for e.g. red cells and platelets count) of the hematological parameter in the newly diagnosed CLL patients. This may be attributed to the fact that some of the patients were with stage B (which is associated with no anemia or thrombocytopenia) and other patients with stage C (which is characterized by anemia and thrombocytopenia) (Graig *et al*., 2010; Freireich 2010). Therefore, the overall picture reflected an interplay between values attributed to each stage.

3.2.1.2 Biochemical Profile:

The only significant alterations observed in the untreated CLL patients were those of slight increased total bilirubin, albumin and uric acid levels compared to control subjects.

Follow-up analysis of the data obtained from those patients in response to (RFC) chemoimmun therapy for a period range of 1-8 months and a median of 4.5 months revealed mild, yet significant, elevation in serum levels of FBG, Urea, ASTand ALT compared to controls. Furthermore, (RFC) therapy failed to correct the increased albumin and uric acid levels observed in the untreated CLL patients (Table 3-4. A).

Table (3-4. A) Biochemical profiles in control and newly diagnosed (untreated and treated) Iraqi CLL patients: A follow up study.

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Treated (4.5months) (n=14)
Fasting blood glucose (mg/dl)	87±19.41 [a]	100±44.92 [ab]	111±38.6 [b]
HbA1c	4.96±0.72 [a]	5.74±2.12 [a]	5.75±1.21 [a]
Cholesterol (mg/dl)	190±24.86 [a]	220±36.08 [a]	188±54.0 [a]
Triglycerides (mg/dl)	194±29.12 [a]	186±22.41 [a]	183±21 [a]
Urea (mg/dl)	32.8±6.80 [a]	38.4±7.74 [ab]	43.2±8.0 [b]
Creatinine (mg/dl)	0.94±0.17 [a]	1.00±0.51 [a]	1.71±1.55 [a]
AST(iu)/l	6.15±2,16 [a]	8.15±2.24 [ab]	10.35±3.8 [b]
ALT (iu)/l	6.89±1.76 [a]	6.47±1.77 [a]	21.14±24.13 [b]
Total bilirubin (mg/dl)	0.92±0.18 [a]	1.08±0.30 [b]	0.99±0.19 [ab]
Albumin (gm/l)	42.2±4.98 [a]	52.1±14.56 [b]	51.0±12 [b]
Uric acid (mg/dl)	5.2±0.99 [a]	7.9±0.70 [b]	7.0±0.89 [b]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

With the exception of the alterations in FBG level, the biochemical changes observed in both newly diagnosed and treated CLL patients were within the reference values established by the manufactures of the kits utilized in the present analyses. For example, the normal reference values for total bilirubin, albumin, and uric acid were (0.2-1.0) mg/dl, (35-50) gm/l, and (2.3-8.20) mg/dl, respectively. (Table 3-4. A).

Similarly, the elevated level of AST (normal value 10-50 IU/l), ALT (reference values 8-60 IU/l), and urea (normal value 18-53 mg/dl) observed in the treated CLL patients, yet remained within the reference values published by the kits manufactures (Table 3-4. A).

Accordingly, the above-mentioned alterations may confer no clinically significant abnormalities in both renal and hepatic functions in both newly diagnosed and treated CLL Patients.

Further analyses of the harvested data from the untreated newly diagnosed CLL blood samples revealed that the (5) diabetic patients out of (18) patients (27.8%) with hyperglycemia and increased HbA1c levels (Table 3-4. B). This finding will be discussed within its context together with the comparable changes observed in carbohydrate metabolism in the old treated CLL patients in section 3.2.2.

Table (3-4. B) Fasting blood glucose and HbA1c in control, and untreated newly diagnosed Iraqi CLL patients

Parameter	Control (n=19)	Newly diagnosed CLL Patients (n=18)	
		Diabetic (n=5)	Non diabetic (n=13)
Fasting blood glucose (mg/dl)	87±19.41 [a]	154±55.00 [b]	87±19.9 [a]
HbA1c%	5.0±0.7 [a]	8.22±2.7 [b]	4.81±0.7 [a]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

However, when the present patients were analysed (on the basis of the presence or absence of diabetes) into 2 groups, diabetes did not seem to have confounding effects on other biochemical Parameter and, for that matter, on other studied Parameter (Appendix 2, 3, 4, and 5).

3.2.1.3 Antioxidant profile

The serum level of the antioxidant glutathione (GSH) was significantly decreased ($P<0.05$), and that of the lipid peroxidation byproduct, malondialdehyde (MDA) was significantly increased ($P<0.05$) in the untreated CLL patients compared with healthy controls (Table 3-5).

Table (3-5) Antioxidant profile in control and newly diagnosed (untreated and treated) Iraqi CLL patients: A follow up study.

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Treated (4.5months) (n=14)
Glutathione (GSH) (µg/ml)	26.45±3.78 [a]	6.45±2.85 [b]	7.52±1.78 [b]
Malondialdehyde MDA (nmol/ml)	2.09±0.94 [a]	4.09±1.08 [b]	3.55±0.80 [c]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

Glutathione (GSH) is an important non-enzymatic antioxidant preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Humphries *et al.*, 2013). GSH protect cells against toxic free radicals involved in the synthesis and repair of DNA, and serves as a reductant antioxidant in oxidation reduction resulting in the formation of oxidized GSH (Wu *et al*,

2004). Therefore, the observed decreased GSH levels in CLL subjects may reflect depletion of non-enzymatic antioxidant reserve and the presence of endogenous oxidative stress. Evidence in available suggesting the presence of increased oxidative stress in CLL (Zhou *et al*., 2003; Collado *et al*., 2012; Gangemsi *et al*., 2012; Gaman *et al*., 2014).

The decreased serum levels of reduced GSH in the present study agrees with that of Bakan *et al*., (2003) who reported a lower serum GSH levels in patients with CLL compared with controls. This finding may be attributed to the over-production of ROS in CLL cells. The high ROS content in CLL cells could be pro-tumorigenic, but also increase the susceptibility of cancer cells to cell death above certain threshold levels (Nogueira and Hay, 2013). ROS could facilitate cancer development by direct oxidative damage to DNA, induction of lipid peroxidation or oxidative damage leading to structural alteration in the DNA (Ralph *et al*., 2012). Furthermore, ROS- induced oxidative stress could also attenuate immune responses by causing dysfunctions and even apoptosis of NK-and t-cells, suggesting its role in immune-escape (Poschke *et al*., 2011).

Follow-up analysis of untreated CLL patients for a median of 4.5 months of RFC therapy failed to normalize both GSH and MDA levels (Table 3-5), suggesting the persistence of endogenous oxidative stress in the treated CLL patients on one hand, and the lack of antioxidant activity of RFC (Rituximab, Fludarabine, and cyclophosphamide) combination therapy on the other hand. To the best of our knowledge, there is no evidence in the available literature suggesting that these anti-leukemic drugs possess any antioxidant activities. Furthermore, evidence is available demonstrating that rituximab (De Rosa *et al*., 2015), fludarabine + cyclophosphamide (Goncalves *et al*., 2009) are capable of inducing ROS-induced oxidative stress in biological systems.

3.2.1.4 Immunological profile

3.2.1.4.1 CD Marker

Lymphocytes of the newly diagnosed CLL patients expressed significantly increased expression of CD5, CD38 and Zap-70 compared to healthy controls (Table 3-6; Figure 3.2).

Table (3-6) Immunological profile in control and newly diagnosed (untreated and treated) Iraqi CLL patients: A follow up study.

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Treated (4.5months) (n=14)
CD-5 %	7.33±7.31 [a]	69.73±14.83 [b]	51.14±15.04 [b]
CD-38 %	5.46±6.80 [a]	39.92±15.53 [b]	26.34±13.44 [b]
ZAP-70 %	7.09±8.32 [a]	46.89±19.15 [b]	9.84±12.78 [a]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

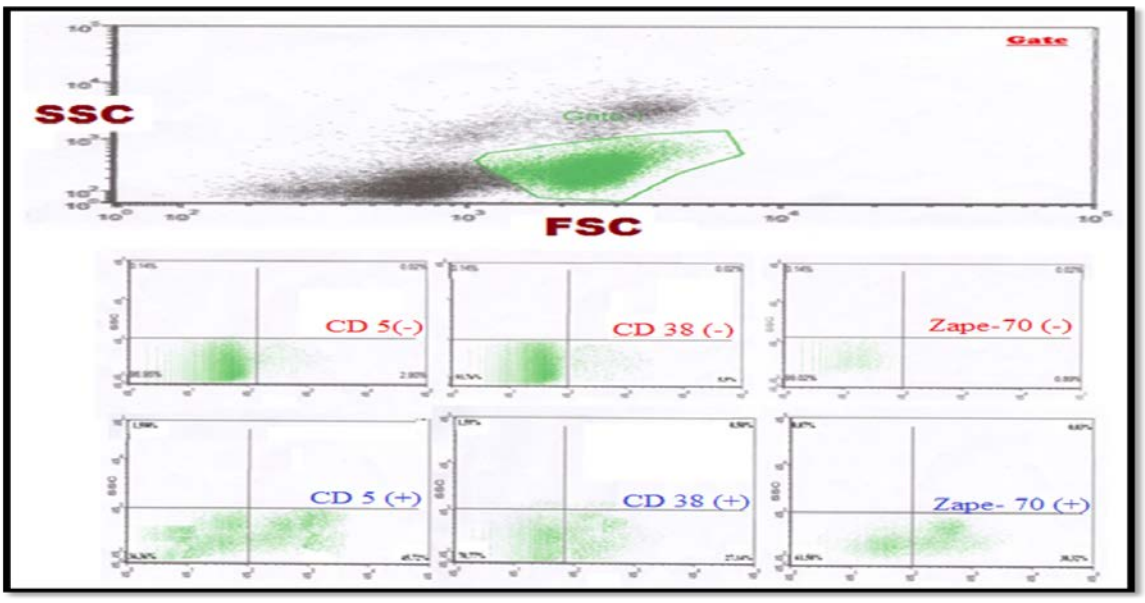


Figure (3-2) The forward and side scatter light signals of the (CD5, CD38 and ZAP-70) in Iraqi patients with CLL and healthy control.

The range of the percent of expression of CD5 in the untreated CLL patients was between 34% (which is the lowest positive value) and 87% with a mean 69.73±14.83, for all the morphologically diagnosed CLL cases which showed positive expression for CD5.

The percent of expression of CD38 range between 28%-75% with a mean of 39.92±15.53 (two of CLL cases showed negative expression of 20% compared with healthy control), as illustrated in table (3-6).

Regarding the intensity of marker expression, the majority of patients showed moderate intensity of expression for CD38. This agreed with results of Gong *et al*. (2001) who demonstrated that the majority of CLL cases showed either moderate or bright expression of CD38, and moderate CD5 expression (Cabezudo, et. al., 1999), regardless of the stage of the disease , suggesting a lack of relation between the stage of the disease and the intensity of expression. On the other hand, Geisler *et al* (1991) demonstrated that low intensity CD38 was associated with shorter survival in CLL.

Regarding the relation of the marker expression to the clinical and hematological Parameter, (Jurisic, et. al., 2008) showed greater CD5 antigen expression ,with higher peripheral blood lymphocyte count. The finding that CD38 expression associated with higher CD5 expression emphasizes that $CD38^{+}$ CLL cases are authentic B-CLL showing a greater disease activity as CD38 had been reported to play a complex role in lymphocyte proliferation (Rossi *et al*., 2010).

In regards to the ZAP-70 range between (24- 90%) with a mean of 46.89±19.15, two patients out of 18 were negative (<24%) for ZAP-70 as compared with control.

ZAP-70 positivity (>30%) for previously untreated and asymptomatic patients is associated with a more unfavorable median survival (6–10 years), while a negative ZAP-70 is associated with a median survival of more than 15 years. (Crespo, et. al., 2003; Kröber, et. al., 2006).

3.2.1.4.2 Interleukins

By the same token, serum level of IL-6 and IL-10 were significantly elevated in newly diagnosed CLL patients compared to controls (Table 3-7).

Table (3-7) Proinflammatory Cytokines in control and newly diagnosed (untreated and treated) Iraqi CLL patients: A follow up study.

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Treated (4.5months) (n=14)
IL-6 pg/ml	0.50±0.31 [a]	2.53±1.98 [b]	1.36±0.98 [b]
IL-10 pg/ml	48.31±22.89 [a]	614±301 [b]	101.4±49.3 [c]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

The levels of IL-6 in the serum of untreated patients with CLL were increased compared with healthy control, ranged between (1.3-6.9) pg/ml. Various studies reported that IL-6 levels in the serum increase in CLL above the range in normal control subjects, however, leukemic cells do not appear to be the source of IL-6 (Luis *et al*., 2015). Several investigators have suggested that IL-6 in CLL inhibits proliferation but

prolongs survival (by suppressing apoptosis) of CLL cells (Aderka *et al*., 1993; Reittie *et al*., 1996).

The range of the IL-10 expression of the newly diagnosed CLL patients was between (212 pg/ml) value and (987 pg/ml) which is the highest positive value with a mean 614±301, with all the morphologically diagnosed CLL cases showed positive expression for IL-10. Various studies reported that IL-10 levels were increased in CLL (Denizot *et al*., 1999;Vassilakopoulos *et al*., 2001) and were associated with poor prognosis (Vassilakopoulos *et al*. 2001). Several studies also revealed a significant elevation in IL-6 and IL-10 plasma levels of CLL patients in comparison to healthy control group, (Fayad *et al*.,2001; Guney *et al*.,2009).

Such increase in the level of IL-10 might be due to its production by malignant cells or by different cells of the immune system, including T and B lymphocytes, macrophages and monocytes (Hadi *et al*., 2013). RFC chemoimmun therapy, for a median of 4.5 months) for the newly diagnosed CLL patients normalized only ZAP-70 levels, but not other Parameter Tables (3-6) and (3-7).

Tsimberidou *et al*., (2009) demonstrated that the current treatments for CLL (RFC) induce apoptosis in CLL cells but lead to significant immune-suppression and patients often develop drug resistance. Other study on CLL demonstrated that the higher levels of ZAP-70 expression are associated with shorter time to treatment and poorer survival, However, the routine clinical measurement of ZAP-70 expression is difficult and non-standardized (Crespo *et al*., 2003).

3.2.2 General features in the treated CLL patients:

In addition to the (18) untreated CLL patients, data were also obtained from another 37 CLL patients who were previously diagnosed and received therapy for a period ranged from 4 months - 8.5 years and median of (21.5 months). Unlike the previous group of CLL patients, those treated patients lack pretreatment data concerning the various Parameter investigated in the present study.

In general, detailed analyses of the various studied Parameter revealed that a median of (21.5 months) of therapy produced comparable results to those obtained following a median of 4.5 months of (RFC) chemoimmun therapy to the newly diagnosed CLL patient's data (Tables 3-8, 3-9, 3-10,3-11 , and 3-12).

Table (3-8) Hematological profile in control, newly diagnosed and old treated Iraqi CLL patients (for a median 21.5 months).

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Old treated (21.5 months) (n=37)
WBCs (10^9/L)	6±1.39 [a]	85±65.38 [b]	11±19.61 [a]
Lymphocytes (10^9/L)	2.3±0.64 [a]	48.9±25.99 [b]	5.1±6.77 [a]
RBCs (10^{12}/L)	4.5±0.56 [a]	3.6±0.80 [b]	3.9±0.76 [b]
Hb (g/dl)	12.9±1.40 [a]	11.5±1.68 [b]	11.9±1.75 [b]
PCV (%)	40±5.50 [a]	36±4.39 [b]	35±6.23 [b]
PLT (10^9/L)	320±69 [a]	168±71.31 [b]	192±1.73 [b]
ESR(mm/hr.)	15±9.31 [a]	42±15.74 [b]	28±7.41 [c]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

Table (3-9. A) Biochemical profiles in control, newly diagnosed and old treated Iraqi CLL patients (for a median 21.5 months).

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Old treated (21.5 months) (n=37)
Fasting blood glucose (mg/dl)	87±19.41 [a]	100±44.92 [ab]	122±51.22 [b]
HbA1c %	4.9±0.72 [a]	5.6±2.06 [ab]	6.8±3.47 [b]
Cholesterol (mg/dl)	190±24.86 [a]	220±36.08 [a]	216±42.0 [a]
Triglycerides (mg/dl)	194±29.12	186±22.41 [a]	196±38.0 [a]
Urea (mg/dl)	32.8±6.80 [a]	38.4±7.74 [b]	38.2±6.47 [b]
Creatinine (mg/dl)	0.94±0.17 [a]	1.00±0.51 [a]	1.10±0.28 [a]
AST(iu)/l	6.15±2,16 [a]	8.15±2.24 [ab]	9.68±7.53 [b]
ALT (iu)/l	6.89±1.76 [a]	6.47±1.77 [a]	8.86±3.44 [b]
Total bilirubin (mg/dl)	0.92±0.17 [a]	1.08±0.30 [b]	1.12±0.16 [b]
Albumin (gm/l)	42.2±4.98 [a]	52.1±14.56 [b]	50.8±11.62 [b]
Uric acid (mg/dl)	5.20±0.99 [a]	7.98±0.70 [b]	7.08±0.80 [b]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

Analysis of the biochemical data of the old treated CLL patients revealed that (11) diabetic out of 37(29.1%) of an old patients showed hyperglycemia and elevated HbA1c levels, thus supporting the presence of diabetes type II (based on patients history), among these patients. (Table 3-9. B).

Table (3-9. B) Fasting blood glucose and HbA1c in control, Diabetic, and euglycemic old treated Iraqi CLL patients

Parameter	Control (n=19)	Old treated CLL patients Median (21.5) months	
		Diabetic (n=11)	Nondiabetic (n=26)
Fasting blood glucose (mg/dl)	87±19.41 [a]	185±48.29 [b]	87±24.39 [a]
HbA1c%	5.0±0.7 [a]	10.7±3.4 [b]	4.9±0.74 [a]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

Collectively, (16) out of (55) CLL patients involved in this study (both untreated newly diagnosed and old treated) showed type II diabetes, with increased of (29%). This percentage is slightly higher than that reported by Sawitsky *et al*., (1977) who observed a prevalence of (22%) of CLL patients with diabetes mellitus prior to therapy.

The reason for this high prevalence in Iraqi patients is unknown, however, it may be explained by the finding of Castillo *et al*., (2012) who suggested that the increased risk of leukemia seemed to be distinct depending on the geographic area of study. They reported that the risk of leukemia is elevated in Asian and American not in European studies.

On the other hand, Habib and Rojna (2013) reported that, overall, (8-18)% of cancer patients have diabetes.

Several lines of evidence are available in the literature documenting the comorbidity of diabetes (especially type II diabetes) with different types of cancers (Giovannucci *et al*., 2010; Habib and Rojna, 2013; Xu *et al*., 2014; Gristina *et al*., 2015), as well as comorbidity of diabetes with different types of leukemia, including chronic myeloid leukemia (CML)

(Breccia *et al*., 2005; Ono *et al*., 2012; Racil *et al*., 2013), acute lymphoblastic leukemia (ALL) (Yetgin *et al*., 1998; Suzuki *et al*., 2011) as well as chronic lymphocytic leukemia (CLL) (Fortuny *et al*., 2005; Khan *et al*., 2008; Castillo *et al*., 2012).

The above-mentioned epidemiologic studies, based on cohort studies and meta analyses, were consistent in their suggestion that diabetes, especially type II DM, is associated with increased risk of cancer. However, the exact pathophysiological mechanism(s) responsible for such association remains unsettled.

Several possibilities have been postulated to explain the likely mechanism(s) involved in promoting incidence of cancer in diabetes. First, the possibility of a direct causal relationship between type II diabetes and CLL has been excluded by Khan *et al*., (2008) who hypothesized that diabetes may not play a causal role in the etiology of CLL. Instead, they suggested that underlying pathophysiological mechanism(s) of both disorders may include shared genetic or environmental susceptibility factors.

Second, Giovannucci *et al*., (2010) suggested that cancer incidence is associated with diabetes as well as diabetes risk factors or diabetes treatment.

Third, the possibility of an indirect biological link between diabetes and cancer received much attention. Among these biological links were hyperglycemia, HbA1c, insulin resistance and hyperinsulinemia, increased production of inflammatory cytokines, and over-production of insulin-like growth factor in type II diabetes that could favor malignant transformation of cells and also progression of tumors (Reviewed by Giovannucci *et al*., 2010; Xu *et al*., 2014; Gristina *et al*., 2015).

Some of these biological links, such as HbA1c, was suggested as a potential risk for different types of cancers independent of diabetes (de Beer and Liebenberg, 2014; Muc-Wierzgon *et al*., 2014).

Needless to say, the limitation of the present study is the small number of patients involved, a fact that limits my ability to draw firm conclusions about either the possible mechanisms responsible for the comorbidity of diabetes and CLL or the clinical significance of the elevated HbA1c in the present study. Therefore, further studies are warranted using large number of patients to investigate whether or not diabetes, or for that matter, HbA1c could be utilized as a potential risk factor for the incidence of CLL in Iraqi patients.

Table (3-10) Antioxidant profile in control, newly diagnosed and old treated Iraqi CLL patients (for a median 21.5 months).

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Old treated (21.5 months) (n=37)
Glutathione (GSH) (µg/ml)	26.45±3.78 [a]	6.45±2.85 [b]	7.52±1.78 [b]
Malondialdehyde MDA (nmol/ml)	2.09±0.94 [a]	4.09±1.08 [b]	3.24±1.78 [c]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

Table (3-11) Immunological profile in control, newly diagnosed and old treated Iraqi CLL patients (for a median 21.5 months).

Parameter	Control (n=19)	CLL Patients	
		Newly diagnosed (n=18)	Old treated (21.5 months) (n=37)
CD-5 %	7.33±7.31 [a]	69.73±14.83 [b]	69.76±15.6 [b]
CD-38 %	5.46±6.80 [a]	39.92±15.53 [b]	33.07±18.97 [b]
ZAP-70 %	7.09±8.32 [a]	46.89±19.15 [b]	7.99±10.50 [a]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

Table (3-12) Proinflammatory Cytokines profile in control, newly diagnosed and old treated Iraqi CLL patients (for a median 21.5 months).

Parameter	Control (n=19)	CLL Patients	
		Newly Diagnosed (n=18)	Old treated (21.5 months) (n=37)
IL-6 pg/ml	0.50±0.31 [a]	2.53±1.98 [b]	2.07±1.67 [b]
IL-10 pg/ml	48.31±23.89 [a]	614±301 [b]	85.5±63.4 [c]

Values are expressed as mean ± standard deviation (SD) . Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

3.3 Molecular Profile in CLL patients:

This part of research has been conducted on the partial IgVH region with amplified 240 bp using specific primers VH FR2 and JH JHE, after

converted RNA (total) being extracted from MNCs to cDNA in one step RT-PCR, for preparation the specific region for sequencing. Twenty subjects were randomly selected and involved in this experiment, eight newly diagnosed CLL patients (4 males and 4 females) and eight old treated CLL patients who are currently receiving treatment, (4 males and 4 females), and four healthy control (2 males and 2 females), which they are one from male and female. (Figure 3-3).

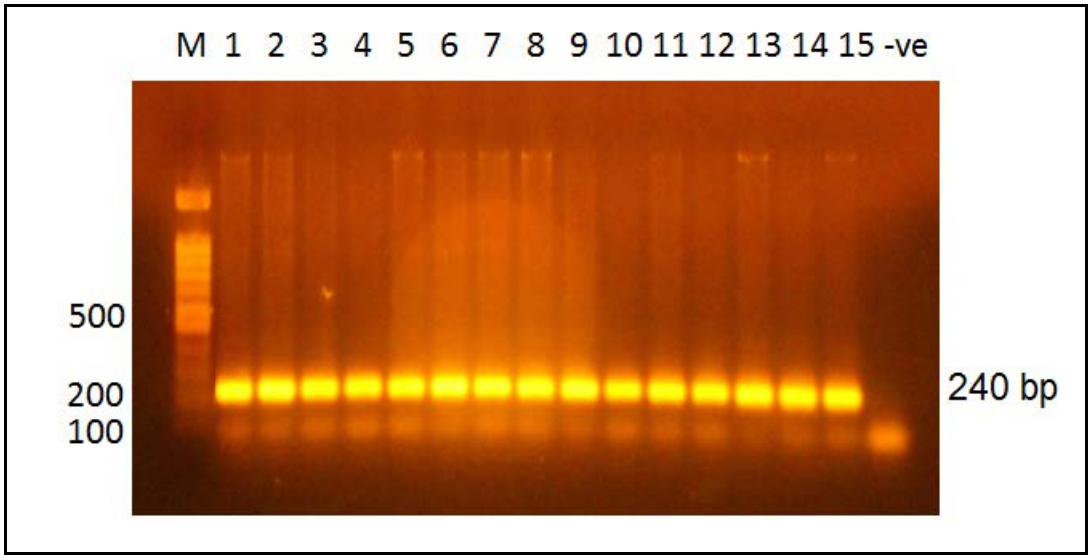


Figure (3-3): Gel electrophoresis for PCR amplification of IGHV gene showing band with molecular size (240bp). Electrophoresis was performed on 1.5 % agarose gel and run with a 100-volt current for 2 hrs. Lane M:100 bp ladder. From 1 to 15 lane were patients and control samples. -ve: negative control.

In normal B-cells, any immune complex or antigen induced germinal center GC enlargement formation of new ones as small B-cells surrounding GC, appearance of memory B-cells and development of Ig production cells of high affinity. During this particularly frenetic bout of cell division, somatic hypermutation of B-cell Ig genes occurs encoded

by unmutated germ-line genes represent a form of evolutionary memory so-called 'natural antibody'. Randomly, some mutated daughter cells will have higher affinity for antigen, some the same or lower and others perhaps none at all. Similarly, mutations in the framework regions may be 'silent' or, if they disrupt the folding of the protein, give rise to nonfunctional molecules (Küppers, 2004; Dooley *et al*., 2006).

Clones use either mutated or unmutated IGHV genes (Fais *et al*., 1998), with distinct clinical courses, the IGHV repertoire in CLL is not random and differs between U-CLL and M-CLL as well as between CLL cells and normal B cells (kipps *et al*., 2000).

Mutations of VH genes of Ig are identified by comparing DNA sequences of genes in B cells with corresponding genes in the germline. DNA sequences of B cells differing by □2% from its germline counterpart are considered mutated (Schroeder *et al*., 1994).

Extensive molecular investigations of the B cell receptor (BCR) indicated that 60%–65% of CLLs carry immunoglobulin heavy-chain variable (IGHV) genes with evidence of somatic hypermutation in their variable regions, a process that occurs in the germinal center and may modify BCR affinity for antigens (Klein, et. al., 2008).

IGHV unmutated CLL cells are typically BCR signaling competent whereas IGHV mutated CLL cells respond weakly or not at all to BCR crosslinking induced by anti IgM antibodies **(**Tsimberidou *et al*., 2009**)**.

The mutational status of IgVH is also associated with specific genomic aberrations; for instance, del (17p13) or del (11q23) occurs more frequently in patients with unmutated IgVH CLL (unmut-IgVH), whereas mutations such as 13q- as sole aberrancy are generally associated with mutated IgVH (mut-IgVH) CLL (Krober *et al*., 2006).

3.3.1 DNA Sequencing

Evaluation of biological prognosticators was centralized in few reference laboratories, IGHV mutational status was performed as previously reported (Bomben *et al*., 2009).

The immunoglobulins expressed by CLL B cells are highly restricted, suggesting they are selected for binding either self or foreign antigen of the immunoglobulin heavy-chain variable (IGHV) genes expressed in CLL (Mauerer *et al*., 2005).

In general, CLL patients that express unmutated Ig heavy chain (IGHV) genes have a worse prognosis than those who express mutated IGHV genes **(**Tsimberidou *et al*., 2009**)**

For DNA sequencing, PCR products were performed and send to Macrogen Company/Korea. Data were analysed using immunoglobulins and T cell receptor sequences database provided from NCBI using IgBLAST tool.

Results of sequencing reveled that healthy controls showed unmutated IGVH, figure (3-4).

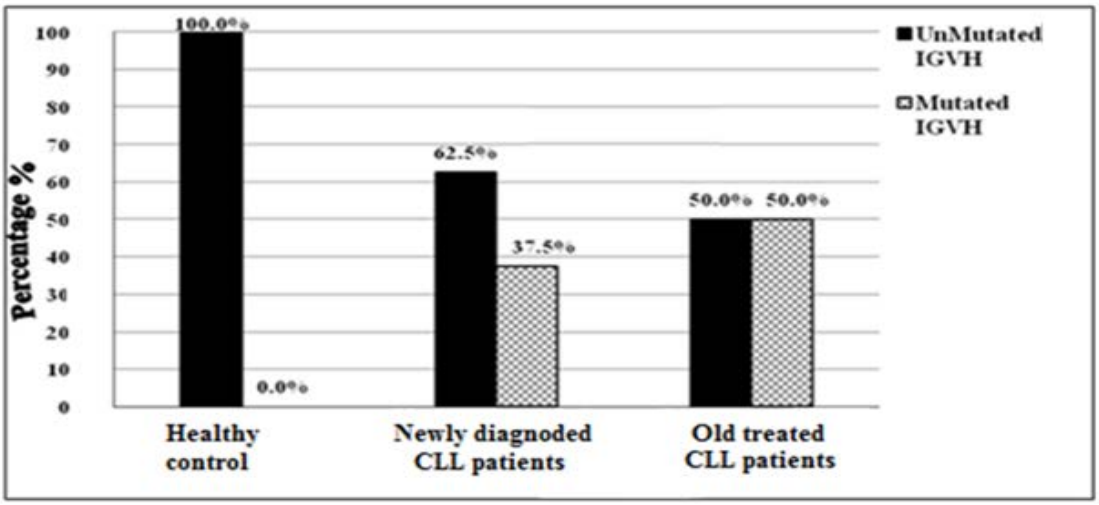
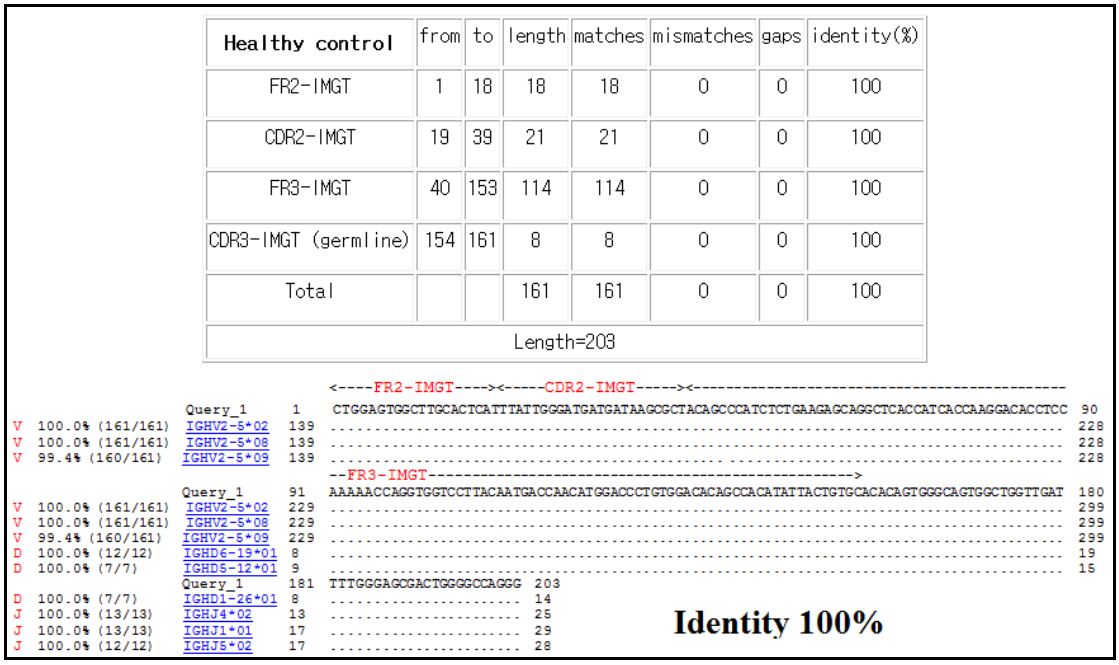


Figure (3-4) Mutated and unmutated IGVH in Iraqi CLL patients with healthy controls.

Furthermore, figure (3-5) showed that healthy controls have sequencing of IGVH completely identical to immunoglobulin and T-cell receptor (IgBLAST).



Healthy control	from	to	length	matches	mismatches	gaps	identity(%)
FR2-IMGT	1	18	18	18	0	0	100
CDR2-IMGT	19	39	21	21	0	0	100
FR3-IMGT	40	153	114	114	0	0	100
CDR3-IMGT (germline)	154	161	8	8	0	0	100
Total			161	161	0	0	100
Length=203							

Figure (3-5): Sequence analysis summary of IGVH in Iraqi healthy control (IgBLAST)

Results of IGVH sequencing the newly diagnosed untreated CLL patients revealed that, 3 (37.5%) out of (8) have mutated IGHV genes, at the position (FR3-IMGT) with identity (99.1%) (Figure 3-4, 3-6). As following:

A-Mismatch one base from fragment length (207)bp at a total identity (99.4%).

B-**Mismatch** one base from fragment length (203)bp at a total identity (99.4%).

C- **Mismatch** one base from fragment length (191)bp at a total identity (99.3%).

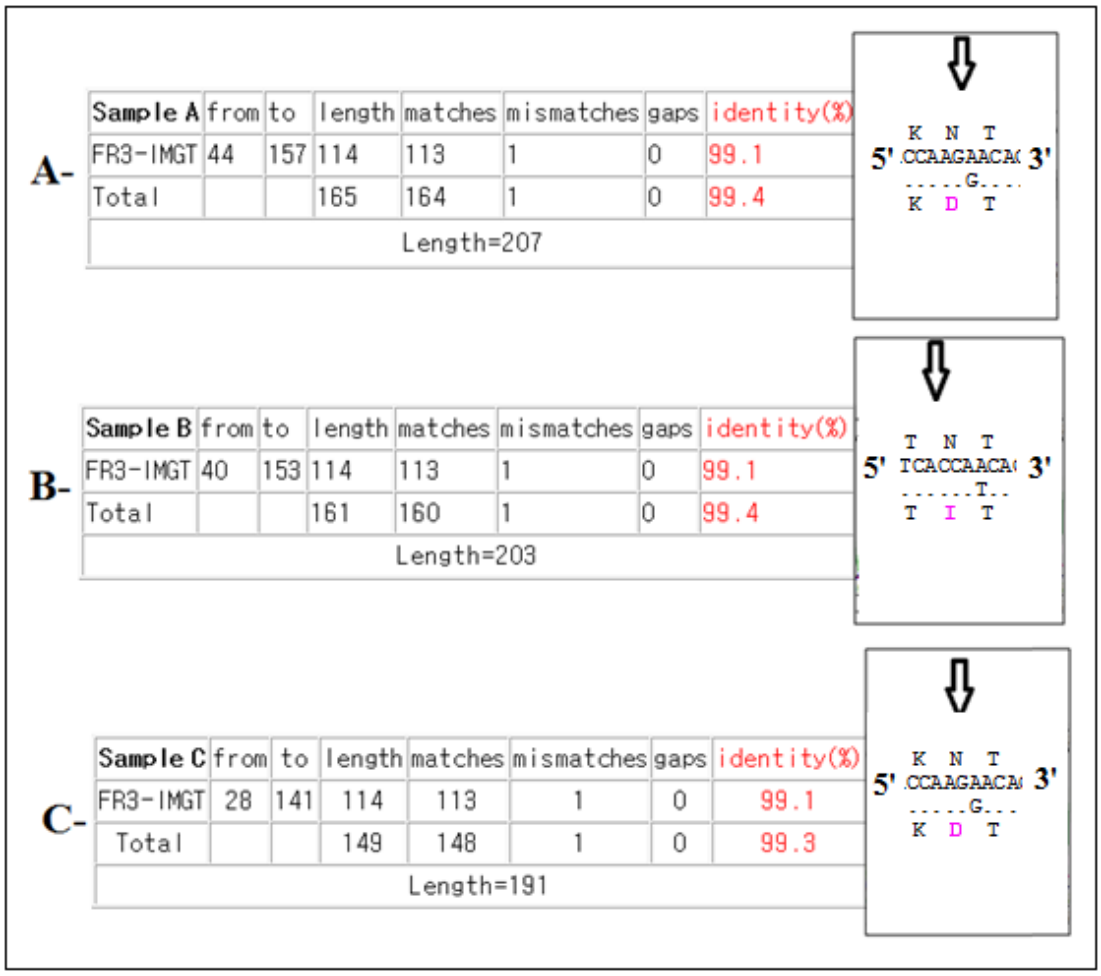


Figure (3-6): Sequence analysis summary of IGVH in the newly diagnosed untreated Iraqi CLL patients (IgBLAST)

DNA sequence in the treated CLL patients revealed that, four of them (50%) have mutated IGHV genes, at the position (FR3-IMGT) with identity (99.1%)(Figure 3-7). As following:

A-Mismatch one base from fragment length (185)bp at a total identity (99.4%).
B-Mismatch one base from fragment length (198)bp at a total identity (99.4%).
C- Mismatch one base from fragment length (180)bp at a total identity (99.4%).
D- Mismatch one base from fragment length (186)bp at a total identity (99.3%).

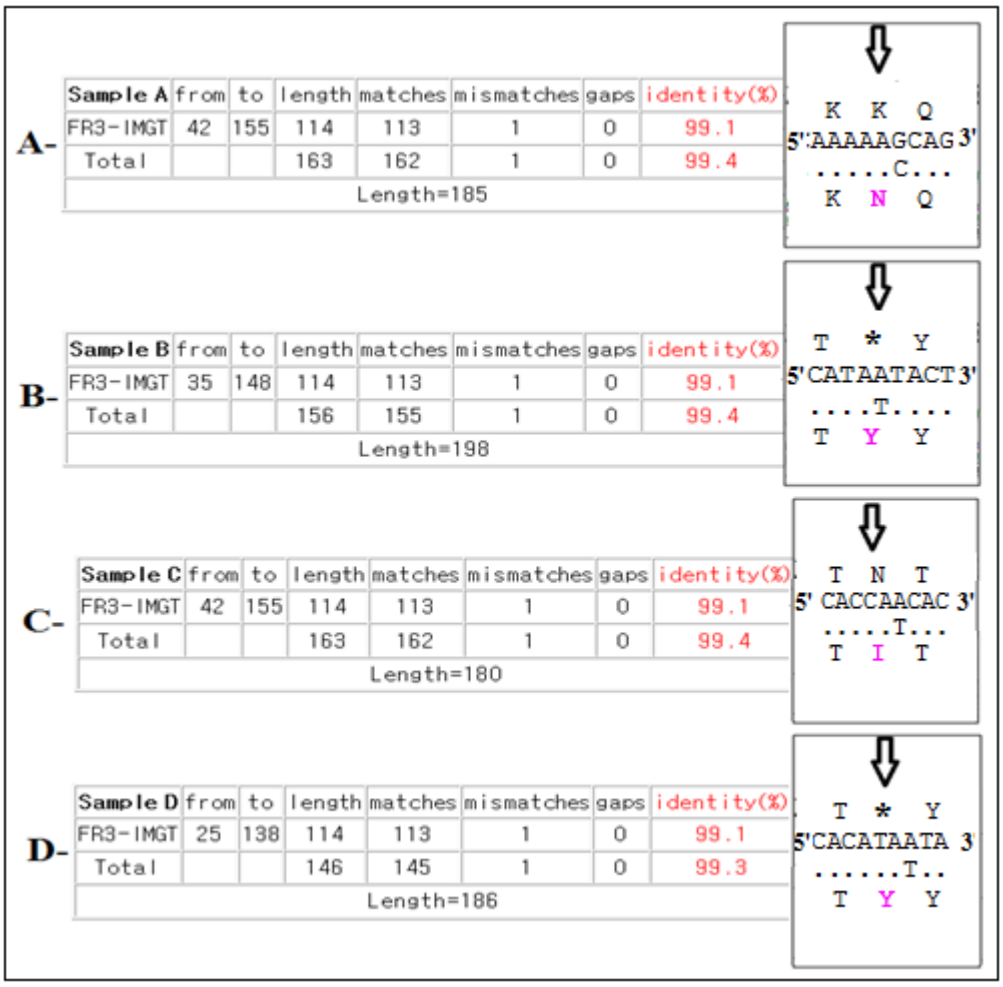


Figure (3-7): Sequence analysis summary of IGVH in the treated Iraqi CLL patients (IgBLAST)

However, CD38 expression and IgVH mutation status are less highly associated (Thunberg *et al*., 2001). CD38 expression may change over the disease course (Hamblin *et al*., 2000). Currently, the best surrogate marker of IgVH mutation status is ZAP70. The expression of ZAP70 in CLL cells has been shown to correlate well with unmutated IgVH genes and poor clinical outcome (Crespo *et al*., 2003). ZAP70 expression with IgVH mutation status ranges from 75 to 90% and its expression seems to be stable over time (Orchard *et al*., 2004).

3.3.2 Molecular analysis of genetic using RT-PCR

Because these studies have focused on different prognostic factors, it is not surprising that they have identified independent sets of potential biomarkers. Novel biologic and genetic markers are providing tools to aid in the better prediction of responses, disease progression, and survival in patients with CLL. Unmut-IgVH is clearly an independent predictor of outcome in CLL patients. The correlation between unmut-IgVH and an increased risk for clonal evolution suggests that the presence of unmut-VH is required to induce karyotypic instability (Tobin *et al*., 2003).

Unmut-IgVH does not appear to independently predict for chemoresistance, but patients with unmut-IgVH CLL are reported to have shorter remissions, the prognosis of unmut-IgVH patients remains poor, even after high dose chemotherapy (Lin *et al*., 2007).

3.4 Evaluation of laboratory Data:

Analysis of the present data using the coefficient of variation gave values that covered the range of (1%-100%), although the majority of them were within the acceptable values (between 5% and 15) (Snedecor and Cochran, 1976), indicating the presence of large variability among the laboratory data. Variability in the present data could be caused by two types of factors. The first, are common factors that are likely to apply to both control and leukemic groups, such as experimental error as well as the biological variation in the values of any of the measured Parameter. The second factors could be attributed to abnormalities restricted to the leukemic state and it is severity (notably the stage of the disease).

It was clear that leukemic data were not uniform or classical but, in fact, varied considerably and covered a range from mild abnormalities to

severe ones. The presence of such variations in the leukemic patients in the present study is likely an important factor responsible for the finding that the coefficient of variation was higher in the leukemic patients than in control subjects.

Therefore, although I acknowledge the presence of considerable variability in the presence data, this has not prevented the detection of many highly significant differences between control and leukemic groups.

Conclusions and Recommendations

Conclusions

The present study reached the following conclusions:

1. Hyperglycemia and elevated HbA1c level (suggestive of diabetes) were present in approximately 29% of the CLL patients, regardless of the disease stage and the duration of combination therapy (Rituximab, Fludarabine and cyclophosphamide) (RFC), thereby confirming the comorbidity of diabetes with CLL state. This finding may suggest the presence of a possible biologic link, such as HbA1c, between the two conditions through, as yet, unknown mechanism(s).
2. The reduction in the serum levels of glutathione, along with the elevation in serum MDA levels of CLL patients suggest the presence of an increased endogenous oxidative stress in CLL patients.
3. The inability of (RFC) chemotherapy to normalize both serum GSH and MDA levels in treated CLL patients is suggestive of a lack of an antioxidant activity of these drugs, and the persistence of endogenous oxidative stress in treated CLL patients.
4. The serum levels of IL-6 and IL-10 are higher in CLL patients compared to control. Furthermore, the expressions of CD5, CD38, and ZAP-70 were also higher in CLL patients than in controls. RFC therapy managed only to normalize ZAP-70 expression.
5. The fact that FRC therapy managed to normalize both absolute lymphocyte count and ZAP-70 expression in CLL patients suggests a possible correlation between those two parameters.
6. Sequence analysis of IGVH gene revealed that 3 out of the 8 tested newly Diagnosed CLL patients and 4 out 8 tested treated CLL patients had mutated IGVH, while none of the 4 tested healthy controls showed mutated IGVH.

Recommendations

Using a large number of patients, further studies are needed to investigate :

- The role of environmental and genetic factors in the severity of CLL in Iraqi Patients.
- The profile of immunophenotypes and cytokines in CLL patients to include more immune profiles that may have a potential in CLL treatment.
- The detailed antioxidant profile in CLL patients, and to assess whether or not the inclusion of some natural antioxidant nutrients, e.g. vitamin E or C, with the standard RFC chemotherapy can augment the efficacy of RFC therapy in CLL patients.
- The detailed biochemical profile concerning carbohydrate metabolism in diabetic CLL patients in order to support or nullify the hypothesis that diabetes may increase the incidence of CLL. Also, to investigate whether or not elevated HbA1c levels could be utilized as a potential risk factor for the incidence of CLL in Iraqi patients.
- The degree of correlation between the various studied parameters in the present study.
- The present studied parameters in each stage of the CLL, i.e., stages A, B and C, in an attempt to determine if there is a stage dependent alterations in these parameters.

Reference And Appendix

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Appendix 1: Data sheet

Subject oN :( )

-Subject name: **Date: / /20**

1- Gender: ( ). 2- Age: ( ) years.

3- Old CLL: ( ): Type of treatment: chemotherapy ( ).

NEW CLL case ( ): No treatment before.

Healthy Control: ( ).

4- Staging at diagnosis (Binet system):

Binet stage A ( ), Binet stage B ( ), Binet stage C( ).

5-Dress: phone no.

6- Duration of disease: ( ) years: ( ) Months.

7-Any other diseases:

-Diabetes ( ): Duration of diabetes:

- High Blood Pressure ( ):

Obesity : ( ), Smoking ( ).

9- History of present illness yes: ( ): NO ( ).

10- Investigations:

NO.	Tests	Results
1		
2		
4		
5		
6		

Clinical examination: ( ). Presence of hepatomegaly: ( ).

Presence of LN enlargement: ( ). Presence of splenomegaly: ( ).

- **Hematological profile in control, old treated diabetic and non diabetic CLL patients.**

Parameters	Control(n=19)	Old treated CLL Patients	
		Diabetic(n=11)	Non Diabetic (n=26)
WBCs (10^9/L)	6.7±1.39 [a]	6.7±4.97	14.1±23.68 [a]
Lymphocytes (10^9/L)	2.3±0.64 [a]	4.3±5.17 [a]	5.6±7.54 [a]
RBCs (10^{12}/L)	4.5±0.56 [a]	4.0±0.63 [ab]	3.9±0.83 [b]
Hb (g/dl)	12.97±1.40 [a]	11.51±1.19 [b]	12.09±1.67 [ab]
PCV (%)	40±5.5 [a]	35±6.32 [a]	36±6.27 [a]
PLT (10^9/L)	320±69 [a]	159±81 [b]	199±67 [b]
ESR(mm/hr.)	15±9.31 [a]	30±3.75 [b]	27±8.67 [b]

Values are expressed as mean ± standard deviation (SD). Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

- **Biochemical profiles in control, old treated diabetic and non diabetic CLL patients.**

Parameters	Control (n=19)	Old treated CLL Patients	
		Diabetic (n=11)	Non Diabetic (n=26)
Cholesterol (mg/dl)	190±24.86 [a]	220±37.69 [a]	212±44.50 [a]
Triglycerides (mg/dl)	194±29.12	188±21.91 [a]	200±44.65 [a]
Blood urea (mg/dl)	32±6.80 [a]	40±5.15 [b]	36±5.54 [ab]
S. Creatinine (mg/dl)	0.94±0.17 [a]	1.19±0.31 [b]	1.06±0.26 [ab]
AST(iu)	6.15±2.16 [a]	9.30±4.32	9.88±8.83 [a]
ALT (iu)	6.89±1.76 [a]	10.0±4.88 [b]	8.28±2.30 [ab]
Total serum bilirubin (mg/dl)	0.92±3.05 [a]	1.11±2.95 [b]	1.12±2.79 [b]
Albumin (gm/l)	42±4.98 [a]	48±10.65 [b]	52±12.23 [b]
Serum uric acid (mg/dl)	5.2±0.99 [a]	7.0±077 [b]	7.1±0.83 [b]

Values are expressed as mean ± standard deviation (SD). Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

- **Antioxidant profile in control, old treated diabetic and non diabetic CLL patients.**

Parameters	Control (n=19)	Old treated CLL Patients	
		Diabetic (n=13)	Non Diabetic (n=24)
Glutathione (GSH) (µg/ml)	26.45±3.78 [a]	7.45±2.85 [b]	8.52±1.78 [b]
Malondialdehyde MDA (nmol/ml)	2.09±0.94 [a]	3.09±1.08 [b]	4.24±1.78 [c]

Values are expressed as mean ± standard deviation (SD). Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

- **Immunological profile in control, old treated diabetic and non diabetic CLL patients.**

Parameters	Control (n=19)	Treated CLL Patients	
		Diabetic (n=13)	Non Diabetic (n=24)
CD-5 %	7.33±7.31 [a]	71.0±18.49 [b]	69.12±14.30 [b]
CD-38 %	5.46±6.80 [a]	32.69±17.70 [b]	33.28±19.95 [b]
ZAP-70 %	7.09±8.32 [a]	9.96±10.0 [a]	6.84±10.68 [a]

Values are expressed as mean ± standard deviation (SD). Value with (a) is normal.
Values with different letters within each parameter are significantly different (P<0.05)

- **Proinflammatory Cytokines profile in control, old treated diabetic and non diabetic CLL patients.**

Parameters	Control (n=19)	Treated CLL Patients	
		Diabetic (n=13)	Non Diabetic (n=24)
IL-6 pg/ml	0.50±0.31 [a]	1.60±1.24 [ab]	2.32±1.83 [b]
IL-10 pg/ml	48.31±23.89 [a]	100.46±85.78 [b]	77.2±48.44 [ab]

Values are expressed as mean ± standard deviation (SD). Value with (a) is normal.
Values with different letters within each parameter are significantly different ($P<0.05$)

الملخص

سرطان الدم اللمفاوي المزمن هو خلايا دم لمفاويه خبيثة [مونوكلونل] تتميز بتراكمها بمراحلها الصغيرة والناضجة في الدم ونخاع العظام والأنسجة الأخرى، و هذه الخلايا اللمفيه من فئة B تتطور وتتكاثر بطريقة خاطئة مما يؤدي إلى اضطراب في المناعة، وعادة ما تتميز هذه الخلايا عن بعضها بالمستضدات اللمفاوية المرتبطة، الفروع (CD5,CD38 and ZAP-70) كطريقة مستخدمة للكشف عنها هي جهاز التدفق الخلوي.

الهدف من هذه الدراسة هو التحري عن التغيرات في التحاليل المناعية والكيمياء الحيوية ، والجزيئية في مرضى سرطان الدم اللمفاوي المزمن لمرضى العراقيين بهذا المرض.

وأجريت هذه الدراسة على (55) مريض بسرطان الدم اللمفاوي المزمن في المركز الوطني لبحوث وعلاج إمراض الدم في الجامعة ألمستنصريه.

حيث أن المرضى قد شملوا (18) مريض مصاب حديثا، من ضمنهم (12) رجل و (6) نساء، بمتوسط عمر (12±55) سنه ومعدل عمر (40-80) سنه. وحسب نظام التدرج ل- Binet في مرضى سرطان الدم اللمفاوي المزمن ، كان (10) من المرضى هم ضمن المستوى (B) و(8) من المرضى هم ضمن المستوى (C). و شملت هذه الدراسة أيضا (37) مريض بسرطان الدم اللمفاوي المزمن معالج، من ضمنهم (28) رجل و (9) نساء بمتوسط عمر (57 ± 9) سنه ومعدل عمر (45-71) سنه. وأيضا (23) مريض من هذه ألمجموعه كانوا ضمن المستوى (B) و (14) من المرضى ضمن المستوى (C) حسب نظام التدرج إلى Binet. وكذلك تضمنت الدراسة أيضا 19 شخص طبيعي أصحاء من ضمنهم تسعه رجال و عشرة نساء بمتوسط عمر (61 ± 17) سنه ومعدل عمر (30-90) سنه.

وقد أظهرت فحوصات صورة الدم الكاملة لمرضى سرطان الدم اللمفاوي المزمن للمصاب حديثا زيادة واضحة في عدد كريات الدم البيضاء مع فقر دم خفيف الشدة و قله في عدد الصفائح الدموية مع زيادة ملحوظة بمعدل ترسيب الكريات الدم الحمراء بالمقارنة مع القراءات للأشخاص الأصحاء. و بعد متابعه نتائج التحاليل إلى المرضى المصابين حديثا بعد أعطائهم جرع العلاج من ((Rituximab, Fludarabine, Cyclophosphamide (RFC) بفترة زمنية (1-8) شهر و بمعدل (4.5) شهر.

وقد لوحظ ان التغيرات الهامة في الاختبارات الكيمياحيوية في المصابين حديثا كانت زيادة في مستوى الألبومين و حامض اليوريك في بلازما الدم و و جد أيضا أن العلاج (RFC) لم يرجعهما إلى المستوى الطبيعي بالمقارنة مع الأصحاء. وأوضحت النتائج ايضا وجود انخفاض هام في مستويات الكلوتاثيون في البلازما للأشخاص المصابين حديثا بالمرض. علاوة على ذاك، وجد أيضا زيادة هامة من المستوى الأساسي للمالونداليديهايد MDA، حيث ان العلاج(RFC) لم يستطع من معادلتهما، مما يشير إلى وجود إلى زيادة في مستوى الأكسدة الداخلي في مرضى سرطان الدم اللمفاوي المزمن. وعلاوة على ذلك، فقد اظهر خمسة مرضى

من (18) المشخصين حديثا (27.8٪) بانهم مصابين بالسكري (النوع الثاني)، حيث تبين بان لديهم زيادة هامة في سكر الدم ومستوى HbA1c في الخلايا الحمراء.

وأظهرت الفروع اللمفاوية (CD5 و CD38 وZAP-70) زيادة هامة في الأشخاص المصابين حديثا بالمرض، ,ويماثلها بالزيادة (IL-6 و IL-10) في بلازما الدم للأشخاص المصابين حديثا. حيث إن العلاج (RFC) لم يستطع سوى ارجاع الزيادة في إنتاج ZAP-70 فقط الى المستوى الطبيعي.

وأما نتائج التحليل الجزيئي في RT-PCR أظهرت بان هنالك 3/ 8لديهم طفرة في IgVH من الأشخاص المصابين حديثا بالمرض، و 8/4 لديهم طفرة في IgVH من المعالجين، ولم تظهر أي طفرة ملحوظة في الأشخاص الطبيعيين.
وهنالك أيضا 37 مريض معالج قديما من مرضى سرطان الدم اللمفاوي المزمن لفترة تمتد من 4 أشهر إلى 8.5 سنة وبمعدل 21.5 شهر، اظهروا نتائج مشابه إلى نتائج المرضى المصابين حديثا والمتابعين بالفترة (4.5) شهر بواسطة العلاج (RFC).

وعلاوة على ذلك، فقد اظهر (11) مريضا من أصل 37 (29.7٪) من المعالجين بانهم مصابين بالسكري النوع الثاني حيث اظهروا ارتفاع السكر في الدم وفي مستويات HbA1c في الكريات الدم الحمراء بالمقارنة مع مستوى سكر الدم في اقرانهم غير المصابين بالسكري من المعالجين.

وبالاستنتاج، فان هذه الدراسة أظهرت وجود مختلف التغيرات في المقاييس المناعية والكيمياحيوية، والجزيئية في مرضى سرطان الدم اللمفاوي المزمن. والاستنتاج المثير للاهتمام كان وجود (16) من أصل (55) مريض بسرطان الدم اللمفاوي المزمن (أي 29٪) لديهم مرض السكري من النوع الثاني و المتميز بـارتفاع في الكلوكوز في الدم و HbA1c في خلايا الدم الحمراء. وتوصي هذه الدراسه بإجراء المزيد من الدراسات لتقييم أهميتها السريرية وعما إذا كان ممكنا أو لم يكن ممكنا اعتبار ارتفاع مستوى HbA1c كعامل خطورة لحدوث سرطان الدم اللمفاوي المزمن في المرضى العراقيين.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الإحيائية

التغيرات الكيموحيوية والمناعية والجزيئية لمرضى أبيضاض الدم اللمفاوي المزمن لعينة من المرضى العراقيين

أطروحة

مقدمة إلى كلية العلوم/ جامعة النهرين
كجزء من متطلبات نيل درجة دكتوراه فلسفة في علوم التقانة الإحيائية

من قبل

حيدر صادق عبد الحسن الظاهري
بكالوريوس في التقانة الإحيائية - جامعة النهرين- 2003
ماجستير في التقانة الإحيائية - جامعة النهرين- 2007

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