Hematological profiles and Human Leukocyte Antigen-class II Alleles in Systemic Lupus Erythrematosus among Sudanese Patients

الدراسات الدموية و الجينية لمرضى الذئبة الحمراء في السودان

By
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Dedication

To my parents

To my brothers

To my husband

To my kids (Ahmed & Juwan)
Acknowledgment

I would like to express my sincere gratitude and appreciation to:

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Abstract:

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production that can result in damage to multiple organ systems. The present study aimed to investigate Human Leukocyte Antigen class II in Systemic lupus erythromatous (SLE) Sudanese patients, the Anti nuclear auto antibodies profile in patient, the antiphospholipid autoantibodies as well as hematological abnormalities.

Eighty SLE patients and sixty matching ethnicity and sex healthy controls were enrolled in present study from Rheumatology Clinic at the National Ribat University Hospital, Medical Military Hospital and Private Clinic. Demographic data (name, sex, age and the tribe) were obtained from patients and Blood samples were collected in EDTA containers to be used for HLA typing by using polymerase chain reaction (PCR) technique, Anti nuclear auto antibodies profile by using Immunobblott technique, antiphospholipid antibodies by using ELISA technique and measurement of hemoglobin, total white blood cells and platelets by using Sysmex.

In present study there are 76 females and 4 males, female to male ratio was 19:1, Afro-asiant tribes were the most affected with disease than Nilo-Saharan tribes. The result of HLA class II, DR & DQ alleles were typed from DNA sample of all patients and controls, DRB1* 15 was carried by 23% of patients and 13% of control with p.value 0.01, DQB1* 05 was carried by 22% of patients and 13% of control with p.value 0.03, DRB1* 13 was carried by 1 2% of patients and 29 % of control with p.value 0.001, DQB1* 06 was carried by 28% of patients and 43% of control with p.value 0.005.

All patients were positive for ANA (100%), where as 73% were positive for anti smith antibody and 42% positive for dsDNA antibody.
The results of antiphospholipid antibodies were 38% and 22% positive of SLE patients for anticardiolipin antibody and Anti-β2-glycoproteinI antibody respectively.

Hematological abnormalities as following: anemic patients (<12g/dl) was 68%, leukopenic patients (<4.0×10⁹/l) was 40% and the thrombocytopenic patients (<100×10⁹/l) was 7%.

From these results we conclude that: DRB1*15 and DQB1*05 alleles may be considered as risk alleles with relative risk 2.1 and 1.7 respectively. While DRB1*13 and DQB1*06 may be considered as protective alleles with relative risk 0.43 and 0.64 respectively.

There are different levels of antiphospholipid antibodies in Sudanese patients, particularly, anticardiolipin antibodies were found more frequently than Anti-β2-glycoproteinI antibody antibodies.

According to present study Anti ANA, anti-Sm and anti dsDNA may be considered as important serological markers for the diagnosis of Sudanese patients with SLE. The hematological abnormalities were not differing from other different diseases.
الخلاصة:

هذه الدراسة تهدف لاختبار النسيج الجيني من النوع الثاني والاجسام المضادة النوويه ومستضدات الدهون الفسفوريه وكذلك التغيرات الدمويه في مرضى الذئبة الحمراء السودانيين. التحق ثمانين مصابا بالذئبة الحمراء وستين شخص معافي مطابقا للجنس والقبيله في هذه الدراسة. قد تم جمع العينات في حاويه بها مانع للإجلط (EDTA) وتستخدم هذه العينات في فحص النسيج الجيني بواسطة تقنية التفاعل التضاعفي التسلسلي وفحص الأجسام المضادة النوويه بواسطة تقنية اللطخته المناعيه وكذلك الكشف عن مستضدات الدهون الفسفوريه بواسطة تقنية الاليزا وفحص التغيرات الدموية باستخدام تقنيه اتوماتيكيه.

نتيجة النسيج الجيني النوع الثاني لكل عينات الحمض النووي في المرضي والاصحاء كانت كالتالي:

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<td>51*5</td>
<td>32%</td>
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نسبة الأجسام المضادة النوويه وجدت بنسبة 5.2% (S,M) وجد بنسبة 32% وكذلك مستضد الحمض النووي وجد بنسبة 32% , فيما يختص بالتغيرات الدمويه هناك مستويات معتدله من مستضدات (ACE) و (LA) , وكذلك نستنج ان الأجسام المضاده النوويه و مستضدات الجين المضاده الدمويه يعتبروا من الدلالات المصليه الهامه في تشخيص المرض السودانيين المصابين بالذئبه مع حساسيه وخصوصيه عالية , ولقد كان فقر الدم أكثر التغيرات الدمويه حدوثا في المرضي بلبه نقص في كريات الدم البيضاء.
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Abbreviations

SLE  systemic lupus erythromatous
HLA  human leukocyte antigen
MHC  major histocompatibility complex
APL  anti phospholipid antibody
ACL  anti cardiolipin antibody
$\beta_2$ GPL1  beta two glycoprotein one
LAC  lupus anticoagulant
CBC  complete blood count
DNA  Deoxyribonucleic acid
ANA  anti nuclear autoantibody
ELISA  enzyme-linked immunosorbent assay
IB  immunoblotting
SSP  sequence specific primering
SSOP  sequence specific oligonucleotide probing
AID  autoimmune disease
ESR  erythrocyte sedimentation rate
dsDNA  double strand deoxyribonucleic acid
RNP  ribonuclear protein
Sm  smith antigen
SSA  soluble substance A
SSB  soluble substance B
AMA  mitochondrial M2 antigens
PCNA  Proliferating cell nuclear antigen
SCL 70  systemic scerolosis 70
IFA  indirect immunoflourecent assay
DILE  drug induced lupus erythromatous
ARA  American Rheumatism Association
snRNA  small nuclear ribonucleic acid
Chapter one

1. Introduction

1.1 Systemic lupus Erytheromatous (SLE)
Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production that can result in damage to multiple organ systems. Although, the aetiology of SLE remains unknown, multiple genes as well as environmental factors are reported to account for the differing prevalence of SLE and its complications in various ethnic groups (D'Cruz et al. 2007; Morel 2007; Tsuchiya et al. 2007).

1.1.1 Human leukocyte Antigen (HLA) in SLE
Recent data suggest that the polymorphic genes of the major histocompatibility complex (MHC) may exert their influence in the outcome and expression of the disease, and several HLA-DR or DQ markers or residues, have been implicated in the appearance of specific autoantibodies (Azizah et al. 2001; Endreffy et al. 2003; Morimoto et al. 2000; Shankarkumar et al. 2003; Sirikong et al. 2002; Vargas-Alarcon et al. 2001).

1.1.2 Anti phospholipid antibodies in SLE
Approximately 30-40% of SLE patients have antiphospholipid antibodies (APL) (Svenungsson et al. 2010).
These antibodies directed towards phospholipids are associated with venous and arterial thrombosis and/or pregnancy loss. The most commonly detected antibodies are anti cardiolipin (aCL), but other such as lupus-like anti coagulant (LAC) and β-2-glycoprotein I (β₂ GPL) do occur (Love and Santoro 1990).
Further more the presence of these antibodies has been shown to correlated with presence of HLA-DRB1*04 in SLE (Kapitany et al. 2009).
1.1.3 Hematological abnormalities in SLE
Hematological changes, including anaemia, leucopenia and thrombocytopenia, occur in more than one half of the patients with this disease. Anaemia is the most common hematological abnormality seen in SLE. Its possible causes are anaemia of chronic disease (ACD), auto-immune haemolytic anaemia and hypoplastic anaemia. Leucopenia affects both granulocytic and lymphocytic lines and may be caused by autoantibodies. Patients with SLE also have increased risk of thrombosis (Robak et al. 1995).

1.2 Rationale
The etiology of SLE is thought to be multifactorial including environmental, hormonal and genetic factors. SLE has a worldwide distribution, with high predilection in young females showing heterogeneous clinical expression (Granados et al. 1996). HLA region genes have been implicated in susceptibility to the disease (Hartung et al. 1992). The precise genetic cause of the association has been difficult to define because of the high degree of polymorphism within the HLA genes.
Systemic lupus erytheromatous and its relation to human leukocyte antigen (HLA) has been exhaustly studied in Africa, Asia, Europe and USA. In fac tmeager data are available on SLE and its association with HLA typing in Sudan. The study of HLA associated genes may help in early detection of SLE and the susceptibility in family members as well as in the diagnosis of the disease in our society. Behind understanding the mechanism and pathogenesis of the disease also this study may helps in development of novel therapeutic targets in the future.
1.3 Objectives

1.3.1 General objectives
To assess the hematological parameters and HLA class II in Sudanese SLE patients.

1.3.2 Specific objectives
1. To detect HLA Class II DRB1, DQB1 in patients and compare with control group.
2. To detect anticardiolipin (IgG, IgM) and Anti-β2-glycoproteinI (IgG, IgM) autoantibodies in systemic lupus erythematosus patients (SLE).
3. To evaluate anti nuclear autoantibodies ANA) in all patients.
4. To estimate hemoglobin, leukocyte, platelets and erythrocyte sedimentation rate in all patients.
Chapter two

2. literature review

2.1 Systemic Lupus Erytheromatous (SLE)

2.1.1 Introduction to SLE
Systemic lupus erythematosus (SLE) is a prototype of autoimmune diseases affecting predominantly women. It is characterized by a multisystem organ involvement because of dysregulation of self-reactive B cells leading to autoantibody production, immune complex deposition and complement activation with tissue damage (Anaya 2006).
The more common clinical features seen in patients with SLE include, skin and joint diseases, haematological abnormalities, renal disease and neuropsychiatric complications (Hochberg 1997; Ramal et al. 2004).

2.1.2 Historical background
Although the term “lupus erythematosus” was introduced by 19th-century physicians to describe skin lesions, it took almost 100 years to realize that the disease is systemic and spares no organ and that it is caused by an aberrant autoimmune response (Duarte C et al., 2011).

2.1.3 Pathogenesis
The etiology of SLE remains unknown. Immune dysregulation with polyclonal B-cell activation appears to drive the production of self-reactive auto-antibodies. The production of auto-antibodies seems to be the sentinel event in the pathogenesis of SLE, the development of which may precede the clinical manifestations of SLE by years (Arbuckle et al. 2003).
The clinical heterogeneity of this disease is mirrored by its complex aetiopathogenesis, which highlights the importance of genetic factors and individual susceptibility to environmental
factors. The majority of patients have elevated levels of auto antibodies, directed in particular against nuclear components such as nucleosomes, DNA and histones, and it is generally accepted that at least some of these have a directly pathogenic role, either by precipitating as immune complexes in target organs or by cross-reacting with other functionally relevant antigens. The presence and persistence of these auto antibodies indicate an abnormality in tolerance, which results from a combination of abnormal handling of auto antigens following apoptosis, and deranged function of T and B lymphocytes (Manson and Rahman 2006).

### 2.1.4 Epidemiology of SLE

The cause of (SLE) is unknown, the disease affect predominantly females (9:1 over males) of childbearing age. In a blinded, randomized, controlled trial, menopausal women with lupus who received hormone-replacement therapy containing conjugated estrogens and progesterone had a risk of a mild-to-moderate disease flare that was 1.34 times the risk among women who received placebo (P = 0.01)(Buyon et al. 2005). However, trials of hormonal treatments for lupus, such as dehydroepiandrosterone, have been disappointing (Chang et al. 2002).

Many drugs cause a variant of lupus called drug-induced lupus. Drug-induced lupus erythematosus (DI-LE) is defined as an entity characterized by clinical manifestations and immunopathological serum findings similar to those of idiopathic lupus but which is temporally related to drug exposure and resolves after withdrawal of the implicated drug (Marzano et al. 2014).

Ultraviolet radiation is the most obvious environmental factor linked to lupus. A photosensitive rash is a criterion of the American College of Rheumatology for the classification of the disease (Tan et al. 1982; Hochberg 1997).

### 2.1.5 Prevalence of SLE

There is wide variation in disease prevalence in different ethnic populations with a 3–4 times increased prevalence in African–American or Afro-Caribbean populations
compared with Caucasians born and raised in the same community (Johnson et al. 1995; McCarty et al. 1995). The prevalence ranges from 20 to 150 cases per 100,000 population, with the highest prevalence reported in Brazil, and appears to be increasing as the disease is recognized more readily and survival increases. In the United States, people of African, Hispanic, or Asian ancestry, as compared with those of other racial or ethnic groups, tend to have an increased prevalence of SLE and greater involvement of vital organs. The 10-year survival rate is about 70% (Pons-Estel et al. 2010). Reliable data about the prevalence of SLE in Sudan are difficult to come by.

### 2.1.6 Diagnostic criteria of SLE

The clinical heterogeneity of the disease forced the establishment of 11 criteria, American College of Rheumatology Criteria for the Diagnosis of Systemic Lupus Erythematosus (SLE), with 4 needed for the formal diagnosis of systemic lupus erythematosus (SLE) (Tan et al. 1982).

**Table 1.2** 1997 Update of the 1982 American College of Rheumatology revised criteria for classification of systemic lupus erythematosus

<table>
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<tr>
<th>Criteria</th>
<th>Description</th>
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<tr>
<td>1. Malar Rash</td>
<td>Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds</td>
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<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions</td>
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<td>3. Photosensitivity</td>
<td>Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation</td>
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<td>4. Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration, usually painless, observed by physician</td>
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<tr>
<td>5. Nonerosive arthritis</td>
<td>Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion</td>
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</table>
| 6. Pleuritis or pericarditis | 1. Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion  
2. Pericarditis--documented by electrocardigram or rub or... |
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<th>7. Renal disorder</th>
<th>evidence of pericardial effusion</th>
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<tr>
<td>1. Persistent proteinuria &gt; 0.5 grams per day or &gt; than 3+ if quantitation not performed</td>
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<tr>
<td><strong>OR</strong></td>
<td></td>
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<td>2. Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed</td>
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<tr>
<th>8. Neurologic disorder</th>
<th>1. Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance</th>
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<tr>
<td><strong>OR</strong></td>
<td>2. Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance</td>
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<th>9. Hematologic disorder</th>
<th>1. Hemolytic anemia--with reticulocytosis</th>
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<td><strong>OR</strong></td>
<td>2. Leukopenia--&lt; 4,000/mm$^3$ on ≥ 2 occasions</td>
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<td></td>
<td><strong>OR</strong></td>
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<tr>
<td></td>
<td>3. Lymphopenia--&lt; 1,500/ mm$^3$ on ≥ 2 occasions</td>
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<td><strong>OR</strong></td>
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<td></td>
<td>4. Thrombocytopenia--&lt;100,000/ mm$^3$ in the absence of offending drugs</td>
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<th>10. Immunologic disorder</th>
<th>1. Anti-DNA: antibody to native DNA in abnormal titer</th>
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<td></td>
<td><strong>OR</strong></td>
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<td>2. Anti-Sm: presence of antibody to Sm nuclear antigen</td>
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<td><strong>OR</strong></td>
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<td>3. Positive finding of antiphospholipid antibodies on:</td>
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1. an abnormal serum level of IgG or IgM anticirodiolipin antibodies,
2. a positive test result for lupus anticoagulant using a standard method, or
3. a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test.

| 11. Positive antinuclear antibody | An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs |

### 2.1.7 Genetic factors of SLE

The concordance rate for lupus is 25% among monozygotic twins and approximately 2% among dizygotic twins (Sullivan 2000). These rates indicate that a genetic contribution is important, but it is not sufficient to cause the disease. Many genes that probably contribute to lupus have been identified by means of whole-genome scans from families in which multiple members have lupus (Namjou et al. 2007; Wakeland et al. 2001).

Genes of the major histocompatibility complex (MHC), particularly HLA-A1, B8, and DR3, have been linked to lupus (Walport et al. 1982).

The response of a T lymphocyte to an antigen is triggered when a receptor molecule on the surface of the T cell recognizes a complex formed by the antigen and an MHC peptide on the surface of an antigen-presenting cell. Different types of cells within the immune system, such as B cells, macrophages, and dendritic cells, can function as antigen-presenting cells. The MHC genotype determines which MHC molecules are available to the antigens that are present and thus how well the antigens can be recognized by T cells. For this reason, particular MHC genes are associated with a risk of an immune response to self antigens and hence a risk of diseases such as lupus.

Null alleles that cause a deficiency of one of the early complement components — C1q, C2, or C4 — are a strong risk factor for lupus (Walport 2002).

Family studies have identified genes that are more likely to occur in patients with lupus than in their healthy relatives (Wakeland et al. 2001).
Many of these genes encode components of the immune system. For example, Wakeland and colleagues (Wakeland et al. 2001), have identified genetic loci that promote lupus in mice (Morel et al. 2000). These loci, designated Sle 1, Sle 2, and Sle 3, contain genes that mediate the loss of immunologic tolerance to nuclear autoantigens, B-cell hyperactivity, and T-cell dysregulation, respectively. (Wakeland et al. 2001)

The Sle 1 cluster contains genes similar to those in regions 1q21–23 and 1q41 of human chromosome 1 that have been linked to lupus in humans (Wakeland et al. 2001).

2.1.8 Laboratory investigation

2.1.8.1 Hematological finding

Anemia:

It is a common hematological abnormality in SLE that is defined as hemoglobin levels of < 12g/dL. It is categorized into the following: anemia of chronic disease (ACD), which is the most common form (60%-80%), iron deficiency anemia (IDA), autoimmune hemolytic anemia (AIHA), and anemia due to chronic renal insufficiency.

Anemia is a frequent occurrence in SLE. Multiple mechanisms contribute, including inflammation, renal insufficiency, dietary insufficiency, and hemolysis (Nossent and Swaak 1991) (Keeling and Isenberg 1993) (Liu et al. 1995; Voulgarelis et al. 2000).

Leukopenia:

Leukopenia is common in SLE. And has been noted in approximately 50% of patients (Keeling & Isenberg 1993; Nossent & Swaak 1991). It is a typical feature of SLE which may occur as a result of lymphopenia, neutropenia or a combination of both. The prevalence of lymphopenia in SLE ranges from 20 to 81% and its degree may correlate with disease activity. Both T and B lymphocytes are reduced, while natural killer cells are typically increased (Schur et al., 2012).
Immunosuppressive agents like azathioprine or cyclophosphamide have the potential to worsen leukopenia via bone marrow suppression, which is less common (Bashal 2013).

Thrombocytopenia

It has a reported prevalence ranging from 7 to 30% in large series of patients with SLE (Hepburn et al. 2010). Increased peripheral destruction of platelets and presence of anti-platelet antibodies, is the most likely pathogenic mechanism (Bashal 2013).

Mild thrombocytopenia (platelet counts between (100-150) X 10^9/1 has been noted in 25- 50% of patients; while counts of less than 50 X 109/1 occur in only 10%((Keeling & Isenberg 1993; Nossent & Swaak 1991).

2.1.8.2 Serology finding

Antinuclear antibodies (ANAs)

Any antibody to nuclear components is an ANA. Most patients with ANAs do not have SLE, but most people with SLE have ANAs. The most common screening test is IFA on rodent liver or human epithelial (HEp2) tissue (van Venrooij et al. 1991) although ELISA tests are available (Emlen and O'Neill 1997; Froelich et al. 1990).

Anti dsDNA antibodies

Anti-dsDNA test is highly specific for SLE and these antibodies not found in patients with other rheumatic diseases (Goulvestre 2006). Usually, 60–80% of patients with active SLE have a positive anti-dsDNA test (Arbuckle et al. 2001). For many patients with anti-dsDNA antibodies, the increased titer is a useful measure of disease activity. (Arbuckle et al. 2001; (Swaak et al. 1979).

Anti-dsDNA antibodies are assumed to be quite specific for SLE activity (Isenberg et al. 1987) (Hahn 1998) (Spronk et al. 1995).

Thus, they are seen uncommonly in patients with other diseases or in normal persons and have a specificity for SLE of nearly 100%. Weakly positive anti-dsDNA results may occur in patients who do not have SLE. The percentage of SLE patients who
have anti-dsDNA antibodies (ie, the sensitivity of the test) varies in published series from 25% up to 85% (Kavanaugh et al. 2000a).

**Anti histone antibodies**

Around 50–80% of patients with SLE have IgG and IgM antihistone antibodies detectable by immunoblotting (IB) or ELISA. ELISAs are described that detect antibodies to total histones or to subfractions (H1, H2a, H2b, H3, and H4) (Aitkaci et al. 1981; Rubin et al. 1982), but the clinical specificity is not well established for any subfraction. Titres of antihistone antibody might reflect disease activity, but are not specific for SLE and cannot distinguish drug induced SLE from idiopathic SLE (Massa et al. 1994; Molden et al. 1986). The typical laboratory profile of systemic Drug Induced Lupus Erytheromatous (DILE) consists of positive antinuclear antibodies (ANA) and antihistone antibodies (Marzano et al. 2009).

**Anti Ribosomal P antibodies**


**Anti SSA-SSB Antibodies**

Ro (SS-A) and La (SS-B) are ribonucleoproteins that have been implicated in protein transcription and translation. Autoantibodies to these antigens were first described Antibodies to Ro (SS-A) and La (SS-B) are found in SLE and Sjogren’s syndrome. Neither is specific for SLE, but both are very useful when anti-dsDNA is absent (Sanchez-Guerrero et al. 1996).
Sm/RNP Antibodies

Anti-Sm test is highly specific for SLE and rarely found in patients with other rheumatic diseases (Stinton and Fritzler 2007). However, only 30% of patients with SLE have a positive anti-Sm test (Santos et al. 2007).

Anti-Sm autoantibodies are relatively specific for SLE and can be used as an aid in diagnosis. Higher titers of anti-Sm are more specific for SLE.

Anti-nRNP antibodies, which are commonly tested for in conjunction with anti-Sm, are present in 30% to 40% of SLE patients. However, anti-nRNP antibodies are not specific for SLE and are not useful for establishing the diagnosis of SLE (Kavanaugh et al. 2000).

Antiphospholipid antibodies

Antiphospholipid antibodies (aPL) are a heterogeneous group of autoantibodies that include anticardiolipin (aCL), lupus anticoagulant (LA) and anti-b2 glycoprotein I (ab2-GPI) (Arvieux et al. 1991; Galli et al. 1990; Keeling et al. 1992; Matsuura et al. 1990; McNeil et al. 1990; Roubey et al. 1995; Viard et al. 1992). Approximately 30-40% of SLE patients have antiphospholipid antibodies (APL) (Svenungsson et al. 2010).

Lupus anticoagulant antibodies are identified by coagulation assays, in which they prolong clotting times. In contrast, anticardiolipin antibodies and anti–b2 glycoprotein I antibodies are detected by immunoassays that measure immunologic reactivity to a phospholipid or a phospholipid-binding protein (Levine et al. 2002).

The pathogenic role of aCL antibodies in the induction of thrombosis and fetal loss has been clearly demonstrated in experimental animal models (Blank et al. 1991; Pierangeli et al. 1995).
2.1.9 Treatment of SLE

Patients with SLE are treated with nonsteroidal antiinflammatory drugs, antimalarial agents, glucorticoids, and immunosuppressive drugs, including cyclophosphamide, azathioprine, methotrexate, and mycophenolate mofetil. The choice of the drug is determined largely by the severity of the disease and the function of the involved organ. Antiinflammatory drugs: inhibitors of cyclooxygenase-2 have been claimed to promote the death of autoreactive T cells (Xu et al. 2004). The antimalarial agent hydroxychloroquine has therapeutic value and limited toxicity. It inhibits the function of toll-like receptors that contribute to autoimmunity. (Sun S Cyclophosphamide pulses (intravenous infusions every month or bimonthly at lower doses) are effective in the treatment of lupus nephritis, although there are serious potential side effects, including bone marrow suppression, infections, and gonadal suppression (Illei et al. 2001).

Mycophenolate mofetil: has considerable therapeutic value with few side effects (Contreras et al. 2004; Radhakrishnan et al. 2010), but its long-term effects with respect to the preservation of kidney function are unproven (Boumpas et al. 2010).
2.2 Human leukocyte Antigen (HLA)

2.2.1 introduction to HLA

The term HLA refers to the Human Leucocyte Antigen System, which is controlled by genes on the short arm of chromosome six. The HLA loci are part of the genetic region known as the Major Histocompatibility Complex (MHC) (Hugh et al., 1984). The MHC has genes (including HLA) which are integral to normal function of the immune response. The essential role of the HLA antigens lies in the control of self-recognition and thus defense against microorganisms. The HLA loci, by virtue of their extreme polymorphism ensure that few individuals are identical and thus the population at large is well equipped to deal with attack (McDevitt 1985). Because some HLA antigens are recognized on all of the tissues of the body (rather than just blood cells), the identification of HLA antigens is described as “Tissue Typing” or “HLA Typing” (Shankarkumar, 2004).
2.2.2 Classification of HLA
Based on the structure of the antigens produced and their function, there are two classes of HLA antigens, termed accordingly, HLA Class I and Class II. The overall size of the human MHC complex is of approximately 3.5 million base pairs (Figure 2.2). Within this the HLA Class I genes and the HLA Class II genes each spread over approximately one third of this length. The remaining section, sometimes known as Class III, contains loci responsible for the complement, hormones, intracellular peptide processing and other developmental characteristics (Sanfilippo F& Amos DB et al., 1986). Thus the Class III region is not actually a part of the HLA complex, but is located within the HLA region, because its components are either related to the functions of HLA antigens or are under similar control mechanisms to the HLA genes (de Jong et al. 2003).

Fig. 2.2 The HLA gene complex(after permission from Narinder and Gurvinder.2003)
2.2.2.1 HLA Class I antigens
The cell surface glycopeptide antigens of the HLAA,- B and -C series are called HLA Class I antigens (Roitt M et al., 1998). A listing of the currently recognized HLA Class I antigens are expressed on the surface of most nucleated cells in the body. Additionally, they are found in a soluble form in plasma and are adsorbed onto the surface of platelets. Erythrocytes also adsorb HLA Class I antigens to varying degrees depending on the specificity (e.g. HLA-B7, A28 and B57 are recognizable on erythrocytes as the so called “Bg” antigens). Immunological studies indicate that HLA-B (which is also the most polymorphic) is the most significant HLA Class I locus, followed by HLA-A and then HLA-C. There are other HLA Class I loci (e.g. HLA-E, F, G, H, J, K and L), but most of these may not be important as loci for “peptide presenters” (Lotteau 1992).

Fig 2.3 HLA class I (adopted from Modulators in rheumatic disease, and the biologic therapies, http://www.arthritis.co.za/immunologyupdate.html)
2.2.2.2 *Class II Antigens*

The cell surface glycopeptide antigens of the HLA-DP, -DQ and -DR loci are termed HLA Class II (Sanfilippo et al. 1984).

HLA Class II molecules consist of two chains each encoded by genes in the “HLA Complex” on Chromosome 6. The T Cells which link up to the HLA Class II molecules are Helper (CD4) T cells, thus the “education” process which occurs from HLA Class II presentation, involves the helper-function of setting up a general immune reaction which will involve cytokines, cellular and humoral defense against the bacterial (or other) invasion. This role of HLA Class II, in initiating a general immune response, is the reason why they need only be present on “immunologically active” cells (B lymphocytes, macrophages, etc.) and not on all tissues (Browning and Mc Michael., 1996).

![HLA Class II](http://www.arthritis.co.za/immunologyupdate.html)

**Fig 2.4** HLA class II (adopted from Modulators in rheumatic disease, and the biologic therapies, [http://www.arthritis.co.za/immunologyupdate.html](http://www.arthritis.co.za/immunologyupdate.html))
2.2.3 HLA in transplantation
A person, on the average, will have one-half of the HLA antigens that match with one-half of their mother’s antigens; the other half of the antigens will match with one-half of their father’s antigens. This is particularly important in identifying good “matches” for tissue grafts and organ transplants, such as a kidney or bone marrow transplant (Shankarkumar, 2004).

Human leukocyte antigen (HLA) molecules are expressed on almost all nucleated cells, and they are the major molecules that initiate graft rejection. The system is highly polymorphic, there being many alleles at each individual locus. Three methods for HLA typing are described in this chapter, including serological methods and the molecular techniques of sequence-specific priming (SSP) and sequence-specific oligonucleotide probing (SSOP). HLA matching has had the greatest clinical impact in kidney and bone marrow transplantation, where efforts are made to match at the HLA-A, -B, and -DR loci. In heart and lung transplantation, although studies have shown it would be an advantage to match especially at the DR locus, practical considerations (ischemic times, availability of donors, clinical need of recipients) make this less of a consideration. Corneal grafts are not usually influenced by HLA matching, unless being transplanted into a vascularized (or inflamed) bed (Sheldon and Poulton 2006).

2.2.4 HLA in disease association
Many HLA molecules exist, but some are of special interest because they are more common in certain autoimmune diseases. The HLA region encodes several molecules that play key roles in the immune system. Strong association between the HLA region and autoimmune disease (AID) has been established for over fifty years. Association of components of the HLA class II encoded HLA-DRB1-DQA1-DQB1 haplotype has been detected with several AIDs, including rheumatoid arthritis, type 1 diabetes and Graves’ disease (Gough and Simmonds 2007), the HLA-B27 antigen is found in 80-90% of the patients with ankylosing spondylitis and Reiter’s syndrome and can aid in
the diagnoses of these diseases. HLA-B27 is also present in 5-7% of the persons without autoimmune diseases. Thus, the mere presence of this HLA molecule is not an indication of disease (Shankarkumar. 2004).

2.2.5 HLA association and SLE
Genetic studies have shown that polymorphisms at several loci, including the major histocompatibility complex (MHC), which encodes human leukocyte antigen (HLA), complement proteins, immunoglobulin receptors, cytokines, and other unmapped genes, are associated with SLE(Castro et al. 2008;Forabosco et al. 2006). It has been suggested that genes in the HLA region represent the most important association with this autoimmune disease, specially HLA-DRB1 and HLA-DQB1 (Wakeland et al. 2000 (Gladman et al. 1999;Wong and Tsao 2006). However, the nature of this relationship is obscure, as the degree of association between SLE and specific genes of the MHC region varies considerably from one population to another(Rhodes and Vyse 2007;Smerdel-Ramoya et al. 2005).

More recent studies using DNA typing have identified disease-associated haplotypes and have extended these initial associations to other racial and ethnic groups. The HLA-DR2 association with SLE is accounted for by the HLA-DRB1*1501-DQA1*0102-DQB1*0602 haplotype, which has been reported in Central European Caucasians(Arnett and Reveille 1992), Chinese (Lu et al. 1997), Japanese (Dong et al. 1993), and African-Americans(Reveille et al. 1991;Schur et al. 1990). The HLA-DR3 haplotype bearing HLA-DRB1*0301-DQA1*0501-DQB1*0201 was observed in Central and Western Europeans, and North Americans(Reveille et al. 1991;Gladman et al. 1999;(Reveille et al. 1998). However, in the majority of other ethnic groups studied, no MHC class II associations have been found (Huang et al. 2001;Marintchev et al. 1995;Yao et al. 1994), In Japanese also HLA-DRB1*15:01, HLA-DRB1 *09:01, HLA-DRB1 *08:02 and HLA-DRB1*04:01 were significantly associated with SLE
susceptibility (Shimane et al. 2013), in Tunisians there is appositive association between DRB1*15 and SLE patients (Ayed et al. 2004)
Chapter three

3. Material and methods

3.1 Study area and population
Study was carried out in Khartoum State during the period from 2010 to 2013, eighty SLE patients and sixty controls were collected from Rheumatology Clinic at the National Ribat University Hospital, Medical Military Hospital and Private Clinic during 2010 to 2012, the inclusion criteria of this study were confirmed SLE cases by clinical manifestation according to American College of Rheumatologists (ACR) classification criteria for SLE, also adult male and female patients (>18 years). Seven ml of blood samples were collected into Ethylene diamine tetra acetic acid (EDTA) container for CBC, ANA profile, HLA typing and anti phospholipid antibodies screening test. The data were collected by filled the questionnaire by investigator including demographic data (name, sex, age and the tribe) and clinical data.

3.2 Ethical consideration
Written informed consent was signed by all subjects included in the study. The ethical clearance was obtained from Tropical Medicine Research Institute (TMRI) in March, 2011.

3.3 Sample Preparation
Blood sample was collected in EDTA tube and divided into two parts, the first one (2ml) for CBC and ESR whereas the second one (5ml), was centrifuged at 3000 rpm for 10 minutes at room temperature. After centrifugation, three different fractions were distinguished, the upper clear layer is plasma which was used in serological test; the intermediate layer was Buffy coat which is the leukocyte-
enriched fraction of whole blood, and the bottom layer contains concentrated erythrocytes. The Buffy coat layer was collected carefully using a 1ml sterile disposable pipette, into a micro centrifuge tube for HLA typing, plasma and buffy coat were stored at $-20^\circ C$ until tested.

### 3.4 Complete blood count (CBC)
Total white blood cells, Red blood cells, platelets and blood indices were measured using automated method (Sysmex KX-21N, Germany), ESR was measured using Westergren method.

### 3.5 Antiphospholipid antibodies detection using ELISA assay
Commercial ELISA kits for detection of Anti-cardiolipin antibodies (IgG, IgM and IgA) and Anti-\(\beta\)2-glycoprotein were purchased from Euroimmun, Germany. The procedure was according to manufacturer’s instructions

#### 3.5.1 Principle of ELISA
Highly purified human cardiolipin/\(\beta\)2-glycoprotein I is bound to microwells. Antibodies against this antigen, if present in diluted serum or plasma, bind to the respective antigen. Washing of the microwells removes unbound unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG, IgM & IgA immunologically detects the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate.

An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue colour. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow colour is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of IgG, IgM & IgA antibodies present in the original sample.
3.5.2 Standards of Anti-β2-glycoprotein I
According to manufacturer (EUROIMMUN, Germany), Combined calibrators with IgG, IgM and IgA class antibodies with different concentrations: 200RU/ml, 20RU/ml and 2RU/ml.

3.5.3 Standards of Anti cardiolipin
According to manufacturer (EUROIMMUN, Germany), Combined calibrators with IgG, IgM and IgA class antibodies with different concentrations: 3.3U/ml, 10U/ml, 30U/ml and 90U/ml.

3.5.4 ELISA Procedure
Each plasma sample was diluted 1:151 into sample buffer, in which 10 ul from such sample, calibrator and control was pre-diluted into ready diluted biotin, incubated for at least 10 minutes at room temperature. Then, 100 µl pre-diluted sample plasma, calibrator, and controls were pipetted into a well of micro plate, and the plate was incubated for 30 min at room temperature. After incubation with patient plasma, the content of micro wells were discarded and washed 3 times with 300 µl wash buffer. In the second incubation, 100 ul enzyme conjugate was added to each well, incubated for 30 minutes at room temperature. Moreover, 100 ul chromogen/substrate was added to each well and the plate was incubated for 15 minutes at room temperature. Then, the reaction was stopped using 100ul stop solution and color was measured using spectrophotometer at 450 nm filter using ELISA reader (ASYS/Expert plus, Austria).

3.5.5 Calculations
For quantitative calculation of the results, the concentration of standards used for creating a calibration curve for Anti cardiolipin 4-parameter-fit with lin-log, for Anti-β2-glycoprotein I point to point fit with linear/linear for optical density and concentration was used.
3.5.6 Expected value
Cutoff value for anti-cardiolipin antibodies is 10 IU/ml and for β2 glycoprotein is 20 IU/ml.

3.6 Anti nuclear autoantibodies (ANA) profile
ANA profile was analyzed using blotting technique (Euroimmun, Germany) according to manufacturer’s instructions.

3.6.1 Principle of Immunoblot
Membrane strips containing electrophoretically separated antigen extracts were used as solid phase. The position of the proteins depends on their respective molecular masses.

- If the sample was positive, specific antibodies in the diluted serum sample were attached to antigens coupled to the membrane.
- In a second incubation step, the attached antibodies were reacted with AP-labelled antihuman antibodies.
- In a third step, the bound antibodies were stained with a chromogen/substrate solution which was capable of promoting a color reaction. An intense dark band at the line of the corresponding antigen was appeared if the serum sample was contained specific antibodies.
- Evaluating the band patterns on the incubated membrane strips involved differentiating non-specific from specific antibodies. The number and intensity of the specific bands was decisive for the result “positive/negative“.

3.6.2 Immunoblot procedure
One and half ml of diluted sample was pipette into membrane strip incubation channel (sample was first diluted 1:101,15 μl of serum to 1.5 ml of sample buffer) and incubated for 30 min with shaking, after that 1.5ml of buffer was added into membrane strip incubation channel and aspirate off three times, then 1.5ml of
enzyme conjugate was pipette into membrane strip incubation channel and incubated with shaking for 30 min, then 1.5ml of buffer was added into membrane strip incubation channel and aspirated off three times, after that 1.5 of chromogen/substrate into membrane strip incubation channel was added and incubated with shaking for 10 min, finally the strip was rinsed with distilled water and aspirated off, and evaluated with computerized scan (Euro line scan, Germany).

3.6.3 Expected value
No band  Negative
Very weak band  Borderline
Medium to strong band  Positive
Very strong band  strong Positive

3.7 DNA Extraction
DNA was extracted according to manufacturer’s instructions using Qiagene kit (QIAGEN GmbH, Hilden, Germany)

3.7.1 Principle
Optimized buffers and enzymes lyses samples, stabilize nucleic acids, and enhance selective DNA adsorption to the QIAamp membrane. Alcohol was added and lysates were loaded onto the QIAamp spin column. Wash buffers were used to remove impurities and pure, ready-to-use DNA was then eluted in water or low-salt buffer.

3.7.2 procedure
Twenty two μl QIAGEN Protease (or proteinase K) was pipetted into the bottom of a 1.5 ml microcentrifuge tube.
then 190 μl of buffy coat was added to the microcentrifuge tube, then
200 μl of Buffer AL was added to the sample. Mix by pulse-vortexing for 30 s was performed, after that sample was Incubated at 56°C for 30 min, then Briefly centrifugation to remove drops from the inside of the lid was done, then 200 μl ethanol (96–100%) was added to the sample, and mixed again by pulse-vortexing for 30 s. After mixing, briefly centrifugation to remove drops from the inside of the lid was carried out, then Carefully the mixture was applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. the cap was closed, and centrifuged at (8000 rpm) for 1 min after that QIAamp Mini spin column was placed in a clean 2 ml collection tube, and discard the tube containing the filtrate, then Carefully the QIAamp Mini spin column was opened and 500 μl Buffer AW1 was added without wetting the rim. the cap was closed and centrifuged at (6000 x g (8000 rpm)) for 1 min, the QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded, then Carefully the QIAamp Mini spin column was opened and 500 μl Buffer AW2 was added without wetting the rim. the cap was closed and centrifuged at full speed (at 6000 x g (8000 rpm)) for 3 min, finally the QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. Carefully the QIAamp Mini spin column was opened and 120 μl Buffer AE was added and Incubated at room temperature (15–25°C) for 15 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min.

*All samples were stored at –20°C until used.

3.8 DNA Quantitation
DNA samples were diluted 1/30(15 μl DNA+435 μl D.W) with sterile double distilled water, DNA samples were vortexed and spun down before used.
Purity determined by calculating the ratio of absorbance at 260nm to absorbance at 280nm. Pure DNA had A260/A280 of 1.7-1.9 (adapted from QIA, 1/199)

3.9 HLA-DR and HLA-DQ Typing

Polymerase chain reaction (PCR)-based HLA-DR and HLA-DQ typing and sub-typing was performed using low-resolution kit (One Lambda, Kittridge Street, Canoga Park, USA). All the examinations were prepared according to the manufacturer’s instructions.

According to One Lambda HLA Class II typing kit protocol, the Polymerase Chain Reaction-Single Stranded Polymorphism (PCR-SSP) methodology was based on the principle that using recombinant Taq polymerase, completely matched oligonucleotide primers that more efficiently used in amplifying target sequence. All kit reagents and DNA samples were brought at room temperature and then vortexed. Primer set tray was placed in a PCR tray microtube storage rack and the tray label was removed. 1 μl of deionized water was added to the negative control reaction tube on the primer set tray. Then 2.1 μl of recombinant Taq polymerase (5 units/μl) was added to the Micro SSPTM Dmix tube and vortexed. Then Micro SSPTM D-mix tube was pulse spun in a microcentrifuge to bring all liquid down from sides of the tube. Micro SSPTM D-mix (10 μl) was added to the negative control reaction tube. 60 μl of the DNA sample was then added to the Micro SSPTM D-mix tube, vortexed and again spun. Sample-reaction mixture (10μl) from the Micro SSPTM D-mix tube was added in to each reaction tube of the Micro SSPTM primer set tray except the negative control reaction tube. Then reaction tubes were then sealed with the tray seal. The Micro SSPTM primer set tray was placed in a thermocycler (Biosystem, USA) and PCR program was started. Primer pairs were designed to have perfect matches only with a single allele or group of alleles. Under strictly controlled PCR conditions, perfectly
matched primer pairs resulted in the amplification of target sequences. The primer set tray was removed from the thermocycler and was unsealed gently without splashing the samples. 10 μl of the PCR reaction was transferred to a 2.5% agarose gel and the samples were electrophoresed at (140-150) volts for (3-5) minutes, bands were finally visualized by UV illuminator.

The control primer pair amplifies a conserved region of the Human β-globin gene, which is present in all human DNA samples and was used to verify the integrity of the PCR reaction. The amplified DNA fragments of the specific HLA primer pairs were smaller than the product of the internal control primer pair, but larger than the diffuse, unincorporated primer band. The typing results were interpreted by using the worksheet provided with the trays with assistance of HLA software available from one lamda, Inc, USA.
3.10 Statistic analysis
The data entered and analyzed using SPSS software for analysis (version 20.0), the frequencies of HLA alleles and autoantibodies were obtained by direct counting. frequencies of HLA alleles in SLE patients, control population were compared, using Chi-square tests. A $P$-value $< 0.05$ was considered to be statistically Significant.
Chapter Four

4. Result

4.1 Study Group
A group of 140 individuals were enrolled in this study, 80 were SLE patients and 60 were healthy individuals.

Table 4.1: Distribution of Sudanese SLE patients (n= 80) according to their age and gender

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-34</td>
<td>2</td>
<td>43</td>
<td>45 (55%)</td>
</tr>
<tr>
<td>35-40</td>
<td>1</td>
<td>16</td>
<td>17 (22%)</td>
</tr>
<tr>
<td>41-54</td>
<td>1</td>
<td>17</td>
<td>18 (23%)</td>
</tr>
<tr>
<td>Total</td>
<td>4(4%)</td>
<td>76(96%)</td>
<td>80(100%)</td>
</tr>
</tbody>
</table>

The result showed that the SLE affect female 20 times than male (F:M = 19:1), The majority of patients with SLE was in age between (18-34)ys which resemble about 45% of whole patients.
**Table 4.2** Distribution of ethnic groups of Sudanese SLE patients (n= 80)

<table>
<thead>
<tr>
<th>Tribe</th>
<th>Frequency</th>
<th>percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nilo-Saharan tribes</td>
<td>30</td>
<td>37%</td>
</tr>
<tr>
<td>Afro-asiatic tribes</td>
<td>50</td>
<td>63%</td>
</tr>
</tbody>
</table>

The result showed that the most common affected tribes of patients were the Afro-asiatic tribes.
4.2 Hematology parameters:
Table 4.3: Hematological parameters results in SLE patients which indicated first sign of disease,

<table>
<thead>
<tr>
<th>Variable</th>
<th>Results</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hemoglobin level</strong></td>
<td>&lt;12g/dl</td>
<td>&gt;12g/dl</td>
</tr>
<tr>
<td></td>
<td>68%(54/80)</td>
<td>32%(26/80)</td>
</tr>
<tr>
<td><strong>Total white blood cell account</strong></td>
<td>&lt;4.0×10⁹/l</td>
<td>&gt;4.0×10⁹/l</td>
</tr>
<tr>
<td></td>
<td>40%(32/80)</td>
<td>60(48/80)</td>
</tr>
<tr>
<td><strong>Platelets account</strong></td>
<td>&lt;100×10⁹/l</td>
<td>&gt;100×10⁹/l</td>
</tr>
<tr>
<td></td>
<td>7%(7/80)</td>
<td>93%(73/80)</td>
</tr>
<tr>
<td><strong>Erythrocyte Sedimentation Rate (mm/hr)</strong></td>
<td>&lt;5</td>
<td>5-30</td>
</tr>
<tr>
<td></td>
<td>2%(2/80)</td>
<td>12%(10/80)</td>
</tr>
</tbody>
</table>

The result showed anemia in 68% of patients, leukopenia found in 40%, thrombocytopenia found in only 7% of patients and ESR (31-100 mm/hr) was found in 44%.
4.3 Antinuclear autoantibody

Table 4.4 the frequency of autoantibodies using immunoblott technique in SLE patients

<table>
<thead>
<tr>
<th>Auto antibodies</th>
<th>No. of positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA</td>
<td>80/80</td>
<td>100%</td>
</tr>
<tr>
<td>Anti dsDNA</td>
<td>34/80</td>
<td>42%</td>
</tr>
<tr>
<td>Anti n(rp)</td>
<td>24/80</td>
<td>30%</td>
</tr>
<tr>
<td>Anti smith</td>
<td>58/80</td>
<td>72%</td>
</tr>
<tr>
<td>Anti SSA</td>
<td>29/80</td>
<td>36%</td>
</tr>
<tr>
<td>Anti SSB</td>
<td>13/80</td>
<td>16%</td>
</tr>
<tr>
<td>Anti Ro52</td>
<td>17/80</td>
<td>21%</td>
</tr>
<tr>
<td>Anti nucleosome</td>
<td>6/80</td>
<td>7%</td>
</tr>
<tr>
<td>Anti histone</td>
<td>6/80</td>
<td>7%</td>
</tr>
<tr>
<td>Anti AMA</td>
<td>6/80</td>
<td>7%</td>
</tr>
<tr>
<td>Anti ribosomal p.protein</td>
<td>6/80</td>
<td>7%</td>
</tr>
<tr>
<td>Anti Jo 1</td>
<td>4/80</td>
<td>5%</td>
</tr>
<tr>
<td>Anti centeromere</td>
<td>2/80</td>
<td>2%</td>
</tr>
<tr>
<td>Anti PCNA</td>
<td>2/80</td>
<td>2%</td>
</tr>
<tr>
<td>Anti SCL 70</td>
<td>0/80</td>
<td>0%</td>
</tr>
</tbody>
</table>

measurement of ANA profile using immunoblot, the result show 72% of the patients were positive for anti amith auto antibody, 42% were positive for anti dsDNA antibody and all the patients were negative for SCL70 autoantibody.
4.4 Antiphospholipid autoantibody prevalence
Antiphospholipid autoantibody was determined by detection of the level of Anticardiolipin anti body and Antiβ₂ glycoprotein antibody in SLE patients plasma.

Table 4.5: measurement of Antiphospholipid antibodies using ELISA. *

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Percentage of positive</th>
<th>Tire of antibody/ IU/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticardiolipin</td>
<td>38%(30/80)</td>
<td>Low(11-20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium(21-80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High(&gt;80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53%(16/30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40%(12/30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7%(2/30)</td>
</tr>
<tr>
<td>Antiβ₂ glycoprotein</td>
<td>22%(18/80)</td>
<td>Low(21-30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium(31-90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High(&gt;90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16%(3/18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56%(10/18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28%(5/18)</td>
</tr>
</tbody>
</table>

The result showed that 38% of the patients had Anticardiolipin antibody and 22% of the patients had Antiβ₂ glycoprotein antibodies,

* These result was published (Gafar A Rana et al., 2014).
Figure 4.1: Showed the titre of Anticardiolipin and Antiβ₂ glycoprotein antibodies in SLE patients’ plasma.
4.5 DNA concentration and purity

Table 4.6: Concentration and ratio of the DNA extracted by QIAGEN kit (QIAGEN USA) from the blood samples of SLE patients and control, measured by Eppendorf Plus.

<table>
<thead>
<tr>
<th>DNA conc./purity</th>
<th>No. of cases</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA conc. (μg/μl)</td>
<td>80</td>
<td>50</td>
<td>257</td>
<td>98.3</td>
<td>41.39</td>
</tr>
<tr>
<td>DNA ratio (A$<em>{260}$/A$</em>{280}$)</td>
<td>80</td>
<td>1.3</td>
<td>1.9</td>
<td>1.62</td>
<td>.172</td>
</tr>
</tbody>
</table>

The result showed that the maximum concentration of DNA was 257 μg/μl and the lower concentration was 50 μg/μl where as the maximum DNA ratio was 1.9 and the minimum DNA ratio was 1.3.
4.6 Gel electrophoresis of the PCR.SSP product

Figure 4.2: Showed a 96 wells gel electrophoresis result of three samples of SLE patients (4 rows for each patients, i.e. 32 well2 for each patient sample) each well showed one band of internal control (β.globin) except well number H1, H5 and H9 which were negative controls. Another band may occur in case of positive result.
4.7 HLA alleles frequency

**Figure 4.3**: Frequency of 12 HLA-DR alleles in the studied group tested for these gene, showed the highest frequency of DRB1*15 and the lowest frequency of DRB1*12.
Figure 4.4: Frequency of the 5 HLA-DQ alleles in the studied group tested for these gene, showed the highest frequency of DRQ1*06 and the lowest frequency of DQB1*04.
<table>
<thead>
<tr>
<th>HLA allele</th>
<th>Study group</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SLE patients</td>
<td>Control</td>
</tr>
<tr>
<td>DRB1*01</td>
<td>4%</td>
<td>2%</td>
</tr>
<tr>
<td>DRB1*03</td>
<td>12%</td>
<td>13</td>
</tr>
<tr>
<td>DRB1*04</td>
<td>9%</td>
<td>10%</td>
</tr>
<tr>
<td>DRB1*07</td>
<td>13%</td>
<td>10%</td>
</tr>
<tr>
<td><strong>DRB1*08</strong></td>
<td><strong>14%</strong></td>
<td><strong>6%</strong></td>
</tr>
<tr>
<td>DRB1*10</td>
<td>5%</td>
<td>4%</td>
</tr>
<tr>
<td>DRB1*11</td>
<td>10%</td>
<td>11%</td>
</tr>
<tr>
<td>DRB1*12</td>
<td>3%</td>
<td>1%</td>
</tr>
<tr>
<td><strong>DRB1*13</strong></td>
<td><strong>12%</strong></td>
<td><strong>29%</strong></td>
</tr>
<tr>
<td>DRB1*14</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td><strong>DRB1*15</strong></td>
<td><strong>23%</strong></td>
<td><strong>13%</strong></td>
</tr>
<tr>
<td>DRB1*16</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>DQB1*02</td>
<td>19%</td>
<td>23%</td>
</tr>
<tr>
<td>DQB1*03</td>
<td>24%</td>
<td>21%</td>
</tr>
<tr>
<td><strong>DQB1*04</strong></td>
<td><strong>6%</strong></td>
<td><strong>0%</strong></td>
</tr>
<tr>
<td><strong>DQB1*05</strong></td>
<td><strong>22%</strong></td>
<td><strong>13%</strong></td>
</tr>
<tr>
<td><strong>DQB1*06</strong></td>
<td><strong>28%</strong></td>
<td><strong>43%</strong></td>
</tr>
</tbody>
</table>

The result showed 23% of the patients had DRB1*15 compared to 13% of the control group with P.value 0.01 (P <0.05), and 22% of SLE patients had DQB1*05 compared to 13% of the control group with P.value 0.03 (P <0.05), while DRB1*13 allele was found to be protective as only 12% of SLE patients had the allele but 29% of control group had the allele with P.value 0.001 (P <0.05).

* not significant value.
4.8 HLA alleles (Risk & protective)

Table 4.8: Risk alleles (DRB1*15, DRB1*08, DQB1*05 and DQB1*04) in SLE patients compared to control individuals.

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Study group</th>
<th>P.value</th>
<th>RR*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>patient</td>
<td>control</td>
<td></td>
</tr>
<tr>
<td>DRB1*15</td>
<td>23%</td>
<td>13%</td>
<td>0.01</td>
</tr>
<tr>
<td>DRB1*08</td>
<td>14%</td>
<td>6%</td>
<td>0.02</td>
</tr>
<tr>
<td>DQB1*05</td>
<td>22%</td>
<td>13%</td>
<td>0.03</td>
</tr>
<tr>
<td>DQB1*04</td>
<td>6%</td>
<td>0%</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 4.9: Protective alleles (DRB1*13 & DQB1*06) in the control individuals compared to SLE patients:

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Study group</th>
<th>p.value</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>patient</td>
<td></td>
</tr>
<tr>
<td>DRB1*13</td>
<td>29%</td>
<td>12%</td>
<td>0.001</td>
</tr>
<tr>
<td>DQB1*06</td>
<td>43%</td>
<td>28%</td>
<td>0.007</td>
</tr>
</tbody>
</table>

*RR = relative risk, 1.0 = indicate probability is identical in two groups, <1.0 = indicate probability is less likely to occur in patients, >1.0 = indicate probability is more likely to occur in patients.
Chapter five:

5. Discussion, conclusion and recommendation

5.1 Discussion

The human leukocyte antigen (HLA) system includes genes involved in graft-vs-host rejection and in immune response. The discovery that HLAs are associated with several diseases led to appealing developments both in basic biomedical research and in clinical medicine, and offered the opportunity to improve the understanding of pathogenesis and classification of diseases, as well as to provide diagnostic and prognostic indicators (Cassinotti et al. 2009).

This is first study attempted in Sudan and can be considered as base line study for further studies in genetic profile of SLE.

For this study, 80 patients with SLE were enrolled. The main age of the patients in current study is approximately similar to other different population as in Tunis (Houman et al. 2004) and Saudi Arabia (Al Arfaj and Khalil 2009) and South Africa (Carey R et al., 2008), female to male ratio was 19:1 which is differ from other studies in Egypt (8:1) (Mohamed A et al., 2006) Tunis 11:1 (Houman et al 2004) and Saudi Arabia 10:1 (Al Arfaj & Khalil 2009), this difference may be due to miss diagnose of disease in males or the prevalence of SLE in Sudanese males is really less than the other population, also this ratio is in agreement with other studies done in South Africa 19:1 (Carey R et al., 2008), and Dubai 20:1 (AlSaleh et al. 2008).
Hematological abnormalities were the same as the results found in the previous studies and ACR criteria (Hochberg. 1997).

The diagnosis of SLE is based on clinical features and laboratory criteria. In addition to the many types of autoantibodies identified in the sera of patients with SLE, the ANA, dsDNA, and Sm antibodies are considered to be the most important laboratory tools for the diagnosis and management of SLE patients (Kavanaugh et al. 2000b; Lam and Petri 2005).

In the present study, we used a group of Sudanese patients who were clinically diagnosed with SLE, who required antinuclear testing to confirm the clinical diagnosis.

ANA displayed a sensitivity of 100% in the Sudanese patients with SLE, which was similar to previous reports in Tunis (Houman et al. 2004), Saudi (Alballa 1995) and Malay (Maraina et al. 2004).

Anti-dsDNA test is highly specific for SLE and not found in patients with other rheumatic diseases (Goulvestre. 2006), Usually, 60–80% of patients with active SLE have a positive anti-dsDNA test (Arbuckle et al. 2001).

The frequency of anti-dsDNA was 42% in this study group, which is lower than the frequency found in the neighboring countries like Saudi Arabia (93%) (Alballa. 1995), and United Arab Emirates (97%) (Alsaleh et al. 2008), the difference may be due to disease activity because not all our patients in active stage of disease, and the frequency is similar as in Afro-American and Hispanics (Alarcon et al. 1999)..

The anti-Sm antibodies are virtually diagnostic of SLE (Al-Jabri et al. 2009). The frequency of anti-sm was 72% in this Sudanese study group, which is different than the frequency found in the other countries like Oman 83%(Al-Jabri et al.2009), Egypt (60%) (Mohamed A et al., 2006), Tunis (49%) (Houman et al. 2004)
and South Africa (42%) (Carey R et al., 2008), this difference may be due to the technique used in measuring the antibody, study population and the ethnic-racial differences, and the frequency is similar as found in Nigeria 75% (Adelowo et al. 2012).

In the present study, the prevalence of anti cardiolipin auto antibodies in SLE Sudanese patients was 38%, this is in agreement with (Basiri et al. 2013) study that done in Iranian SLE patients (36%) and was higher than the prevalence detected in Turkish SLE patients (27%) (Define et al. 2008), and lower than that reported in Bangladeshis SLE patients (62%) (Anupam et al. 2009).

Recently, it has been reported that the detection of antibodies to β2-GPI is a better Indicator in APS manifestations than the detection of aCL (Fanopoulos et al. 1998). In the present study, the prevalence of a anti-β2-glycoprotein I antibodies in SLE Sudanese patients was 21%, The prevalence of anti-beta2 GPI in Colombian (21%) and Spaniards (18%) patients with SLE (Cucurull et al. 1999) which is similar to that observed in our study, In the other hand the prevalence of anti-beta2 GPI in Turkish patients (83%) (Mehmet et al., 2007) which is higher than the present study.

In different populations, the difference of results may be due to the modality used in the measurement of antibodies, the study population, ethnic-racial differences and variation of positive criteria with respect to investigators (Aguirre et al. 2001).

The DNA isolated from blood specimens of patients and control group, was measured by spectrophotometer at wave length 260nm for DNA and 280 for protein, the ratio calculated from two reading. The minimum and maximum concentrations were 50 µg/µl and 257 µg/µl respectively and the mean was 98.3 µg/µl. The DNA ratio was in range 1.3-1.9 with mean 1.62.

The concentrations and ratio of all DNA specimens were within the accepted range used in PCR-SSP technique according to protocol of Micro SSP HLA DNA typing kit (One Lambda Inc. USA).
DRB1*15, DRB1*08, DQB1*05 and DQB1*04 were considered as risk alleles for SLE, with relative risk 1.42, 1.52, 1.37 and 2.10 respectively. Different investigators were reported the same results such as Saleh Al-Motwee et al., 2013 (Al-Motwee et al. 2013), Khaled Ayed et al., 2004, Shimane et al., 2013, Reveille et al., 1998, Sirikong et al., 2002, and Pan et al., 2009 (Pan et al. 2009), who showed a positive association between DRB1*15 in Saudi, Tunisians, Japanese, African Americans, Thais and Taiwanese in SLE patients respectively. Shimane et al., 2013 and Reveille et al., 1998 were reported that DRB1*08 associated with Japanese and Hispanics SLE patients respectively. Also DRB1*1503, and DRB1*08 alleles were more frequently found in, African Americans, and Hispanics, respectively (Reveille JD el al., 1998).

In the present study DRB1*13 and DQB1*06 were considered as protective alleles for SLE, This is in agreement with Furukawa et al. (2014) (Furukawa et al. 2014) and (Vasconcelos et al. 2009) who reported protective association between the carrier frequencies of HLA-DRB1*13 allele in SLE in Japanese and Portuguese population respectively, This agreement of our molecular data and the data of other previous studies may be useful in molecular diagnose of SLE and early detection of disease in our society.

The HLA system shows phenomenon called linkage disequilibrium, in which certain alleles occur together in the same haplotype more often in some population. The most extreme example is in Caucasians where the HLA-A1, B8, DR3 (DRB1*0301), DQ2 (DQB1*0201) haplotype is so conserved that even the alleles at the complement genes (Class III) can be predicted with great accuracy. Similar haplotypes are observed in selected caste groups and tribal groups of India (Shankarkumar et al., 1999). Also, at HLA Class II, this phenomenon is so pronounced, that the presence of specific HLA-DR alleles can be used to predict the HLA-DQ allele with a high degree of accuracy before testing. Because of
linkage disequilibrium, a certain combination of HLA Class I antigen, HLA Class II antigen and Class III products will be inherited together more frequently than would normally be expected. It is possible that these “sets” of alleles may be advantageous in some immunological sense, so that they have a positive selective advantage (Shankarkumar. 2004).
5.2 conclusions
In conclusion,

- Regarding the genetic profile:
  DRB1*15, DRB1*08, DQB1*05 and DQB1*04 may be considered as risk alleles and DRB1*13 and DQB1*06 can be considered as protective alleles.

- Regarding the immunological profile:
  anti ANA, anti-Sm and anti dsDNA are significantly important serological markers for the diagnosis of Sudanese patients with SLE and There is moderate levels of aPLs antibodies in SLE Sudanese patients.

5.3 Recommendations
- Change the policy of diagnose of SLE by introducing anti smith to be considered as more specific immunological marker, antiphospholipid antibodies to be tested in all SLE patients, also introduce the molecular diagnose of SLE.

- HLA typing investigations on families with history of SLE for screening the disease which minimize mortality and morbidity in our society.
Chapter six

6. Reference

References


Froelich, C.J., Wallman, J., Skosey, J.L., & Teodorescu, M. 1990. Clinical value of an integrated ELISA system for the detection of 6 autoantibodies (ssDNA, dsDNA, Sm, RNP/Sm, SSA, and SSB). *J.Rheumatol.*, 17, (2) 192-200 available from: PM:2108246


Appendix I

ELISA wash buffer:
The wash buffer is 10x concentrate
Wash buffer 100ml
Distilled water 900ml

Reagent and equipment for gel:

10% TBE 1X
Tribase 5.4gm
Boric acid 2.75gm
EDTA 0.36gm
D.W 500ml

2.5% agarose
Agrose 1.25gm
10%TBE 50ml

Ethidium bromide (10µl/ml) 25 µl
Appendix II

HLA class II in SLE patients

Questionnaire

Serial No. : …………………
Name:…………………………………………………………

Sex: ……………………………………..            Age: ……………………….
Tribe: ……………………………
Tel No. : ………………………………

Clinical manifestation:

………………………………………………………………………………………
………………………………………………………………………………………
………………………………………………………………………………………

Investigations:

CBC:
Hb:                                 TWBCs:                           PLts:

ANA profiles:
ANA   Anti AMA
Anti ribosomal p.protein   Anti Jo 1
Anti centeromere   Anti PCNA
Anti SCL 70   Anti dsDNA
Anti nrp   Anti smith
Anti SSA   Anti SSB
Anti Ro52   Anti nucleosome
Anti histone

Other investigations: