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Association of Human Leukocyte Antigen (-DR3,-DR4,-B27) and Autoantibodies profile among Autoimmune hepatitis patients

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By

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{قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا
إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ
الْحَكِيمُ }

صدق الله العلي العظيم

(البقرة الآية 32)

Dedication

To the spirit of my father and mother.....

To those who always supported me.....

My family

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Last, I am indeed grateful to my family for their understanding, and their invaluable help accomplishing this work.

Abdulrazzaq Abdulah

Supervisor's Certification

We certify that this **thesis (Association of Human Leukocyte Antigen (-DR3,-DR4,-B27) and Autoantibodies profile among Autoimmune hepatitis patients)** was prepared under our supervision at the Department of Microbiology, College of Medicine / Al-Qadisiya University, as a partial fulfillment of the requirement for the degree of philosophy in Science of Medical Microbiology.

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We, the members of examining committee, certify after reading this thesis { **Association of HLA(-DR3,DR4, B27) and autoantibodies profile among autoimmune hepatitis Patients** } and after examining the student in its contents, it was found adequate for the degree of PhD of Science in Medical Microbiology.

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Abbreviations:

Abbreviations term	Meaning
Ad-2D6	Adenovirus-vector expressing humanCYP2D6
AIH	Autoimmune hepatitis
AILD	Autoimmune liver disease
ALT	Alanine transaminase
AMA	Antimitochondrial antibodies
ANA	Antinuclear antibodies
APC	Antigen presenting cells
ASC	Autoimmune sclerosing cholangiti
ASMA	Anti smooth muscle antibody
AST	aspartate aminotransferase
CI	Confidence interval
CTLA- 4	Cytotoxic T lymphocyte antigen-4 gene
CYP2D6	Cytochrome P450 2D6
DEPC	Diethylpirocarbonate
DILI	Drug induce liver injury
FTCD	Formimino transferase cyclodeaminase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
IAIHG	International Autoimmune Hepatitis Group
IFN	Interferon
INR	International normalised ratio
LKM	Liver-kidney-microsomal antibody
MHC	Major histocompatibility complex
OR	Odds ratio
p-ANCA	Perinuclear anti-neutrophylic cytoplasm antibodies
PBC	Primary biliary cirrhosis
PSC	Primary sclerosing cholangitis
SD	Standard deviation
SLA	Anti-soluble liver antigen
SLE	Systemic lupus erythematosis
SMA	Smooth muscle antibodies
T1DM	Type 1 diabetes mellitus
TNF	Tumor necrosis factor

Summary

Autoimmune hepatitis (AIH) is a chronic, immunologically mediated inflammatory liver disorder of unknown etiology. AIH is so far thought to be an auto-immune disease. This disease has revealed characteristics regarding predisposing factors and auto-antibodies profile.

One of the genetic predisposing factors is thought to be HLA-DR3 and DR4 genes. In addition, there is an overlapping in auto-antibodies production during the course of disease.

The present study aimed to investigate the frequencies of HLA-DR3, DR4 and HLA-B27 genes and evolution of (Anti-nuclear antibody (ANA), Soluble liver antigen (SLA), Anti mitochondria antibody (AMA) and Liver cytosolic (LC1)) in a group of 60 Iraqi patients (19 males and 41 females) who have been already diagnosed with AIH in the GIT Center of Al-Diwaniyah Teaching Hospital, Gastrointestinal tract (GIT) center in Al-Hussain hospital / Karbala, and Gasteroentestinal and Hepatology Teaching Hospital/Baghdad compared to a group of apparently healthy (control group N= 50) individuals (16 males and 34 females).

The Real-time PCR was used for the HLA-genes and ELISA was also used to estimate the auto antibodies quantitatively in all subjects of this study. The age of the patients were ranged from 7-69 years in AIH group and from 10-67 years in healthy controls group. The median value concentration of ANA (U/ml) were; 20.9 and 4.8 among AIH patients and healthy group respectively, with significant difference ($P< 0.001$). An interesting receiver operating characteristic values (ROC)

(0.804) found for this auto-antibody as moderate diagnostic test in this aspect.

The anti-Soluble liver antigen (SLA) auto-antibody occurred in median concentration of 8.3U/ml and 5.2 U/ml among AIH patients and healthy group respectively. It was significantly differed in the two groups when compared to each other; with high significant difference (P = 0.005) in the differentiation of the AIH patients group from healthy controls group with ROC (0.655).

For anti-Liver kidney microsomal auto-antibody, the median concentration of 7.5 U/ml and 3.6 U/ml among AIH patients and healthy group, respectively, statistically significant difference when compared among the two studied groups, both classes had provided ROC (0.644).

On the context of genotyping of HLA- genes, DR3, DR4, and B27 were found to be different in their frequencies significantly among AIH patients that created high etiological fraction of 0.504, 0.583, and 0.129 respectively compared to healthy controls, with odd ratio (OR) 7.35 for DR3, high OR 8.0 for DR4 and 2.23 for B27.

The frequencies of these genes in AIH patients are highly significant, which are different between patient group, for DR3 compared to healthy group which 58.3% and 16% respectively, and very high significantly differed between patient group for DR4 compared to healthy group which 66.7% and 20% respectively, while no significant differed between patient group compared to healthy group for B27 which 23.3% and 12% respectively.

Moreover, we have studied the validity topic of auto-antibodies parameters represented by sensitivity, specificity and accuracy. The clinical relevant of auto-antibodies parameters have also been estimated

using positive predictive values (PPV) and negative predictive values (NPV). The highest validity parameters of Anti-nuclear antibody (ANA), Soluble liver antigen (SLA) and Liver kidney microsomal (LKM) autoantibody occurred at cut-off values of 9.20 U/ml, 7.85 U/ml and 4.70 U/ml, respectively, where used to predict AIH, whereas, the total serum bilirubin, direct serum bilirubin, SGPT, SGOT and Alkaline phosohatase cut off were 1.15, 0.65, 35.5, 33.5 and 92.5(U/ml) for detection of the diseases in this study.

Also this study showed significant difference of association between HLA-DR3 and other autoantibodies (AMA and SLA) with p value (0.006 and 0.001), respectively, while there are no significant differences association between the HLA-DR3 and other autoantibodies.

From the result of this study, it was concluded that the strong evidence that HLA-DR3 and DR4 genes are common predisposing factor and play a major role in the pathogenesis for AIH disease, and the ANA, SLA, and LKM auto-antibodies profile AIH also are common predisposing factor.

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Introduction and Literatures Review

1. Introduction and Literatures Review

1.1. Introduction

Autoimmune liver disease (AILD) consists of autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). It is characterised by the immune mediated injury to the hepatocytes and bile ducts, hepatocyte injury is the predominant features in autoimmune hepatitis and biliary injury (Wang, and Zheng, 2013).

Women are affected more frequently than men with a sex ratio of around 4:1(van Gerven *et al*., 2014). In women a bimodal age pattern is usually seen, one in the late teens and one around the menopause but it should be stressed that disease can develop in all age groups and both genders (Gronbaek *et al*., 2014).

Autoimmune hepatitis (AIH) is a serious autoimmune liver disease that is characterized by a progressive destruction of the liver parenchyma and the development of chronic fibrosis (Czaja, 2015). An estimated 100,000 to 200,000 persons are currently affected by AIH in the USA (Czaja, 2006) and, according to the World Health Organisation, AIH has an annual incidence of approximately 2 in 100,000 individuals and a prevalence 15 cases per 100,000 persons worldwide (World Health Organization, 2016).

Thus, the prevalence and incidence of AIH is similar as for the two other major autoimmune liver diseases, primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). As in most autoimmune diseases, AIH has a female predominance (sex ratio, 3.6:1). It occurs in children and adults of all ages and affects several ethnic groups (Manns *et al*., 2010).

It is also associated with other autoimmune conditions such as hypothyroidism, ulcerative colitis, type 1 diabetes mellitus, rheumatoid arthritis, celiac disease or skin disorders such as vitiligo. Therefore, obtaining a detailed history especially family history of autoimmune conditions is important in the assessment of patients with potential AIH diagnosis (Zachou *et al*., 2013).

Blood tests can show signs of hepatitis with raised alanine transaminase (ALT, U/L) (usually less than 500 U/L), aspartate transaminase (AST, U/L) and occasionally bilirubin (mmol/L). Typical immunology profile in AIH patients are raised IgG with positive antinuclear antibody (ANA), anti-smooth muscle antibody ASMA (in type 1 AIH) and anti- Liver-kidney-microsomal LKM (in type 2 AIH). Patients with acute presentation of AIH should be monitored for synthetic function such as international normalised ratio (INR) and albumin.

Liver biopsy is recommended at the time of presentation to establish the diagnosis as well as to guide the treatment, however in patients presenting with acute hepatitis who has suspicious diagnosis of AIH, the treatment should not be delayed (Manns *et al*., 2010).

As regards the immunogenetic background of AIH, HLA-DR3 (recently split into DR17 and DR18) and HLA-DR4 are associated with type 1 AIH. In Japan, HLA-DR4 is frequently found in AIH patients, as has been shown in European or North American Caucasoid patients. However, HLA-DR3–positive AIH is quite rare, because the prevalence of DR3 is extremely rare in the normal Japanese population. In a report on North American patients, the clinical features of HLA-DR4–positive AIH differed from those of HLA-DR4–negative patients. In addition, the clinical features of AIH in elderly patients differed from those of

younger patients (Miyake *et al*., 2007). Recently, a lower frequency of HLA-DR4 and a higher frequency of histologically acute hepatitis were reported in adolescent and early adulthood AIH (Furumoto *et al*., 2015).

1.1.1. Aim and objectives

In Iraq, the autoimmune hepatitis is highly increasing compared with the last two decades. Shedding light on the prevalence of certain HLAs and the validation some auto-antibodies among Iraqi patients with autoimmune hepatitis was the aim of this study.

Our objectives were:

1- The evaluation of the risk of having AIH by three selected HLA; HLA-DR3,-DR4,-B27 using real time PCR.
2- The validation of some routinely –used, selected serological markers (ANA, AMA, SLA, LC1, and LKM) among AIH patient.
3- The diagnostic value and the strength of the linear correlation between studied parameters.
4- Assessment of HLA genotype impact on autoimmune profile of patients with AIH.

1.2. Literatures review

1.2.1. Autoimmune Diseases

Autoimmune diseases are a broad range of related diseases as a clinical syndrome caused by the activation of T-cells, or B-cells, or both, in the absence of a continuing infection or other discernible cause, in which a person‘s immune system produces an inappropriate response against its own cells, tissues, and/or organs, resulting in inflammation and damage (World Health Organization, 2007). The development of autoimmune diseases depends on a combination of genetic factors and environmental factors. There are over 80 different autoimmune diseases, and these ranges from common to very rare diseases. Most autoimmune diseases are thought to be polygenic, involving more than one gene. Autoimmune diseases appear to be either systemic (e.g. case of systemic lupus erythematosis, SLE) or organ-specific as in the case of type 1 diabetes mellitus (T1DM), (Hantoosh, 2015; Khlebos, 2013).

1.2.2. Autoimmune Hepatitis

Autoimmune liver disease (AILD) is an umbrella term for diseases caused by immune mediated reaction to either hepatocytes or bile ducts. Regulatory T cells (T-reg) play an important and essential role in the maintenance of homeostasis and prevention of autoimmune responses. Autoimmune hepatitis (AIH) is caused by an immune mediated injury of the hepatocytes and characterized by presence of lobular and interface hepatitis on liver histology, presence of hyper gammaglobulinaemia and high titer of antinuclear antibodies (ANA) and other antibodies such as (ASMA, LKM, and SLA) in the serum with elvated transferase level on liver biochemistry (Wang and Zheng, 2013).

Autoimmune hepatitis (AIH) is an immune mediated, chronic inflammatory disease of unknown aetiology, mainly affecting the hepatocytes. It was first defined in 1950 by Waldenstrom when he described a chronic hepatitis in young woman which eventually lead to liver cirrhosis (Strassburg and Manns 2011). It is characterized by the morphological changes of interface hepatitis on liver biopsy, hypergammaglobulinaemia and the presence of high circulating ANA in the serum. It is more common in women (around 75%) and can affect at any age from young to elderly (Zachou *et al*., 2013; Aizawa and Hokari 2017).

Autoimmune hepatitis (AIH) was first described in 1951 (Waldenstrom, 1952) as a chronic hepatitis of young women with hypergamma- globulinemia in the absence of cirrhosis, which responds well to adrenocorticotrophic therapy (ACTH). In 1956 the association with anti-nuclear antibodies (ANA) was discovered and the term ‘‘lupoid hepatitis’’ was created (Mackay *et al*., 1965). Autoimmune hepatitis (AIH) is the third most common inflammatory chronic liver disease in man preceded by HBV infection and HCV infection (Czaja, 2005a).

Autoimmune hepatitis (AIH) is a relatively uncommon disorder, which can lead to a potentially fatal liver dysfunction. Although AIH shows prominent predilection towards young women, it can affect males and females in all ages. The median age of diagnosis is 39 year in man and 49 years in women (Al-Chalabi *et al*., 2008). In the majority of cases the disease follows an insidious, fluctuating course, and presents with non-specific clinical symptoms such as malaise, anorexia, jaundice and arthralgias. Especially in older patients, well established liver

cirrhosis is often present at the time of diagnosis. In smaller group of cases the initial presentation is dominated by symptoms of acute or fulminate hepatitis (Strassburg and Manns, 2006).

Autoimmune hepatitis is an inflammatory liver disease primarily affecting women and is characterized by elevated transaminase levels, the presence of specific auto antibodies, raised IgG, and interface hepatitis on histology. Although the aetiopathogenesis of AIH is unclear, several autoimmune pathways have been proposed which are largely based on auto reactive CD4+ T lymphocytes recognizing a liver-specific auto-antigen (Manns *et al*., 2010).

Autoimmune hepatitis may present acutely in approximately 40% of cases and may resemble acute viral hepatitis (Manns *et al*., 2010). It is a non-resolving chronic liver disease that affects mainly women and is characterized by hyper gammaglobulinaemia even in the absence of cirrhosis, circulating autoantibodies, association with human leukocyte antigens (HLA) DR3 or DR4, interface hepatitis on liver histology, and a favorable response to immunosuppression. The disease, if untreated, often leads to cirrhosis, liver failure and death (Zachou *et al*., 2013 and Manns *et al*., 2015a).

Autoimmune hepatitis is a chronic, progressive, and sometimes fluctuating necroinflammatory liver disorder of unknown origin (Unnithan *et al*., 2004). It is characterized by immunologic and autoimmune features, including the presence of circulating autoantibodies and elevated serum globulin levels, a heterogenous clinical picture, and a response to therapeutic immunosuppression. In addition, there is a significant predisposition of the female sex and a significant association with the presence of HLA DR3 and DR4 alleles among affected patients (AL- Obeidy *et al*., 2008).

The main circulating autoantibodies, are anti-nuclear antibodies (ANA), anti-smooth muscle antibodies (ASMA), anti-liver kidney microsome antibodies (anti-LKM) and anti liver cytosol antibody (anti-LC). (Manns, *et al*., 2010). The detection of non organ and liver related auto antibodies remains the hallmark for the diagnosis of the disease in the absence of viral, of chronic hepatitis or hepatic injury (AL–Mammori *et al*., 2012).

Table (1-1): A summary of the phenotypic markers of autoimmune liver disease (Andrew, 2014).

Parameter	Autoimmune hepatitis	PSA	PBC
Age	***All age group***	***>45 years***	***Usually >40***
Gender	***Female:Male Type1 4:1 Type 2. 9:1***	***Female:Male 9:1***	***Female:Male 3:7***
ANA	***1:40 titre in 70-80% Disease specific***	***Disease specific ANA in 30-50%***	***Non-specific ANA in 70-80%***
Anti-LKM 1	***3-4% (categorises as type 2 AIH)***	***Not present***	***Not present***
Panca	***90%***	***Not present***	***26-94%***
Interface hepatitis	***Characteristic***	***Occasionally present***	***Occasionally present***
Response to immunosuppression	***Yes***	***No***	***No***

PSA: Primary sclerosing cholangitis, **PBC**: Primary biliary cirrhosis

Anti mitochondrial antibody characterize classical , type 1 Autoimmune hepatitis, ASMA are also frequently detected in type 1 as well as p-ANCA in some cases , Although AMAs are specific and sensitive diagnostic markers for primary biliary cirrhoses (PBC) also can be detected in about 15% of AIH patients in association with type 1,

and type 3 with specific liver proteins. Liver Kidney Microsome (LKM) is more specific, can be subdivided into two subtypes (2a, and 2b (if the patients have HCV antibody)) (AL–Mammori *et al*., 2012) table (1-1).

1.2.3. Clinical presentations of AIH

Most patients present with nonspecific symptoms such as fatigue, arthralgia and anorexia at the time of presentation. About 25% of patients present asymptomatically (Liberal *et al*., 2014) and the majorities of patients present late with symptoms of portal hypertension and decompensate cirrhosis. Recent systematic review mentioned that around 25 % of elderly patients (age 60 to 65) were more likely to present asymptomatically (Chen *et al*., 2014).

Autoimmune hepatitis can also present during pregnancy or postpartum period. Physical examination can either be normal, or show hepatomegaly, splenomegaly or signs of chronic liver disease. In some patients, AIH present as acute severe hepatitis and rarely, they progress to fulminant form and require liver transplantation (Strassburg and Manns, 2011).

1.2.4. Types of autoimmune hepatitis

There are two classical types of AIH and probably a type III AIH:

- **Type I autoimmune hepatitis:** is characterized by the presence of smooth muscle antibody (SMA) and/or ANA in the serum. Type I disease occurs at any age but very often has a bimodal distribution. 78% of patients are women (female-to-male ratio, 3.6:1), and 41% have concurrent extra-hepatic immunologic diseases, including autoimmune thyroiditis (12%), Graves disease (6%), ulcerative colitis (6%) rheumatoid arthritis, pernicious anaemia, systemic sclerosis, Coombs' positive

haemolytic anaemia, idiopathic thrombo-cytopaenic purpura, leukocytoclastic vasculitis, nephritis, erythema nodosum, and fibrosing alveolitis (1% each). The occasional presence of ulcerative colitis compels cholangiography to exclude PSC (Muratori *et al*., 2009).

Forty percent of patients with severe type I disease has an acute onset of symptoms, and the disease may present in a fulminant fashion. Typically, patients with an acute presentation often have clinical signs of cirrhosis with ascites, oesophageal varices, with thrombocytopaenia, hypoalbuminemia, hypergammaglobulinaemia and histological changes of cirrhosis that indicate chronic liver disease. This acute presentation with changes reflecting chronic liver disease reflects a pre-existent subclinical disease that is unmasked by disease progression or a spontaneous exacerbation. 8% of patients have no features of chronicity and are initially indistinguishable from an acute viral or toxic hepatitis (Buchel, 2007).

- **Type II Autoimmune hepatitis:** is characterized by the presence of anti-LKM 1 in the serum and is far less prevalent than type I. Type II AIH afflicts mainly children between 2 and 14 years old and only 20% of patients are adults. Type II has clinical, laboratory, genetic and prognostic differences from type I (Muratori *et al*., 2009).
- **Type III autoimmune hepatitis**: is characterized by the presence of an anti-soluble liver antigen/ liver/pancreas (anti-SLA/LP). Patients with type III AIH usually lack ANA and anti-LKM1, but commonly have SMA (35%),

mitochondrial antibodies (22%), rheumatoid factor (22%) and antibodies to liver-membrane antigen (26%).

Three types of anti-liver-kidney microsomal (anti-LKM) antibodies have been described in inflammatory liver diseases. The anti-LKM-1 antibody is a serologic marker of Type II AIH. The target of the anti-LKM-1 antibodies is the cytochrome 450 2D6 oxidase (CYP2D6), which can be localized to hepatocytes and to proximal renal tubular cells. Due to their similar staining patterns, anti-LKM antibodies can be occasionally confused with anti-mitochondrial antibodies (AMA) on indirect immunoflourescence stains. However, the anti-LKM-1 pattern lacks reactivity to distal renal tubules and to stomach parietal cells. Anti-LKM-3 antibodies are directed against the microsomal glucuronosyl- transferase (UGT1A), and can be only seen in 10% of Type 2 AIH patients (Vitozzi *et al*., 2004).

The ANA distribution pattern and antigens detected in AIH are different from those seen in primary biliary cirrhosis (PBC), but can overlap with drug induced, alcoholic, or HCV hepatitis. Although non-specific, ANA represent an important diagnostic criterion of AIH, which is also incorporated into the AIH scoring system. Neither the ANA patterns nor the ANA titers however, have been shown to correlate with disease progression, therapeutic response or need for transplantation (Vitozzi *et al*., 2004).

Anti-soluble liver antigen (anti-SLA) antibodies can be detected in 10-50% of Type 1 AIH patients. Anti-SLA antibodies (table 1-2) are directed against the UGA repressor serine t-RNA cytoplasmic antigen (Vitozzi *et al*., 2004). While in adults anti-SLA is specific for Type 1 AIH, in children it could be associated with either Type 1 or Type 2

disease. High anti-SLA antibody titers are seen in severe AIH (Manns and Vogel, 2006). Whether the elevated anti-SLA levels actively contribute to hepatocyte death, or they are simply induced by the release of more intracellular antigens, still needs to be determined. In cases when serology for anti-SMA, ANA, and anti-LKM is negative, anti-SLA can help to establish the diagnosis. Some consider positive anti-SLA serology the hallmark of Type 3 AIH, but this category is not recognized by the official classification systems (Manns and Vogel 2006).

Table (1-2): Prevalence of commonly detected autoantibodies in autoimmune hepatitis (Invernizzi *et al*., 2007).

Antibody	Antigen	AIH	Overlap with other diseases
ANA	***Multiple nuclear targets***	***AIH-1(70-80%)***	***Drug, alcohol, hepatitis***
Anti-SMA	***Various cytoskeletal components***	***AIH-1(80%)***	***HCV, PBC***
Anti-LKM	***Cyp2D6***	***AIH-2(100%)***	***HCV***
Anti-SLA	***UGA repressor t-RNA associated protein***	***AIH-1, AIH-2 in pediatric,(AIH3 ?)***	***HCV, autoimmune cholangitis***
Anti-LC1	***Fomiminotansferase cyclodeaminase***	***AIH-2(30-50%), AIH-1***	***HCV***
Panca	***Unknown***	***AIH-1(65-95%)***	***PSC, HCV***

Type I AIH is associated with the HLA- DR3 or the HLA- DR4 serotypes in northern European and North American patients. The

HLA-DR3 haplotype is associated with early-onset, severe AIH, while HLA-DR4 associates with milder disease that has a better response to corticosteroid therapy (Krawitt, 2006). HLA-DR3 occurs more frequently in type I AIH in South America, and is associated with poor response to therapy (Czaja *et al*., 2002). Susceptibility in Japanese patients is linked to HLA-DR4 (Czaja, 2005a).

On the other hand, type II AIH has been associated with the HLA-DQ alleles in Canadian and French paediatric patients, while HLA-OR seems to be protective in northern European caucasians (Krawitt, 2006).

Antibodies to liver cytosol type 1 antigen (anti-LC1) are found in over 30% of Type 2 AIH patients. These antibodies directed against the 58 kD formimino transferase cyclodeaminase (FTCD) protein. Anti-LC1 shows significant correlation with disease activity and could be used as a marker of residual inflammation (Manns and Vogel, 2006).

1.2.5. Etiology of autoimmune hepatitis

The etiology of autoimmune hepatitis is unknown, though both genetic and environmental factors are likely to be involved. An immune response targeting liver auto antigens is thought to initiate and perpetuate the liver damage. Several genetic factors interact to influence susceptibility to AIH, clinical manifestations, response to treatment and overall prognosis. The strongest genetic associations are found within genes of the human leukocyte antigen (HLA) region (the human major histocompatibility complex, MHC) which located on the short arm of chromosome 6, which are involved in the presentation of antigenic peptides to T cells, and are therefore implicated in the initiation of an adaptive immune response (Liberal *et al*., 2011).

The aetio- pathogenesis of AIH is poorly understood, but there is some evidence to suggest that a numerical and functional impairment of T-regulatory cells may be involved, in addition, a variety of genetic and environmental factors are involved in the development of the disease, including viruses (Saadi, and Hula, 2015).

According to several observations, attention has been focused on the possibility that genetic background could play a crucial role in the susceptibility to AIH. The importance of participation genetic factors in the disease is currently based on the increased risk of autoimmune disease in first- degree relatives of patients with AIH however few reports denoted that 15% of siblings and 10% of offspring were observed (Vergani and Mieli-Vergani, 2008), while increased frequency of specific genetic markers as certain human leukocyte antigen (HLA) has been reported in patients group other than in general population. Therefore; several studies in different areas were undertaken to test the possibility of association between this disease and one or several of the HLA, as suggested by Czaja and associates (Czaja, 2013b). Positive association with the Ag (HLA-A1) was reported in Caucasian patients, while another study reported other Ags (AL-Obeidy *et al*., 2009a).

There are particularly strong associations within the HLA-DR locus (Donaldson, 2004), with the HLA-DR3 and -DR4 molecules conferring susceptibility to AIH-1 in Europe and North America. The associations with HLA -DR3 and -DR4 are considered strong enough to contribute to the diagnosis of AIH according to the revised diagnostic scoring system designed by the International Autoimmune Hepatitis Group (IAIHG) (Alvarez *et al*., 1999). HLA-DR7 and -DR3 confer susceptibility to AIH type-2. Patients positive for HLA- DR3 have a more aggressive form of the disease with worse overall prognosis (Ma

et al., 2006). The HLA-DR3 haplotype is associated with early-onset, severe AIH, while HLA-DR4 associates with milder disease that has a better response to corticosteroid therapy (Lim *et al* 2001; Krawitt 2006).

HLA-DQB1has also been linked to the development of AIH-2, although this allele is in linkage disequilibrium with DRB1, both associated with AIH-2 (Djilali-Saiah *et al*., 2004; de Boer *et al*., 2014). On the other hand, type II AIH has been associated with the HLA-DQBl alleles in Canadian and French pediatric patients, while HLA-DR2 seems to be protective in northern European Caucasians (Krawitt, 2006).

HLA-DR is associated with type II AIH in Brazil and Germany. German patients furthermore had higher frequencies of HLA-DR and HLADQ (Bittencourt *et al.,* 2003; Teufel *et al*., 2006).

As the case with most other autoimmune diseases, AIH shows a female preponderance of 4: 1 in Caucasoid populations. There is some evidence suggesting that hormonal differences, gender specific responses, exposure to drugs and toxins contribute to the higher prevalence in women. Environmental agents, infections and pregnancy could also play a role in triggering disease. Despite the definite female preponderance, there seems to be no difference in the clinical expression of AIH between sexes (Czaja, 2007; McFarlane, 2008).

1.2.6. Gender effects

The female predisposition for autoimmune hepatitis and autoimmune disease in general is unexplained (McFarlane and Heneghan, 2004). HLA DR4-positive women with type 1 autoimmune

hepatitis has a greater variety of HLA DR4 alleles associated with their disease than HLA DR4-positive men (Czaja and Donaldson, 2002).

Women may thereby have a greater facility to be sensitized to self or foreign antigens than men. They may also be exposed to unique antigens, and/or they may respond to common antigens against, which men do not react. Triggering factors unique to the female state may include drugs, toxins, infections, and environmental antigens. Pregnancy is a unique state of women and protracted exposures to fetal cells or antigens in the maternal circulation may stimulate autoreactivity through microchimerism. Estrogen levels do modulate immune reactivity, and they may be contributory to the autoimmune propensity in women. High estrogen levels favor a type 2 cytokine response which drives activated immunocytes towards antibody production and an anti-inflammatory effect. Normal or low estrogen levels promote a type 1 cytokine response which drives the clonal expansion of tissue infiltrating cytotoxic T cells and causes liver damage. The female propensity for autoimmune hepatitis is apparent among children and among pre- and post-menopausal adults (Béland *et al*., 2009).

1.2.7. Epidemiology of AIH

Autoimmune hepatitis occurs in all races and in all geographical areas and considered relatively rare, as its prevalence ranges from 16 to 18 cases per 100,000 inhabitants in Europe (Delgado *et al*., 2013; van Gerven *et al*., 2014; Ngu *et al*., 2010). For instance, prevalence rates of 42.9 cases per 100,000 and 24.5 cases per 100,000 inhabitants have been reported in Alaska natives (Hurlburt *et al*., 2002), and New Zealand (Ngu *et al*., 2010).

The reported prevalence of AIH ranges from 10 to 17 per 100 000 in Europe and appears to be similar to that of primary biliary cirrhosis (Werner *et al*., 2008). AIH has been described in many ethnic groups and seems to be a worldwide disease (Verma *et al*., 2007). Women are affected 3-4 times more frequently than men. Although initially thought to be particularly prevalent in young women, the disease appears to affect all age groups and, in the UK, may actually be more common in older than in younger patients (Al-Chalabi *et al*., 2006).

Autoimmune hepatitis is described as a disease of young women, the patient group in which the disease was initially reported. A female predilection has been confirmed in almost all studies with a female to male ratio of around 3:1 across the world. The age of manifestation of AIH varies greatly from as early as the first year of life up until the eighties (Lohse and Mieli-Vergani, 2011).

The relationship between drug induce liver injury (DILI) and AIH is complex and not fully understood (Weiler-Normann, and Schramm, 2011; Castiella *et al*., 2014).

1.2.8. Associated autoimmune conditions

Autoimmune hepatitis is associated with the presence of a wide variety of other autoimmune or immune-mediated diseases. Actually, concurrent autoimmune diseases are common in patients with AIH and mirror the full range of known autoimmune diseases. Therefore, an extended diagnostic screening for other autoimmune diseases, especially autoimmune thyroiditis, seems reasonable in patients with AIH, both at diagnosis and at regular intervals during follow-up (Teufel *et al*., 2010). In addition to the patient being affected by immune-mediated diseases, their occurrence is also more frequent in first-degree

relatives of AIH patients, and therefore a careful family history should be undertaken (Panetta *et al*., 2012;Gronbaek *et al*., 2014).

Concomitant extrahepatic autoimmune disorders are found in approximately 13% of the AIH cases. These are most prevalent in the pediatric and in elderly patient groups (Strassburg and Manns, 2006). The list of rheumatic conditions frequently observed in AIH includes rheumatoid arthritis, Sjogren's syndrome, autoimmune pericarditis, mononeuritis multiplex and systemic lupus erythematosus. Felty's syndrome, defined by the classical triad of neutropenia, splenomegaly, and rheumatoid arthritis can also is associated with AIH (Czaja, 2007).

Autoimmune hepatitis is detected in 10-20% of patients with the Autoimmune-Endocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) syndrome (Vogel *et al*., 2002).

1.2.9. Occurrence and ethnic variability

Autoimmune hepatitis has a global occurrence, and it has been described in African Americans, native Alaskans, Arabs, Asians, Europeans, Iranians, South Americans, and subcontinental Indians. Its incidence among white northern Europeans is 1.9 cases per 100,000 persons per year, and its point prevalence is 16.9 cases per 100,000 persons per year (Boberg, 2002).

In the United States, autoimmune hepatitis affects 100,000 to 200,000 persons, and it accounts for 5.9% of the liver transplantations in this country and 2.6% of the liver transplantations in Europe (Strassburg, 2013). The frequency of autoimmune hepatitis among patients with chronic liver disease in North America is between 11% and 23%. Its prevalence among Alaskan natives is 43 per 100,000

population and higher than that reported elsewhere (Hurlburt *et al*., 2002).

Race may affect disease severity as well as occurrence. Cirrhosis is present at accession more commonly in black North American patients with autoimmune hepatitis than in white North American patients (85% versus 38%), and hepatic synthetic function is decreased more frequently (Lim *et al*., 2001). Alaskan natives have a higher frequency of acute icteric disease, asymptomatic illness, and advanced fibrosis at presentation than non-native counterparts (Hurlburt *et al*., 2002). Japanese patients typically have mild, late onset disease that can respond to non-steroidal medication such as ursodeoxycholic acid (Nakamura *et al*., 1998).

South American patients are younger than white North American counterparts, and they have more severe laboratory abnormalities (Czaja *et al*., 2002). African, Asian and Arab patients have early age onset disease, and they have a higher frequency of laboratory findings, greater occurrence of biliary changes on histological examination, and poorer initial response to standard therapy than other ethnic groups (Zolfino *et al*., 2002). These findings suggest that geographical location and ancestry affect occurrence and behavior of the disease. Interwoven into the natural history of the disease in each racial group and geographical region are cultural and socioeconomic factors that remain unsorted. Differences in the consequences of the liver disease must be correlated with delays in diagnosis or difficulties in accessing medical care that are region-specific (Czaja *et al*., 2005).

1.2.9. Pathogenic mechanisms

The etiology of AIH remains unknown and fundamental questions regarding disease pathogenesis remain to be resolved. It is generally

believed that AIH occurs in a genetically susceptible host as the consequence of an exaggerated immune reaction towards hepatic tissue. Such a response can occur when effectors lymphocyte responses are abundant and inappropriate leading to tissue damage, or, alternatively, when there is a numerical and/or functional defect in regulatory T cells (Treg) controlling such responses. This defect is more obvious at disease presentation than during treatment induced remission, where a partial recovery is observed (van Gerven *et al*., 2016).

Autoimmune hepatitis carries all features of an autoimmune disease: genetic predisposition, association with other autoimmune diseases, spontaneous disease fluctuations, autoantibodies, and auto-reactive T-cells, inflammatory infiltrate, and a good response to immuno- suppression. As in other autoimmune diseases, the etiology is not understood, nor the factors that may trigger a flare, and those that may lead to spontaneous remissions in some patients. The exact aetiopathogenesis of AIH is still unknown. It is a complex process that interlinking environmental and genetic factors in a susceptible host. The most common environmental trigger thought to cause AIH is viral infection (Makol *et al*., 2011).

The most popular hypotheses evoke a constellation of interactive factors that include a triggering agent, a genetic predisposition, and various determinants of autoantigen display, immunocyte activation, and effectors cell expansion (Czaja, 2001; Vergani *et al*., 2002).

Autoimmune Hepatitis is a disease of the liver that occurs when the body's immune system attacks cells of the liver. Anomalous presentation of human leukocyte antigen (HLA) class II on the surface of hepatocytes, possibly due to genetic predisposition or acute liver infection, causes a cell-mediated immune response against the body's

own liver, resulting in autoimmune hepatitis. This abnormal immune response results in inflammation of the liver, which can lead to further complications including cirrhosis (Liberal *et al*., 2015).

Histological examination of the liver tissue of patients with AIH reveals a large number of mononuclear cells, macrophages, plasma cells and lymphocytes infiltrating the portal tract and the adjacent parenchyma. T lymphocytes expressing the alph/ *B* T cell receptor predominate and the majority of these cells are positive for the CD4 helper/inducer phenotype and/or the CD8 cytotoxic/suppressor phenotype. A substantial number of natural killer (NK) cells, macrophages, and B cells can also be found (Bogdanos and Christen, 2008). Both T cells and B cells play an important role in the adaptive immune response to both self and non-self-antigen (Hubscher and dams, 2010).

Regulatory T cells (Treg) play an essential role in the homeostasis and prevention of autoimmune conditions (Wang and Zheng, 2013). In AIH, the number of Tregs is normal but their function is impaired (Hubscher and dams, 2010).

Autoimmune hepatitis (AIH) is initiated by the presence of auto antigen peptide onto antigen presenting cells (APC) which activates T helper cells (Th0) due to interleukins (IL 2 and 4). Upon activation of Th0, it can differentiate into Th1 and Th2 cellular pathway (figure 1-1). Th1 produces IL2 and Interferon gamma (IFN-r) which subsequently activates CD8 lymphocytes (Vergani *et al*., 2002).

Most patients present with nonspecific symptoms such as fatigue, arthralgia and anorexia at the time of presentation. About 25% of patient presents asymptomatically (Liberal *et al*., 2014).

1.2.9.1. Natural killer T (NKT)

NK T-cells are produced in the bone marrow rather than the thymus, lack antigen-specific receptors, and produce interferon (IFN-γ) and tumor necrosis factor (TNF-α). They are inhibited by cells with normal expression of the major histocompatibility complex (MHC) and by inhibitory receptors activated by glycolipid. Conversely, they target cells with aberrant MHC expression, defend against cells altered by viruses or cancer, and seem to promote hepatic regeneration. The immunoregulatory cytokines orchestrate immunocyte differentiation through cross-regulatory actions and result in cellular and humoral mechanisms of liver cell injury (Washington and Manns 2012).

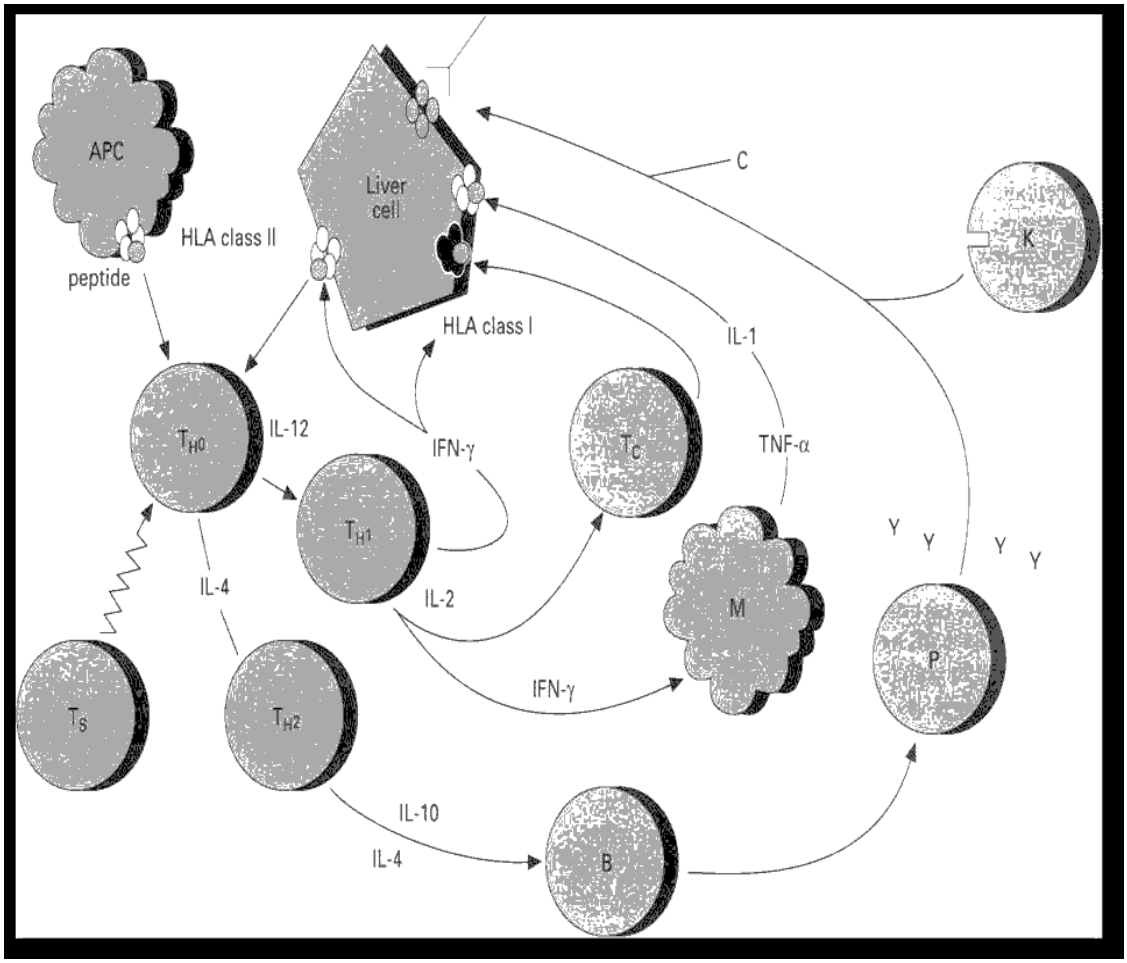


Figure (1-1): Pathogenesis of Autoimmune hepatitis (Corrigan *et al*., 2014).

1.2.9.2. Immune mechanisms

The liver is regarded as a lymphoid organ with unique immunological properties. Because of its location and function, the liver is continuously exposed to a large antigenic load that includes pathogens, toxins, tumor cells, dietary, and self-antigens. The liver contains large numbers of phagocytic cells, antigen presenting cells (APC) and lymphocytes and is a site for the abundant production of cytokines, complement components and acute phase proteins. The intrinsic lymphocyte population mainly resides in the portal tracts but is also scattered throughout the parenchyma, consisting of both cells of the innate (natural killer T cells, killer cells, and macrophages) and the adaptive (T and B cells) arms of the immune system. The blood entering the liver from the gut is rich in bacterial and dietary antigens that intermingle with lymphocytes (Manns *et al*., 2015b).

Immunoregulatory mechanisms are required to determine whether an antigen encounter will result in immunological unresponsiveness (tolerance) or reactivity. Liver autoimmunity implies loss of self tolerance. Programmed cell death - apoptosis - which is responsible for the normal turnover of hepatocytes and the elimination of liver cells and unwanted lymphocytes in inflammatory pathologies is also relevant to the breakdown and/or maintenance of liver tolerance. First, death by apoptosis allows for non-inflammatory elimination of cell components in contrast to necrosis, which is pro-inflammatory and potentially autoantigenic. Second, apoptosis is the mechanism whereby the immune system is “cleansed” of autoreactive T and B lymphocytes as illustrated by the process of “activation induced cell death”.

Various mechanisms have been proposed to account for the onset of an autoimmune liver response with no single initiating event being

able to explain all instances of autoimmunity. Two general conditions, however, should prevail: self reactive B and T lymphocytes must exist in the immunological repertoire and autoantigens must be presented in conjunction with MHC class II molecules by APC (Yeoman *et al*., 2009).

1.2.9.3. Cellular autoimmunity

The histological picture of interface hepatitis with its striking infiltrate of lymphocytes, plasma cells, and macrophages was the first to suggest an autoaggressive cellular immune attack in the pathogenesis of AIH. Whatever is the initial trigger, this massive recruitment of activated inflammatory cells is likely to cause damage. Immunohistochemical studies have identified a predominance of T lymphocytes mounting the α/β T cell receptor. Amongst the T cells, a majority are positive for the CD4 helper/ inducer phenotype, and a sizeable minority for the CD8 cytotoxic phenotype. Lymphocytes of non-T cell lineage are fewer and include (in decreasing order of frequency) natural killer cells (CD16/CD56 positive), macrophages, B cells, and plasma cells. The involvement of natural killer T cells is the focus of ongoing studies. There are different possible pathways that an immune attack can follow to inflict damage on hepatocytes (Diego *et al*., 2008).

1.2.10. HLA Associations

AIH is a “complex trait” disease, i.e. a condition not inherited in a Mendelian autosomal dominant, autosomal recessive, or sex-linked fashion. The mode of inheritance of a complex trait disorder is unknown and involves one or more genes, operating alone or in concert, to increase or reduce the risk of the trait, and interacting with

environmental factors. Susceptibility to AIH is imparted by genes within the major histocompatibility complex (MHC) - the human leukocyte antigen (HLA) region - on the short arm of chromosome 6, (figure 1-2), especially genes encoding HLA DRB1 alleles. Since the role of class II MHC molecules is to present peptide antigens to CD4 T cells, HLA class II antigen presentation with ensuing T cell activation is likely to be involved in the pathogenesis of AIH (Vergani and Mieli-Vergani, 2008).

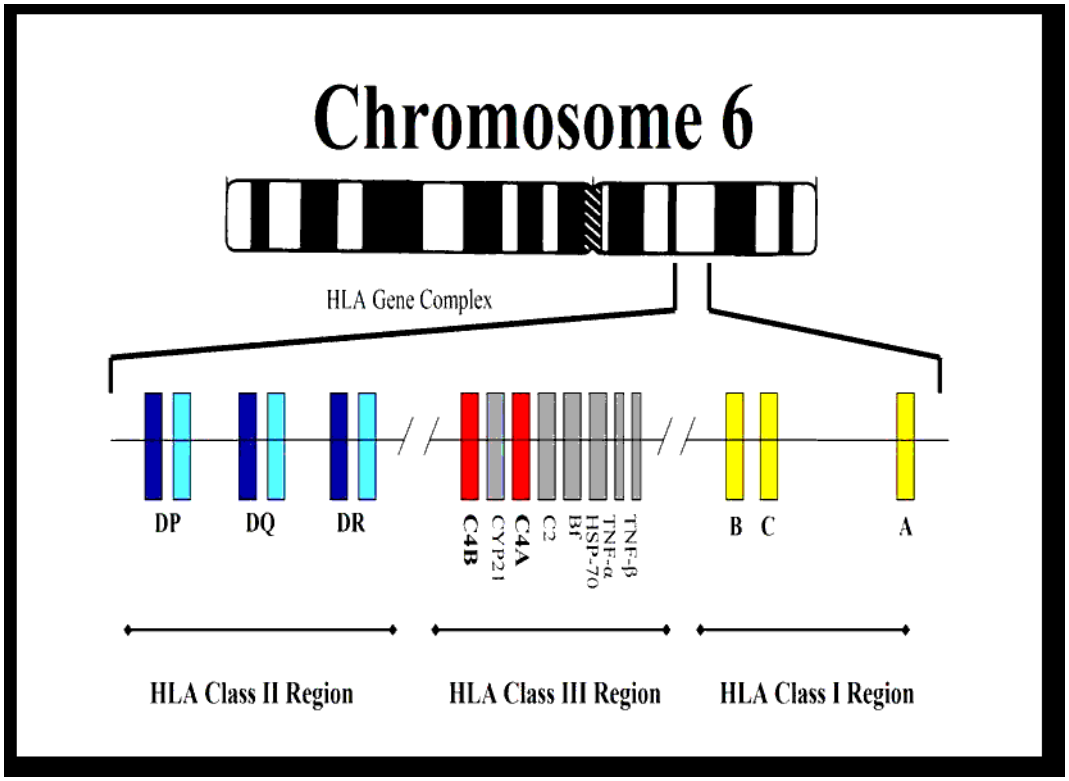


Figure (1-2): a simplified map of the HLA region on human chromosome 6. Showing the major gene of classI, II, and III (adapted from Jonna *et al.*, 2011).

Genetic factors affect the occurrence, clinical expression and treatment outcome of type 1 autoimmune hepatitis. HLA DR3 is the main susceptibility factor in white northern European and North American patients, and HLA DR4 is a secondary but independent risk

factor. Eighty-five percent of white patients from these regions have HLA DR3, DR4 or DR3-DR4. Different geographical regions and ethnic groups have different susceptibility factors. HLA DR3 occurs rarely in the Japanese population, and HLA DR4 is the principal risk factor for autoimmune hepatitis in this ethnic group. HLA DR4 is also the principal susceptibility factor for autoimmune hepatitis in mainland China (Qiu and Ma 2003).

1.2.11. Genetic of AIH

Genetic autoimmune promoters inside and outside the MHC may also affect the occurrence of autoimmune hepatitis either in synergy with the principal susceptibility alleles (epistasis) or in lieu of them (Czaja and Donaldson, 2002).

Polymorphisms of the tumor necrosis factor-α gene (*TNFA*- *α*), and the cytotoxic T lymphocyte antigen-4 gene (*CTLA-4*) have been associated with increased immune reactivity and disease severity in type 1 autoimmune hepatitis in white North American and northern European patients (Djilali-Saiah *et al*., 2004). These promoters are host-related and not disease-specific. Constellations of them in varying combinations may affect the occurrence, clinical phenotype, and outcome of autoimmune hepatitis. Their occurrence and impact may also vary by geographical region and ethnic group (Bittencourt *et al*., 2003).

Other genetic promoters that have been implicated in the pathogenesis of autoimmune hepatitis include polymorphisms of the vitamin D receptor (*VDR*) gene, point mutation of the tyrosine phosphatase CD45 gene (Vogel *et al*., 2002). Polymorphisms of the Fas gene (tumor necrosis factor receptor super family-6 or TNFRSF6) and polymorphisms of the MHC class I chain gene. Cytokine

imbalances perhaps related to genetic polymorphisms that control cytokine production and receptor function are undoubtedly important in affecting the cascade of immune-mediated interactions resulting in hepatocyte injury. Transforming growth factor-β (*TGF-β*) has been implicated as an important protective mechanism against autoimmune hepatitis by suppressing infiltration of the liver with autoreactive T cells (Schramm *et al*., 2003).

1.2.12. The role of autoantibodies in AIH

Antinuclear antibody (ANA), SMA, and anti-LKM1 constitute the standard repertoire of autoantibodies that are assessed in autoimmune hepatitis. Antibodies to soluble liver antigen/liver pancreas (anti-SLA/LP), perinuclear anti- neutrophil cytoplasm antibodies (pANCA), endomysium, and tissue transglutaminase are ancillary markers that are useful in evaluating patients who are seronegative for the standard battery (Czaja and Norman, 2003).

Other autoantibodies that have been described in autoimmune hepatitis, which are either not generally available, investigational in nature, or of limited clinical value are antibodies to Asialoglycoprotein receptor (anti-ASGPR), actin, chromatin, liver cytosol type 1, double-stranded DNA, histones, Saccharomyces cerevisiae, and lactoferrin (Czaja *et al*., 2005), table (1-3).

Antibodies to SLA/LP are present in 26% of patients with autoimmune hepatitis who are otherwise seronegative, and they are useful in reclassifying cryptogenic chronic hepatitis as autoimmune hepatitis (Baeres *et al*., 2002). These autoantibodies also identify individuals who have more severe disease than seronegative counterparts and who invariably relapse after corticosteroid withdrawal (Czaja *et al*., 2002).

Since anti-SLA/LP is closely associated with HLA DR3, they may be surrogate markers of a genetic propensity for relapse or refractory disease (Czaja *et al*., 2002).

Analysis of the immunoprecipitated RNAs extracted from cell is the most powerful, sensitive and specific method to detect anti- SLA/LP autoantibodies, but an enzyme-linked immunosorbent assay (ELISA) is available as a commercial kit and performance parameters between the methods are comparable. Perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) and immunoglobulin-A (IgA) antibodies to endomysium (EMA) and tissue transglutaminase (tTG) are ancillary markers of autoimmune hepatitis that are available in the clinical laboratory (Czaja and Norman, 2003).

1.2.13. Subclassification and autoantibody patterns

Based on the differences in clinical, serological and genetic characteristics AIH can be divided into at least two major subtypes (table 1-2) Type 1 disease is characterized by the presence of circulating anti smooth muscle antibodies (anti-SMA). However, positive anti-SMA serology in itself is not diagnostic of AIH, as it can be seen in other inflammatory liver diseases as well as in various rheumatoid conditions. The anti-SMA antibodies react with various cytoskeletal antigens, among which F-actin, especially in a polymerized form, is thought to be the most specific for AIH. While other anti-SMA antibodies are often detected in HBV and HCV hepatitis, they usually do not react with the F-actin epitopes. On rodent kidney sections indirect immunoflourescence staining of vessels, glomeruli, and tubules corresponds to the distribution of F-actin, and therefore it is indicative of Type 1 AIH. The serum level of F-actin antibodies can also be

measured with ELISA based tests (Czaja *et al*., 1999; Lapierre *et al*., 2007).

Table (1-3): Clinical and laboratory characteristic findings in the different subtypes of autoimmune hepatitis.

	Type 1 AIH	Type 2 AIH
Characterastic antibodies	**Anti-SMA,ANA,pANCA**	**Anti-LKM-1**
Age of onset	**Peaks at 10-20,45-70**	**2-14**
Gender distribution	**78% female**	**89% female**
IgG increase	**Pronounced**	**Modrate**
IgA decrease	**Absent**	**Occasinal**
Association with HLA haplotypes	**B8,DR3,DR4**	**B14,DR3**

1.2.14. Diagnosis of Autoimmune Hepatitis

The diagnosis of AIH requires the presence of characteristic clinical features and exclusion of other chronic liver conditions, such as viral hepatitis, drug-induced hepatitis, fatty liver disease; alcohol related liver disease, Wilson's disease, alpha-1-antitrypsin deficiency, or hemochromatosis (Ashima *et al*., 2011).

1.2.14.1. Laboratory Features

Laboratory studies typically show elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (levels are generally <500 U/L), but on rare occasions can range

between 500– 1000 U/L. Some patients may have high conjugated bilirubin and alkaline phosphatase necessitating exclusion of extrahepatic biliary obstruction, cholestatic forms of viral hepatitis, drug-induced disease, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). The alkaline phosphatase rarely exceeds 4 X normal and generally remains <2 times normal. Another characteristic laboratory feature of AIH is hypergammaglobulinemia, with a selective increase in IgG (Ashima *et al*., 2011).

1.2.14.2. Autoantibodies

The characteristic circulating autoantibodies seen in AIH include ANA, SMA, and (LKM-1) autoantibodies. They are helpful in diagnosis as well as for classification of AIH into type 1 and type 2 diseases (Czaja, 2010).

1.2.14.3. Histological Diagnosis

The histologic hallmark of AIH is a lymphoplasmacytic periportal infiltrate invading the limiting plate, also called piecemeal necrosis or “interface hepatitis” that eventually progresses to lobular hepatitis (Makol *et al*., 2011). There is often an abundance of plasma cells and eosinophils are frequently present. The portal lesion typically spares the biliary tree. A lobular, or panacinar hepatitis is also frequently observed. Fibrosis is present in all but the mildest forms of AIH. It causes distortion of the hepatic lobule and the appearance of regenerative nodules, resulting in cirrhosis. Many patients with acute presentation may have chronic features on liver biopsy indicating a subclinical phase of disease and several patients with mild clinical disease may have advanced fibrosis on biopsy (Feld *et al*., 2005).

Liver biopsy is recommended at the time of presentation to establish the diagnosis as well as to guide the treatment, however in patients presenting with acute hepatitis who has suspicious diagnosis of AIH, the treatment should not be delayed (Manns *et al*., 2010).

Typical histological findings in AIH consist of prominent periportal lymphoplasmacytic infiltrate, interface hepatitis and lobular hepatocyte necrosis/dropout. In a long standing process a cirrhotic pattern can be present. While the picture of extensive hepatocyte dropout with mild inflammatory infiltrate is more suggestive of an adverse drug reaction mediated liver injury, negative infectious and drug history with a prominent plasma cell infiltrate favors AIH. (Manns *et al*., 2010).

Although the above described microscopic findings are not specific, in the appropriate clinical setting they strongly support the diagnosis of AIH. Moreover, liver biopsy continues to remain the "gold standard" for grading and staging disease activity (Eapen and Roberts-Thomson, 2006).

1.2.14.4. Radiology

There are no specific imaging techniques to confirm the diagnosis of autoimmune hepatitis. In adults with both AIH and inflammatory bowel disease IBD, cholangiographic changes suggestive of PSC are present in up to 44% patients and may affect therapy and prognosis. In children with AIH, autoimmune sclerosing cholangitis can be present with or without inflammatory bowel disease (Czaja, 2010).

1.2.14.5. Blood Tests

Laboratory tests in AIH are usually consistent with a chronic active liver injury pattern. Marked elevation of liver transaminases and moderate hyper bilirubinemia are commonly detected in the active

phase of the disease. Elevated serum IgG immunoglobulins as well as various circulating autoantibodies are characteristically seen in this immune response mediate process. Positive Coombs test in the AIH patients is often associated with autoimmune hemolytic anemia. False positive VDRL reactivity could also be occasionally detected (Manns and Vogel 2006).

Blood tests can show signs of hepatitis with raised alanine transaminase (ALT, U/L) (usually less than 500 U/L), aspartate transaminase (AST, U/L) and occasionally bilirubin (mg/dL). Typical immunology profile in AIH patients are raised Ig G with positive ANA, ASMA (in type 1 AIH) and LKM (in type 2 AIH). Patients with acute presentation of AIH should be monitored for synthetic function such as international normalized ratio (INR) and albumin as well as mental status or sign of hepatic encephalopathy since some patients can progress to acute liver failure rapidly and will need liver transplantation. Viral hepatitis, toxins, drugs should be excluded in patient presenting with acute or chronic form of hepatitis (Al-Chalabi *et al*., 2008).

The diagnosis of AIH primarily relies on the scoring system which was established by the International Autoimmune Hepatitis Group in 1993, and was revised in 1999 (Alvarez *et al*., 1999). This highly complex scoring system incorporates several clinical and histochemical criteria including sex, medication history, liver histology, transaminase levels and autoantibody titers. The scoring system is highly sensitive (89%) in detecting AIH patients. Since the introduction of the revised criteria, the system can better differentiate between cholestatic and hepatocytic disorders, and therefore its specificity (89.5%) has also significantly improved (Talwalkar *et al*., 2002).

In patients who are negative for conventional antibodies and AIH is strongly suspected, additional tests can be done including perinuclear antineutrophil cytoplasmic antibodies (pANCA), actin (anti-actin), soluble liver antigen (anti- SLA), asialoglycoprotein receptor (anti-ASGPR), chromatin, and liver cytosol type 1 (anti-LC1) (Ashima *et al*., 2011).

1.2.15. Complications of AIH

In principle the complications of AIH are the same as in any other acute or chronic progressive liver disease. In acute presentations the risk of liver failure and infectious complications are predominant and may be aggravated by immunosuppressive treatment. In chronic disease, especially in patients undiagnosed or insufficiently treated, complications of cirrhosis occur. In particular, hepatocellular carcinoma (HCC) is a known consequence of AIH-related cirrhosis although its occurrence in association with AIH is significantly less frequent compared to most other causes of liver cirrhosis (van Gerven *et al*., 2014; Gronbaek *et al*., 2014).

A recent population based study showed that the risk of hepatic and extra-hepatic malignancy was significantly increased in AIH patients (Ngu *et al*., 2012). Studies from Denmark, Germany, Netherlands, UK, USA and Japan identified male gender as a particular risk factor, and the presence of cirrhosis was a universal prerequisite for HCC development, which was observed in the at risk cirrhotic population at a rate of 1–2% per year (van Gerven *et al*., 2014).

In addition to complications of the liver disease, complications of long-term immunosuppression need to be considered, and the two risks may associate. Of note, extra-hepatic malignancies of diverse cell types

occur in 5% of patients in an unpredictable fashion with non-melanoma skin cancers being the most common (Czaja, 2013b).

Some patients without proper treatment, AIH progresses to cirrhosis and eventually Hepatocellular carcinoma (HCC). The presence of cirrhosis at diagnosis or during treatment and the need for long-term immunosuppressive therapy have been observed as risk factors for malignant transformation (Migita *et al*., 2012). In addition risk factors for HCC furthermore include male gender, advanced stage disease, portal hypertension as ascites and esophageal varices (Czaja, 2013a). HCC occurs in 1%-9% of AIH patients, which is less frequently compared to patients with chronic viral hepatitis. Imaging with ultrasonography or computed tomography should be conducted every 6-12 month. In patients who develop liver failure, liver transplantation needs to be considered (Malekzadeh *et al*., 2012). When AIH is indicated for transplantation, transplanted patients, practically compared to other chronic liver diseases, have an excellent 5 year survival of between 78%-91%. The recurrence rate of AIH after initial successful transplantation is problematic and occurs in around 30% of patients (Liberal *et al*., 2012).

As a chronic liver disease, AIH has similar complications. Indeed, at first evaluation, cirrhosis developed in almost 33% of affected subjects. Therefore, a timely and correct diagnosis seems mandatory in an attempt to stop the progression of chronic hepatitis to cirrhosis, decompensate disease and the development of portal hypertension and ultimately hepatocellular carcinoma (HCC) (Gatselis *et al*., 2015).

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Equipment and Instruments: The instruments and equipments used in this study are listed in table (2-1).

Table (2-1) instrument &equipments with their remarks:

No.	Equipment	Company	Country
1	ARCHITECT plus	Abbott	USA
2	Beakers	AMSCO	Germany
3	Centrifuge	EPPENDORF	Germany
4	Cylinders	AMSCO	Germany
5	Deep freezer	GFL	Germany
6	Disposable syringe 5ml and 3ml	STERILE EO	UK
7	EDTA tube (anticoagulant tube)	AFNA-DISPO	Jordan
8	Eppendorf tubes	Sterellin Ltd.	UK
10	Exispin centrifuge	BIONEER	Korea
11	Flasks	AMSCO	Germany
12	High Speed Cold centrifuge	Eppendorf	Germany
13	Incubator	Mammert	Germany
14	Micropipette injector	EPPENDROF	Germany
15	Micro-plate washer.	BIOTEK	USA
16	Micro-titer plate reader (450 nm filter)	BIOTEK	USA

17	Miniopticon Real-Time PCR	BioRad	USA
18	Multichannel micropipette reservoir	SIAMED	Germany
19	Nanodrop Spectrophotometer	THERMO	USA
20	Oven	Mammert	Germany
21	PCR tubes	BIONEER	Korea
22	Plain tubes	AF CO-Dispo	Jordan
23	Printer	EPSON MODEL P172B	Indonesia
24	Refrigerator	Sanyo Medical	Japan
25	Sensitive Balance	Sartorius	Germany
26	Stop watch	Junahans	Germany
27	Vortex	CYAN	Belgium
28	Water bath	Mammert	Germany

2.1.2. ELISA Kits

Table (2-2): Kit contents for detecting ANA, AMA, SLA and LKM.

No.	KIT	Contents	Company/Country
1	ANA, AMA, SLA, and LKM ELISA Test Kit 96 test	1.sample buffer 2.wash buffer 3. cut-off calibrator 4. calibrators 5. TMB substrate 6. Stop solution 7. Conjugate reagent 8. Negative control 9. positive control 10. Microtiterplate	AESKULISA / Germany
2	LC1 ELISA Test Kit 24 test	1.Dot strip 2. wash buffer 3.Sample diluent 4.Conjugate 5. substrate 6.Incubation tray for 8 strips	HepAK Dot / Germany

2.1.3. Molecular Kits

Commercially provided chemicals for real time PCR work used in the study, their companies and countries of origin are shown in the following table (2-3).

Table (2-3): Real time PCR kits with their remarks

No.	Kit	Company	Country
1	AccuPrep®Genomic DNA extraction kit (Whole Blood)	**Geneaid**	**USA**
	1. Proteinase K 25 mg 2 vial		
	2. Binding buffer (GC) 25 ml		
	3. Washing buffer 1 (W1) 40 ml		
	4. Washing buffer 2 (W2) 20 ml		
	5. Elution buffer (EL) 30 ml		
	6. Binding column		
	7. Collection tube 2 ml		
2	**AccuPrep® 2X GreenStar qPCR Master Mix kit**	**Bioneer**	**Korea**
	(2X) Syber green dye 4 tubes		
	DEPC water		
	Adhesive film		

2.1.3.1. Designing Primers

MHC–HLA alleles specific primers that used in this study were designed by using HLA-alleles specific sequence from NCBI-GenBank 111database (HLA-DR3: Genbank code: NT-167244.2, HLA-DR4: Genbank code: AH002824.2 and HLA-B27 Genbank code : M12967.1) and primer 3 plus design online, they were provided by Bioneer Company, Korea as shown in the following table (2-4).

Table (2-4): primers sequence with orientation and the PCR product size

HLA allele specific primer	Sequence		PCR Size
HLA-DR3	F	TTGTTGGGGTTCACAAGTGG	80bp
	R	AAGCCACAAGCCTGTTTTCC	
HLA-DR4	F	ATCCAGGCAGCATTGAAGTC	124bp
	R	ACTGTTTCCAGCATCACCAG	
HLA-B27	F	AATCTGCATGTTCGCTGTGC	97bp
	R	TCAACACCAAATGGGCACAG	

Genbank code: HLA-DR3: NT-167244.2, HLA-DR4: AH002824.2, and HLA-B27: M12967.1

2.1.4. Chemicals

The chemicals and biological material used in this study are listed in table (2-5).

Table (2-5): chemicals and biological material with their remarks

No.	Chemicals	Company	Country
1	Free nuclease water	Bioneer	Korea
2	Absolute Ethanol	BDH	England
4	Distilled water	Al-Rafidain	Iraq

2.1.4.1. Biochemical kits: The entire biochemical tests in this study were done by automated devise ARCHITECT plus (abbott).

2.1.4.2. Kit total serum bilirubin

The TSB assay is used for the quantitative analysis of total bilrubin in human serum or plasma in the adult and neonates.

- **Kit content**

Reagent	Reactive ingredient	Concentration
R1	Surfactants	4.51%
	HCL	8.204 g/L
R2	2,4-dichloroaniline	0.81 g/L
	HCL	5.563 g/L
	Sodium nitrate	0.345 g/L
	Surfactants	1.96%

2.1.4.3. Alkaline phosphatase

The alkaline phosphatase assay is used for the quantitative analysis of alkaline phosphatase in human serum or plasma.

- **Kit content**

Reagent	Reactive ingredient	Concentration
R1	2-amino-2-methylpropanol Magnesium Zinc sulfate	1.2 mol/L 7.2mmol/L 3.6mmol/L
R2	4-nitrophenyl phosphate	171.6 mmol/L

2.1.4.4. Aspartate aminotransferase (AST): The aspartate aminotransferase used for the quantitation analys of aspartate aminotransferase in human serum or plasma.

- **Kit content**

Reagent	Reactive ingredient	concentration
R1	*B*- NADH Malate dehydrogenase lactate dehydrogenase *L*- Aspartate	o.16 mg/ml 0.64 U/ml 0.64 U/ml 232mmol/L
R2	4-Ketoglutarate *L*- Aspartate	51.3 mmol/L 100 mmol/L

2.1.4.5. Alanine aminotransferase (ALT): The alanine aminotransferase assay is used for the quantitation analys of alanine aminotransferase in human serum or plasma.

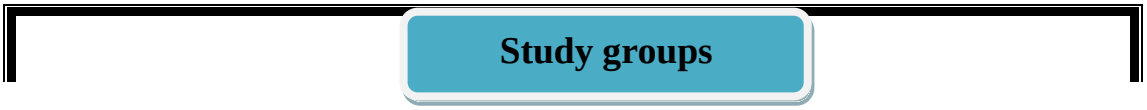
- **Kit content**

Reagent	Reactive ingredient	Concentration
R1	*B*- NADH lactate dehydrogenase *L*- Alanine	o.16 mg/ml 0.64 U/ml 292mmol/L
R2	4-Ketoglutaric acid *L*- Alanine	77 mmol/L 1000 mmol/L

2.2. Methods

2.2.1. Study design

This study was conducted at the Department of Microbiology and parasitology/ College of Medicine/ University of Al Qadisyia in Iraq. It was carried out during the period from March 2016 to December 2016. Subjects that were enrolled in this study were categorized into two groups, group1 (60 patients of autoimmune hepatitis) and group 2 (50 healthy control). The study design (figure 2-1) is illustrated in the flow chart below:



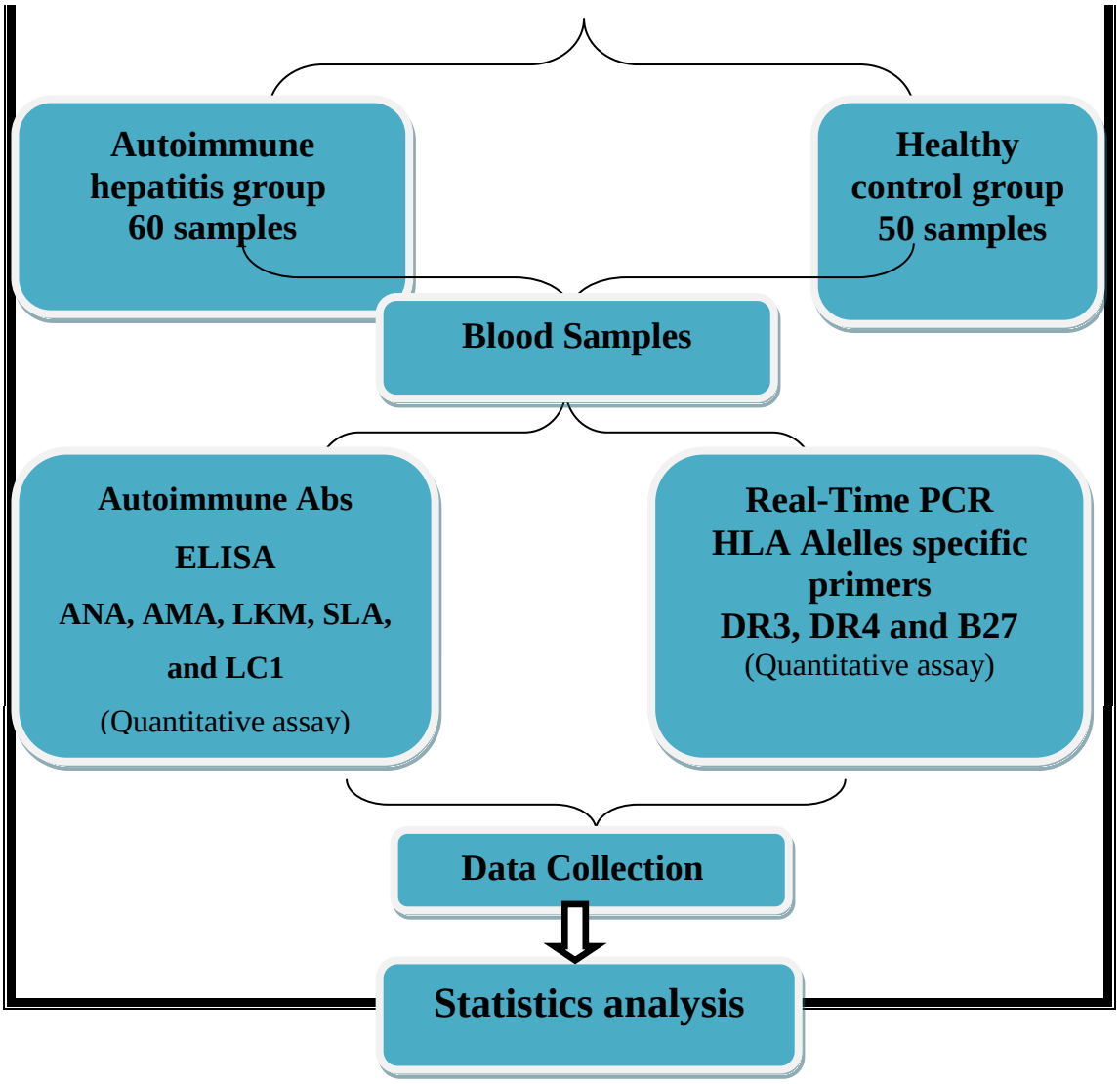


Figure (2-1) A chart illustrating the study design

2.2.2. Autoimmune hepatitis Patients Group

Sixty Iraqi patients' with autoimmune hepatitis from AL-Diwaniyah GIT centre, Gastrointestinal and liver hospital/ Baghdad, and Karbala GIT center had been recruited for this study. All subjects of the patients group selected randomly include (19) males and (41) females, have been clinically diagnosed with autoimmune hepatitis clinically by

physicians, and by laboratory tests (biochemical tests, and immune assay tests) with an age range of (7-69) years with a mean age (40.6) year old. The time from which the disease had been clinically diagnosed was from 1 month up to 12 years.

All patients show negative serological evidence of chronic viral hepatitis B and C by enzyme-linked immunoassay. There was no evidence for illicit drug abuse, excessive alcohol intake or exposure to hepatotoxic drugs.

2.2.3. Control group

For the purpose of comparisons, the control group consisted of 50 healthy control individuals (15 males and 35 females) for age (10-67) years old, sex and ethnic back ground (Iraqi Arabs) were selected who had no history or clinical evidence of hepatitis or any chronic disease and obvious abnormalities according to laboratory findings of biochemical tests and immune assay tests who were considered as control. All of them had negative family history of autoimmune hepatitis.

2.2.4. Blood samples collection

Blood samples were collected by vein puncture. Eight milliliters (ml) of venous blood were withdrawn from each patient and control using disposable syringes under aseptic condition. Six milliliters from each sample were transferred to 10 milliliters sterile Gel tube and centrifuged at 3000 rpm for 5 minutes and some of the separated serum was used for biochemical tests and the other separated serum are divided into several Eppendorf tubes and immediately frozen at –20 ºC till further use to avoid repeated thawing and freezing. The remaining (2

ml) were transferred into tube with K_2-EDTA (ethylene diamine tetra acetic acid) (2.5 mg / ml), kept at –20 ºC for molecular study of HLA genes (DR3, DR4, and B27).

2.3. Enzyme Linking Immunoisorbant assay (ELISA).

ELISA assay was used for detection and measurement of auto-antibodies specific for (anti-ANA, AMA, SLA, and LKM) (Aesku Diagnostics Microform ring 2.55234 Wendelsheim Germany) and (HepAK Dot Generic assay GmbH D-15827 Dahlewltz Germany) for LC1 were used in this work. The kits contained the following reagents:

2.3.1. For ANA, AMA, SLA, and LKM

A. Reagents to be reconstituted:

1. Sample buffer (5X): 1 vial, 20 ml -5X concentrated (capped white: yellow solution).
2. Washing buffer 50X: 1 vial, 20 ml -50X concentrated (capped white: green solution).

B. Reagents to use

1. Negative control: 1 vial, 5 ml - capped green: colerless solution.
2. Positive solution: 1 vial, 5 ml - capped red: yellow solution).
3. Cut-off calibrator: 1 vial, 1.5 ml - capped blue: yellow solution.
4. Calibrators: 6 vail, 1.5 ml – capped white: yellow solution.
5. Conjugate, IgG: 1vail, 15 ml capped blue: blue solution.
6. TMB Substrate: 1vail, 15 ml capped black: colerless solution.
7. Stop solution: 1vail, 15 ml capped white: colerless solution.
8. Microtiter plate: 12x8 well strip.

C. Principle of the test

Serum samples diluted 1:101 were incubated in the micro plates coated with the specific antigen. Patient's antibodies, if present in the specimen, would bind to the antigen. Afterwards, anti-human immunoglobulin's conjugated to horseradish peroxidase (conjugated) were incubated and reacted with the antigen –antibody complex of the samples in the micro plate. Addition of TMB-substrate solution generated an enzymatic colorimetric (blue) reaction, which was stopped by diluted acid (color change to yellow).The rate of color formation from the chromogenic is a function of the amount of conjugate bound to the antigen –antibody complex and this is proportional to the initial concentration of the respective antibodies in the patient sample (Shan *et al*., 2002).

2.3.2. For LC1

A. Reagents to use

1-Dot strip: 24 numbered strips coated with 4 specific antigen

2-Wash buffer: 10X sufficient for 400ml solution- 40 ml concentrate: capped blue.

3-Sample diluents: 40ml capped yellow, yellow solution.

4- Conjugate: antihuman-IgG coupled with alkaline phosphatase: 40ml capped black, red solution.

5-Substrate nitroblue tetrazolium with bromo-chloroindolyl-phoshate: 40ml capped black, black bottle.

6- Incubation tray for 8 strips: 3X.

B-Principle of the test

HepAK Dot is a sensitive immunodot assay for the determination of IgG antibodies to M2, LKM, LC1, and SLA in human serum or plasma respectively.

HepAK Dot includes 24 numbered test strips with 6 dots fixed on a plastic support. Dots are coated with purified M2, LKM, LC1, and SLA. Two test dots serve as positive and negative controls.

Patient's sera and strips were incubated in the test tray. During the first incubation antibodies of the patient sample bind to the target antigens immobilized on the solid-phase of the strips, the unbound serum components are removed by a washing step.

Bound antibodies react specifically with anti-human-IgG conjugated to alkaline phosphatases which convert the colourless substrate solution to a dark purple precipitating dot.

2.3.3. Preparations prior to pipetting: "According to the manufacturing instructions"

A. Diluting the concentrated reagents:

The concentrated sample buffer was diluted 1:5 with distilled water (e.g.20 ml plus 80 ml). The concentrated wash buffer was diluted 1:50 with distilled water (e.g.20 ml plus 980 ml) for ANA, SLA, AMA, and LKM.

B. Samples:

Serum samples were diluted 1:101 with sample buffer (1X) (e.g. 1000 µl samples buffer (1X) +10 µl serum). The diluted preparation was mixed well.

C. Washing:

A quantity of 20 ml of diluted wash buffer (1X) was prepared per 8 well or 200 ml per 96 wells (e.g. 4ml concentrate plus 196 ml distilled water). Automated washing: excess volumes required were considered for setting up the instrument and dead volume of robot pipette. Manual washing liquid from wells were discarded by inverting the plate. The Microwell frame was knocked with wells downside vigorously on clean adsorbent paper. Diluted wash buffer (300 µl) was pipetted into each well, and after 20 second the whole procedure repeated twice again.

D. Microtiter plates:

The number of wells required for the test was calculated. Unused wells were removed from the frame, replaced and sealed tightly in the provided plastic bag, together with desiccant, and stored in fridge at 2-8 ºC.

2.3.4. Assay Steps: "According to the manufacturing instructions"

2.3.4.1. FOR ANA, AMA, SLA, and LKM

1. For each patient's diluted serum, 100 µl was pipetted into the designated micro wells.
2. For each calibrators or cut –off calibrator and negative and positive controls, 100 µl was pipetted into the designated wells.
3. The micro plate was incubated for 30 minutes at 20-32 ºC.

4. The wells were washed 3x with 300 µl washing buffer (diluted 1:50).
5. A volume of 100 µl of the conjugate was pipetted into each well.
6. The micro plate was incubated for 30 minutes at 20-32 ºC.
7. The wells were washed 3x with 300 µl washing buffer (diluted 1:50).
8. A volume of 100 µl of TMB substrate was pipetted into each well.
9. The micro plate was incubated for 30 minutes at 20-32 ºC. And protected from intense light.
10. A volume of 100 µl stop solution was pipetted into each well, using the same order as for the substrate.
11. The micro plate was incubated for 5 minute minimum.
12. The micro plate was agitated carefully for 5 seconds.
13. Absorbance was read at 450 nm (optionally 450/620 nm) within 30 minute (Wies *et al*., 2000).

2.3.4.2. Assay Steps for LC1

1. All reagents was bring to room temperature (18-25 ºC) before used and mixed gently without causing foam.
2. The strips placed with the reactive side up (labels on the top) into the respective well. 2ml of solution was dispensing into the respective well.
3. The tray was covered and incubated at room temperature for 10 minute.
4. The solution in the wells was discarded by slowly inverting the plate. The edges of the tray dried with absorbent paper.

5. A volume of 1.5ml of sample was adding and 10µl of patient serum or plasma was added to the respective wells.
6. The tray was covered and incubated at room temperature for 30 minutes with shaking.
7. Each well washed three times, three minutes with 1.5ml of wash solution with shaking. The solution in the wells was discarded by slowly inverting the plate and the edges of the tray were dried with absorbent paper.
8. A volume of 1.5ml of conjugate was added to each well. The tray was covered and incubated at room temperature for 30 minutes with shaking.
9. The step 7 was repeated and added 1.5ml of substrate to well.
10. The plate was covered, incubated 10-12 minutes, while shaking, and the step 7 was repeated.
11. The strips were collected from the well and dried the membrane by pressing briefly the reactive side of the strip onto absorbent paper. The strips were interpreted after approximately 30 minutes (Johnson and McFarlane, 1993).

2.3.5. Calculation of the Data

- **For ANA, AMA, SLA, and LKM**

For qualitative interpretations were read the optical density of the cut-off calibrator and patient samples. Patients OD with OD of the cut-off calibrator were compared. For qualitative interpretation it has been recommend considered sera within a range of 20% around the cut-off

value as equivocal. All samples with higher ODs have been considered positive, samples with lower ODs has been considered negative.

Negative: OD patient < 0.8 x OD cut-off

Equivocal: 0.8 x OD cut- off < OD patient < 1.2 x OD cut-off

Positive: OD patient > 1.2 x OD cut –off.

A quantitative interpretation, of the standard curve, can be achieved by plotting the optical density (OD) of each calibrator (y-axis) with respect to the corresponding concentration values in U/ml (x-axis). For best result, it is recommended that log/line coordinates and 4-parameter is fit. From the OD of each sample read the corresponding antibody concentrations expressed in U/ml?

Normal range <12 U/ml

Equivocal range 12-18 U/ml

Positive result > 18 U/ml

- **Evaluation of result for LC1**

Results should be interpreted only after strips have been dried for at least 30 minutes. The positive control must be positive in all cases. The correlation of the dot ensures that the test has been run correctly and the kit components are not degraded. If the positive control dot shows no coloration the result cannot be interpreted.

The negative control demonstrated the extent of non specific antibody binding of the sample in the test, the coloration of the dot corresponds minimal intensity above which a sample is considered positive.

The test dots are coated with autoantigens and detect specific antibody binding of the sample in the test. The color intensity of the test dot depending on the titer of specific antibody binding in the sample.

The patient sample is positive concerning a certain antibody if the test dot coloration is stronger (more intense) than the negative control.

2.3.6. Standard curve plotting

For anti ANA, AMA, SLA, and LKM, standard curve was done as illustrated in figure (2-2, 2-3, 2-4, 2-5) respectively, they graphed by plotting known standard concentration against their correspondent optical densities. They were used to quantitative and qualitative the test samples for these entire assay.

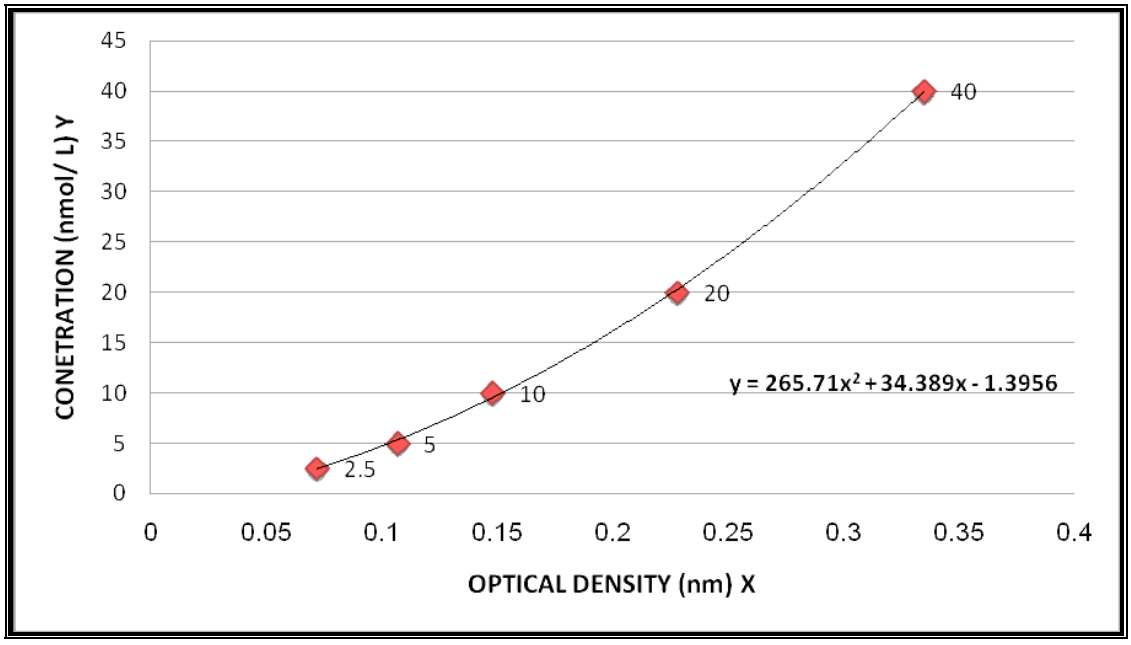


Figure (2-2): The standard curve of ANA

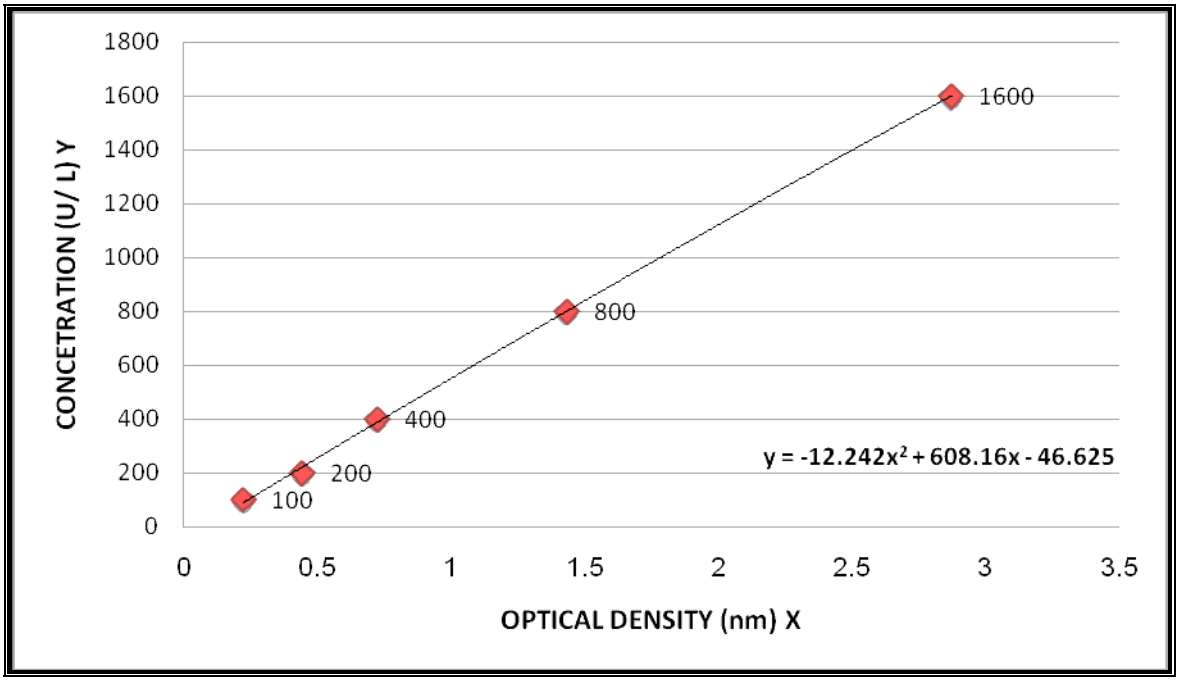


Figure (2-3): The standard curve of AMA

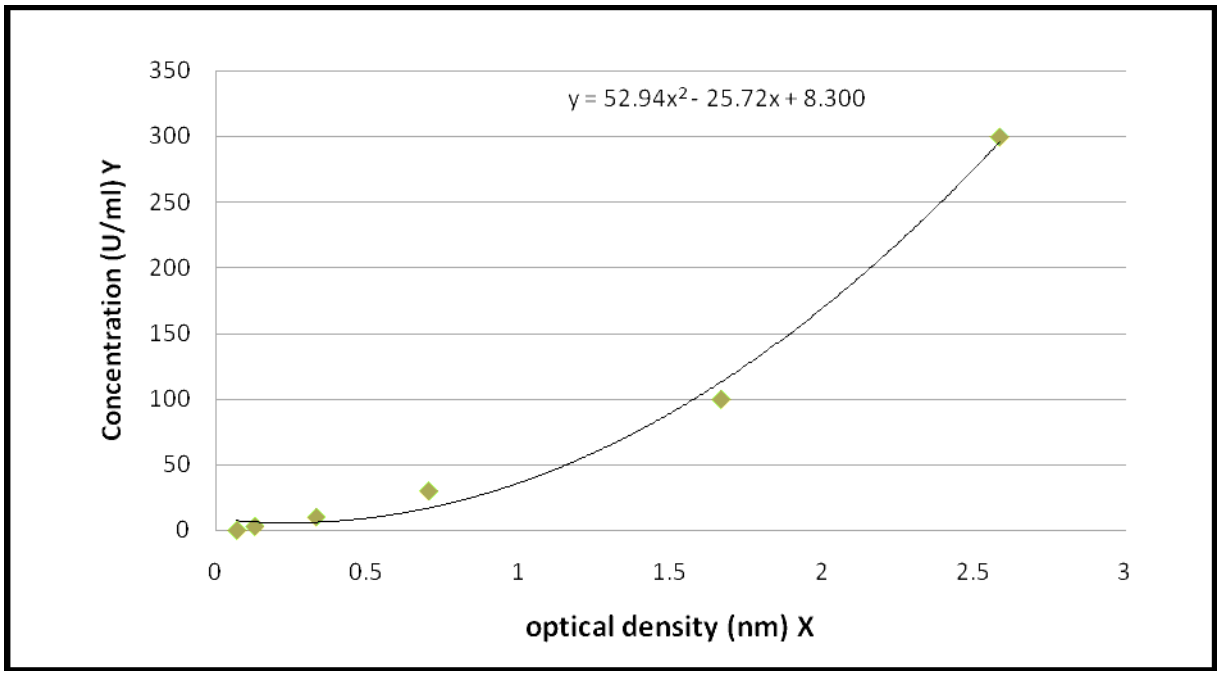


Figure (2-4): The standard curve of SLA

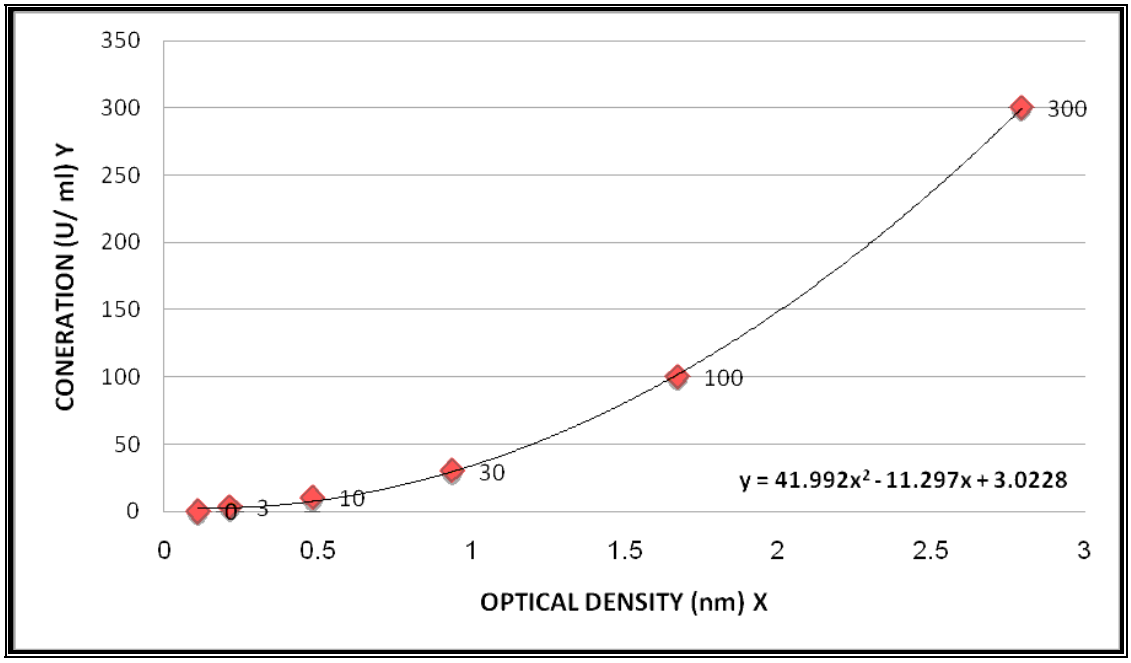


Figure (2-5): The standard curve of LKM

2.4. Real-Time PCR

Real-Time PCR technique was performed for the detection of HLA-DR3, HLA-DR4, and HLA-B27 genes in blood samples of autoimmune hepatitis, patients group, as wells as in healthy control group, this technique was carried out according to the method described by (Gersuk and Nepom, 2007), as following steps:

2.4.1. Genomic DNA Extraction

Genomic DNA from blood samples were extracted by using Genomic DNA mini kit extraction kit (Frozen Blood) Geneaid. USA, and done according to company instructions as following steps:

1. A 200µl of frozen blood was transferred to sterile 1.5ml microcentrifuge tube, and then added 30µl of proteinase K and mixed by vortex. And incubated at 60 ºC for 15 minutes.

2. After that, 200µl of lysis buffer was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70 ºC for 15 minutes, and inverted every 3 minutes through incubation periods.

3. 200µl absolute ethanol were added to lyses and immediately mixed by shaking vigorously.

4. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000 rpm for 5 minutes. And the 2 ml collection tube containing the flow-through was discarded and placed the column in a new 2 ml collection tube.

5. 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.

6. 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube.

7. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.

8. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre-heated elution buffer were added to the center of the column matrix.

9. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

2.4.2. Genomic DNA Profiling

The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of blood genomic DNA was added to measurement.
4. The purity of DNA was determined by reading the absorbance in Nanodrop spectrophotometer at 260 nm and 280 nm, so the DNA has its absorption maximum at 260 nm and the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; if the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

2.4.3. Real- Time PCR master mix preparation

Real-Time PCR master mix was prepared by using AccuPrep® 2X GreenStar qPCR PreMix kit (Bioneer. Korea), and done according to company instructions as following table (2-8).

These qPCR master mix reaction components that mentioned in table (2-8) above were added into standard strip qPCR. Then all tubes vortex for mixed the components and centrifuge for 3000rpm for 3 minutes in Exispin centrifuge, after that transferred into Miniopticon Real-Time PCR BioRad USA.

Table (2-6): Real-Time PCR master mix was preparation

qPCR master mix	Volume
2X Syber green master mix	12.5 µL
Genomic DNA template	5µL
Forward primers (10pmol)	1µL
Reverse primers (10pmol)	1µL
DEPC	5.5 µL
Total	25µL

2.4.4. Real-Time PCR Thermocycler Conditions

qPCR Thermocycler conditions was designed for HLA specific primers according to primer annealing temperature and qPCR Syber green kit instructions as following in table (2-13):

Table (2-7): Program the PCR setting

Step	Condition	Cycle
Pre-Denaturation	95 °C 1 min	1
Denaturation	95 °C 10 sec	40
Annealing/Extension	60 °C 30 min	
Detection (Scan)		
Melting curve	60-95 °C	1

2.4.5. Real-Time PCR Data Analysis

qPCR data analysis was performed by interpretation of threshold cycle number that explain the positive amplification of HLA alleles in patients and healthy control samples.

2.5. Statistics analysis

Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning method and inconsistencies were remedied. An expert statistical advice was sought for. Statistical analyses were done using SPSS version 21 computer software (Statistical Package for Social Sciences) in association with Microsoft excel 2010. To measure the strength of association between 2 categorical variables, such as the presence of certain HLA genotype and disease status the odds ratio (OR) was used. For example OR for the association between having a specific genotype and having autoimmune hepatitis equals the ratio of the odds of having the specific genotype versus lacking it among cases to the similar odds among controls. The ROC method was used to evaluate the performance of a quantitative test in differentiating between a disease status (or an outcome) and a second comparison group (Emery, 1976; Kleinbaum *et al*., 2007).

Chapter Three

Results

3. Result

3.1. Distribution of patients according to the age and gender

As shown in table (3-1), the current study revealed an age range of (7-69) years in autoimmune hepatitis patients with mean age (mean= 40.6 ± SD 14.9), whereas the age range of healthy group were (10-67) years with mean age (36.7 ± SD 15.0).

Table (3-1): Distribution of studied groups according age and gender

Parameters	Healthy controls		Autoimmune hepatitis		P (t-test)
Age (years)					**0.17 [NS]**
Range	(10 to 67)		(7 to 69)		
Mean	36.7		40.6		
SD	15.0		14.9		
SE	2.1		1.9		
N。	50		60		
Gender	**N**	**%**	**N**	**%**	**P (Chi square) = 1 [NS]**
Female	34	68	41	68.3	
Male	16	32	19	31.7	
Total	50	100	60	100	

SD: Standard deviation, N: number and NS: no significant, SE;standard error

Also table (3-1) showed no obvious statistically significant differences in mean age between cases and controls**.**

Data of the current study showed that there were females predominant, where the female showed 34(68%) and 41(68.3%) for the control and case group, respectively while the male showed 16(32%) and 19(31.7%) for the control and case group respectively, with ratio of male to female in this study was 1:2.8.

3.1.2. According to the duration of disease.

The results presented in table (3-2) shows the duration intervals of autoimmune hepatitis among the patients, it ranged from less than 1 year 25(41.7%), to more than 3 years 15(25%), while age intervals 1-2 years was 20(33%).

Table (3-2): Frequency distribution of cases group according duration of the disease.

AIH Patients		
Duration of the disease in years-categories	**No.**	**%**
< 1	25	41.7
1-2	20	33.3
3+	15	25.0
Total	60	100

3.2. Evaluation of Auto-antibodies:

3.2.1. Anti-Nuclear antibody (ANA)

The median concentrations of ANA antibody has significantly different (P < 0.001) between autoimmune hepatitis patient (20.9 U/ml) and healthy control (4.8 U/ml), as shown in table (3-3).

Moreover, table (3-3) and figure (3-1) shows the higher range, median, and inter-quartile ranges for this antibody among the two studied groups.

In AIH patients, the range of ANA level was (0.2 to 164.2 U/ ml) while in the healthy control was (0.02 to 20.4U/ml). The ANA autoantibody occurred in 45(75%) among 60 of the AIH patients.

Table (3-3): Different (ANA) level parameters among study groups.

Anti- Nuclear antibody (ANA U/ml)	Study group		P value
	Healthy controls	**Autoimmune hepatitis**	**< 0.001**
Range	(0.02 to 20.4)	(0.2 to 164.2)	
Median	4.8	20.9	
Inter-quartile range	(2.6 to7.5)	(8.35 to59.8)	
No	50	60	

The diagnostic and differentiating value of this auto-antibody has been also calculated using Receiver operated characteristics curve (ROC; specificity, sensitivity, accuracy and positive/ negative predictive values.

In the differentiation of autoimmune hepatitis patients from healthy control, this test has revealed on ROC area value of (0.804) as shown in (figure 3-2, and Table 3-9),

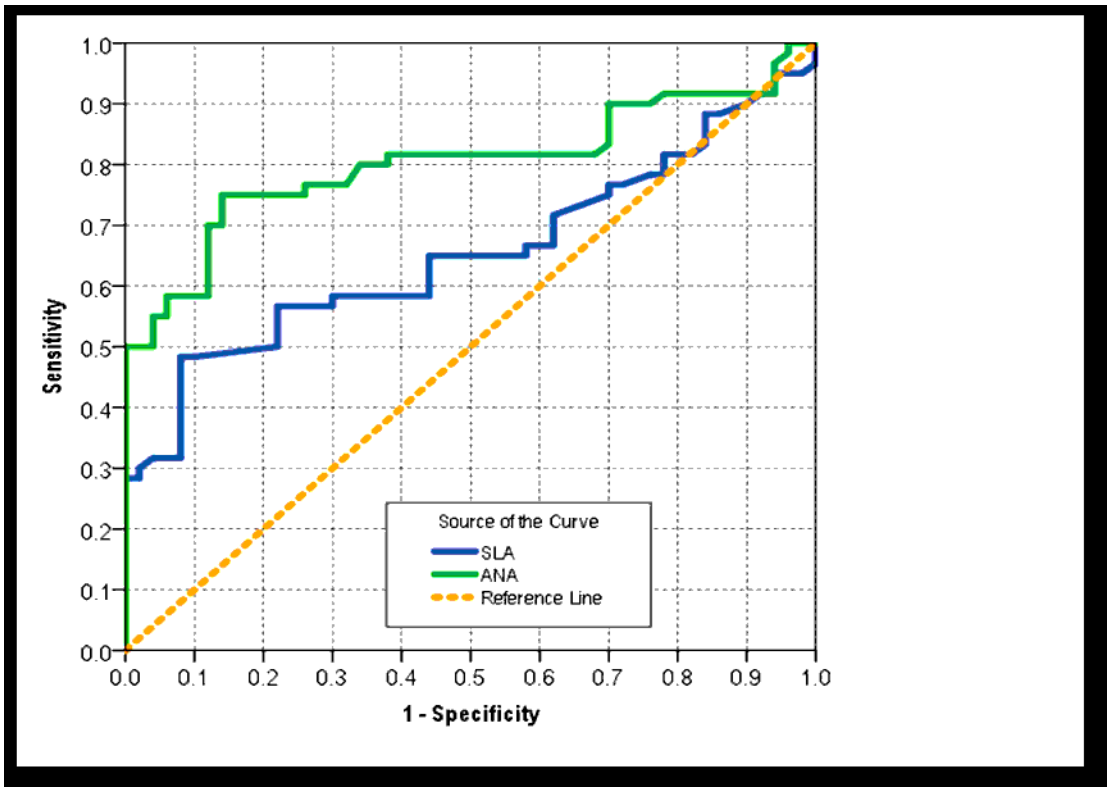


Figure (3-1): Receiver operated characteristics curve (ROC) showing the trade-off between sensitivity (true positive rate) and 1-specificity (false negative rate) for ANA and SLA when used as test to predict a diagnosis of autoimmune hepatitis differentiating it from healthy controls.

3.2.2. Anti-Soluble liver antigen (SLA)

The different parameters of anti- SLA antibody over the two studied groups are presented in table (3-4). It shows statistically significant differences in the median level of this auto-antibody (P 0.005) in the studied groups. It has risen in AIH patients (8.3 U/ml) compared to healthy control group (5.2 U/ml). Moreover, the level range of Anti-SLA was (0.2 to 174.3 U/ml) in AIH group and (0.8 to 17.8 U/ml) among healthy control individuals. The presence of SLA among AIH group in this study was 33(55%), the distribution of all of two studied group subjects over the different levels of this auto-antibody is

demonstrated in figure (3-3), where the highest number of patient were occurred within the inter-quartile range of (4.15- 19.5 U/ml) compared with lower one of healthy control (3.8 - 7.8 U/ml) .

Table (3-4): Different (anti-SLA) level parameters among study groups.

Anti-Soluble Antibody (SLA U/ml)	Study group		P value
	Healthy controls	Autoimmune hepatitis	0.005
Range	(0.8 to 17.8)	(0.2 to 174.3)	
Median	5.2	8.3	
Inter-quartile range	(3.8 to 7.8)	(4.15 to 19.5)	
N。	50	60	

Using ROC curves to evaluate the diagnostic value of this auto-antibody, table (3-9) and figure (3-2) revealed a high ROC value (0.655) for this class of auto-antibody when used as test to predict AIH differentiating them from healthy control. So, a moderate diagnosis value for this test may be concluded.

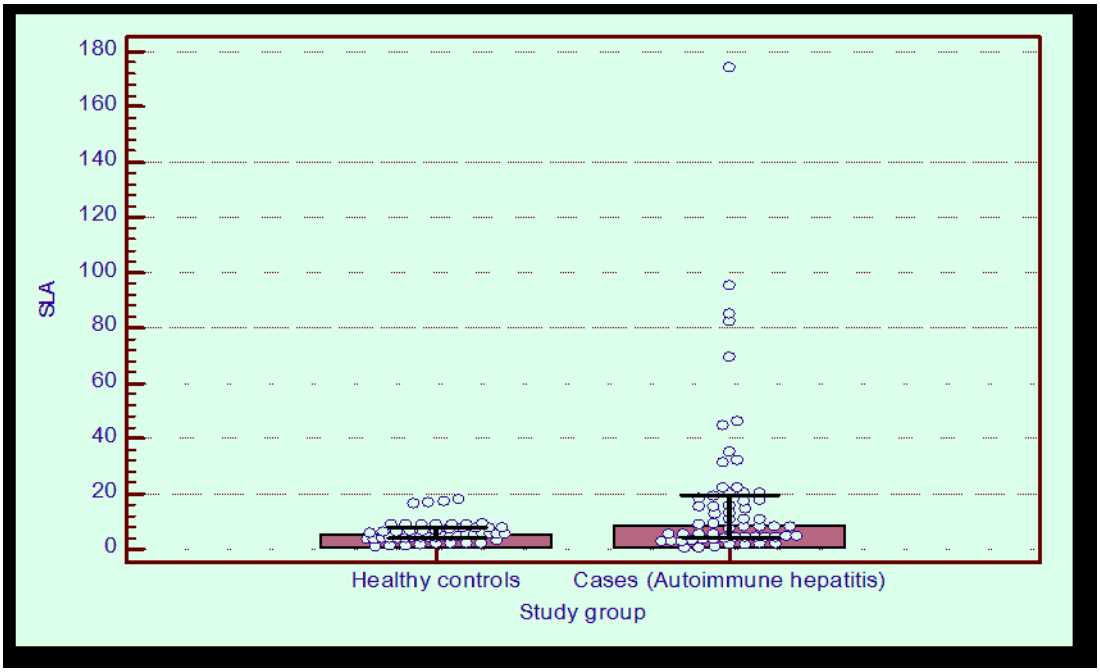


Figure (3-2): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) of SLA.

3.2.3. Anti-Liver kidney microsomal (LKM)

The median titers of LKM for the two study groups evaluated in this work are shown in table (3-5) and figure (3-4). There were significant differences in the median, range, and inter-quartile range of this autoantibody between the two groups studied. The median range in health group was (3.6U/ml) ranged in (0.2-16.8U/ml) while the median of case group was (7.5 U/ml) ranged in (0.3-104.3 U/ml). The results of the present study revealed highly significant differences (p 0.01) comparing the two groups. The level (positives) of LKM autoantibody among the AIH patients is 38(63%).

When using ROC curves to evaluate the diagnostic value of this auto-antibody, revealed a ROC value (0.644) for this class of auto-antibody when used as test to predict AIH differentiating them from healthy control, table (3-9), figure (3-5).

Table (3-5): Different (anti-LKM) level parameters among study groups.

Anti-Liver kidney antibody (LKM U/ml)	Study group		P value
	Healthy controls	Autoimmune hepatitis	0.01
Range	(0.2 to 16.8)	(0.3 to 104.3)	
Median	3.6	7.5	
Interquartile range	(1.7 to 8.3)	(1.7 to 18.99)	
N	50	60	
Mean rank	46.9	62.7	

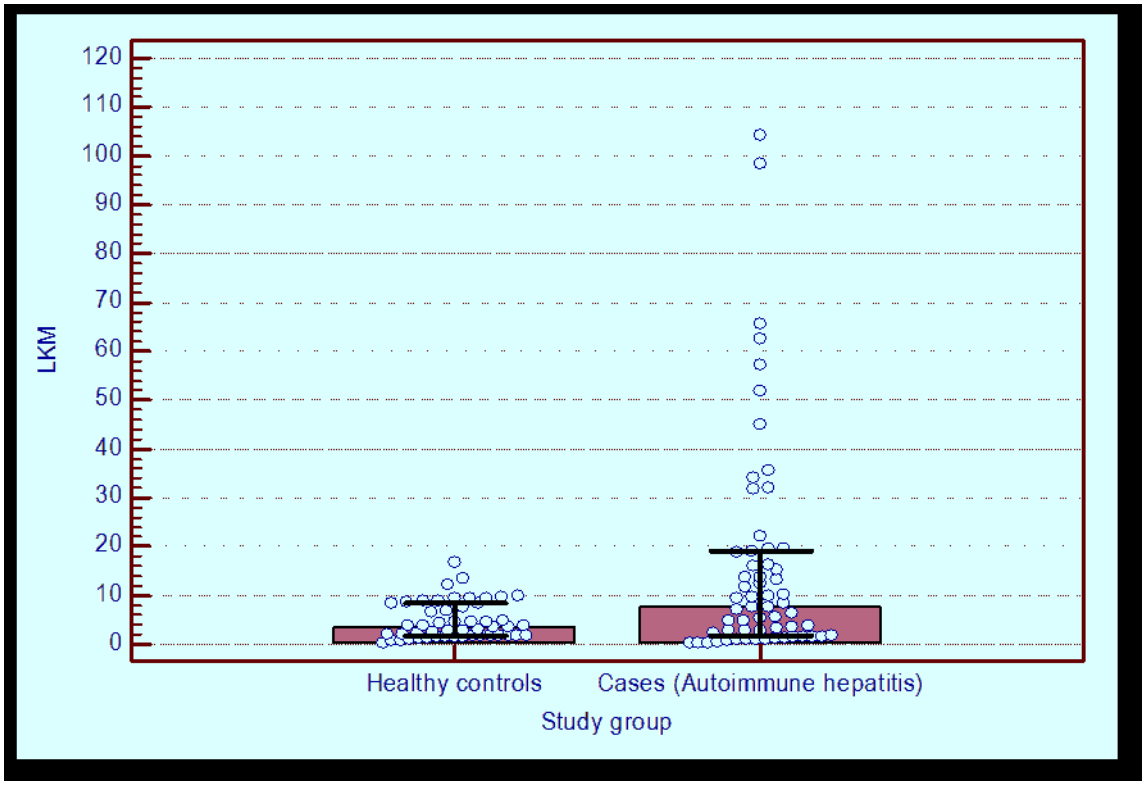


Figure (3-3): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) of LKM.

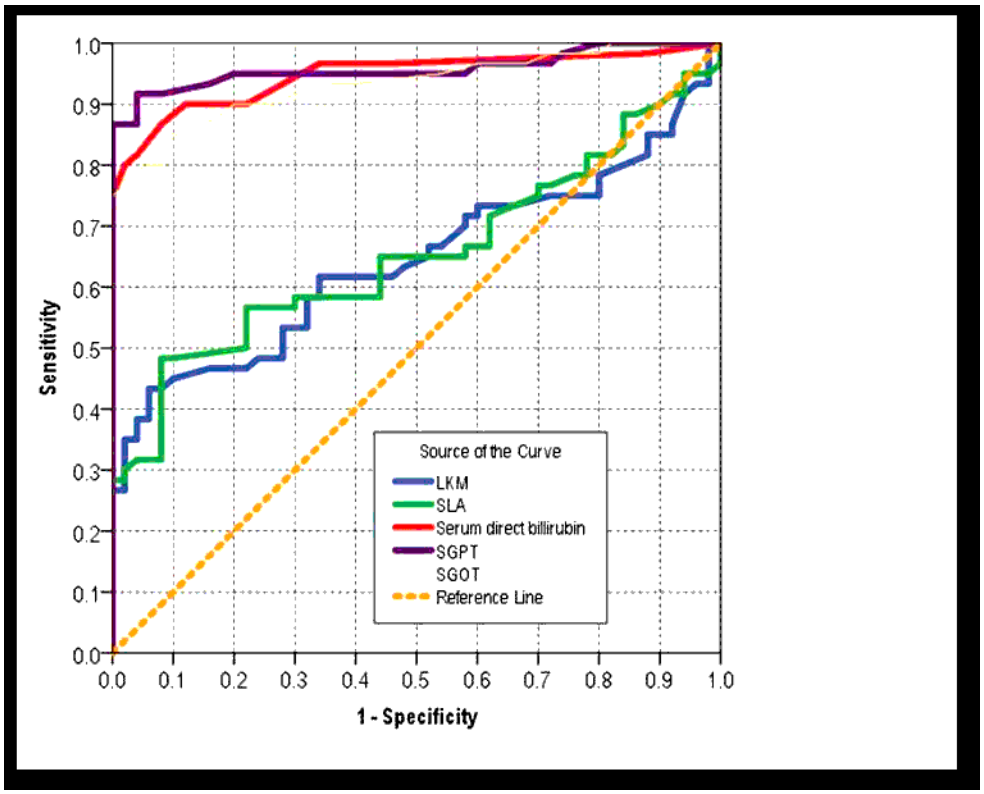


Figure (3-4): ROC curve showing the trade-off between sensitivity (true positive rate) and 1-specificity (false negative rate) for LKM when used as test to predict a diagnosis of autoimmune hepatitis differentiating it from healthy controls.

3.2.4. Anti-Mitochonderial Antibody (AMA).

The median concentrations of AMA antibody has no significant differences (P 0. 33) among autoimmune hepatitis patient (4.7 U/ml) which approximate it is median among healthy control (3.8 U/ml), as shown in table (3-6). Moreover, table (3-6) and figure (3-6) shows no higher differences in range, median, and inter-quartile ranges for this antibody among the two studied groups.

Table (3-6): Different (anti-AMA) level parameters among study groups.

Anti-Mitochonderial antibody (AMA U/ml)	Study group		P value
	Healthy controls	Autoimmune hepatitis	0.33[NS]
Range	(0.1 to 52)	(0.1 to 90.5)	
Median	3.8	4.7	
Interquartile range	(1.8 to 7.2)	(1.05 to 14.8)	
N	50	60	

The diagnostic and differentiating value of this auto-antibody has been also calculated using ROC curves; specificity, sensitivity, accuracy and positive/ negative predictive values. In the differentiation of autoimmune hepatitis patients from healthy control, this test has revealed an ROC area of (0.554, p 0.33[NS]) (figure 3-14 and table 3-9).

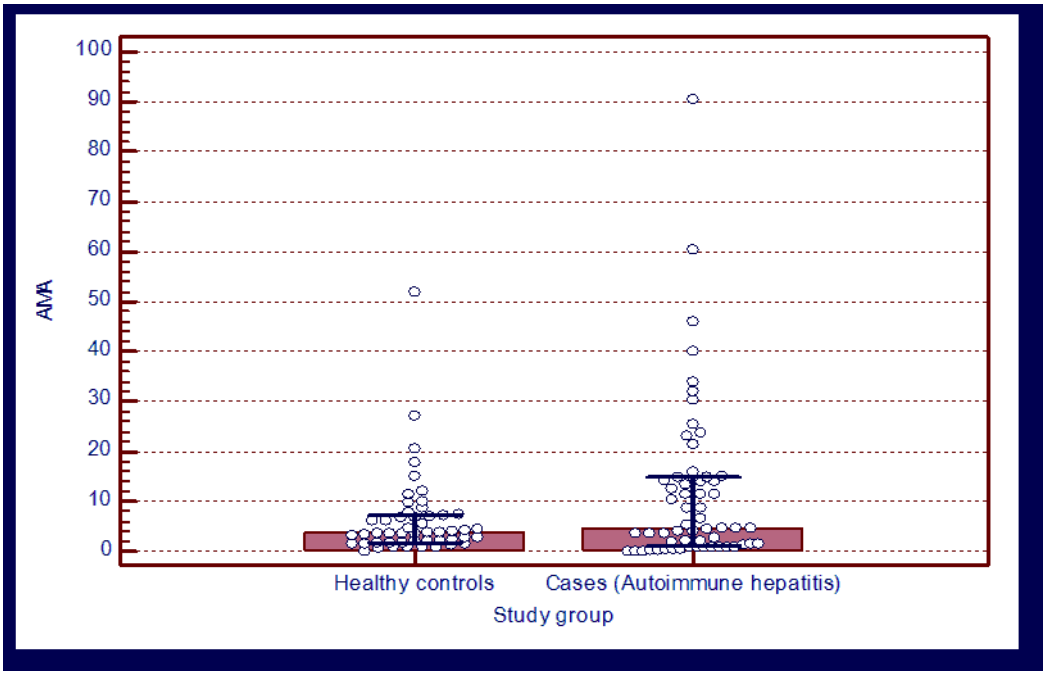


Figure (3-5): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) AMA.

3.2.5. Anti-Liver cytosolic Antibody (LC1)

Table (3-7) shows the prevelance of anti liver cytosolic-1 antibody, the number of positive LC1 in this study were 6(10%) in AIH patients and the OR 95% CI ratio were 12.05(1.43 - 101.5) and p value (0.022), this autoamtibody shown no significant differences when compared the case group with health control group, figure (3-7), with etiologic fraction (0.092).

Table (3-7): The risk of being a case (autoimmune hepatitis) by LC1.

LC1	Study group							
	Healthy controls		Autoimmune hepatitis					
	No	%	No	%	OR	95% CI OR	P	EF
Negative	50	100.0	54	90.0				
Positive	0	0.0	6	10.0	12.05	(1.43 - 101.5)	0.022	0.092

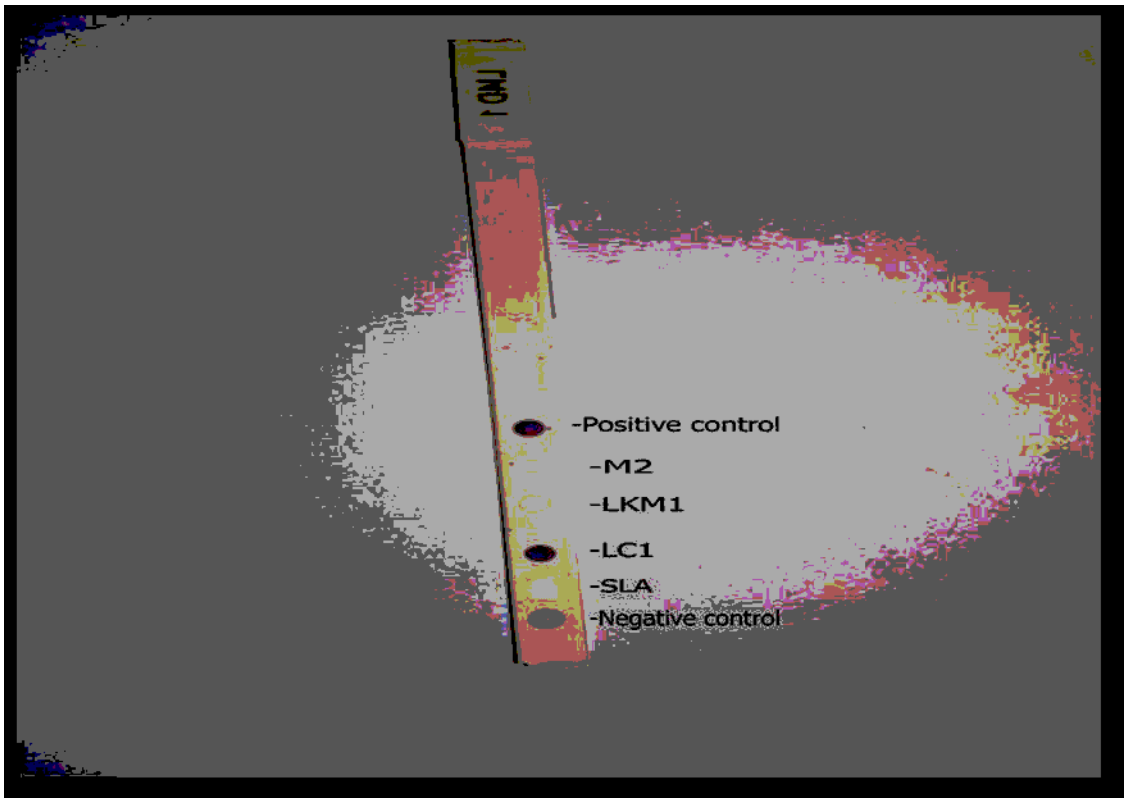


Figure (3-6): the positive LC1 by HepAK Dot method

3.3. Biochemical test

In the current study, all biochemical tests (Total serum bilirubin, Direct serum bilirubin, SGPT, SGOT, and Alkaline phosphatase), median concentrations has significant ($P < 0.001$) among autoimmune hepatitis patient more than among healthy control, as shown in table (3-8). Also table (3-8) and fig. (3-8), fig. (3-9), fig.(3-10), fig.(3-11) and fig.(3-12) show the higher range, median, and inter-quartile ranges for this biochemical tests among the two studied groups.

Table (3-8): The case-control difference in median of selected measurements.

Biochemical test (mg/dL)	Study group		P Value
	Healthy Control N(50)	Autoimmune hepatitis N(60)	
Total serum billirubin(mg/dL)			**<0.001**
Range	(0.4 to 1.2)	(0.8 to 25.2)	

Median	0.8	4.1	
Interquartile range	(0.7 to 0.9)	(2.3 to 8.8)	
Serum direct billirubin(mg/dL)			**<0.001**
Range	(0.1 to 0.9)	(0.1 to 16.7)	
Median	0.2	1.7	
Interquartile range	(0.2 to 0.4)	(0.95 to 3.6)	
SGPT(U/L)			**<0.001**
Range	(7 to 43)	(17 to 943)	
Median	23.0	132.5	
Interquartile range	(17 to 29)	(82.5 to 303.5)	
SGOT(U/L)			**<0.001**
Range	(7 to 54)	(12 to 942)	
Median	21.0	89.0	
Interquartile range	(15 to 28)	(54.5 to 233)	
Alkaline phosphatase(U/L)			**<0.001**
Range	(11 to 106)	(45 to 981)	
Median	66.5	222.0	
Interquartile range	(48 to 81)	(109.5 to 373.5)	

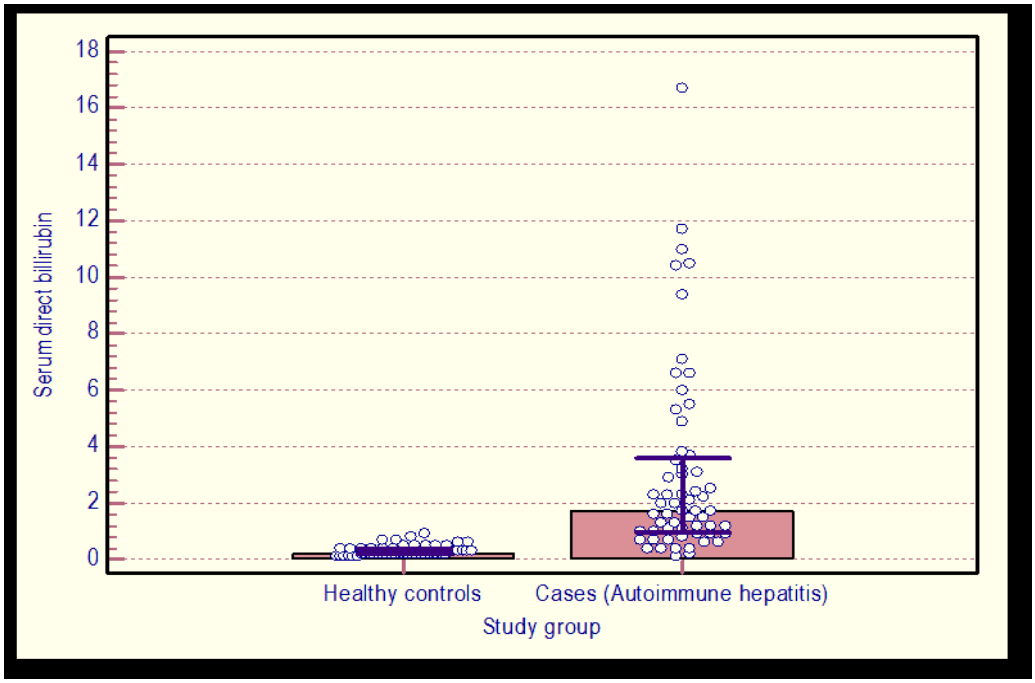


Figure (3-7): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) direct serum billirubin.

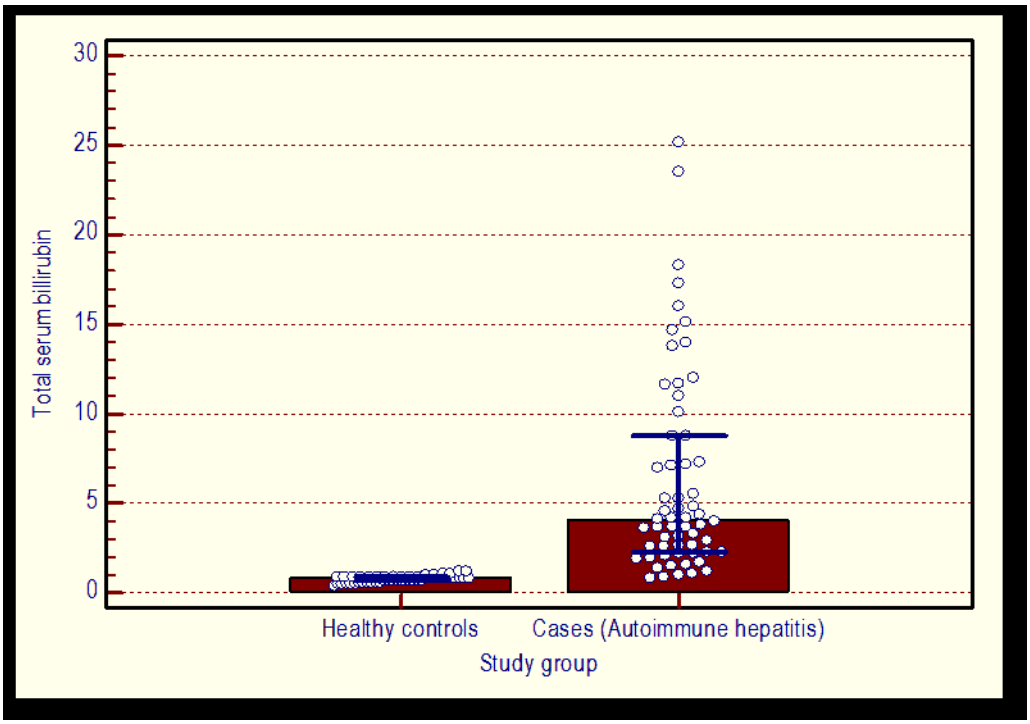


Figure (3-8): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) serum total billirubin.

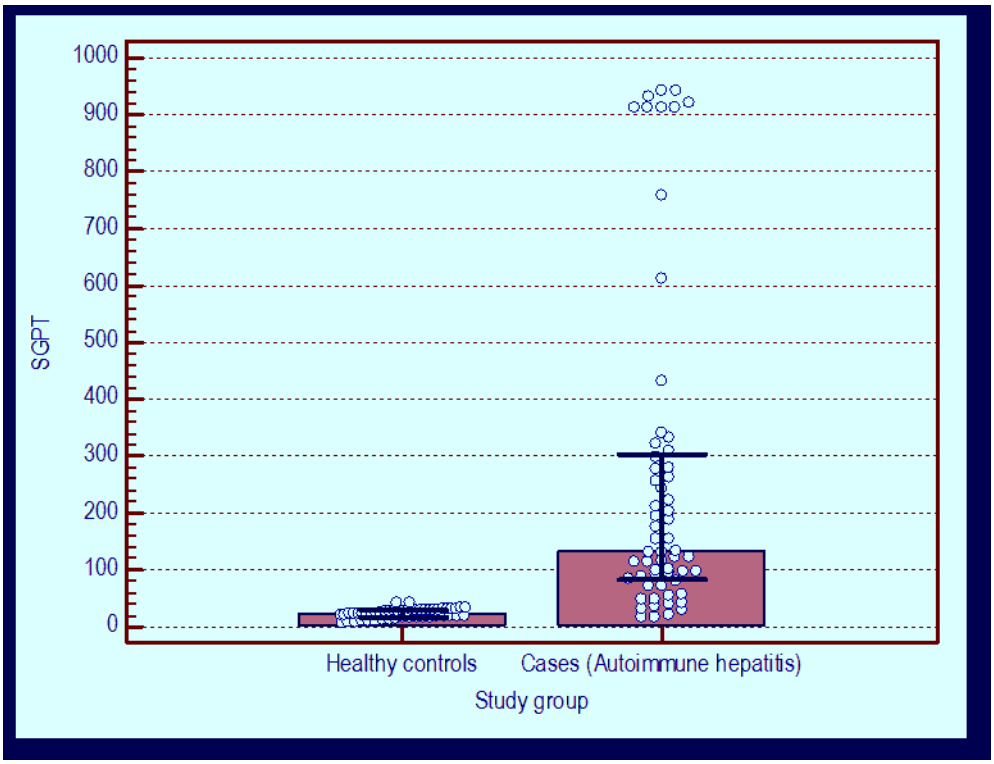


Figure (3-9): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) serum SGPT.

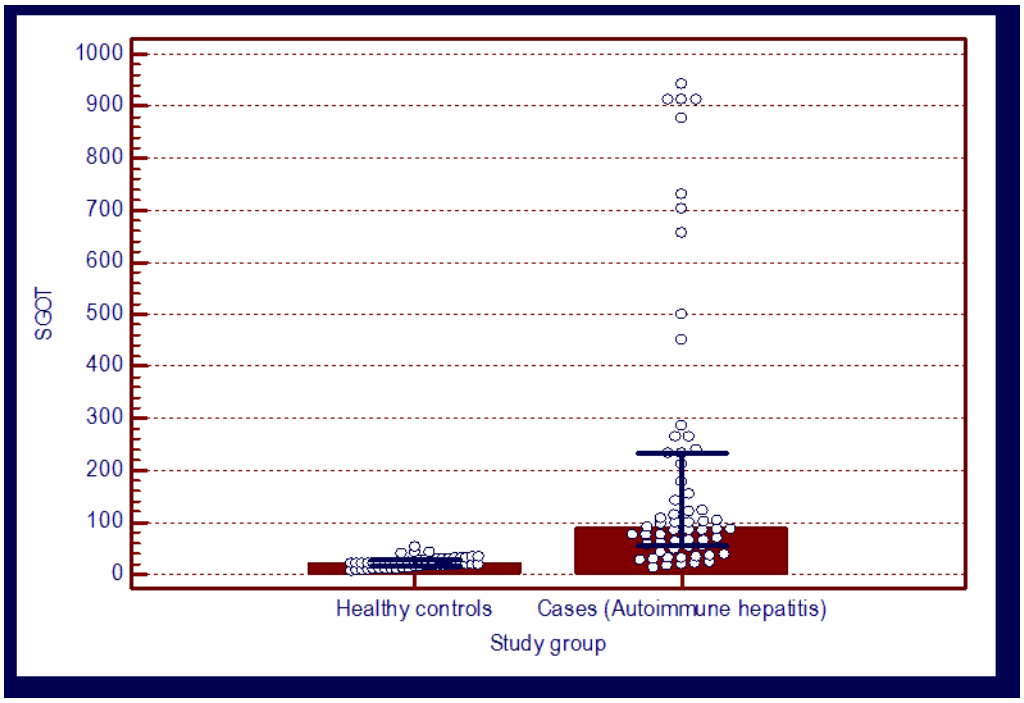


Figure (3-10): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) serum SGOT.

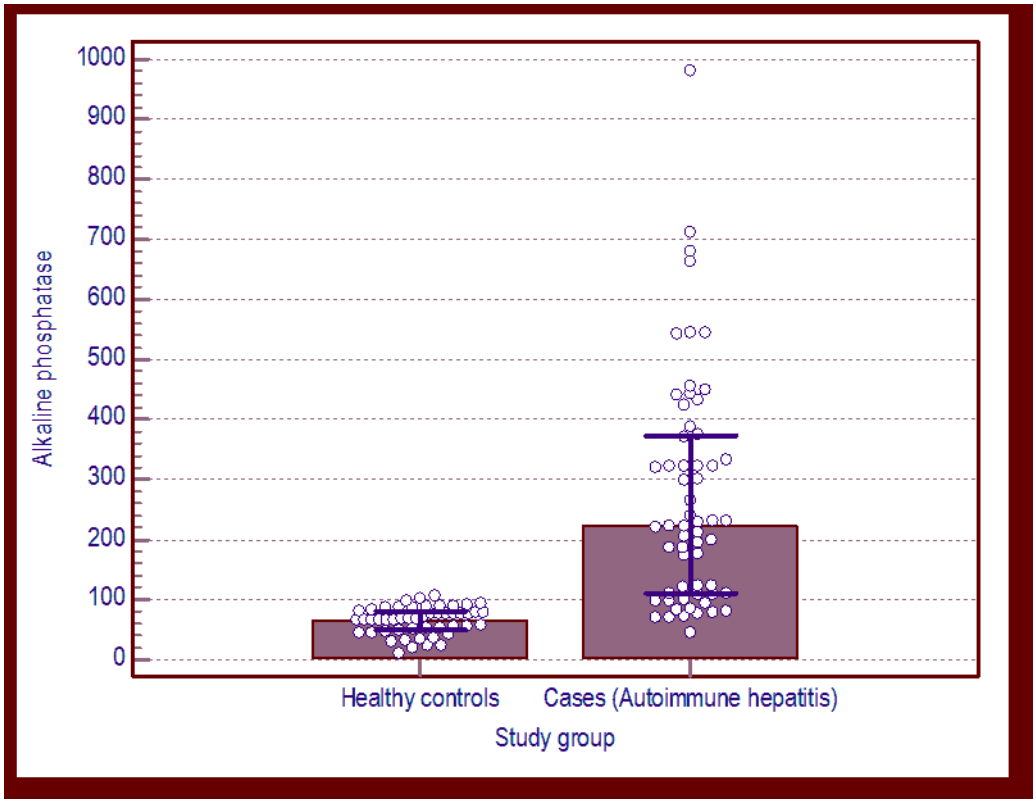


Figure (3-11): Dot diagram with error bars showing the case-control difference in median (with its inter-quartile range) serum Alkaline phosphatase

Total serum total bilirubin, SGPT, Serum direct bilirubin, alkaline phosphatase and SGOT qualified as test for diagnosing autoimmune hepatitis differentiating it from healthy controls (ROC area > 0.9), table (3-9), figure (3-13) and figure (3-14).

Serum ANA was a very good test with an ROC area of (0.804), while Serum SLA and LKM provided a test with marginal validity for differentiating between cases and healthy controls.

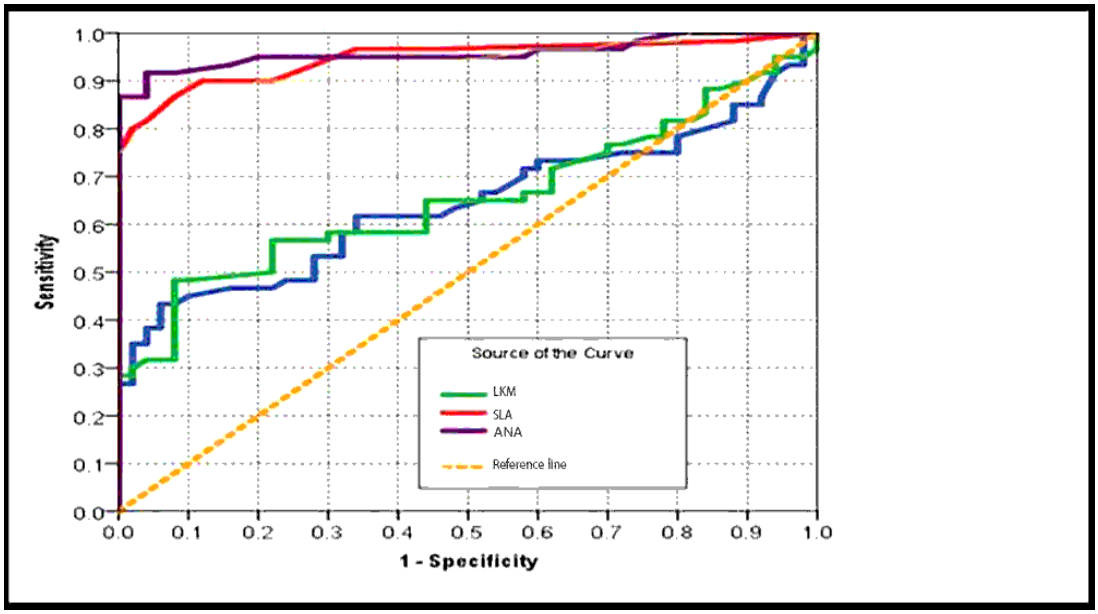


Figure (3-12): ROC curve showing the trade-off between sensitivity (true positive rate) and 1-specificity (false negative rate) for selected measurements when used as test to predict a diagnosis of autoimmune hepatitis differentiating it from healthy controls.

Table (3-9): Receiver operated characteristics curve (ROC) area for selected measurements when used as test to predict a diagnosis autoimmune hepatitis differentiating them from healthy controls.

Auto-antibody (U/ml)	**Area under ROC**	**P**
ANA	**0.804**	**<0.001**
SLA	**0.655**	**0.005**
LKM	**0.644**	**0.01**
AMA	**0.554**	**0.33[NS]**

The auto-antibodies are influenced by age factor; (table 3-10) indicates a results difference in the prevalence of antibodies in relation to age group. In this study the median with age intervals <30 of AMA higher than the median of age intervals (30-49 and 50+ years), but failed to reach statistically significant (P 0.96).

Table (3-10): Median concentration of autoantibodies in AIH patients in relation to age groups.

Autoantibodies	**Age group (years)**			**P Value**
	< 30 N(14)	**30-49 N(31)**	**50+ N(15)**	
AMA				**0.96[NS]**
Range	(0.3 to 60.3)	(0.1 to 46)	(0.1 to 90.5)	
Median	6.0	4.7	4.0	
Interquartile range	(1.2 to 14.8)	(0.9 to 14.1)	(0.5 to 23.8)	
LKM				**0.45[NS]**
Range	(0.3 to 19.7)	(0.3 to 104.3)	(0.3 to 98.3)	
Median	5.9	9.5	7.5	
Interquartile range	(2.4 to 12.4)	(1.6 to 31.7)	(1.6 to 18.89)	
SLA				**0.13[NS]**
Range	(0.2 to 35)	(0.2 to 174.3)	(0.8 to 95.3)	

Median	4.5	13.1	7.9	
Interquartile range	(3.6 to 10.5)	(4.2 to 20.1)	(4.3 to 22.2)	
ANA				**0.06[NS]**
Range	(0.2 to 164)	(0.7 to 164.2)	(3.4 to 131.7)	
Median	10.4	37.0	33.3	
Interquartile range	(3.3 to 22.6)	(11.7 to 87.2)	(9.7 to 51.7)	

Table (3-11) shows the Prevalence of autoantibodies in AIH patients in relation to duration of disease, there is an increase the probability of auto-immune hepatitis after less than one years of duration of AIH in all autoantibodies may be concluded. However, there is no statistically significant.

Table (3-11): Median concentration of autoantibodies in AIH patients in relation to duration of disease.

Autoantibodies	Duration of the disease in years-categories			P Value
	< 1 N(25)	1-2 N(20)	3+ N(15)	
AMA				**0.53[NS]**
Range	(0.1 to 60.3)	(0.1 to 34)	(0.5 to 90.5)	
Median	4.7	4.5	4.7	
Interquartile range	(0.8 to 14.1)	(1.05 to 14.35)	(3.6 to 25.4)	
Mean rank	29.4	28.7	34.8	
LKM				**0.23[NS]**
Range	(0.3 to 65.5)	(0.3 to 104.3)	(0.8 to 34)	
Median	4.8	11.1	7.5	
Interquartile range	(1.4 to 11.8)	(5.1 to 33.6)	(1.6 to 16.3)	
Mean rank	26.7	35.7	30	
SLA				**0.08[NS]**
Range	(0.2 to 69.5)	(3.5 to 46.1)	(0.8 to 174.3)	
Median	4.6	11.5	7.9	
Interquartile range	(2.8 to 15.6)	(6.7 to 20.1)	(4.3 to 82.3)	

Mean rank	24.6	35.2	34.2	
ANA				**0.6[NS]**
Range	(0.4 to 164.2)	(0.2 to 109.7)	(1.1 to 131.7)	
Median	28.1	18.1	14.2	
Interquartile range	(11 to 87.2)	(8.35 to 38.2)	(6.5 to 87.2)	
Mean rank	32.8	27.5	30.8	

3.4. Parameters' validity of autoantibodies.

The sensitivity, specificity, accuracy, positive (PPV) and negative (NPV) predictive values have been utilized in the assessment and evaluation of auto-antibodies in diagnosis and differentiation of the two groups investigated in this study, appendix (3).

Receiver operated characteristics curve (ROC) analysis of AMA, LKM, SLA, and ANA assays have established the optimal cut-off values to the four serological markers that give highest sensitivity and specificity for the diagnosis of AIH.

Table (3-12) shows the optimum cut-off values for each of auto-antibodies assayed in the discrimination of AIH patients and healthy control group; they were (8.20, 4.70, 7.85, and 9.20 respectively). The sensitivity, specificity and accuracy were demonstrated for all 4 auto-antibodies in table 3-12.

Positive predictive value at pre-test probability (PPV) 90% for AMA, LKM, SLA, and ANA were (95.3%, 94.2%, 95.9% and 98.0% respectively); negative predictive value at pre-test probability (NPV) were (92.9%, 93.9%, 94.2% and 96.8% also respectively) for each of four auto-antibodies.

Table (3-12): Parameters' validity for auto-antibodies when used as test to predict AIH differentiating them from healthy controls.

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
AMA						
0.20 (Highest sensitivity)	95.0	2.0	52.7	49.2	89.7	78.3
8.20 **(Optimum cut-off)**	45.0	80.0	60.9	69.2	95.3	92.9
56.15 (Highest specificity)	3.3	100.0	47.3	100.0	100.0	90.3
LKM						
0.25 (Highest sensitivity)	100.0	2.0	55.5	50.5	90.2	100.0
4.70 **(Optimum cut-off)**	61.7	66.0	63.6	64.5	94.2	93.9
17.85 (Highest specificity)	26.7	100.0	60.0	100.0	100.0	92.5
SLA						
0.95 (Highest sensitivity)	95.0	2.0	52.7	49.2	89.7	78.3
7.85 **(Optimum cut-off)**	56.7	78.0	66.4	72.0	95.9	94.2
18.05 (Highest specificity)	28.3	100.0	60.9	100.0	100.0	92.6
ANA						
0.17 (Highest sensitivity)	100.0	4.0	56.4	51.0	90.4	100.0
9.20 **(Optimum cut-off)**	75.0	86.0	80.0	84.3	98.0	96.9
21.50 (Highest specificity)	50.0	100.0	72.7	100.0	100.0	94.7
Total serum billirubin						
0.75 (Highest sensitivity)	100.0	44.0	74.5	64.1	94.1	100.0
1.15 **(Optimum cut-off)**	93.3	96.0	94.5	95.9	99.5	99.2
1.30 (Highest specificity)	91.7	100.0	95.5	100.0	100.0	99.1
Serum direct billirubin						
0.15 (Highest sensitivity)	98.3	12.0	59.1	52.8	91.0	98.5
0.65 **(Optimum cut-off)**	86.7	92.0	89.1	91.5	99.0	98.4

0.95 (Highest specificity)	75.0	100.0	86.4	100.0	100.0	97.3
SGPT						
16.5 (Highest sensitivity)	100.0	20.0	63.6	55.6	91.8	100.0
35.5 **(Optimum cut-off)**	91.7	96.0	93.6	95.8	99.5	99.0
45.5 (Highest specificity)	86.7	100.0	92.7	100.0	100.0	98.5
SGOT						
11.5 (Highest sensitivity)	100.0	18.0	62.7	54.9	91.6	100.0
33.5 **(Optimum cut-off)**	85.0	90.0	87.3	89.5	98.7	98.2
54.5 (Highest specificity)	75.0	100.0	86.4	100.0	100.0	97.3
Alkaline phosphatise						
44.0 (Highest sensitivity)	100.0	18.0	62.7	54.9	91.6	100.0
92.5 **(Optimum cut-off)**	85.0	92.0	88.2	91.4	99.0	98.2
107.0 (Highest specificity)	78.3	100.0	88.2	100.0	100.0	97.6

ROC analysis of biochemical test (Total serum bilirubin, direct serum bilirubin, SGPT, SGOT, and Alkaline phosphatase) assays have established the optimal cut-off values to the five biochemical markers that give highest sensitivity and specificity for the diagnosis of AIH.

The optimum cut-off values for each of biochemical markers assayed in the discrimination of AIH patients and healthy control group; they were (1.15, 0.65, 35.5, 33.5, and 92.5 respectively). The sensitivity, specificity and accuracy were demonstrated for all 5 biochemical test in table 3-12.

Positive predictive value at pre-test probability (PPV) 90% was (99.5%, 99.0%, 99.5%, 98.7% and 99.0 respectively); negative

predictive value at pre-test probability (NPV) was (99.2%, 98.4%, 99.0%, 98.2% and 98.2 also respectively) for each of five biochemical test.

3.5. Molecular detection of HLA-DR3, DR4, and B27 genes

There are specific alleles of HLA-class II that associated with susceptibility for development of the AIH disease. The frequency of distribution of class II HLA-DR3, DR4 and class I HLA- B27 alleles for patients as compared with healthy control group in (%, OR, P, EF) are shown in (Table 3-13). A survey of the distribution of HLA-DR3, HLA-DR4 and HLA-B27 genes frequency yielded evidence of positive association between class II alleles and AIH disease. For HLA-DR3, there was a high significant difference in the frequency of this gene, that is, 58.3% vs. 16.0%, with OR: 7.35, and etiological fraction (EF): 0.504 when the patients group compared with healthy control, there was statically difference (P <0.001). The second gene, HLA-DR4 investigated in this study has also showed a very high significant difference as it is expressed in high frequency in AIH disease patients compared with control group; 66.7 vs. 20.0% with OR: 8.0, and etiological fraction: 0.583, the (P <0.001). Moreover, the third gene HLA-B27, are found in low frequencies in patients of AIH disease compared to healthy control groups. The percentages of HLA-B27 genes among AIH disease patients were 23.3% vs. 12.0% with OR 2.23, and etiological fraction (EF 0.129); with no significant difference (P= 0.131).

Table (3-13): The risk of having AIH disease compared to controls in the presence of selected positive HLA-DR3, DR4 & B27.

	Healthy controls (N= 50)		AIH patients (N= 60)					
HLA gene	**N**	**%**	**N**	**%**	**OR**	**95% CI OR**	**P**	**EF**
HLA-DR3								
Negative	42	84.0	25	41.7				
Positive	8	16.0	35	58.3	7.35	(2.95 -18.3)	<0.001	0.504
HLA-DR4								
Negative	40	80.0	20	33.3				
Positive	10	20.0	40	66.7	8.00	(3.33 – 19.2)	<0.001	0.583
HLA-B27								
Negative	44	88.0	46	76.7				
Positive	6	12.0	14	23.3	2.23	(0.79 - 6.33)	0.131[NS]	0.129

Overall predictive accuracy = 88.2%..... P (Model) < 0.001
OR..... Oddes ratio
EF ..... etiological fraction

As shown in table (3-14), after adjusting for the other two tested HLA genes, the presence of HLA-DR3 significantly increased the risk of having the disease by 14.7 times. A positive HLA-DR4 significantly increased the risk of having autoimmune hepatitis by 16.8 times after controlling for the remaining two HLA genes included in the model. A positive HLA-B27 marginally increased the risk of having autoimmune hepatitis by 2.2 times after controlling for the remaining two HLA genes included in the model. The model was statistically significant and accurately predicted the group membership of subjects (controls Vs cases) with 79.1% accuracy, fig. (3-15), fig. (3-16), fig. (3-17), fig. (3-18). fig. (3-19) and fig. (3-20).

Table (3-14): Multiple logistic regressions with the risk of being a case (autoimmune hepatitis) as the dependent (outcome) variable and selected HLA phenotype as the explanatory variables.

HLA- gene	Partial OR	95% confidence interval	P
Positive DR3	14.7	(4.4 to 48.8)	<0.001
Positive DR4	16.8	(5.3 to 53.7)	<0.001
Positive B27	2.2	(0.56 to 8.8)	0.25[NS]

Overall predictive accuracy = 79.1%
P (Model) < 0.001

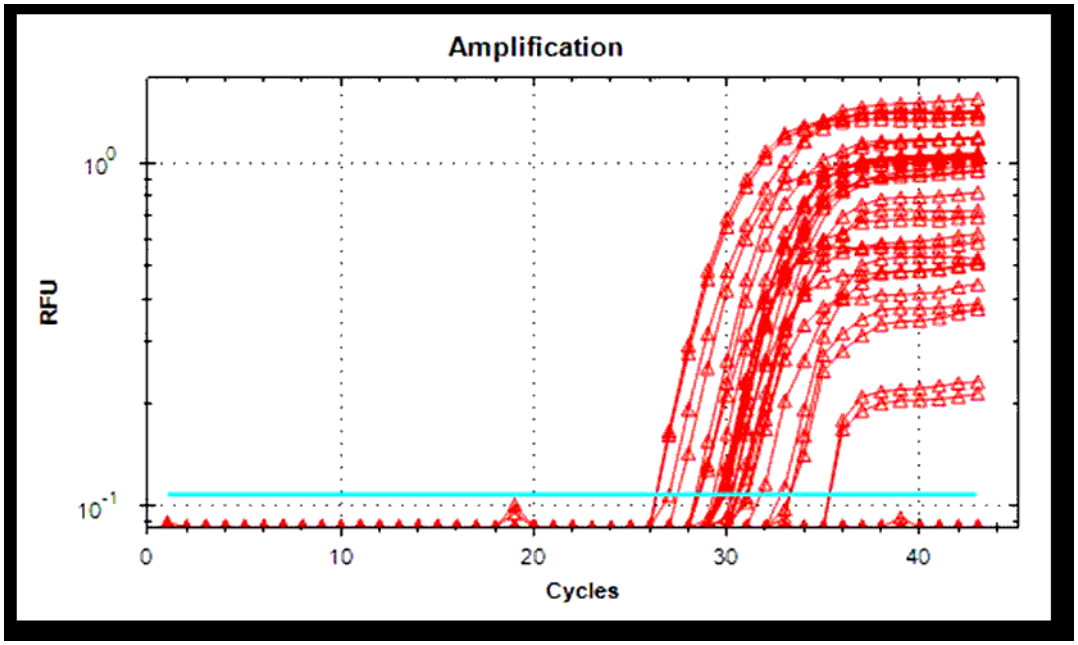


Figure (3-13): Real-Time PCR amplification plot of HLA-DR3 gene in autoimmune hepatitis patients group.

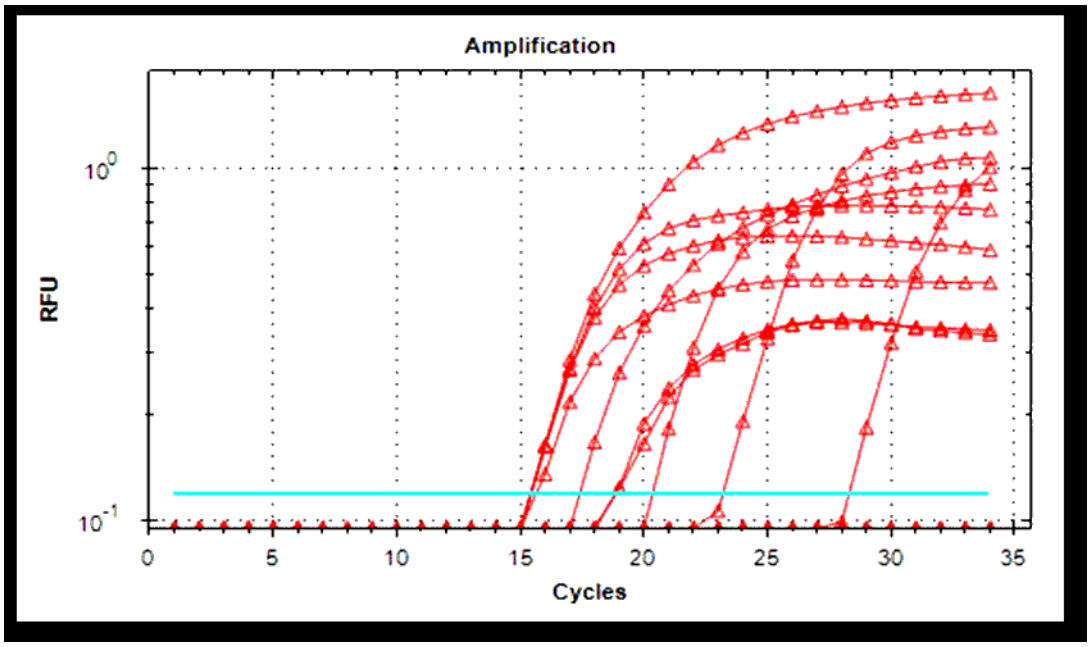


Figure (3-14): Real-Time PCR amplification plot of HLA-DR3 gene in healthy control group.

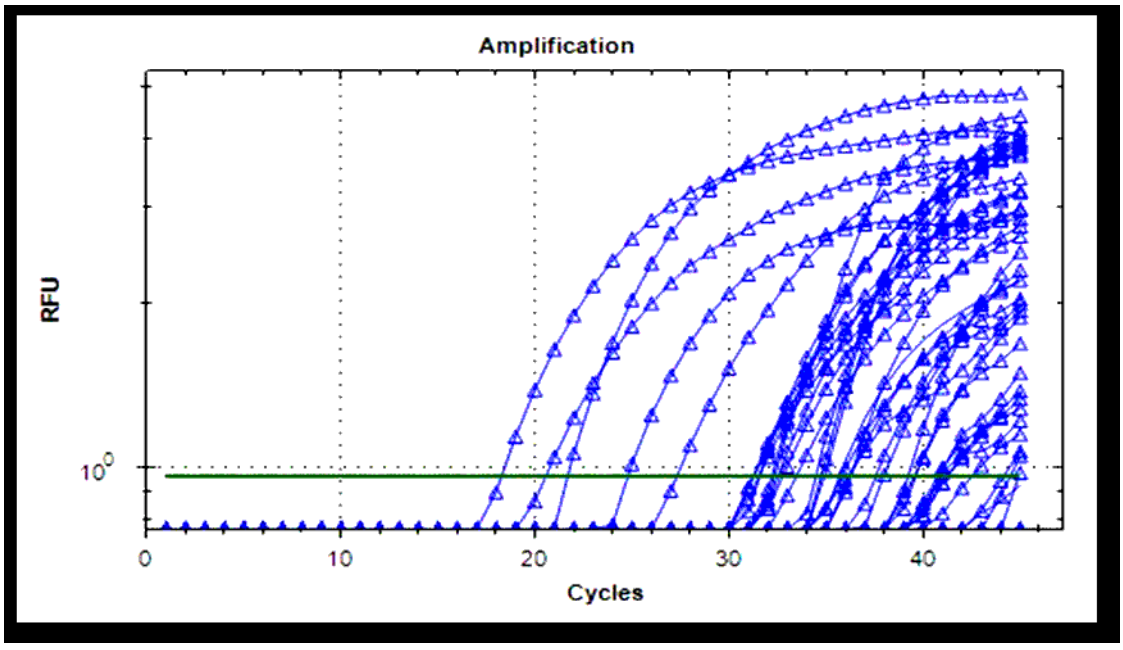


Figure (3-15): Real-Time PCR amplification plot of HLA-DR4 gene in autoimmune hepatitis patients group

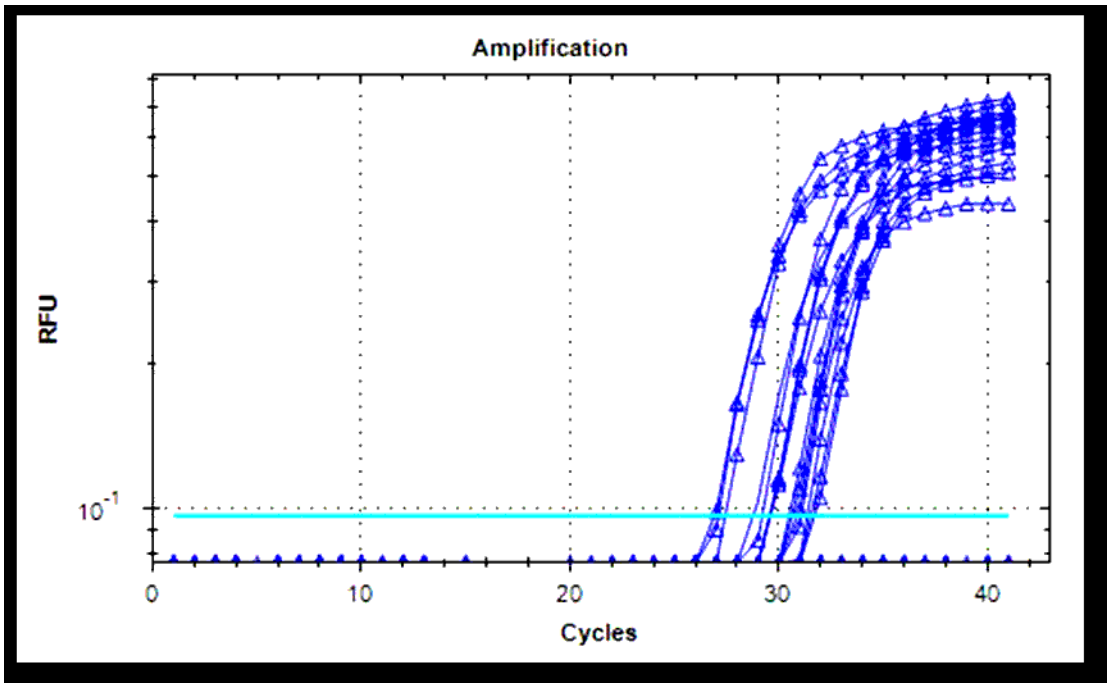


Figure (3-16): Real-Time PCR amplification plot of HLA-DR4 gene in healthy control group.

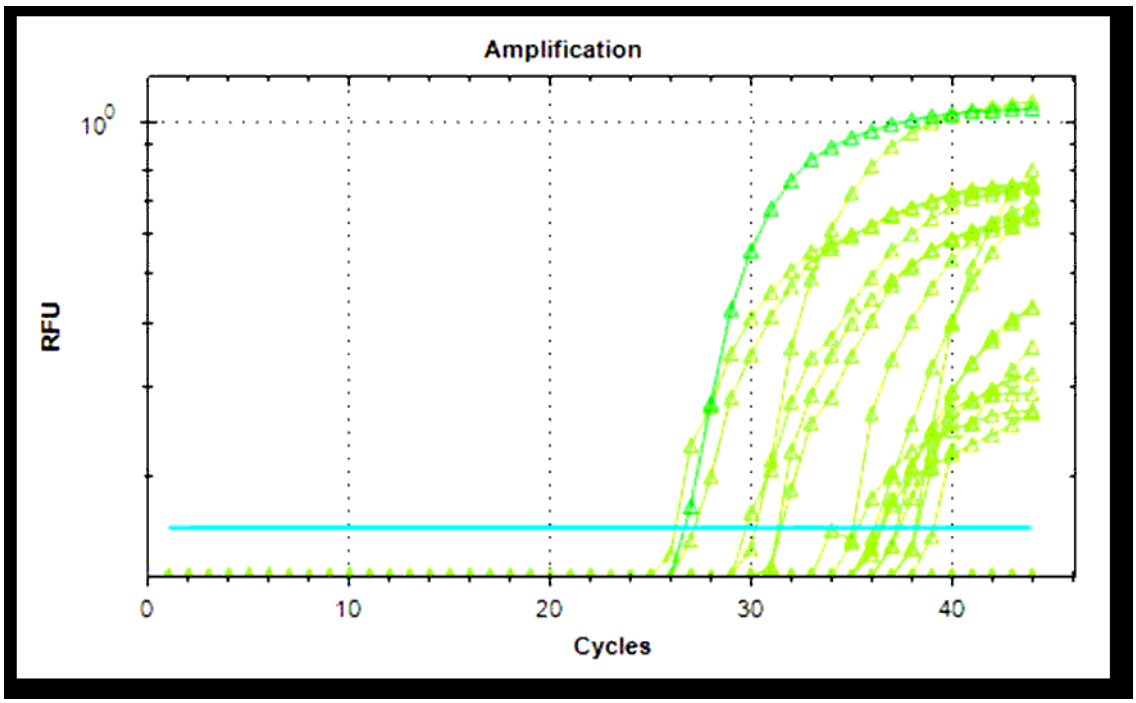


Figure (3-17): Real-Time PCR amplification plot of HLA-B27 gene in autoimmune hepatitis patients group

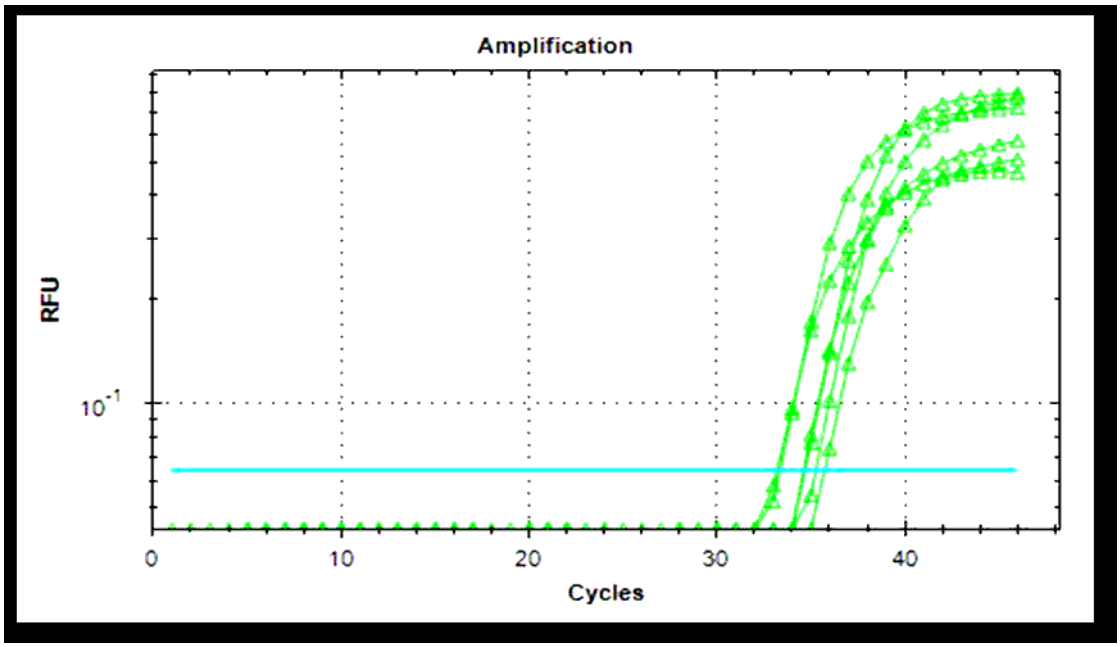


Figure (3-18): Real-Time PCR amplification plot of HLA-B27 gene in healthy control group.

3.6. HLA-DR3, HLA-DR4 and HLA-B27 association with autoantibodies

As shown in table (3-15), the net and independent effect of a set of HLA genes and selected antibodies on the risk of having autoimmune hepatitis was tested in a multiple logistic regression model. Having a positive HLA-DR3 significantly increased the risk of having autoimmune hepatitis by 9.9 times after controlling (adjusting) for the remaining explanatory variables included in the model. A positive HLA-DR4 significantly increased the risk of having autoimmune hepatitis by 17.6 times after controlling for the remaining explanatory variables included in the model. A positive HLA-B27 marginally increased the risk of having autoimmune hepatitis by 2.2 times after

controlling for the remaining two HLA genes and the other explanatory markers included in the model. For each one unit increase in ANA concentration the risk of having the disease is multiplied by 1.11% after adjusting for the remaining explanatory variables included in the model. The remaining three markers had a marginal positive association with the risk of having the disease after controlling for other independent variables included in the model. The model was statistically significant and accurately predicted the group membership of subjects (controls Vs cases) with 88.2% accuracy.

Table (3-15): Multiple logistic regressions with the risk of being a case (autoimmune hepatitis) as the dependent outcome variable and selected HLA genotype and selected markers as the explanatory variables.

	Partial OR	95% confidence interval	P
Positive DR3	9.9	(1.94 to 50.5)	0.006
Positive DR4	17.6	(3.5 to 88.7)	<0.001
Positive B27	2.2	(0.3 to 15.6)	0.45[NS]
AMA	1.05	(0.99 to 1.11)	0.1[NS]
LKM	1.04	(0.97 to 1.12)	0.3[NS]
SLA	1.06	(0.96 to 1.18)	0.23[NS]
ANA	1.11	(1.03 to 1.21)	0.011

Table (3-16) represented the distribution of serum-positive and negative autoantibodies and biochemichals assay in patients with HLA-DR3 risky gene. The proportion of serum-positive AMA in patients with HLA-DR3 risky gene were significant higher (P= 0.006) than those who had serum-negative AMA, the median of positive patients (11.4) vs negative patients (2.1) and also SLA antibody had significant difference with HLA-DR3, the median of positive patients (12.4) compared to negative patients (4.6), p value (0.001) the same result with SGOT the median of positive patients (109) compared to negative patients (77) and (P=0.041).

Also this study showed no statistically significant difference association between HLA-DR3 and other auto-antibody (table 3-16).

Table (3-16): Association of studying assay level with HLA-DR3

	HLA-DR3		
	Negative N(25)	**Positive N(35)**	**P**
AMA			**0.006**
Range	(0.1 to 60.3)	(0.1 to 90.5)	
Median	2.1	11.4	
Interquartile range	(0.8 to 10.3)	(2.7 to 23)	
Mean rank	23.2	35.7	
LKM			**0.08[NS]**
Range	(0.3 to 104.3)	(0.3 to 98.3)	
Median	2.9	9.7	
Interquartile range	(1.3 to 15.3)	(4.8 to 19.7)	
Mean rank	25.8	33.9	
SLA			**0.001**
Range	(0.2 to 44.5)	(0.2 to 174.3)	
Median	4.6	12.4	
Interquartile range	(2.8 to 13.1)	(5.5 to 22.3)	
Mean rank	23.7	35.4	
ANA			**0.93[NS]**
Range	(0.2 to 164.2)	(0.7 to 164)	
Median	14.2	27.4	

Interquartile range	(6.5 to 65.6)	(9.3 to 56.3)	
Mean rank	30.3	30.7	
Total serum billirubin			**0.47[NS]**
Range	(0.9 to 15.1)	(0.8 to 25.2)	
Median	3.7	4.2	
Interquartile range	(2.6 to 5.5)	(2.3 to 11.6)	
Mean rank	28.6	31.9	
Serum direct billirubin			**0.78[NS]**
Range	(0.2 to 7.1)	(0.1 to 16.7)	
Median	2.0	1.6	
Interquartile range	(1 to 2.5)	(0.9 to 4.9)	
Mean rank	29.8	31	
SGPT			**0.13[NS]**
Range	(18 to 943)	(17 to 942)	
Median	122.0	203.0	
Interquartile range	(71 to 195)	(94 to 432)	
Mean rank	26.5	33.4	
SGOT			**0.041**
Range	(12 to 913)	(17 to 942)	
Median	77.0	109.0	
Interquartile range	(34 to 103)	(66 to 285)	
Mean rank	25.1	34.4	
Alkaline phosphatise			**0.94[NS]**
Range	(69 to 544)	(45 to 981)	
Median	239.0	211.0	
Interquartile range	(110 to 321)	(109 to 387)	
Mean rank	30.7	30.4	

The distribution of serum-positive and negative autoantibodies and biochemichales assay in patients with HLA-DR4 risky gene was represented in table 3-17**;** there are no statistically significant difference association between HLA-DR4 and other auto-antibody and biochemichal assay (table 3-17). The same result was occurred with the HLA-B27 when associated with studying assay level table (3-18)

Table (3-17): Association of studying assay level with HLA-DR4

	HLA-DR4		
	Negative N(20)	Positive (40)	P
AMA			**0.84[NS]**
Range	(0.4 to 23.8)	(0.1 to 90.5)	
Median	4.4	6.0	
Interquartile range	(1.4 to 14.8)	(0.85 to 15)	
Mean rank	29.9	30.8	
LKM			**0.49[NS]**
Range	(0.3 to 51.8)	(0.3 to 104.3)	
Median	6.2	8.0	
Interquartile range	(1.55 to 16.6)	(2.35 to 18.995)	
Mean rank	28.3	31.6	
SLA			**0.39[NS]**
Range	(0.8 to 69.5)	(0.2 to 174.3)	
Median	12.6	7.9	
Interquartile range	(4.9 to 18.6)	(3.55 to 21.15)	
Mean rank	33.3	29.1	
ANA			**0.29[NS]**
Range	(0.4 to 139)	(0.2 to 164.2)	
Median	22.8	20.9	
Interquartile range	(6.35 to 37.65)	(8.55 to 164.2)	
Mean rank	27.1	32.2	
Total serum billirubin			**0.81[NS]**
Range	(0.8 to 23.5)	(0.9 to 25.2)	
Median	3.9	4.2	
Interquartile range	(2.65 to 11.65)	(2.25 to 7.25)	
Mean rank	31.3	30.1	
Serum direct billirubin			**0.99[NS]**
Range	(0.1 to 16.7)	(0.2 to 11)	
Median	1.4	2.0	
Interquartile range	(1 to 5.75)	(0.95 to 3.15)	
Mean rank	30.5	30.5	
SGPT			**0.77[NS]**
Range	(22 to 942)	(17 to 943)	
Median	127.5	143.0	
Interquartile range	(75.5 to 467)	(86.5 to 269.5)	
Mean rank	31.4	30	
SGOT			**0.29[NS]**

Range	(17 to 942)	(12 to 913)	
Median	138.5	86.0	
Interquartile range	(45 to 475)	(56.5 to 132)	
Mean rank	33.9	28.8	
Alkaline phosphatise			**0.63[NS]**
Range	(45 to 981)	(70 to 680)	
Median	216.0	225.5	
Interquartile range	(91 to 421.5)	(122 to 352)	
Mean rank	29	31.3	

Table (3-18): Association of studying assay level with HLA-B27

	HLA-B27		
	Negative N(46)	**Positive N(14)**	**P**
AMA	**0.84[NS]**		
Range	(0.1 to 90.5)	(0.3 to 46)	
Median	6.0	3.8	
Interquartile range	(0.8 to 14.1)	(1.9 to 15.1)	
N	46	14	
Mean rank	30.3	31.2	
LKM			**0.68[NS]**
Range	(0.3 to 98.3)	(0.3 to 104.3)	
Median	7.4	11.8	
Interquartile range	(1.6 to 15.3)	(1.8 to 19.7)	
Mean rank	30	32.2	
SLA			**0.92[NS]**
Range	(0.2 to 174.3)	(0.2 to 85)	
Median	9.9	7.9	
Interquartile range	(3.8 to 20.1)	(5.5 to 18.9)	
Mean rank	30.4	30.9	
ANA			**0.11[NS]**
Range	(0.2 to 164.2)	(0.4 to 164)	
Median	16.1	54.7	
Interquartile range	(6.5 to 39.4)	(13.3 to 87.2)	
Mean rank	28.5	37.1	
Total serum billirubin			**0.58[NS]**
Range	(0.8 to 25.2)	(1.2 to 16)	
Median	4.0	4.1	

Interquartile range	(2.2 to 7.3)	(2.9 to 11.7)	
Mean rank	29.8	32.8	
Serum direct billirubin			**0.58[NS]**
Range	(0.1 to 16.7)	(0.4 to 9.4)	
Median	1.8	1.7	
Interquartile range	(0.9 to 3.2)	(1.2 to 5.5)	
Mean rank	29.8	32.8	
SGPT			**0.58[NS]**
Range	(18 to 943)	(17 to 933)	
Median	143.0	127.5	
Interquartile range	(71 to 298)	(115 to 332)	
Mean rank	29.8		
SGOT			**0.97[NS]**
Range	(12 to 942)	(32 to 913)	
Median	99.0	80.5	
Interquartile range	(43 to 233)	(61 to 265)	
Mean rank	30.6	30.3	
Alkaline phosphatise			**0.62[NS]**
Range	(45 to 981)	(72 to 663)	
Median	230.0	202.0	
Interquartile range	(111 to 371)	(98 to 432)	
Mean rank	31.1	28.5	

3.7. Correlations of studied tests in AIH

Table (3-19) revealed significant correlation between SGPT with SGOT (r= 0.817 and P <0.001). Also serum total bilirubin with alkaline phosphatase (r=0.416 and p<0.001)

Table (3-19): Linear correlation coefficient of different parameters in AIH.

	AMA	LKM	SLA	ANA	Total serum billirubin	Serum direct billirubin	SGPT	SGOT	Alk	Age
LKM	r=-0.11 P=0.4[NS]									
SLA	r=0.053 P=0.69[NS]	r=0.001 P=0.99[NS]								
ANA	r=-0.103 P=0.43[NS]	r=0.267 P=0.039	r=0.112 P=0.39[NS]							
TSB	r=-0.049 P=0.71[NS]	r=0.011 P=0.93[NS]	r=-0.18 P=0.17[NS]	r=0.078 P=0.55[NS]						
Direct TSB	r=-0.025 P=0.85[NS]	r=0.065 P=0.62[NS]	r=-0.195 P=0.14[NS]	r=0.076 P=0.57[NS]	r=0.937 P<0.001					
SGPT	r=0.309 P=0.016	r=-0.022 P=0.87[NS]	r=-0.062 P=0.64[NS]	r=0.169 P=0.2[NS]	r=0.173 P=0.19[NS	r=0.168 P=0.2[NS				
SGOT	r=0.212 P=0.1[NS]	r=-0.014 P=0.91[NS]	r=-0.046 P=0.73[NS]	r=0.154 P=0.24[NS]	r=0.264 P=0.042	r=0.271 P=0.036	r=0.817 P<0.001			
ALK	r=0.027 P=0.84[NS]	r=0.029 P=0.83[NS]	r=-0.194 P=0.14[NS]	r=0.011 P=0.94[NS]	r=0.269 P=0.037	r=0.416 P<0.001	r=0.156 P=0.24[NS	r=0.216 P=0.1[NS]		
Age (years)	r=-0.124 P=0.35[NS]	r=0.103 P=0.43[NS]	r=0.257 P=0.047	r=0.189 P=0.15[NS]	r=-0.13 P=0.32[NS	r=-0.135 P=0.3[NS]	r=-0.158 P=0.23[NS	r=-0.165 P=0.21[NS]	r=-0.143 P=0.28[NS]	
Duration Of disease	r=0.166 P=0.2[NS]	r=0.09 P=0.49[NS]	r=0.247 P=0.06[NS]	r=-0.118 P=0.37[NS]	r=-0.042 P=0.75[NS	r 0.035 P 0.79[NS	r=-0.03 P=0.82[NS	r=-0.168 P=0.2[NS]	r=-0.064 P=0.63[NS]	r=0.405 P=0.001

Moreover, there are moderate correlation between AMA with SGOT(R=0.309 P=0.016), SLA with the age (r=0.257 and p=0.047), and LKM with ANA (r=0.267 and p=0.039) the ANA, duration of disease and total serum bilirubin has no significant impact on the result of different parameters in this study.

Table (3-20): Linear correlation coefficient of different parameters in healthy control group.

	AMA	LKM	SLA	ANA	Total serum billirubin	Serum direct billirubin	SGPT	SGOT	Alk
LKM	r=0.156 P=0.28[NS]								
SLA	r=-0.152 P=0.29[NS]	r=0.265 P=0.06[NS]							
ANA	r=-0.161 P=0.27[NS]	r=-0.122 P=0.4[NS]	r=-0.175 P=0.23[NS]						
TSB	r=0.115 P=0.43[NS]	r=-0.021 P=0.88[NS]	r=-0.008 P=0.96[NS]	r=-0.025 P=0.86[NS]					
Direct TSB	r=0.21 P=0.14[NS]	r=0.14 P=0.33[NS]	r=-0.097 P=0.5[NS]	r=0.2 P=0.16[NS]	r=0.248 P=0.08[NS]				
SGPT	r=-0.201 P=0.16[NS]	r=0.144 P=0.32[NS]	r=0.192 P=0.18[NS]	r=-0.127 P=0.38[NS]	r=0.234 P=0.1[NS]	r=0.244 P=0.09[NS]			
SGOT	r=-0.084 P=0.56[NS]	r=-0.081 P=0.58[NS]	r=-0.012 P=0.93[NS]	r=-0.135 P=0.35[NS]	r=-0.23 P=0.11[NS]	r=-0.204 P=0.16[NS]	r=0.065 P=0.65[NS]		
Alk	r=0.174 P=0.23[NS]	r=0.044 P=0.76[NS]	r=-0.027 P=0.85[NS]	r=-0.105 P=0.47[NS]	r=0.051 P=0.72[NS]	r=-0.079 P=0.58[NS]	r=-0.089 P=0.54[NS]	r=0.036 P=0.8[NS]	
Age (years)	r=0.016 P=0.91[NS]	r=-0.216 P=0.13[NS]	r=-0.029 P=0.84[NS]	r=0.108 P=0.45[NS]	r=0.062 P=0.67[NS]	r=0.001 P=0.99[NS]	r=-0.104 P=0.47[NS]	r=-0.072 P=0.62[NS]	r=0.26 P=0.07[NS]

Chapter Four

Discussion

4. Discussion

4.1. The age and gender

Autoimmune hepatitis (AIH) is an inflammatory condition of the liver that can affect ages, sexes, and races of patients (Hennes *et al*., 2008). In the current study the age range of 7-69 years in autoimmune hepatitis patients with mean age (40.6), this result was just similar to that of Mauss *et al*., (2013). Moreover, it was, for some extent comparable to (39.2±11.2) years reported by Manns and Petra, (2000).

Thus in Iraq the frequencies of AIH is in younger age patients which might be attributed to environmental factors, malnutrition and stress or even due to the fact that the life span of Iraqi are lower than that for European (Al-Obaidi, 2008).

Most autoimmune diseases show a striking sex tendency in their incidence, women being affected more frequently than men (Béland *et al*., 2009). AIH has a female predominance (sex ratio, 3.6:1). It occurs in children and adults of all ages and affects several ethnic groups (Christen and Edith 2016). The female was predominant in this study (68.3%) and this comparable with other studies where women represent (73.4%) in Al-Naaiem, (2009), Baranov *et al*., (2003) where women represent (78%) and Sabri (2003).

Also the ratio of male to female which was (1:2.8) in this work which is nearly comparable to that of (1:2.7) reported by Al-Naaiem (2009). However, the ratio 1:2.4 and 1:3 had been reported by other

studies of Manns and Obermyer-Straub (2000) and Sabri (2003), respectively.

These variations may be related to the differences in race and genetic factor in addition to environmental differences. The explanation for predominance of AIH among females more than males may be due to the effect of hormonal differences which activate Th2 and subsequently enhance autoantibodies production (Goldsby *et al*., 2000).

4.2. The Auto-antibodies levels:

In general, regarding the laboratory diagnosis of autoimmune diseases, the detection of autoantibodies alone doesn't mean the presence of neither the disease, nor it is absence exclude it so far. However, these tests, i.e., autoantibodies detection, may accomplish the clinical feature of the case, as do the biochemical tests. The routinely used tests during the course of autoimmune hepatitis were investigated in this study.

To shed light the supportive value of these autoantibodies and on possible association with more candidate genetic factors, as well.

4.2.1. Anti-Nuclear antibody (ANA)

The detection of serum autoantibodies is presumptive evidence for AIH and other autoimmune disorders. Therefore, when compared with autoantibody profile of healthy control, ANA was exclusively present in serum of patients with AIH.

There were significant differences in the median concentration of this autoantibody ($p < 0.001$) among autoimmune hepatitis patient and the level of ANA antibodies among AIH patients in this study was (75%).

Andreas *et al*., (2006) have referred to the positive level of ANA antibodies in 76% of patients with AIH group. Al-Obeidy and Al-Khalidi, (2006) have reported similar results, where the ANA was 80% positive and Al-Naaiem, (2009). However, lower frequencies have been reported by Mauss *et al*., (2013), where it occurred in only (17.6%) and Mohmad *et al*., (2010), where it occurred in only (13.33%) in AIH patients.

Although no specific ANA nuclear antigen has been identified in AIH type 1 (Bogdanos *et al*., 2008). So lower frequencies in some studies may be due to depending on type I AIH or because small number of samples.

4.2.2. Anti-Soluble liver antigen (SLA)

The anti-soluble liver antigen auto-antibodies were first described by Manns et al. in 1987 (Czaja *et al*., 2002). SLAs are directed against tRNAs and can be detected in 47.5% of the AIH patients. These antibodies are highly disease specific for severe forms of AIH type1 and are typically associated with a fatal outcome (Trivedi, and Hirschfield 2000).

There were significant differences in the median concentration of this autoantibody ($p < 0.005$) between autoimmune hepatitis patient and healthy control.

As with the occurrences of other autoantibodies during the course of diverse autoimmune diseases, such materials may be/may be not developed, and if developed, there is a certain extent at which they produced. In other words, some controversial between different studies regarding the presence of anti-SLA, some are in concordance with ours like Muratori *et al*., (2005), but differ from the results of other like Al-

Obeidy and Al-Khalidi, (2006), Al-Obeidy *et al*., (2008), Mohmad *et al*., (2009), who did not show SLA in it is result and AL- Obeidy *et al*., (2009a), this differences may be due to small number of samples .

4.2.3. Anti-Liver kidney microsom (LKM)

The significant differences of LKM among AIH patients (p= 0.01) when compared with health control group. In this study is disagree with AL-Obeidy *et al*., (2009a), the LKM was (16.44%), Mauss *et al*., (2013) the LKM (1.2%) and Muratori *et al*., 2009 (53%), this difference may be due to the small sample size and variation of prevalence of auto-immune diseases among AIH patients in this study.

4.2.4. Anti-Mitochonderial antibody (AMA)

The presence of AMA has been reported in 8–20% of typical AIH patients. Furthermore, AMA- positive AIH is associated with the presence of DRB1∗ 03, DRB1∗ 04, and DRB1∗ 13, the histological features of chronic active hepatitis and probable or improbable pretreatment scores (Hind and Hisham 2012).

There was no significant differences (P < 0. 33) in this autoantiboby among autoimmune hepatitis patient when compared with health control group. Although AMAs are specific and sensitive diagnostic markers for primary biliary cirrhoses (PBC) also can be detected in about 15% of AIH patients in association with type 1, and type 3 with specific liver proteins (AL–Mammori *et al*., 2012) this comparable to Mohmad *et al*., (2010) and Al-Obeidy and Al-Khalidi (2006).

4.2.5. Anti-Liver cytosolic 1(LC1)

This autoantibody had shown no significant differences when compared the case group with health control group (p value 0.022). This result was nearly compatible to AL-Obeidy *et al*., (2009b), were the LC1 was (13.7%) and Muratori *et al*., (2005).

4. 3. The Biochemical test

There were significant differences between the two study groups in the median concentration of all biochemical tests (total serum bilirubin, direct serum bilirubin, SGPT, SGOT, and alkaline phosphatase), ($p < 0.001$) among autoimmune hepatitis patient, this result is in agreement with what reported by some researchers regarding significant statistical association of AIH with AL- Obeidy *et al*., (2008), Al-Obeidy and Al-Khalidi (2006), and disagree with Hassan *et al*., (2013), were (2.1, 74.5, 94) the medain of TSB, SGPT, and SGOT respactively and agree with the medain of alkaline phosphatase (222).

Total serum bilirubin, SGPT, Serum direct bilirubin, alkaline phosphatase and SGOT qualified as an excellent test for diagnosing autoimmune hepatitis differentiating it from healthy controls (ROC area > 0.9). Serum ANA was a very good test with an ROC area of >0.8. Serum SLA and LKM provided a test with marginal validity for differentiating between cases and healthy controls.

4. 4. The diagnostic value of autoantiboies

In the context of diagnosing autoimmune hepatitis differentiating it from healthy controls, the optimum cut-off value for ANA was ≥9.2 which is associated with the highest accuracy (80%). Testing positive at this cut-off value would establish a diagnosis of hepatitis with 84.3% confidence level in a clinical situation where the pretest probability for

having the disease is equal to its absence (50:50 chances, which is the situation where no physician is making a differential diagnosis). Increasing the pretest probability to 90% (the disease being highly probable on clinical grounds only) would increase the yield of the positive test result (confidence in a positive test) to 98%. The highest specificity cut-off value is set at ≥21.5 which is 100% specific. Testing positive at this cut-off value (having a serum ANA ≥ 21.5) would establish a possible diagnosis of hepatitis with 100% confidence level in any clinical situation. The highest sensitivity cut-off value is set at ≥ 0.17, which is 100% sensitive. Testing negative at this cut-off value would exclude a possible diagnosis of hepatitis with 100% confidence level in a clinical situation where the disease is of low probability (pretest probability =10%).

4.5. Relationship between HLA Typing and autoimmune diseases

There is a strong association between some diseases and the major histocompatibility complex (MHC), also known in humans as the human leukocyte antigen (HLA) (Ghodke *et al*., 2005). Encompasses 7.6 Mb on chromosome 6 p21 and is the most gene dense region within the human genome encoding 252 expressed loci (Horton *et al*., 2004).The genetic system of MHC contains more than 70 known genes on the short arm of chromosome 6 and spans about 4 million base pairs of DNA. Many individuals who have certain HLA allele increased vulnerability to certain diseases (Mutar, 2013).

Autoimmune hepatitis (AIH) is the third most common inflammatory chronic liver disease in man preceded by HBV infection and HCV infection (Kalliopi *et al*., 2004). According to several

observations, attention has been focused on the possibility that genetic background could play a crucial role in the susceptibility to AIH. The importance of participation genetic factors in the disease is currently based on the increased risk of autoimmune disease in first- degree relatives of patients with AIH however few reports denoted that 15% of siblings and 10% of offspring (Vergani *et al*., 2008) were observed, while increased frequency of specific genetic markers as certain human leukocyte antigen (HLA) has been reported in patients group other than in general population. Therefore; several studies in different areas were undertaken to test the possibility of association between this disease and one or several of the HLA- Ags, as suggested by Czaja and associates (Czaja and Donaldson, 2000). Positive association with the Ag (A1) was reported in Caucasian patients, while another study reported other Ags (Donaldson *et al*., 2002; Czaja, 2005b).

However, the association between HLA type and most infectious diseases is not very clear because of the multiplicity of factors that can affect their outcome. Nevertheless, in some cases such a relation is obvious, a situation that may be helpful in establishing either the resistance or susceptibility to certain infectious diseases and even a prognosis of the disease progression HLA types found in tropical countries tend to differ a lot from those in temperate parts of the world, thus people susceptibility to disease differ because the viruses found there are different. Some HLA types are known to attack the body own cells causing autoimmune diseases (Larsen and Chester 2004).

4. 6. Human leukocyte antigens and autoimmune hepatitis

The pathogenesis of autoimmune hepatitis (AIH) is a complex one. However, it is believed that a susceptible individual, owing to his genetic background, sex and age, can develop the disease following

exposure to an environmental trigger. Autoimmune hepatitis does not follow a Mendelian pattern of inheritance; hence no single causative genetic locus has been identified. However, several genes, inside and outside the HLA locus, have been linked to an increased susceptibility to AIH (Miyake and Kazuhide 2008).

The current study shows high frequencies of class II HLA-DR3 and HLA-DR4, which appear a high significant difference ($P<0.001$) in AIH group than in control group, while HLA-B27 shown no significant difference ($P=0.131$).

HLA-DR4 is the most important immunogenetic factor responsible for type 1 AIH in Japan (Yoshizawa *et al*., 2005). However, the frequency of HLA-DR4 in other countries ranges widely, from 3 % to 59 % (Shankarkumar *et al*., 2005). In a study of Italian and North American type 1 AIH, HLA-DR4 was found to be less frequent in both Italian type 1 AIH and in control Italian individuals compared with North American counterparts (Muratori *et al*., 2005).

Different results have obtained by different researchers around the world. HLA-DR4 is the most important immunogenetic factor responsible for type 1 AIH in Japan. However, the frequency of HLA-DR4 in other countries ranges widely, from 3 % to 59 % (Teufel *et al*., 2006).

In a study of Italian and North American type 1 AIH, HLA-DR4 was found to be less frequent in both Italian type 1 AIH and in control Italian individuals compared with North American counterparts (Muratori *et al*., 2005).

This study is comparable to Ma and Qiu (2001) in case of HLA-DR4, Furumoto *et al*., (2015), were the frequency of the HLA-DR4 was significantly higher in AIH than in control individuals (59.7 % vs. 41.8

%, P < 0.001), the Odds ratio (95 % Cl) was 2.14 (1.51–3.04), and also nearly comparable with Hassan et al., (2013) in the HLA-B27 Odds Ratio, (Confidence Interval) which 0.39(0.02-0.75), and p value(0.504).

This result was incompatible with Andreas *et al*., (2006) who detected HLA-DR3 and HLA-DR4 in (38%) of German AIH patients and (30%) for –DR3 and (23%) for –DR4 in Italian patients and Czaja *et al*., (2008) were HLA-DR3(45%).

The current study is disagreement with Enriqe *et al*., (2011), were the HLA-DR3 in this study was (p=0.04, and OR 2.6(0.87-7.9, 95%), Ngu *et al*., (2013) were the HLA-DR3 (OR = 2.45, 95% CI 1.65-3.61, p < 0.0001), and with Muratori *et al*., (2005).

HLA-DR3 and HLA-DR4 considered as an essential factor in AIH mechanism was showed highly significant difference (P value 0.001), which resample the frequency of the two genes studied by AL-Obeidy *et al*., (2010).

HLA-DR3 associated with younger age at presentation, diminished response to therapy and more frequent liver failure requiring liver transplantation as compared to HLA-DR4 (Ashima *et al*., 2011).

One previous result found to be in discrepancy with our result that of Amarapurkar *et al*., (2003) who studied HLA Genotyping autoimmune hepatitis in western India and attuned strongly significant association of autoimmune hepatitis was found amongst HLA-B27.

As in other autoimmune diseases, there are primary associations with the HLA class I B8 and class II DR3 and DR52a loci. There is also a secondary association with HLA-DR4 in white patients and a primary association with HLA-DR4 in Asians. With the use of more sophisticated molecular techniques, genotyping has confirmed the disease association with specific loci in the HLA-DR region and

identified specific amino acid sequences in the light chains of the HLA-DR beta molecules as more specific markers Krawitt, (2010).

The course of AIH is also significantly influenced by the HLA antigen profile of the affected individual. Individuals with HLA-DR3 have a lower probability of reaching remission, have more frequent relapses, and require transplantation more often. The HLA- DR4 positive subgroup is characterized by a higher age of onset and a more benign outcome (Furumoto *et al*., 2015).

4.6. Association HLA and Autoantibodies

In the current study there are statistically significant difference association between HLA-DR3 and AMA (P 0.006) and SLA (P 0.001), while HLA-DR4 and HLA-B27 shown no significant difference to other auto-antibody, this compatible with Furumoto *et al*., (2015) in case of ANA and AMA.

This subject has been knocked on for a reason, that is, some HLA molecules have a capacity to present self antigens certain subsets of T cell or B lymphocytes as foreign antigens, as a results an autoimmunity may be triggered and autoantibodies are produced (Mosaad, 2015) for this possible reason, this was subject of intense researches.

Conclusions and Recommendations

Conclusions

1- The HLA-DR3 and DR4 genes are common predisposing factor for AIH patients.

2- In patients, a significant increase was observed in HLA-DR4 gene compared to HLA-DR3 among Iraqi AIH patients.

3- The HLA-B27 gene is uncommon predisposing factor for AIH patients.

4- The ANA, LKM, and SLA autoantibodies are significantly increased, which were observed in AIH patients than healthy controls.

5- High proportion of AMA and SLA autoantibodies were found in the positive HLA-DR3 than positive HLA-DR4.

6- It is possible to use the ANA and SLA autoantibodies as test to predict diagnosis of autoimmune hepatitis differentiating it from healthy controls.

7- LC1 autoantibody is not specific to diagnosis the AIH.

8- There is no relationship between the AIH and age of patients.

Recommendation

1- The adoption of quantitative assays and optimum cut-off values of ANA, LKM, and SLA in the diagnosis of AIH disease.

2- The genotyping of HLA-alleles should incorporate as routine work for the diagnosis and survey issues to predict the present of the different autoimmune diseases.

3- The genotyping of HLA-genes should be study in relative subjects and family history for diagnose of AIH, survey and predict issues of the AIH.

4- The adoption of Sequence Specific Primers (SSP) for DR3 and DR4 genes to determine the sequence of nucleotides among Iraqi AIH patients in future studies.

5- Using the immunohistochemistery technique for more diagnosis of AIH disease.

6- A specialized follow-up by serologic screening for other disease are essential in the management of patients affected by AIH.

7- A good communication should be ensured between the clinicians and the diagnostic lab making the final decision of each suspect autoimmune disease.

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Appendices

Appendix (1): Questionnaire basic information sheet for AIH patients and controls.

Hospital:

Date: **Time:**

Name: **الأسم:**			
ID:		**Dr.name:**	
Patient name:		**Unit:**	
Sex:			
Age:		**Specimen**	
Duration of disease			
Family history	**Father:** **Mother:**	**Liver biopsy result**	**If present**
US	**Spleen,liver,ascitis**		
OGD		**Other AID**	
History	**Drug, Alkohol, Viral hepatitis**		

Appendix (2): Show specific tests for AIH patients and controls.

AIH	Result
Biochemical tests	
TSB	
Indirect	
AST	
ALT	
Alkaline phosphatase	
T.Protein	
S. Albumin	
Gama-globulin	
PT/PTT/INR	
Coombs test	

Serology tests	
LKM	
SLA	
ANA	
AMA	
LC1	
HLA	
HLA-DR3	
HLA-DR4	
HLA-B27	

Appendix 3: Validity parameters for selected measurements when used as test to predict a diagnosis of autoimmune hepatitis differentiating it from healthy controls (Higher values for the measurement being more suggestive of a positive diagnosis).

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
AMA						
0.20 (Highest sensitivity)	95.0	2.0	52.7	49.2	89.7	78.3
0.35	91.7	2.0	50.9	48.3	89.4	68.4
0.45	90.0	2.0	50.0	47.9	89.2	64.3
0.60	86.7	2.0	48.2	46.9	88.8	57.4
0.75	86.7	4.0	49.1	47.4	89.0	73.0
0.85	76.7	8.0	45.5	45.5	88.2	75.5
1.05	75.0	12.0	46.4	46.0	88.5	81.2
1.30	73.3	14.0	46.4	46.0	88.5	82.5
1.45	70.0	16.0	45.5	45.5	88.2	82.8
1.60	70.0	20.0	47.3	46.7	88.7	85.7
1.75	70.0	24.0	49.1	47.9	89.2	87.8
1.85	70.0	26.0	50.0	48.6	89.5	88.6

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
2.00	68.3	26.0	49.1	48.0	89.3	88.1
2.15	65.0	28.0	48.2	47.4	89.0	87.8
2.25	65.0	32.0	50.0	48.9	89.6	89.2
2.50	65.0	34.0	50.9	49.6	89.9	89.7
2.90	63.3	38.0	51.8	50.5	90.2	90.3
3.20	63.3	40.0	52.7	51.4	90.5	90.8
3.45	63.3	42.0	53.6	52.2	90.8	91.2
3.65	58.3	48.0	53.6	52.9	91.0	91.2
3.80	58.3	50.0	54.5	53.8	91.3	91.5
3.95	58.3	54.0	56.4	55.9	91.9	92.1
4.10	55.0	56.0	55.5	55.6	91.8	91.8
4.30	55.0	58.0	56.4	56.7	92.2	92.1
4.50	53.3	60.0	56.4	57.1	92.3	92.0
4.65	53.3	62.0	57.3	58.4	92.7	92.3
5.05	48.3	62.0	54.5	56.0	92.0	91.5
5.45	46.7	62.0	53.6	55.1	91.7	91.3
5.85	46.7	64.0	54.5	56.5	92.1	91.5
6.35	46.7	68.0	56.4	59.3	92.9	92.0
6.60	45.0	68.0	55.5	58.4	92.7	91.8
6.75	45.0	70.0	56.4	60.0	93.1	92.0
6.90	45.0	72.0	57.3	61.6	93.5	92.2
7.10	45.0	74.0	58.2	63.4	94.0	92.4
7.35	45.0	76.0	59.1	65.2	94.4	92.6
7.65	45.0	78.0	60.0	67.2	94.8	92.7
8.20 **(Optimum cut-off)**	45.0	80.0	60.9	69.2	95.3	92.9
8.65	43.3	82.0	60.9	70.7	95.6	92.9
9.25	41.7	82.0	60.0	69.8	95.4	92.7
9.85	41.7	84.0	60.9	72.3	95.9	92.8
10.10	41.7	86.0	61.8	74.9	96.4	93.0
10.85	40.0	86.0	60.9	74.1	96.3	92.8
11.70	35.0	88.0	59.1	74.5	96.3	92.4
12.30	35.0	90.0	60.0	77.8	96.9	92.6
12.95	33.3	90.0	59.1	76.9	96.8	92.4
13.60	31.7	90.0	58.2	76.0	96.6	92.2
14.00	28.3	90.0	56.4	73.9	96.2	91.9
14.45	26.7	90.0	55.5	72.7	96.0	91.7
14.95	21.7	90.0	52.7	68.4	95.1	91.2
15.50	20.0	92.0	52.7	71.4	95.7	91.2
16.85	18.3	92.0	51.8	69.6	95.4	91.0
19.20	18.3	94.0	52.7	75.3	96.5	91.2
20.95	18.3	96.0	53.6	82.1	97.6	91.4
22.15	16.7	96.0	52.7	80.6	97.4	91.2
23.40	15.0	96.0	51.8	78.9	97.1	91.0
24.60	13.3	96.0	50.9	76.9	96.8	90.9

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
26.25	11.7	96.0	50.0	74.5	96.3	90.7
28.75	11.7	98.0	50.9	85.4	98.1	90.9
31.20	10.0	98.0	50.0	83.3	97.8	90.7
33.00	8.3	98.0	49.1	80.6	97.4	90.6
37.00	6.7	98.0	48.2	76.9	96.8	90.4
43.00	5.0	98.0	47.3	71.4	95.7	90.3
49.00	3.3	98.0	46.4	62.5	93.8	90.1
56.15 (Highest specificity)	3.3	100.0	47.3	100.0	100.0	90.3
LKM						
0.25 (Highest sensitivity)	100.0	2.0	55.5	50.5	90.2	100.0
0.45	95.0	2.0	52.7	49.2	89.7	78.3
0.65	93.3	2.0	51.8	48.8	89.6	73.0
0.75	93.3	4.0	52.7	49.3	89.7	84.4
0.85	91.7	6.0	52.7	49.4	89.8	86.6
1.00	86.7	8.0	50.9	48.5	89.4	84.4
1.15	85.0	8.0	50.0	48.0	89.3	82.8
1.25	85.0	12.0	51.8	49.1	89.7	87.8
1.35	81.7	12.0	50.0	48.1	89.3	85.5
1.45	80.0	16.0	50.9	48.8	89.6	87.8
1.55	78.3	20.0	51.8	49.5	89.8	89.3
1.65	75.0	20.0	50.0	48.4	89.4	87.8
1.75	75.0	28.0	53.6	51.0	90.4	91.0
1.95	73.3	34.0	55.5	52.6	90.9	92.0
2.15	73.3	38.0	57.3	54.2	91.4	92.8
2.30	73.3	40.0	58.2	55.0	91.7	93.1
2.45	71.7	40.0	57.3	54.4	91.5	92.7
2.65	71.7	42.0	58.2	55.3	91.7	93.0
2.85	70.0	42.0	57.3	54.7	91.6	92.6
3.00	66.7	46.0	57.3	55.2	91.7	92.5
3.15	66.7	48.0	58.2	56.2	92.0	92.8
3.40	65.0	48.0	57.3	55.6	91.8	92.5
3.65	63.3	52.0	58.2	56.9	92.2	92.7
3.80	61.7	54.0	58.2	57.3	92.3	92.7
4.15	61.7	58.0	60.0	59.5	93.0	93.2
4.50	61.7	60.0	60.9	60.7	93.3	93.4
4.70 **(Optimum cut-off)**	61.7	66.0	63.6	64.5	94.2	93.9
4.85	58.3	66.0	61.8	63.2	93.9	93.4
5.15	58.3	68.0	62.7	64.6	94.3	93.6
5.50	56.7	68.0	61.8	63.9	94.1	93.4
5.95	55.0	68.0	60.9	63.2	93.9	93.2
6.50	53.3	68.0	60.0	62.5	93.8	92.9
6.75	53.3	70.0	60.9	64.0	94.1	93.1
7.00	53.3	72.0	61.8	65.6	94.5	93.3
7.35	51.7	72.0	60.9	64.9	94.3	93.1

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
7.55	48.3	72.0	59.1	63.3	94.0	92.6
7.95	48.3	74.0	60.0	65.0	94.4	92.8
8.40	48.3	76.0	60.9	66.8	94.8	93.0
8.55	46.7	78.0	60.9	68.0	95.0	92.9
8.75	46.7	80.0	61.8	70.0	95.5	93.1
9.20	46.7	84.0	63.6	74.5	96.3	93.4
9.60	45.0	90.0	65.5	81.8	97.6	93.6
9.75	43.3	92.0	65.5	84.4	98.0	93.6
9.85	43.3	94.0	66.4	87.8	98.5	93.7
10.05	41.7	94.0	65.5	87.4	98.4	93.5
11.00	40.0	94.0	64.5	87.0	98.4	93.4
11.95	38.3	94.0	63.6	86.5	98.3	93.2
12.25	38.3	96.0	64.5	90.6	98.9	93.3
12.80	36.7	96.0	63.6	90.2	98.8	93.2
13.40	35.0	96.0	62.7	89.7	98.7	93.0
13.65	35.0	98.0	63.6	94.6	99.4	93.1
14.50	31.7	98.0	61.8	94.1	99.3	92.8
15.65	30.0	98.0	60.9	93.8	99.3	92.6
16.15	28.3	98.0	60.0	93.4	99.2	92.5
16.55	26.7	98.0	59.1	93.0	99.2	92.3
17.85 (Highest specificity)	26.7	100.0	60.0	100.0	100.0	92.5
SLA						
0.95 (Highest sensitivity)	95.0	2.0	52.7	49.2	89.7	78.3
1.20	95.0	4.0	53.6	49.7	89.9	87.8
1.40	95.0	6.0	54.5	50.3	90.1	91.5
1.55	91.7	6.0	52.7	49.4	89.8	86.6
1.70	91.7	8.0	53.6	49.9	90.0	89.6
1.85	90.0	10.0	53.6	50.0	90.0	90.0
1.95	88.3	14.0	54.5	50.7	90.2	91.5
2.35	88.3	16.0	55.5	51.3	90.4	92.5
2.75	86.7	16.0	54.5	50.8	90.3	91.5
2.95	83.3	16.0	52.7	49.8	89.9	89.6
3.25	81.7	18.0	52.7	49.9	90.0	89.8
3.45	81.7	22.0	54.5	51.1	90.4	91.5
3.55	80.0	22.0	53.6	50.6	90.2	90.8
3.65	78.3	22.0	52.7	50.1	90.0	90.1
3.75	78.3	24.0	53.6	50.8	90.3	90.9
3.90	76.7	28.0	54.5	51.6	90.6	91.5
4.05	76.7	30.0	55.5	52.3	90.8	92.0
4.15	75.0	30.0	54.5	51.7	90.6	91.5
4.25	71.7	38.0	56.4	53.6	91.2	92.3
4.35	68.3	38.0	54.5	52.4	90.8	91.5
4.45	66.7	38.0	53.6	51.8	90.6	91.1
4.55	66.7	42.0	55.5	53.5	91.2	91.9

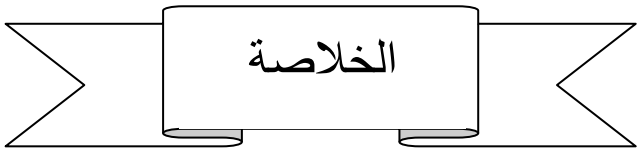
Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
4.65	65.0	42.0	54.5	52.8	91.0	91.5
4.85	65.0	44.0	55.5	53.7	91.3	91.9
5.10	65.0	46.0	56.4	54.6	91.5	92.2
5.25	65.0	52.0	59.1	57.5	92.4	93.0
5.40	65.0	56.0	60.9	59.6	93.0	93.5
5.70	58.3	56.0	57.3	57.0	92.3	92.4
5.95	58.3	58.0	58.2	58.1	92.6	92.6
6.20	58.3	62.0	60.0	60.6	93.3	93.1
6.50	58.3	66.0	61.8	63.2	93.9	93.4
6.90	58.3	68.0	62.7	64.6	94.3	93.6
7.30	58.3	70.0	63.6	66.0	94.6	93.8
7.45	56.7	70.0	62.7	65.4	94.4	93.6
7.65	56.7	74.0	64.5	68.5	95.1	93.9
7.85 **(Optimum cut-off)**	56.7	78.0	66.4	72.0	95.9	94.2
8.25	50.0	78.0	62.7	69.4	95.3	93.4
8.80	48.3	90.0	67.3	82.9	97.8	94.0
9.15	48.3	92.0	68.2	85.8	98.2	94.1
9.90	46.7	92.0	67.3	85.4	98.1	93.9
10.55	43.3	92.0	65.5	84.4	98.0	93.6
11.50	41.7	92.0	64.5	83.9	97.9	93.4
12.75	40.0	92.0	63.6	83.3	97.8	93.2
13.85	38.3	92.0	62.7	82.7	97.7	93.1
14.85	36.7	92.0	61.8	82.1	97.6	92.9
15.35	33.3	92.0	60.0	80.6	97.4	92.5
16.05	31.7	92.0	59.1	79.8	97.3	92.4
16.55	31.7	94.0	60.0	84.1	97.9	92.5
16.90	31.7	96.0	60.9	88.8	98.6	92.7
17.30	30.0	98.0	60.9	93.8	99.3	92.6
17.60	28.3	98.0	60.0	93.4	99.2	92.5
18.05 (Highest specificity)	28.3	100.0	60.9	100.0	100.0	92.6
ANA						
0.17 (Highest sensitivity)	100.0	4.0	56.4	51.0	90.4	100.0
0.30	98.3	4.0	55.5	50.6	90.2	95.6
0.55	96.7	6.0	55.5	50.7	90.2	94.2
0.90	93.3	6.0	53.6	49.8	89.9	89.0
1.30	91.7	6.0	52.7	49.4	89.8	86.6
1.55	91.7	8.0	53.6	49.9	90.0	89.6
1.65	91.7	10.0	54.5	50.5	90.2	91.5
1.86	91.7	18.0	58.2	52.8	91.0	95.1
2.11	91.7	20.0	59.1	53.4	91.2	95.6
2.25	91.7	22.0	60.0	54.0	91.4	96.0
2.45	90.0	24.0	60.0	54.2	91.4	95.6
2.80	90.0	26.0	60.9	54.9	91.6	95.9
3.05	90.0	28.0	61.8	55.6	91.8	96.2

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
3.20	90.0	30.0	62.7	56.3	92.0	96.4
3.35	88.3	30.0	61.8	55.8	91.9	95.9
3.60	83.3	30.0	59.1	54.3	91.5	94.2
3.85	81.7	32.0	59.1	54.6	91.5	94.0
3.95	81.7	36.0	60.9	56.1	92.0	94.6
4.20	81.7	38.0	61.8	56.8	92.2	94.9
4.45	81.7	40.0	62.7	57.6	92.5	95.2
4.55	81.7	42.0	63.6	58.5	92.7	95.4
4.65	81.7	48.0	66.4	61.1	93.4	95.9
4.80	81.7	50.0	67.3	62.0	93.6	96.1
4.95	81.7	52.0	68.2	63.0	93.9	96.2
5.10	81.7	54.0	69.1	64.0	94.1	96.4
5.25	81.7	56.0	70.0	65.0	94.4	96.5
5.70	81.7	58.0	70.9	66.0	94.6	96.6
6.15	81.7	60.0	71.8	67.1	94.8	96.7
6.25	81.7	62.0	72.7	68.2	95.1	96.8
6.35	80.0	62.0	71.8	67.8	95.0	96.5
6.45	80.0	66.0	73.6	70.2	95.5	96.7
6.60	76.7	68.0	72.7	70.6	95.6	96.3
6.85	76.7	72.0	74.5	73.2	96.1	96.5
7.20	76.7	74.0	75.5	74.7	96.4	96.6
7.45	75.0	74.0	74.5	74.3	96.3	96.4
7.85	75.0	76.0	75.5	75.8	96.6	96.5
8.40	75.0	78.0	76.4	77.3	96.8	96.6
8.75	75.0	80.0	77.3	78.9	97.1	96.6
9.00	75.0	84.0	79.1	82.4	97.7	96.8
9.20 **(Optimum cut-off)**	75.0	86.0	80.0	84.3	98.0	96.9
9.50	73.3	86.0	79.1	84.0	97.9	96.7
10.35	71.7	86.0	78.2	83.7	97.9	96.5
11.15	70.0	86.0	77.3	83.3	97.8	96.3
11.40	70.0	88.0	78.2	85.4	98.1	96.4
11.60	68.3	88.0	77.3	85.1	98.1	96.2
12.50	65.0	88.0	75.5	84.4	98.0	95.8
13.35	60.0	88.0	72.7	83.3	97.8	95.2
13.50	58.3	88.0	71.8	82.9	97.8	95.0
13.80	58.3	90.0	72.7	85.4	98.1	95.1
14.10	58.3	94.0	74.5	90.7	98.9	95.3
15.60	55.0	94.0	72.7	90.2	98.8	94.9
17.45	55.0	96.0	73.6	93.2	99.2	95.0
18.05	53.3	96.0	72.7	93.0	99.2	94.9
18.70	51.7	96.0	71.8	92.8	99.1	94.7
19.60	50.0	96.0	70.9	92.6	99.1	94.5
20.20	50.0	98.0	71.8	96.2	99.6	94.6
21.50 (Highest specificity)	50.0	100.0	72.7	100.0	100.0	94.7

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
Total serum billirubin						
0.75 (Highest sensitivity)	100.0	44.0	74.5	64.1	94.1	100.0
0.85	98.3	66.0	83.6	74.3	96.3	99.7
0.95	96.7	88.0	92.7	89.0	98.6	99.6
1.05	95.0	92.0	93.6	92.2	99.1	99.4
1.15 **(Optimum cut-off)**	93.3	96.0	94.5	95.9	99.5	99.2
1.30 (Highest specificity)	91.7	100.0	95.5	100.0	100.0	99.1
Serum direct billirubin						
0.15 (Highest sensitivity)	98.3	12.0	59.1	52.8	91.0	98.5
0.25	96.7	54.0	77.3	67.8	95.0	99.3
0.35	96.7	66.0	82.7	74.0	96.2	99.4
0.45	90.0	78.0	84.5	80.4	97.4	98.6
0.55	90.0	88.0	89.1	88.2	98.5	98.8
0.65 **(Optimum cut-off)**	86.7	92.0	89.1	91.5	99.0	98.4
0.75	81.7	96.0	88.2	95.3	99.5	97.9
0.85	80.0	98.0	88.2	97.6	99.7	97.8
0.95 (Highest specificity)	75.0	100.0	86.4	100.0	100.0	97.3
SGPT						
16.5 (Highest sensitivity)	100.0	20.0	63.6	55.6	91.8	100.0
17.5	98.3	26.0	65.5	57.1	92.3	99.3
18.5	96.7	28.0	65.5	57.3	92.4	98.7
19.5	96.7	32.0	67.3	58.7	92.8	98.9
20.5	96.7	38.0	70.0	60.9	93.3	99.0
21.5	96.7	40.0	70.9	61.7	93.5	99.1
22.5	95.0	42.0	70.9	62.1	93.6	98.7
23.5	95.0	54.0	76.4	67.4	94.9	99.0
24.5	95.0	56.0	77.3	68.3	95.1	99.0
26.0	95.0	58.0	78.2	69.3	95.3	99.1
27.5	95.0	62.0	80.0	71.4	95.7	99.1
28.5	95.0	70.0	83.6	76.0	96.6	99.2
29.5	95.0	80.0	88.2	82.6	97.7	99.3
31.0	93.3	84.0	89.1	85.4	98.1	99.1
32.5	91.7	92.0	91.8	92.0	99.0	99.0
33.5	91.7	94.0	92.7	93.9	99.3	99.0
35.5 **(Optimum cut-off)**	91.7	96.0	93.6	95.8	99.5	99.0
38.5	90.0	96.0	92.7	95.7	99.5	98.9
41.0	88.3	96.0	91.8	95.7	99.5	98.7
42.5	86.7	96.0	90.9	95.6	99.5	98.5
45.5 (Highest specificity)	86.7	100.0	92.7	100.0	100.0	98.5
SGOT						
11.5 (Highest sensitivity)	100.0	18.0	62.7	54.9	91.6	100.0
12.5	98.3	20.0	62.7	55.1	91.7	99.1
14.0	98.3	22.0	63.6	55.8	91.9	99.2

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
15.5	98.3	26.0	65.5	57.1	92.3	99.3
16.5	98.3	28.0	66.4	57.7	92.5	99.3
17.5	96.7	32.0	67.3	58.7	92.8	98.9
18.5	96.7	38.0	70.0	60.9	93.3	99.0
19.5	96.7	40.0	70.9	61.7	93.5	99.1
20.5	95.0	46.0	72.7	63.8	94.1	98.8
21.5	93.3	60.0	78.2	70.0	95.5	98.8
22.5	93.3	64.0	80.0	72.2	95.9	98.9
24.5	91.7	68.0	80.9	74.1	96.3	98.7
26.5	91.7	70.0	81.8	75.3	96.5	98.7
27.5	90.0	74.0	82.7	77.6	96.9	98.5
28.5	90.0	76.0	83.6	78.9	97.1	98.6
29.5	90.0	80.0	85.5	81.8	97.6	98.6
31.0	88.3	82.0	85.5	83.1	97.8	98.4
32.5	85.0	88.0	86.4	87.6	98.5	98.1
33.5 **(Optimum cut-off)**	85.0	90.0	87.3	89.5	98.7	98.2
35.0	83.3	92.0	87.3	91.2	98.9	98.0
37.5	81.7	92.0	86.4	91.1	98.9	97.8
39.5	80.0	92.0	85.5	90.9	98.9	97.6
41.5	80.0	94.0	86.4	93.0	99.2	97.7
46.0	78.3	98.0	87.3	97.5	99.7	97.6
51.5	76.7	98.0	86.4	97.5	99.7	97.4
54.5 (Highest specificity)	75.0	100.0	86.4	100.0	100.0	97.3
Alkaline phosphatase						
44.0 (Highest sensitivity)	100.0	18.0	62.7	54.9	91.6	100.0
46.0	98.3	22.0	63.6	55.8	91.9	99.2
47.5	98.3	24.0	64.5	56.4	92.1	99.2
49.0	98.3	26.0	65.5	57.1	92.3	99.3
51.5	98.3	28.0	66.4	57.7	92.5	99.3
53.5	98.3	30.0	67.3	58.4	92.7	99.4
54.5	98.3	32.0	68.2	59.1	92.9	99.4
55.5	98.3	34.0	69.1	59.8	93.1	99.5
57.0	98.3	36.0	70.0	60.6	93.3	99.5
61.5	98.3	38.0	70.9	61.3	93.5	99.5
65.5	98.3	46.0	74.5	64.6	94.2	99.6
66.5	98.3	50.0	76.4	66.3	94.7	99.6
68.0	98.3	56.0	79.1	69.1	95.3	99.7
69.5	96.7	56.0	78.2	68.7	95.2	99.3
70.5	95.0	56.0	77.3	68.3	95.1	99.0
71.5	95.0	58.0	78.2	69.3	95.3	99.1
74.0	93.3	58.0	77.3	69.0	95.2	98.7
76.5	91.7	68.0	80.9	74.1	96.3	98.7
77.5	91.7	70.0	81.8	75.3	96.5	98.7

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability = 50%	PPV at pretest probability = 90%	NPV at pretest probability = 10%
79.5	90.0	74.0	82.7	77.6	96.9	98.5
81.5	88.3	76.0	82.7	78.6	97.1	98.3
82.5	88.3	78.0	83.6	80.1	97.3	98.4
83.5	86.7	78.0	82.7	79.8	97.3	98.1
85.5	85.0	78.0	81.8	79.4	97.2	97.9
87.5	85.0	84.0	84.5	84.2	98.0	98.1
88.5	85.0	86.0	85.5	85.9	98.2	98.1
89.5	85.0	88.0	86.4	87.6	98.5	98.1
91.0	85.0	90.0	87.3	89.5	98.7	98.2
92.5 **(Optimum cut-off)**	85.0	92.0	88.2	91.4	99.0	98.2
95.0	83.3	94.0	88.2	93.3	99.2	98.1
97.5	81.7	94.0	87.3	93.2	99.2	97.9
99.0	80.0	96.0	87.3	95.2	99.4	97.7
101.0	78.3	96.0	86.4	95.1	99.4	97.6
104.0	78.3	98.0	87.3	97.5	99.7	97.6
107.0 (Highest specificity)	78.3	100.0	88.2	100.0	100.0	97.6



مرض التهاب الكبد المناعي يعتقد انه أحد أمراض المناعة الذاتية. هذا المرض يكشف خصائص عوامل التهيئة والاجسام المضاده.

يعتقد ان احد العوامل التهيئة الوراثية هو جينات مستضدات الكريات البيضاء البشرية دي ار 3 و دي ار 4. بالأضافة ان هناك تداخل في انتاج الاجسام المضاده خلال دورة المرض.

تهدف الدراسة الحالية الى التحقق من تكرار جينات دي ار 3 , دي ار 4 وبي 27 وتطور اضداد الاجسام المضاده (ANA, AMA, SLA و LKM) في مجموعة من 60 مريض عراقي (41 ذكر و 19 انثى) الذين تم تشخيص مرضى التهاب الكبد المناعي بالفعل في مركز الجهاز الهظمي/ مستشفى الديوانية التعليمي و مركز الجهاز الهظمي/ مستشفى الحسين/كربلاء و مستشفى الجهاز الهظمي/ بغداد مقارنة لمجموعة من الأصحاء (المجموعة الضابطة عدد 50) شخصا والتي تضم (34 ذكر و 16 انثى).

استعمل Real time PCR لدراسة التركيب الوراثي لجينات مستضدات الكريات البيضاء البشرية مع استخدام تقنية الاليزا لتقدير كمية الاجسام المضادة في كل الأشخاص

الخاضعين للدراسة. كان عمر المرضى متراوحا بين 7 - 69 سنة في مجموعة مرضى التهاب الكبد المناعي ومن 8 - 67 سنة في مجموعة الأصحاء وحساب المتوسط الحسابي للأضداد ANA (U/ml) كانت 20,9 و4,8 بين مرضى التهاب الكبد المناعي و مجموعة الاصحاء بالترتيب مع فارق احصائي معنوي (P<0،001)، ومعيار مهم قيمة ROC (المنطقة تحت المنحنى AUC0،804) وجدت لأضداد هذه الاجسام المضاده الذاتية كأختبار تشخيصي معتدل في هذا الجانب.

أضداد الاجسام المضاده الذاتيه SLA حدثت في متوسط تركيز 8,3 في مجموعة المرضى و 2, 5 في مجموعة الأصحاء، وقد اختلف كثيراً في المجموعتين بالمقارنة مع بعضها البعض مع فارق عالي كبير (P= 0،005) في التفريق بين مجموعة المرضى عن مجموعة الأصحاء و المنطقة تحت المنحنى (AUC 0،655).

أضداد الاجسام المضاده الذاتيه LKM حدثت في متوسط تركيز 7,5 في مجموعة المرضى و 6, 3 في مجموعة الأصحاء، مع فارق معنوي (P= 0،01) بين المجموعتين

تم قياس اضداد AMA ولا يوجد فرق ذو دلالة أحصائية بالمقارنة بين المجموعتين المدروستين، والمنطقة تحت المنحنى لكل من الصنفين كانت (0،554).

في سياق التنميط الجيني لمستضدات الكريات البيضاء البشرية دي ار 3 و دي ار 4 وبي 27قد وجدَ اختلافاً في تكراراتهما بشكل كبير بين مرضى التهاب الكبد المناعي للجزء المسبب للمرض 0,504 ,0,583و 0,129 بالترتيب مقارنة مع مجموعة الاصحاء، مع تزايد النسبه الأرجحيه 7,35 للـ دي ار 3 و نسبة أرجحيه عاليه (8) للـ دي ار 4و2,23 لل بي 27 . بينما ترددات هذه عاليه الجينيات كانت تختلف بشكل كبير بين مجموعة المرضى للـ دي ار 3 مقارنةً مع مجموعة الاصحاء التي (58,3% و 16% بالترتيب)، و اختلاف بشكل عالي وملحوظ بين مجموعة المرضى للـ دي ار 4 مقارنةً مع مجموعة الاصحاء التي (66,7% و 20% بالترتيب)، وكانت ليست ذات فرق معنوي بالنسبة لل بي 27 (23,3% و 12% بالترتيب) على التوالي في مجموعة مرضى التهاب الكبد المناعي و الاصحاء.

علاوة على ذلك درسنا موضوع صحة هذه المعلمات ممثلةُ بالحساسية، النوعية والدقة. الصلة السريرية لهذه المعلمات كما تم تقديرها باستخدام القيم التنبئية الإيجابية (PPV) والقيم التنبئية السلبية (NPV). وقد وقعت على أعلى معايير صلاحية لقيم SLA, ANA, LKM(7,85, 4,70 و 9,20 على التوالي)، حيث تستخدم للتنبؤ لمرض التهاب الكبد المناعي ، بينما قيمةTotal serum bilirubin , Direct serum bilirubin , SGPT, SGOT

Alkaline phosohatase بلغ (1,15 , 0,65, 5, 35 , 5, 33 و 5, 92) تم الحصول على تلك القيم في الكشف عن وجود المرض.

من نتائج هذه الدراسة، تم التوصل الى دليل قوي ان جينات مستضدات الكريات البيضاء البشرية دي ار 3 و دي ار 4 هي عوامل مهيئه شائعه وتلعب دوراً رئيسياً في أمراضية التهاب الكبد المناعي وتداخل أضداد (ANA, SLA و LKM) في التهاب الكبد المناعي هي عوامل مهيئه شائعه.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة القادسية
كلية الطب

علاقة مستضدات الخلايا البيضاء البشرية دي ار نمط3, دي ار نمط4و بي 27وصورة بعض الاضداد الذاتية في مرضى التهاب الكبد المناعي

اطروحة مقدمة إلى
مجلس كلية الطب – جامعة القادسية
كجزء من متطلبات نيل درجة فلسفة الدكتوراه

في علم الأحياء المجهرية الطبية

من قبل

عبدالرزاق عبدالله طاهر

ماجستير احياء مجهرية طبيه/ كلية الطب 2012

بإشراف

الأُستاذ الدكتور

الأُستاذ المساعد الدكتور

حمادي عبطان الهلالي

اسامه طاهر العبيدي

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