Sudan University of Science and Technology

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Evaluation of Interleukin-17 Level among Sudanese Hepatitis B Patients in Khartoum State

تقييم مستوي المادة الخلوية 17 وسط المرضي السودانيين بالتهاب الكبد الوبائي النوع ب بولاية الخرطوم

A Dissertation Submitted in Partial Fulfillment for the Requirements M.Sc Degree in Medical Laboratory Science (Microbiology).

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قال تعالي:

الأية

وَ فِي~ أَنفُسِكُمْ أَفَلاَ تُبْصِرُونَ(21)

صدق الله العظيم

سورة الذاريات الآية [21)

DEDICATION

To my beloved parents who made me what I'm today

To my respectful brothers and sister

To my lovely friends

To whom will find it beneficial work

To those who have made it possible ….. Teachers

Acknowledgments

Piously my gratitude and prayers to **ALMIGHTY ALLAH** for the mercy that followed me during the long way of this research and granted me ability to accomplish this work. I would like to express my special thanks and my sincere gratefulness to **Dr. Kawthar Abdelgaleil MohammedSalih** for her immense effort not only to accomplish this work but also to inculate the researcher's soul on me. Special thank to my colleague **Ali Hamdan Mohammed** and **Wafaa Alabed** for their efforts and boundless support. Then I would like to thank my parents and friends who have helped me with their valuable suggestions and guidance has been helpful in various phases of the completion of the project.

Abstract

HBV remains a major global pathogen that cause acute and chronic hepatitis , liver cirrhosis , and hepatocellular carcinoma, IL-17 is pro-inflammatory cytokines play an important role in regulation of viral infections .

This case-control study was aimed to evaluate the Interleukin-17(IL-17) level in Sudanese patients infected with Hepatitis B virus(HBV) compared to healthy control group in Khartoum state during period from March to October 2019.

Sixty subjects, selected randomly in this study, with age varies from 17-50 years , 30 subjects are hepatitis B patients as case group with mean of age 29.7±9.57 years and 30 subjects as control group with mean of age 32.4±9.5 years are age and sex matched healthy control group.

Three milliliter of Venous blood sample were collected in EDTA container from each subjects and separated using centrifuge to obtain plasma. IL-17 concentration was measured by Enzyme Linked Immunosorbent Assay (ELISA) in Ibn Sina University research center. The data was analyzed using statistical package of social science programme (Version 20) . students T test and one way ANOVA test were used to compare between means. *P.value* significant when ≤0.05 .

Mean level of IL-17 was significantly increased in HBV patients (16.28± 9.57pg/ml) when compared to control group (7.47±8.48 pg/ml) with (*p.value* 0.00).

mean level of IL-17 in hepatitis B patients was lower in males than females, which is statistically insignificant *p.value* 0.328 , and in healthy control IL-17 mean level was higher in female than male , which is statistically significant (*P.value* 0.017).

Also, there was no statistically difference between IL-17 level in acute and chronic hepatitis B patients(*P.value* 0.223).

Mean of IL-17 in patients who had didn't use treatment was higher than patients who used treatment with insignificant statistical correlation (*P. value* was 0.673).

According to jaundice: there was no significant difference in IL-17 level between patients with symptoms of jaundice and patients have no symptoms of jaundice (*p. value* 0.854).

Mean of IL-17 in patients who didn't receive blood transfusion was higher than patients who received blood transfusion mean with insignificant statistical correlation (*P.value* was 0.691).

One way ANOVA test showed insignificant correlation between age group and IL-17 (*P.value* 0.061). In correlation of IL-17 level with age in two group *p.value* were 0.190 HBV patients and 0.076 in control group.

This study concluded that IL-17 concentration may be a useful prognostic marker for HBV infection.

مستخلص البحث

يظل فيروس الكبد البائي النوع ب احد العوامل المسببة للأمراض العالمية التي تسبب التهاب الكبد الحاد و المزمن, تليف الكبد, و سرطان الكبد. المادة الخلوية 17 من المواد الخلوية المؤيدة للالتهابات يلعب دورا هاما في تنظيم العدوى الفيروسية.

هذه دراسة الحالة والضابطة هدفت إلى تقييم مستوى المادة الخلوية 17 لدى المرضى السودانيين المصابين بالتهاب الكبد ب الفيروسي مقارنة مع الأفراد الطبيعيين بولاية الخرطوم في الفترة من مارس حتى أكتوبر 2019.

اختير ستين فردا عشوائيًا لهذه الدراسة و كانت أعمارهم تتراوح من 50-17، 30 مريضا من مرضى التهاب الكبد ب الفيروسي متوسط أعمارهم 29.7±9.57 سنوات و 30 من الأفراد الطبيعيين متوسط أعمارهم 32.4±9.5 سنوات متوافقين في العمر والجنس.

سحبت3 مللتر عينة دم وريدية من كل مشارك في أنبوبة تحتوي عل EDTA و فصلت باستخدام الطرد المركزي وحصل على البلازما. قيس تركيز المادة الخلوية 17 عن طريق فحص الممتز المناعي المرتبط بالإنزيم في مركز أبحاث جامعة ابن سينا. حللت البيانات باستخدام الحزمة الإحصائية للمجتمع (نسخة 20). استخدم أنوفا (ANOVA) و اختبار T لمقارنة الأوساط و كانت القيمة المطلقة متوافقة عند اقل من 0.05 .

كان هناك ارتفاع ذو دلالة إحصائية للوسط الحسابي للمادة الخلوية 17 في المرضي بالتهاب الكبد ب الفيروسي 16.28±9.57 بيكوجرام\مل مقارنة بالأفراد الطبيعيين بيكوجرام\مل 7.47±8.48 (القيم الاحتمالية 0.00) .

متوسط المادة الخلوية 17في مرضي التهاب الكبد ب الفيروسي اقل في الرجال من النساء, حيث لا توجد فروق ذات دلالة إحصائية (القيم الاحتمالية 0.328) بينما في الأفراد الطبيعيين اعلي في النساء من الرجال , حيث توجد فروق ذات دلالة إحصائية (القيم الاحتمالية0.017) . أيضا لم يكن هناك فرق إحصائي بين التهاب الكبد البائي الحاد و المزمن (القيمة الاحتمالية 0.223) .

متوسط المادة الخلوية 17 في المرضى الذين لم يتلقوا علاجًا و المرضى الذين تلقوا علاجًا لم يكن ذو دلالة إحصائية (القيمة الاحتمالية 0.673). لم يكن هناك فرق إحصائي بين المرضى الذين عانوا من أعراض اليرقان و الذين لا يعانون منها (القيمة الاحتمالية 0.854). و أيضا لم يكن هناك فرق إحصائي بين المرضى الذين لم يتم لهم نقل دم والمرضى الذين تم لهم نقل دم (القيمة الاحتمالية 0.691) .ليس هناك علاقة بين معدل المادة الخلوية 17 والعمر في المجموعتين (القيم الاحتمالية 0.190) في مرضي التهاب الكبد البائي و 0.076 في الأفراد الطبيعيين).

وخلصت هذه الدراسة إلى أن تركيز المادة الخلوية 17 قد يكون علامة نذير مفيدة لعدوى التهاب الكبد ب الفيروسي.

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List of Abbreviations

ALT	Alanine transaminase
APOBEC	Apolipoprotein B mRNA editing enzyme . catalytic
cccDNA	Covalently closed circular DNA
CCL	Family of small cytokines
CHB	Chronic hepatitis B
CTLs	Cytotoxic T cells
CXCL	Type of chemokines
CXCR	Chemokine receptors
DCs	Dendritic cell
DDB1	Damage specific DNA binding proteins 1
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Food and drug administration
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HepaRG	Human bipotent progenitor cell
HNF	Hepatocyte nuclear factor
HSPG	Heparan sulfate proteoglycan
IFN-β	Interferon- beta
IFN-α	Interferon- alpha
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1 β	Interleukin 1 beta
IL-1 α	Interleukin 1 alpha
IL-10	Interleukin 10
IL-12	Interleukins 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-15R	Interleukin 15 receptor
IL-17	Interleukin 17
IL-18	Interleukin 18
IL-1Ra	Member of the interleukin 1 cytokines family
IL-2	Interleukin 2
IL-21	Interleukin 21
IL-21R	Interleukin R
IL-23	Interleukin 23
IL-36 β	Interleukin 36 beta
IL-36 α	Interleukin 36 alpha
IL-36 γ	Interleukin 36 gama
IL-36Ra	Interleukin 36 receptor antagonist
IL-38	Interleukin 38
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-7R	Interleukin 7 receptor

IL-7R α	Interleukin receptor alpha
IL-9	Interleukin 9
ILs	Interleukins
INF-γ	Interferon-gama
ISG	Interferon-induced gene
mRNA	Messenger Ribonucleic acid
NK	Natural killer
NTCP	Sodium taurocholate contransporting polypeptide
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein -1 polypeptide-like
RANTES	Regulated on activation normal T cell expressed and Secreted
RNA	Ribonucleic acid
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th17	T helper 17
Th2	T helper 2
Th9	T helper 9
TIR	Toll-interleukin receptor
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TNFR3	Tumor necrosis factor receptor 3
TNF-α	Tumor necrosis factor alpha
WHO	World health organization

CHAPTE ONE

INTRODUCTION

Chapter One

1.Introduction

1.1.Introduction

HBV is an important human pathogen that has caused chronic infections worldwide (Ott *et al*., 2012). Recent data obtained from a modeling study has shown that the global prevalence of hepatitis B surface antigen (HBsAg) was 3.9% in 2016, corresponding to an estimated 290 million infections worldwide (Wu *et al*., 2018). HBV mainly infects hepatocytes and causes a wide spectrum of clinical manifestations (Bernal *et al*., 2010; Glebe and Bremer , 2015).

HBV infection causes a broad spectrum of liver diseases ranging from acute to chronic hepatitis B infection with no biochemical evidence of liver injury to progressive chronic hepatitis B, which may advance to liver cirrhosis, liver failure, and hepatocellular carcinoma (Yu *et al*., 2016).

According to the report of Global Burden of Disease Study 2013, between 1990 and 2013, occurred a 63% increase in the global viral hepatitis deaths, passing from the tenth (in 1990) to seventh (in 2013) leading cause of death worldwide (Stanaway *et al*., 2016). There are approximately 400 million HBV carriers in the world complications such as fulminant hepatic failure, cirrhosis and hepatocellular carcinoma develop annually in 250 000 of them. While the majority of adult infections remit, chronicity develops in 5−10% of cases .Adaptive immune response, which develops against HBV (a hepatotropic non-cytopathic virus) plays a key role in infection control. After the virus is taken into the liver cells infection ensues and host immune response is initiated which result in cytokines secretion and designation of immune response according to them (Ozguler *et al*., 2015).

In recent years, cytokines have become a research hotspot in knowing the pathogenesis of chronic hepatitis and liver cirrhosis. Interleukin 17 (IL17) is a cytokine produced by a newly defined subset of helper T cells. The IL17 family of cytokine has been reported to be involved in many immune processes , most notably

in inducing and mediating proinflammatory responses (Du *et al*., 2013). Interleukin-17 (IL-17) can participate in hot's anti infection immunity and resist extracellular pathogens (Yang *et al*., 2018). Abundant evidence suggests a potential correlation between IL-17-producing cells (i.e Tc17 cells or Th17 cells) or IL-17 levels and a more sever diseases stage of HBV progression (Zhang *et al*.,2018).

1.2.Rationale

HBV infection is a global health concern. Infection with HBV impacts the liver and results in wide range of illnesses. It can be transmitted through transfusion of infected blood , exposure to secretion , and a needle stick or cut by a contaminated sharp object (Hamad *et al*., 2019). HBV infection is considered endemic in Sudan, different prevalence rates were observed in different states (Badawi *et al*., 2018).

IL-17-secreting Th 17 cells, which have been verified as closely associated with the development and severity of liver damage in patients with the hepatitis B (Huang *et al*.,2012). IL17 was recently found to be extensively involve in the pathogenesis of chronic liver disease and antiviral immunity (Wang *et al*., 2011). IL-17 is implicated in inflammatory liver damage after HBV infection . Studies have demonstrated that there is good positive correlativity between the levels of serum IL-17 in hepatitis B patients and the progression of hepatocirrosis. IL-17 play an important role in the development of CHB. It is mediates viral response after HBV infection .IL17 may be help to give new insights in treating CHB , and may provide the basis for immune intervention therapy (Yang *et al*., 2018). No published data about IL-17 among HBV infection in Sudan .

1.3.Objectives

1.3.1.General objectives

-To evaluate plasma level of Interleukine-17 among Sudanese HBV disease patients in Khartoum State.

1.3.2.Specific objectives

-To measure level of IL17 in HBV disease patients and in healthy using ELISA.
-To compare between plasma levels of IL17 in HBV patients and normal controls .
-To correlate IL17 level with possible risk factors e.g age ,gender , treatment , blood transfusion , jaundice and duration of disease.

CHAPTER TWO

LITERATURE REVIEW

Chapter Two

2.Literature review

2.1. Background

The HBV remains a major global pathogen that cause acute and chronic hepatitis , liver cirrhosis , and hepatocellular carcinoma (Trepo *et al*., 2014). It is belongs to the Orthohepadna genus of Hepadnaviridae family of virus and has a unique replication strategy where in it replication . Its 3.2 kb DNA genome using an RNA intermediate via reverse transcription (Sandhu *et al*., 2017). The world health organization (WHO) estimate that there are over 240 million chronically infected people worldwide . The chronic form of this infection is associated with a variety of clinical manifestations, ranging from an asymptomatic carrier state to severe liver disease , including cirrhosis and hepatocellular carcinoma (HCC) (WHO 2015). People are infected reside in low-income and middle-income countries particularly in Africa (Meriki *et al*., 2018).

The risk of developing chronic HBV infection after acute exposure ranges from 90% in newborns of HBeAg-positive mothers to 25%-30%in infants and chil- dren under 5 to less than 5% in adults. In addition, immunosuppressed persons are more likely to develop chronic HBV infection after acute infection.(Norah , 2018).

Approximately 360 million people are chronic carriers of viral hepatitis B and more than one million die every year as a result of acute fulminant hepatic failure (Carlson and Perl, 2014).

viruse may be transmitted from individuals having acute or chronic infection, hepatitis B virus is found mainly in the blood, vaginal secretions, semen and serous fluids of an infected individual (CDC, 2012).

Hepatitis B vaccine provide protection against HBV infection and all its related complication which include chronic hepatitis , fulminant hepatitis , liver cirrhosis and hepatocellular carcinoma (HCC) (Meriki *et al* ., 2018).

2.2. Structural biology

Mature HBV is an enveloped , icosahedral viruse. It packages a circular dsDNA genome with gaps in both strands and a reverse transcriptase covalently bound to the

5' end of the antisense strand ; it also package a number of host proteins.(zlotnick , 2017).

There are three different viral structure that were observed in serum of HBV-infected patients by electron microscopy are dane particle (42nm) , spherical (20nm) and filamentous (22 nm) (Nese *et al*., 2015). All of three particle have a common HBsAg on there surface. The spherical and filmentous particles are composed of HBsAg and host-derived lipids without HBV gentome; thus they are non-infectious (Doo *et al*., 2010). On the other hand Dane particle is a 42 nm sphere which is complete infectious HBV virion . the core region of Dane particle contains a small , circular , partially double-stranded DNA molecule and viral DNA polymerase that surrounded by nucleocapsid .Assembled hepatitis B core antigens (HBsAg) build nucleocapsid that is covered with a lipid envelope containing HBsAg (Nese *et al*., 2015). The cholesterol-rich composition of the lipid envelope is required for viral infectivity. During budding of the nucleocapsid from the endoplasmic reticulum , the nucleocapsid induced ordered and condensed arrangement of the three different surface glycoproteins : L(large), M(middle), S(small) on the envelope membrane(Baumert *et al*., 2014). The nucleocapsid structure of HBV contains HBV genome with is 3.2 Kilobase (kb) in length and partially double-stranded relaxed circular DNA (rcDNA) molecule. Nucleocapsid is formed by composition of 240 viral capsid proteins with 27 nm diameter in an icosahedral structure , and it contains single copy of viral genome DNA and viral polymerase enzyme covalently attached to 5' end of the negative chain , (Nese *et al*., 2015).

2.3. Epidemiology

Hepatitis B is common and dangerous infectious disease . There is distinct geographical variation of HBV prevalence and incidence rates in the Eastern Mediterranean region .In the Eastern Mediterranean region , around 4.3 million individuals are infected with hepatitis B. HBV infection rates vary from 0.6% in Iraq to higher than 8% in Sudan (Hamad *et al* .,2019). HBV infection is widespread , with prevalence >8% in parts of sub-saharan Africa such as west Africa-particularly , Burkina faso , Ivory Coast , Gambia , Ghana , Guinea , Liberia , Mali , Mauritania , Niger , Nigeria , Senegal , Sierrta Leone , Southern Sudan ,Angola ,Uganda and Somalia .and intermediate prevalence (2-7.99%) is present in some regions of the

eastern Mediterranean (e.g Tunisia) , Central Asia (e.g Kazakhstan) , Southeast Asia (e.g Thailand , Bhutan and Bangladesh) , China , parts of South America (e.g Colombia) and in some European countries (e.g Albania Bulgaria , Romania and Turkeya) . A low prevalence is present (<2%) in some part of North America (e.g the United State ,Canada and Mexico) in some European countries (e.g Belgium ,Czech Republic ,Denmark , F rance) and in Australia .(Stasi *et al*., 2017).

In the middle East and the Indian subcontinent , WHO estimate that about 2%-5% of the population are chronically affected with hepatitis B as compared to less than 1% of the population in western Europe and North America(WHO 2015). The prevalence of HBV in Gulf countries is around 3.5% and 4.25% , as reported from Kuwait and Saudi Arabia , respectively (Habibzadeh , 2014). In fact , a 2017 study from Saudi Arabia showed a higher rate of HBV seroprevalence reaching 7.9% (Al-Humayed , 2017). Variation in the prevalence rates reflects the variation in the risk factors associated with the infection in each country . Despite the few studies on the hepatitis B situation in the Arabic world , the prevalence of chronic HBV infection was found to be decreasing in some Arabic countries , such as Arabian Gulf , Lebanon , Egypt and Libya (Hamad *et al*., 2019).

2.4.Replication

HBV chronically infects hepatocytes. It replicates by reverse transcription of an RNA intermediate , the pregenome. Nuclear cccDNA , formed from the incoming relaxed circular viral DNA , serves as the transcriptional template .(Trepo *et al*., 2014). Progeny genomes are formed by reverse transcription , which occurs within viral nucleocapsids in the cytoplasm of infected cells . (Liu *et al*., 2015). Nucleocapsids with mature viral DNA are either assembled into viral envelopes and exported from the infected hepatocyte or , if needed , transported to the nucleus to amplify cccDNA copy number .Envelpe proteins are also secreted as subviral particles , hepatitis B surface antigen (HBsAg), as are large numbers of virus-like particles with empty nucleocapsids (Hu and Seeger 2015).

While the broad outline of infection and replication are clear, gaps in knowledge still exist at the molecular level 1t still do not know all of the steps in disassembly of viral nucleocapsids , delivery of the viral genome into the cell nucleus , and cccDNA

formation .(Hu , 2018).cccDNA transcription is clearly dependent upon liver specific transcription factors , but the roles of virus proteins in HBV transcription are still nuclear, HBx is needed for efficient transcription from cccDNA, but the mechanism is not yet known . While ti is likely that HBx recruits a cellular proteins to the Cullin4-DDB1 E3 ligase , the nature of the cellular proteins remain elusive .(Buendia ; Neuveut 2015). Packaging into nucleocapsids of pregenomic RNA and revers transcriptase are essential for viral DNA synthesis , and packaging of a variety of cellular factors , including a kinase , chaperones and members of the APOBEC family of proteins , have also been reported (Seeger and William , 2016).

2.5.Antiviral therapy

Interferon therapy may induce immune clearance of HBV , but this is rare . Nucleoside analogue inhibitors of HBV reverse transcription are effective at suppressing virus replication , which leads to an amelioration of immune pathogenesis , presumably due to reduced numbers of infected hepatocytes (Gish *et al*., 2015). However , infected hepatocytes almost always persist at low levels , possibly because inhibition of cccDNA synthesis is incomplete , and infection typically rebounds it therapy is stopped , or if there is a breakthrough of drug resistant HBV . This warrants a search for more effective cocktails that not only inhibit reverse transcription of most HBV variants , but also target other steps in viral DNA synthesis (e.g nucleocapsid assembly . (Locarnini and Zoulim , 2016) . In addition , efforts are being made to employ nucleases (e.g Case9) for degradation of cccDNA . Finally , it would be desirable to have a therapy that could effectively induce immune clearance and / or immune control in all HBV carriers , though approaches to this goal , aside from limited success with interferon therapy , have so far not been promising (Seeger and William 2016).

2.6.Genotype

HBV can be classified into at least 8 genotypes with a divergence of more than 8% of nucleotide sequences (Kurbanov *et al*., 2010). There is a strong relationship between HBV genotype and geography worldwide , and genotype has been shown to influence the natural history and , in turn , transmission patterns of hepatitis B infection , (Lin and Kao 2015).

Genotype also influences the progression of viral infection through phases, which . in turn , determines infectivity and age at transmission or infection (Maclanchlan and Cowie , 2015). HBV genotypes and subgenotypes may also have relevance to hepatitis B control efforts through vaccination . Mismatch between the strain used to derive hepatitis B vaccine (serotype adw) and that wich is prevalence in a given population may result in increased vaccine escape and reduced efficacy at a population level (Davies *et al*., 2013).

2.7.Transmission

The virus owes its remarkable diffusion to its environmental resistance (about 7 days at room temperature) . However , after introduction of the vaccination , significant reduction in its transmission has been detected (Stasi *et al*., 2017). Although , outbreaks continue to occur in the centers of chronic dialysis . Since 1980, the anti-HBV vaccination was recommended for all subjects in serum-negative dialysis and staff . However , in long-term dialysis , patients have a poor immune response to the HBV vaccine in comparison to healthy subjects ; in fact , the rate of immunization and anti-HBs titer is lower after completion of the vaccination program. (Fabrizi *et al*., 2013).

World wide in 2010 , 1.2 million (range , 0.3-2.7 million) of injection drug users were HBsAg-positive , with a weighted overall prevalence of 8.4% . The HBsAg prevalence was measured in 59 countries (harbouring 73% of worls's population), midpoint prevalence estimates of 5-10% in 10 countries (Stasi *et al*., 2017).

The risk of mother-to-child transmission is related to the state of HBV replication of the mother that correlated with the presence of hepatitis B e antigen(HBeAg). In fact , 90% of HBeAg-positive mothers transmit HBV infection to their newborns compared to 10%-20% of HBeAg-negative mothers(Navabakhsh *et al*., 2011).

Heterosexual-and especially homosexual-activity is an important rout of transmission , HBsAg prevalence estimate range from<1% to 4% in men who have sex with men in 3 of the 34 countries (Hahne *et al*., 2013). Prisoner are at high risk of HBV infection due to social and environmental risk factors , in addition to specific features of the prison environment (i.e. restricted space and overcrowding) . In prison , prevention programmes for vaccination against HBV could be both a challenge and an

opportunity; many people who do not otherwise have access to health care can be reached and followed for a certain period , although the environment itself presents an increase risk for HBV transmission (Stasi *et al*., 2016).

2.8.Immunopathology

Hepatitis B virus engages different immune components over time as it progresses through its pathogenesis. It is primarily a concerted action of interferon-γ (INF-γ) and cytolytic $CD8^+$ T cells that target infected hepatocytes during acute phase of infection. Interferons have an important role during acute HBV infection as the infected hepatocytes begin production of IFN-α /β that inhibits viral packaging (Sandhu *et al*., 2017).NK cells have been suggested to have a role in acute HBV although there is conflicting evidence about their contribution depending on the stage of pathogensis of the patients (Schuch *et al*., 2014). In patients with acute HBV infection , there is reduce activation of NK cells that exhibit decrease cytolytic activity that coincided with peak viremia (Sandhu *et al*., 2017). Furthermore , chronic HBV patients showed decrease NK cell activation markers and reduced IFN-γ and TNF-α production (Tjwa *et al*., 2011). Interestingly , NKT cells , a group of immune cells that share properties with NK cells and signal though T cell receptor (TCR) to lipids , were activated early during HBV infection by lipids generated due to HBV infection and contributed in priming T and B cells (Zeissing *et al*., 2012). Kupffer cells , tissue resident macrophages within the sinusoidal compartment of liver , act as first line of defense against pathogens invading the liver . HBV is know to infect hepatocytes in the liver but no direct infection of kupffer cells has been observed although uptake of HBsAg by these cells has been reported (Boltjes *et al*., 2015).Adaptive immune response , particularly the HBV- specific $CD8^+$ T-cells response , have been suggested to play an important role in viral clearance (Chung and Rung 2017). A recent study showed that the IFN-γ-mediated release of CXCL9 by kupffer cells would augment and retention of HBV-specific $CD4^+$ T cells through CXCR3 and promote their apoptosis , which facilitated the antigen-specific tolerance during HBV persistence (Zegn *et al*., 2016).

Once chronic infection has been established , the patients may experience repetitive liver damage , characterized by an elevated serum ALT level. It is still unclear how the adaptive immune response leads to liver damage but does not completely clear the virus. The comparison of liver-infiltrating HBV-specific $CD8^+$ T cells between

patients with and those without a high viral load accompanied with liverdamage revealed a high frequency of intrahepatic HBV-specific $CD8^+$ cells in patients without hepatic immunopathology. In patients with active hepatitis , virus-specific $CD8^+$ T cells were less frequent among liver infiltrating $CD8^+$ T cells , but the absolute number was similar , owing to the large amount of cellular infiltration (Chung and Rung 2017).

The other study also showed that the HBV-specific CTLs were also present in PBMCs from immune-tolerant patients (Kennedy *et al*., 2012). These data suggest that HBV-specific CTLs exist in patients with CHB but that they are not quantitatively and qualitatively strong enough to clear the virus . analysis of HBV-specific $CD8^+$ T cells in the liver and the peripheral boold showed that intrahepatic cells expressed higher levels of programmed cell death protein 1 (PD-1) than their peripheral counterparts (Fisicaro *et al*., 2017). Which suggests that these CTLs would undergo exhaustion in a liver microenvironment with congnate antigen expression. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is also highly expressed on HBV-specific CTLs among PBMCs from patients with CHB (Schurich *et al*., 2011) .In addition to expression of multiple coinhibitory molecules on $CD8^+$ T cells ,a recent study based on an analysis of PBMCs from patients with CHB showed that exhausted HBV-specific $CD8^+$ T cells have substantial downregulation of various cellular processes centered on extensive mitocodiral alterations (Fisicaro *et al*., 2017).

2.9.Diagnosis

Serological markers for HBV infection consist of HBsAg , anti-HBs , HBeAg , anti-HBe and anti-HBc IgM and IgG . The identification od serological markers allows : to identify patients with HBV infection ; to elucidate the natural course of chronic hepatitis B (CHB) ; to assess the clinical phases of infection ; and to monitor antiviral therapy (CDC 2011) .HBsAg is the serological hallmark of HBV infection. After an acute exposure to HBV , HBsAg appears in serum within 1 to 10 weeks . Persistence of this marker for more than 6 months implies chronic HBV infection (Eun and Young 2016).Several studies have reported the association between transcription activity of cccDNA in the liver and serum HBsAg levels (Thompson *et al*., 2010). Difference in the serum HBsAg levels during the different phases of infection indicate the distribution of cccDNA during the respective phase of the disease . The serum

HBsAg titer are higher in patients with HBsAg-positive CHB than in HBeAg-negative CHB (Jaroszewicz *et al*., 2010). Monitoring of quantitative HBsAg levels predicts treatment response to interferon and disease progression in NBeAg-negative CHB patients with normal serum alanine aminotransferase levels (Martinot-Peigoux *et al*., 2013).

Anti-HBs is known as a neutralizing antibody , and confers long-term immunity .In patients with acquired immunity through vaccination , anti-HBs is the only serological marker detected in serum . In the past HBV infection , it is present in concurrence with anti-HBc IgG . Occasionally , the simultaneous appearance of HBsAg and anti-HBs has been reported in patients with HBsAg positive (Eun and Young , 2016). In most cases , anti-HBs antibodies are unable to neutralize the circulating viruses , thus these patients are regarded as carriers of HBV , HBeAg and anti-HBe had been used to known infectivity and viral replication , but their use for this purpose has mostly been replaced by HBV DNA assay . HBeAg to anti-HBe seroconversion is related to the remission of hepatic disease (Deny and Zoulim 2010).however , active viral replication is sustained in som patients with HBe seroconversion due to mutations in the pre-core and core region that inhibit or decrease the production of HBeAg . (Krajden and Petric 2014).

HBcAg is an interacellular presence in infected hepatocyte, thus it is not identified in the serum . During acute infection , anti-HBc IgM and IgG emerges 1-2 weeks after the presence of HBsAg along with raised serum aminotransferase and symptoms . after 6 months of acute infection , anti-HBc IgM wears off. Anti-HBc IgG continues to detec in both patients with resolved HBV infection and CHB. some HBsAg-negative individuals are positive for anti-HBC IgG without anti-HBs , in this situation, it should be considered isolated anti-HBc positive. It can be seen in three conditions. First , it can be predominantly seen as IgM class during the window period of acute phase. Second, after acute infection had ended , anti-HBs has decreased below the cutoff level of detection. Third , after several years of chronic HBV infection , HBsAg has diminished to undetectable levels. If the result of markers show isolated anti-HBc positive , anti-HBc IgM should be checked in order to assess the possibility of recent HBV exposure. HBV DNA assay shuld be tested in chronic liver disease patients to find out occult HBV infection characterized by existence of detectable HBV DNA without serum HBsAg (Eun and Young , 2016).

HBV DNA is a directed measurement of the viral load , which reveals the replication activity of virus. It is detectable at the early stage of infection (1 month after HBV infection) and increases up to peak level (more than 10^8 copies/ml) approximately 3 months after the exposure to HBV and then gradually diminishes in chronic infection Or disappears at the recovery from HBV infection . (Eun and Young , 2016). As the prevalence of serologically negative HBV infection (HBeAg-negative CHB and occult HBV infection) has increased , HBV-DNA detection has obtained more a wareness in clinical medicine (Datta *et al*., 2014). There are two principles of techniques to identify and quantify HBV DNA: singal amplification such as hybrid capture and branced DNA technology ; target amplification such as polymerase chain reaction (PCR) (Caliendo *et al*., 2011). Real-time PCR can detect wide dynamic range of viral load (lower range , 10-15 IU/mL; upper range, 10^7-10^8 IU/mL). For this reason , it has come to be the standard method to detect and quantify HBV DNA in clinical setting . Furthermore , it can be fully automated and dose not generate carry-over contamination (Eun and Young 2016).

2.10. Cytokines

Cytokines are a cell-signaling group of low molecular weigh extracellular polypeptides /glycoprotiens synthesized by different immune cells , mainly , by T cells , neutrophils and macrophages , which are responsible to promote and regulate immune respose (i.e activity , differentiation , proliferation and production of cells and other cytokines (Ma *et al*., 2016). Cytokines are involved in a broad array of biological activities including innate and adaptive immunity , development of cellular and humoral immune responses , induction of the inflammatory response , regulation of hematopoiesis , control of cellular proliferation and differentiation , besides having roles in tissue regeneration and healing of wounds .(Dhama , 2016).

2.10.1.Cell produces cytokines

Cytokines are proteins produced and secreted by a variety of cells including stromal cells , fibroblasts , and endothelial cells . (Vazquez *et al*., 2015). The production and release of cytokines from innate immune cells are critical responses to inflammation and infection in the body . Innate immune cells comprise populations of white blood cells such as circulating dendritic cells (DCs) , neutrophils , natural killer (NK) cells , momocytes , eosinophils , and basophils , along with tissue-resident mast cells and machrophages . (Lwasaki ; Medzhitov 2010). Residing at the frontline of defence in immunity , these cells control opportunistic invasion by a wide range of viral , fungal , bacterial and parasitic pathogens , in part by releasing a plethora of cytokines and chemokines to communicate with other cells and thereby to orchestrate immune response . This array of soluble mediators secreted by different innate immune cells includes TNF , IFNγ , interleukins IL-1β, IL-4, IL-6, IL-10, IL-12, IL-18, CCL/4RANTES, and TGFβ (Lacy and Stow 2011).

Th1 subset secreted IL-2 , IFNγ , and TNF , and is responsible for many classic cell-mediated function including activation of cytotoxic T lymphocytes and macrophages (Siransy *et al* ., 2018). The Th2 subset secreted IL-4, IL-5 , IL-6 , IL-9 , IL-10 , IL-13 , and regulates B-cell activity and differentiation (Owen *et al*., 2013). The Th17 subset is primarily involved in recruiting leukocyres and incducing inflammation and they secreted IL-17 (Abbas *et al*., 2015).

The Th9 subsets secreted IL-9 which have a role in the induction and the pathogenesis of atopic disease , antiparasite immunity and immune pathological disease of the gut (Kaplan *et al*., 2015).

2.10.2.Types of cytokines

2.10.2.1.Interferons (IFN)

Interferons (IFN) are glycoproteins belonging to the family of cytokines and have antiviral , antitumor , and immunomodulatory activities.(Garbe *et al*., 2011). It's classified based on receptor binding and structural and biochemical differences.(Kaur , 2015).

2.10.2.2. Tumor necrosis factor (TNF)

Is a cytokine that had the name derived from it discovery in 1975 as a molecule that caused in- vitro necrosis of tumors. Shortly there after, it was observed that TNF expression was promoted by immune system cells. These discoveries were important to a posterior characterizing of the TNF superfamily and the TNF receptor superfamily, which has more than 40 members, being the most outstanding TNF-α (commonly named as TNF) and TNF-β (also named lymphotoxin), but also including cytokines and membrane proteins that have similar sequence homologies and a homotrimeric pyramidal structure (e.g. CD40 ligand, FAS ligand, OX40 ligand, GITR ligand and other several proteins). The binding of this family of cytokines with their respective receptors triggers especially inflammatory reactions (Ma *et al*., 2016) .

TNF-β, a type II transmembrane protein, is an important key in the development of lymph nodes and Peyer's patches, and also for the maintenance of secondary lymphoid organs. The expression of TNF-β is mainly stimulated by lymphocytes. TNF-α will be better described in the following topics (Drutskaya *et al*., 2010). Although it were discovered many receptors along the decades, two are best known: TNFR1 (55 kD) and TNFR2 (75 kD). Both receptors are plasma membrane trimmers, while TNFR1 is expressed by most human cells and TNFR2 is mainly produced by immune system cells. It is important to mention

that TNFR2 have a higher affinity to TNF. They are related to inflammatory reactions, so that a cytokine bind to the receptor, it induces the recruitment of proteins that are important for the process (Drutskaya *et al*., 2010 ; Kalliolias and Ivashkiv , 2016) .

2.10.2.3.Interleukins

Interleukins (ILs) are a group of secreted proteins with diverse structures and functions. These proteins bind to receptors and are involved in the communication between leukocytes. They are intimately related with activation and suppression of the immune system and cell division. The interleukins are synthesized mostly by helper CD4+ T lymphocytes, monocytes, macrophages and endothelial cells. (Sims and Smith 2010 ; Akdis *et al*., 2011 ; Abbas *et al*., 2015). Interleukins are named as IL plus a number. Previously, different names were used to refer to the same IL. For instance, IL-1 was called lymphocyte-activating factor, mitogenic protein or T cell replacing factor III. In order to standardize the nomenclature, in 1979, during the Second International Lymphokine Workshop, the term interleukin was introduced. After that, the interleukins started being named consecutively according to the date of their discovery (Akdis *et al* ., 2011) There have been identified 40 interleukins so far and some of them are further divided into subtypes (e.g. IL-1α, IL-1β). These ILs are grouped in families based on sequence homology and receptor chain similarities or functional properties. (Akdis *et al*., 2011; Abbas *et al*., 2015 ; Wang *et al* ., 2016 ; Catalan-Dibene *et al*., 2017).

2.10.2.3.1.The interleukin-1 family

Interleukin-1 family is composed by 11 cytokines: 7 ligands with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ), 3 receptor antagonists (IL-1Ra, IL-36Ra and IL-38) and 1 anti-inflammatory cytokine (IL-37) (Akdis *et al*., 2011; Garlanda *et al*., 2013). The interleukin-1 family started with only two components: IL-1α, IL-1β. Over the years, new IL with similar behavior and/or structure were discovered and added to the family. All the agonists members of this family show pro-inflammatory activity. These cytokines share a common C-terminal three-dimensional structure with a typical β-trefoil fold consisting of 12-β-strands connected by 11 loops, and have identical positioning of certain

introns. Considering that, it is plausible to affirm that they probably arose from the duplication of a common ancestral gene (Sims and Smith 2010; Dinarello , 2011; Palomo *et al*., 2015) . All members of the family except IL-18 and IL-33 have genes encoding on chromosome 2 in a 400 kb region in human species. Despite the fact that all the cytokines are extracellular, they are synthesized without a hydrophobic leader sequence and are not secreted via reticulum endoplasmic-Golgi pathway, with the exception of IL-1Ra. The secretion mechanism of the other members of the family is still not known. These cytokines bind to closely related receptors, and many of the encoding genes are clustered in a short region of chromosome 2. The receptors contain extracellular immunoglobulin domains and a toll/IL-1 receptor (TIR) domain in the cytoplasmic portion (Sims and Smith , 2010 ; Palomo *et al*., 2015).

2.10.2.3.2.Interleukin-2 family

The IL-2 cytokine family, also known as the common γ-chain family, is composed by ILs 2, 4, 7, 9, 15 and 21. All these ILs bind to the common γc receptor, also called CD132. These cytokines act as growth and proliferation factors for progenitors and mature cells (Sim and Radvanyi , 2014). IL-2 is the first member of the common γ-chain family, previously known as T cell growth factor. This cytokine is mainly produced by CD4+ and CD8+ T cells, but can be also expressed by dendritic cells and NKs. IL-2 has been extensively used as an anti-cancer therapy (Akdis *et al*., 2011; Sim and Radvany , 2014). IL-4 is produced by Th2 cells, basophils, eosinophils and mastocytes. It has two receptors: IL4-R type I, which binds only to IL-4 and is composed by CD124 (IL-4rα) and CD 132; and type II, which binds to IL-4 and to IL-13, and it consists in IL-4Rα and IL-13Rα1. These receptors are spread all over the human body. IL-4 is known to play several different roles, regulating allergic conditions and activating the immune response against extracellular parasites (B cell class switching to IgE). It is the main cytokine to stimulate development of Th2 cells. Dupilimab is an IL-4 receptor antagonist approved in 2017 by FDA for treatment of eczema .(Paul , 2015, Shirley , 2017). IL-7 is a homeostatic cytokine. It can be found essentially in T cells, progenitors of B cells and bone marrow macrophages. As the other members of the family, its receptor

(IL-7R) consists in the common γ-chain fraction, along with another unit, the IL-7Rα (CD127). (Akdis *et al*., 2011; Lundstrom *et al*., 2012). IL-9 is mainly produced by Th2 cells, but it is also expressed in less amounts by eosinophils and by mastocytes of asthmatic patients. Its receptor, IL-9R, is composed by the CD132 and IL-9Rα units. IL-9 is a potent growth factor for T cells and mastocytes, and some of it activities include the inhibition of cytokine production by Th1 cells, IgE production, and mucus secretion by bronchial epithelium. (Kaplan , 2013 ; Zhao *et al*., 2013). IL-15 is structurally homologous to IL-2. The receptor, IL-15R, is composed by the CD132 subunit common to the family, and also by IL-15Rα and IL-2Rβ chains. IL-15 is produced by keratinocytes, skeletal muscle cells, monocytes and activated CD4+ T cells, in response to signals that trigger innate immunity. IL-15 has some identical functions to IL-2 (Abadie and Jabri , 2014). IL-21 is produced by T cells, NKT cells and Th17. The receptor, IL-21, is present in various parts of the human body and consists in CD132 and IL-21R. This cytokine is involved with B cells functions, and also increases the proliferation of CD8+ T cells, NK cells and NKT. IL-21 is currently being studied as anti-cancer therapy (Lin and Leonard , 2017).

Other cytokines In addition to the aforementioned cytokines, other also deserves attention, such as chemokines. The chemokines represent a large family of structurally homologous cytokines that stimulate leukocytes movement and regulate the migration of them from the blood to tissues, in a process named chemotaxis. They control homeostatic immune cells, such as neutrophils, B cells, and monocytes, trafficking between the bone marrow, blood and peripheral tissues. Therefore, they can be classified as chemotactic cytokines (Griffith *et al*., 2014). There are about 50 human chemokines, classified into 4 families according to the location of N-terminal cysteine residues. The two major families are CC and CXC chemokines, (Delves *et al*., 2011).

2.10.3.Mechanisms of action

Virus entry into host cells is the starting point of a productive infection process. Inhibition of viral entry is thus a promising approach to control a virus. Targeting either of the two host factors HSPG or NTCP that are crucial for HBV entry is exploited by cytokines or cytokine-induced mediators. By using HBV-infected

HepaRG cells, we demonstrated that IFN-α-treated cells release factors restricting HBV entry (Xia *et al*., 2014). These factors competed with the virus for binding to HSPG. This study revealed a novel antiviral mechanism of IFN-α: inhibiting HBV infection of neighboring cells by inducing soluble factors that bind to HSPG and block HBV attachment. Recently, cholesterol-25-hydroxylase (CH25H) was identied as an interferon-induced gene (ISG) (Liu *et al*., 2013). Cholesterol-25-hydroxylase converts cholesterol to 25-hydroxycholesterol (25HC), which inhibited entry of a number of enveloped viruses including HBV (Iwamoto *et al*., 2014). The cytokines interleukin (IL)-6 and IL-1β regulate NTCP expression and can thus inhibit HBV entry (Xia and Protzer , 2017). A recent study revealed that HBV entry was inhibited by up to 90% when cells were pretreated with IL-6, resulting in a strong reduction of cccDNA and HBsAg secretion (Bouezzedine *et al*., 2015). In parallel, decreasing NTCP mRNA level led to a strong reduction in NTCP-mediated taurocholate uptake in a dose-dependent fashion. HBV entry and bile acid uptake were restored by lentiviral over expression of NTCP. This indicated that IL-6 inhibits HBV entry through the downregulation of the viral entry receptor NTCP, most likely by inhibiting hepatocyte nuclear factor (HNF) 4α-mediated transcription (Xia and Protzer , 2017).

2.10.4.IL-17

IL-17 was discovered in the 1990 and has since emerged as a remarkably pleiotropic cytokine that contributes in unique ways to the host immune response . IL-17 can be produce by a wide range of immune cell populations , such as TH17 cells (Ma *et al*., 2019), $CD8^+$ T cells, $\gamma\delta$T cells (Kim *et al* ., 2018),natural Killer (NK) cells , natural Killer T (NKT) cells , mast cells (Chen *et al*., 2019) , nutrophils (Lin *et al*., 2011), and group 3 innate lymphoid cells (ILC3) (Wang *et al*., 2018). It's plays a key role in the maintenance of tissue integrity and the generation of protective immune responses to infectious microorganisms , especially at epithelial barrier sites .The proinflammatory properties of IL-17 also make it a crucial mediator of inflammation and imunopathology (Veldhoen , 2017). The surprisingly diverse functions of IL-17 have made it among the more favourable immunotherapeutic target candidates for the treatment of a wide range of diseases , including cancers (Ibrahim *et al*., 2018), autoimmune diseases (Brembilla *et al*., 2018) and infectious diseases (Carney ,

2018). Research is also being done on IL-17 in the regulation of viral infections , where it plays varied and crucial roles. Intriguingly , a body of literature indicates that whil IL-17 is a critical player in host defence by substantially suppressing viral infections , it has also been strongly implicated in the promotion of viral infection and related illness (Ma *et al*., 2019). Viruses may induced tissue damage by promoting fibrosis development , as is particularly true for chronic HBV infections whereby inappropriate activation of IL-17-producing cells plays a critical role in maintaining fibrogenic pathways and diseases progression (Passo *et al*., 2010).

2.11.Relation of IL-17 with HBV

Abundant evidence suggests a potential correlation between IL-17-producing cells (i.e Tc17 cells or Th17 cells) or IL-17 levels and a more sever diseases stage of HBV progression (Zhang *et al*.,2018). While mechanistic studies have shown a causal link between IL-17 production and the development of liver fibrosis . For example , IL-17 expression has been observed to be specifically localized to portal areas and fibrotic septa in patients with HBV infection. (Lin *et al*., 2011) . Interestingly , most of these IL-17 expressing cells are neutrophils , with the number of these cells showing a strong positive correlation with liver fibrosis stage (Macek *et al*., 2016). More specifically , the work of Wang and his colleagues in 2013 , highlighted a critical role of the IL-23/IL-17 axis in the induction of liver damage after HBV infection. In HBV infected patients , IL-23 production by DCs and macrophages stimulates the differentiation of naïve $CD4^+$ T cells into Th17 cells , the primary source of IL-17 in HBV-infected livers (Lee *et al*., 2013). However , the relationship between IL-17 and fibrosis in chronic HBV diseases remains unclear.(Wang *et al*., 2011).

2.12.Previous studies

In China Yang and his colleagues quantified serum IL-17 by ELISA in 213 patients with CHB and 20 healthy controls , levels of IL-17 proteins in CHB patients were higher than those in the healthy control group(p<0.05) (Yang *et al*., 2018).

In 2017 Meng and his colleagues detect levels of IL-17 among chronic HBV infected patients and showed that IL-17 values (2.923±2.310 pg/ml), all enrolled patients has high serum level of IL-17 .(Meng *et al*., 2017).

In West China Hospital of Sichuan University on 2019 Cai and his colleagues evaluate level of IL-17 in 123 patients with HBV , 70 subjects with HBV spontaneous clearance and 59 healthy controls . IL-17 level in HbsAg clearance group strikingly increased compared with CHB . Higher serum level of IL-17 contributed to HBV surface antigen clearance .(Cia *et al*., 2016).

IL-17 level was detected by the ELISA kit in 70 cases of CHB patients and 20 healthy .There were no statistically significant differences in age , gender ratio etc . between CHB group and the healthy control group; the groups were comparable (P>0.05). Compared with the healthy control group IL-17 level in CHB patients was significantly increased (p<0.05) (Song *et al*., 2016).

You and his colleagues showed that chronic HBV infected patients had significantly increased serum level of IL-17 compared with normal individuals (P<0.001). There is marked correlation between the concentration of IL-17 and severity of liver disease and viral replication. Increased serum level of IL-17 correlate positively with the severity of liver disease and active viral replication in chronic HBV infection (You *et al*., 2014).

In 2013 Wang and his colleagues anticipated that IL-17 expression might be enhanced in HBV infected liver tissue , as result of the elevated IL-23 expression .Indeed they found that IL-17 expression was significantly elevated in the livers of CHB as compared to healthy controls . IL-17 level was determined by use Ready-SET-Go ELISA kit (Wang *et al*., 2013).

Du and his colleagues in China 2013 was determined serum IL-17 level by use a double-antibody sandwich ELISA . Average serum IL-17 protein values for CHB was

38.9± 11.34 pg/ml while the control group value 28.2± 7.78 pg/ml .These serum IL-17 level was 37.9% higher in patients with CHB compared to normal controls CHB *P*= 0.0002 .IL-17 expression was found to be increased with increasing degrees of liver fibrosis .This suggests that IL-17 may not only induce the inflammation , but also contribute to disease progression and chronicity (Du *et al*., 2013).

In 2011 Wang and his colleagues measured serum levels of IL-17 in patients of different groups . Compared with the normal control group , the serum concentrations of IL-17 significantly increased in patients with CHB , sever hepatitis B and hepatic cirrhosis (all p-values were < 0.05). this study confirms the enhanced expression of IL-17 in HBV-associated diseases (Wang *et al*., 2011).

In New York 2011 Wang and his colleagues detect role of IL-17 in cirrhotic and non-ciroohotic patients with hepatitis B . IL-17 increased with the severity of fibrosis and cirrhosis (P=0.06 and P=0.09) (Wang *et al*., 2011).

In South-East Iran 2010 Kazemi and his colleagues determine IL17A as chronic disease inducer . The study showed that IL-17A serum levels are significantly higher in cases (12.48 ± 2.00 pg/ml) in comparison to healthy controls (4.43± 0.54 pg/ml) (Kazemi *et al*., 2010).

CHAPTER THREE

MATERIALS AND METHODS

Chapter Three

3.Materials and Methods

3.1.Study design

This is prospective and case control study.

3.2.Study area , setting and duration

Study was conducted in Saba laboratory (hepatitis B virus infections center) in Khartoum state during the period from march to November (2019).

3.3. Study population

Study population consisted of 60 Sudanese individuals of age between 17-50 years , divided into 2 groups as follows-HBV infected made up of 30 patients and 30 control group.

3.4. Inclusion criteria

Patients were considered in HBV infected if they complain from signs and symptoms . control group was age and sex matched apparently health subjects .

3.5. Exclusion criteria

All HBV infected patients with situation that affected cytokine level including physiological factors such as (Pregnancy , smoking and alcohol consumption) and others diseases such as (autoimmune diseases , infectious diseases , allergy , hypersensitivity , cancer , heart failure and Parkinson diseases).

3.6. Sample size

A total of 60 subjects were enrolled in this study . 30 samples were collected from HBV infected patients and 30 samples were collected from healthy volunteer.

3.7. Data collection

Samples were collected randomly , Questionnaire was used to collect demographic , clinical and laboratory data and it was full filled by us .

3.8. Ethical consideration

Permission to carry out the study was obtained from college of Medical Laboratory Science . Permission of laboratory manger was taken before beginning . Every sample was collected after verbal approval by patients and volunteer.

3.9. Sampling

Three ml of venous blood was collected from patients and control in a EDTA container . Then the samples were centrifuged and serum was separated in a sterile container and stored at -20 ºC until analysis . serum level of IL-17 were measured using ELISA .(Biolegend's ELISA MAX^{TM}).

3.10.Principle and procedures

3.10.1. Principle of the ELISA

Biolegend's ELISA MAX^{TM} Deluxe Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human IL-17 specific monoclonal antibody is irst coated on a 96-well plate. Standards and samples are added to the wells , and IL-17 binds to the immobilized capture antibody . Next , a biotinylated anti-human IL-17 detection antibody is added , producing an antibody-antigen-antibody "sandwich". Avidin-horseradish preoxidase is subsequently added , followed by TMB Sustrate Solution , producing a blue color in proportion to the concentration of IL-17 present in sample . Finally , the stop Solution changes the reaction color from blue to yellow , and the microwell absorbance is read at 450 nm with a microplate reader (biolegend , USA , 2016).

3.10.1.1.ELISA Procedure

One hundred µL of diluted capture antibody solution was added to each well , seal the plate and incubate overnight between 2^0 and 8^0 C .Then plate washed 4 times with at least 300µL of wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper . To block the plate by adding 200 µL 1X Assay diluents A to each well , seal the plate and incubate at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly. Plate washed 4

times ; add 100 µL Diluted stanndareds and samples to the appropriate wells . seal the plate and incubate at room temperature for 2 hours with shaking . then wash plate 4 times ; add 100µL diluted detection antibody solution to each well , seal the plate and incubate at room temperature for 1 hour with shaking . wash plate 4 times ; add 100 µL diluted Avidin-HRP solution to each well , seal the plate and incubate at room temperature for 30 minutes with shaking . wash plate 5 times ; soaking for 30 seconds to 1 minute per wash . add 100µL of freshly mixed TMB substrate solution to each well and incubate in the dark for 30 minutes. Add 100µL stop solution to each well . read absorbance at 450 nm and 570 nm within 15 minutes . The absorbance 570 nmm can be substracted from the absorbance at 450 nm .

3.10.1.2.ELISA Washer principle

First the wash solution is pump from the wash bottle , the solution is dispense to the cuvette by short pins , and then the wash liquid is aspirate from the cuvette by long pins , at the end the waste liquid was pumped into the waste bottle by the vacuum pupp.(www.diasource.be 2019).

3.10.1.3.ELISA reader principle

White light produced by the lamps is focused into a beam by the lens and passes through the sample. Part of the light is absorbed by the sample and the remaining light is transmitted . It is filtered by interference filters and focused onto the photodiodes . The photodiode converts the received light into an electrical signal which is transformed into a digital form , from which the microprocessor calculates the absorbance , taking in account of the blank and dichromatic selection .(www.diasource.be 2019).

3.11. Statistical analysis

Data was analyzed by using statistical package for social sciences (SPSS) programme (version 20) using one way ANOVA test and independent T-test for testing difference significance . correlation test to find out correlation . The probability value ≤ 0.05 was considered significance.

CHAPTER FOUR

RESULTS

Chapter Four

4.Results

4.1.Results

Sixty volunteers with age varies from 17-50 years were enrolled in this study,30 subjects are hepatitis B virus patients with mean of age 29.7 ± 9.3 years,17 of them,(56.7%) were males and13(43.3 %) are females. Other 30 subjects are apparently healthy control group with mean of age 32.4 ± 9.5 years, 56.7% are males and 43.3 % are females (Table 4-1).

Mean level of IL-17 was significantly increased in hepatitis B virus patient (16.28± 9.57pg/ml) when compared to control one (7.47±8.48pg/ml) with(*p.value* 0.00) (Table 4-2).

Comparisons between IL-17 levels in hepatitis B patients with sex , IL-17 mean level was lower in males(11.75±8.61pg/ml)than females (13.62±11.64 pg/ml), which is statistically insignificant *p.value* 0.328 . and in healthy control IL-17 level mean was higher in female (10.13±6.15) than male (5.44±3.99) , which is statistically significant (*P.value* 0.017).(Table 4-2).

According to duration of disease high plasma level of IL-17 in patients with acute hepatitis B compared to chronic hepatitis B patients ,mean were 18.3±10.44 and 13.97±8.2 respectively, this difference was statistically insignificant (*P. value* 0.223).

Regarded to treatment showed that insignificantly increased between patients using treatment compared to patient not use treatment (16.4±9.9 and 15.21±9.20 respectively) (*p. value* 0.673).

There was no significant difference in IL-17 level between patients with symptoms of jaundice (17.12±10.25pg/ml) and patients have no symptoms of jaundice (16.15±9.67pg/ml) (*p. value* 0.854).

In blood transfusion IL-17 level was decreased in patients received blood transfusion (14.01±6.68pg/ml) when compared to patients didn't receive blood transfusion (16.63±9.99pg/ml) and this difference was statistically insignificant (*p.value* 0.061).

One way ANOVA test showed insignificant comparison between age group and IL-17 (*P.value* 0.061) (Table 4-4).

There was insignificant positive correlation between IL-17 and age in both hepatitis B virus group and control group (r = 0.065 and *p.value* 0.190 , r = 0.108 and *p.value* 0.076) respectively . (Figures 4-8 , 4-7).

Table(4-1): Gender Distribution among Study Groups.

Gender	Case	Control	Total
Male	17(56.7%)	17(56.7%)	34
Female	13(43.3%)	13(43.3%)	26
Total	30	30	60

Tables(4-2): Comparison Of IL-17 Level and Age between Hepatitis B Patients Group and Healthy Control Group.

Parameter	Hepatitis B virus patients	Healthy control group	*P.value*
IL-17	16.28±9.57	7.47 ±8.48	0.00
Age	29.7± 9.3	32.4 ± 9.5	

**p.value* ≤ 0.05 = significant

Table(4-3): Comparison between Hepatitis B Virus Patients and Different Study Variables.

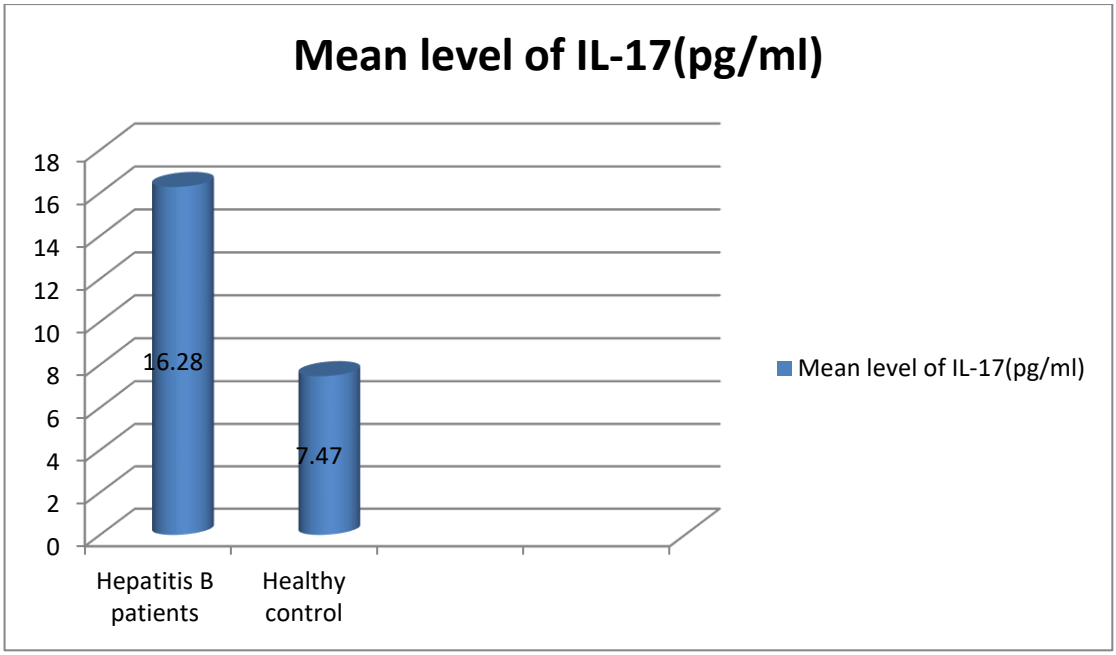
Variables		Mean±STD	*p. value*
Duration	Acute	18.3 ± 10.44	0.223
	Chronic	13.97± 8.2	
Treatment	Yes	16.481 ± 9.93	0.673
	No	15.21 ± 9.20	
Blood transfusion	Yes	14.01± 6.68	0.691
	No	16.63 ± 9.99	
Jaundice	Yes	17.12 ± 10.25	0.854
	No	16.15 ± 9.67	

**p.value* ≤ 0.05 = significant

Table(4-4): Comparisone between IL-17 and Different Age Group

Age groups	Mean±STD	*P.value*
<20	38.8±8.7	0.061
20-45	15.4±13.2	
>45	16.2±9.6	

**P.value*≤0.05= significant



Figure(4-1):Mean Level of IL-17 Concentration in Hepatitis B Patients Group and Healthy Subjects Group.

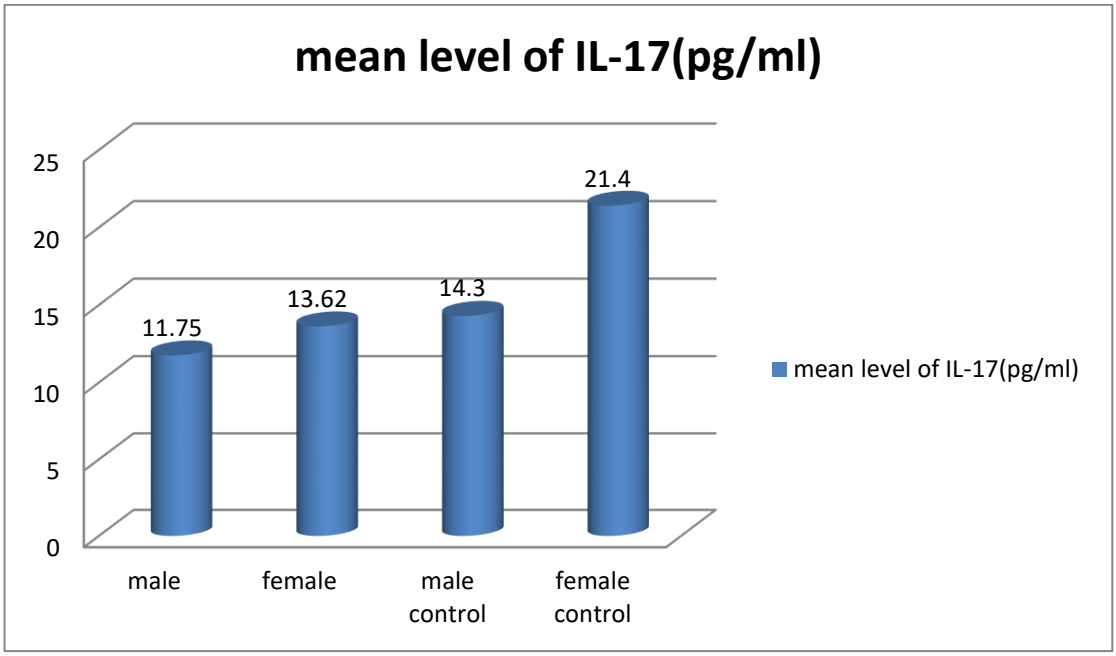
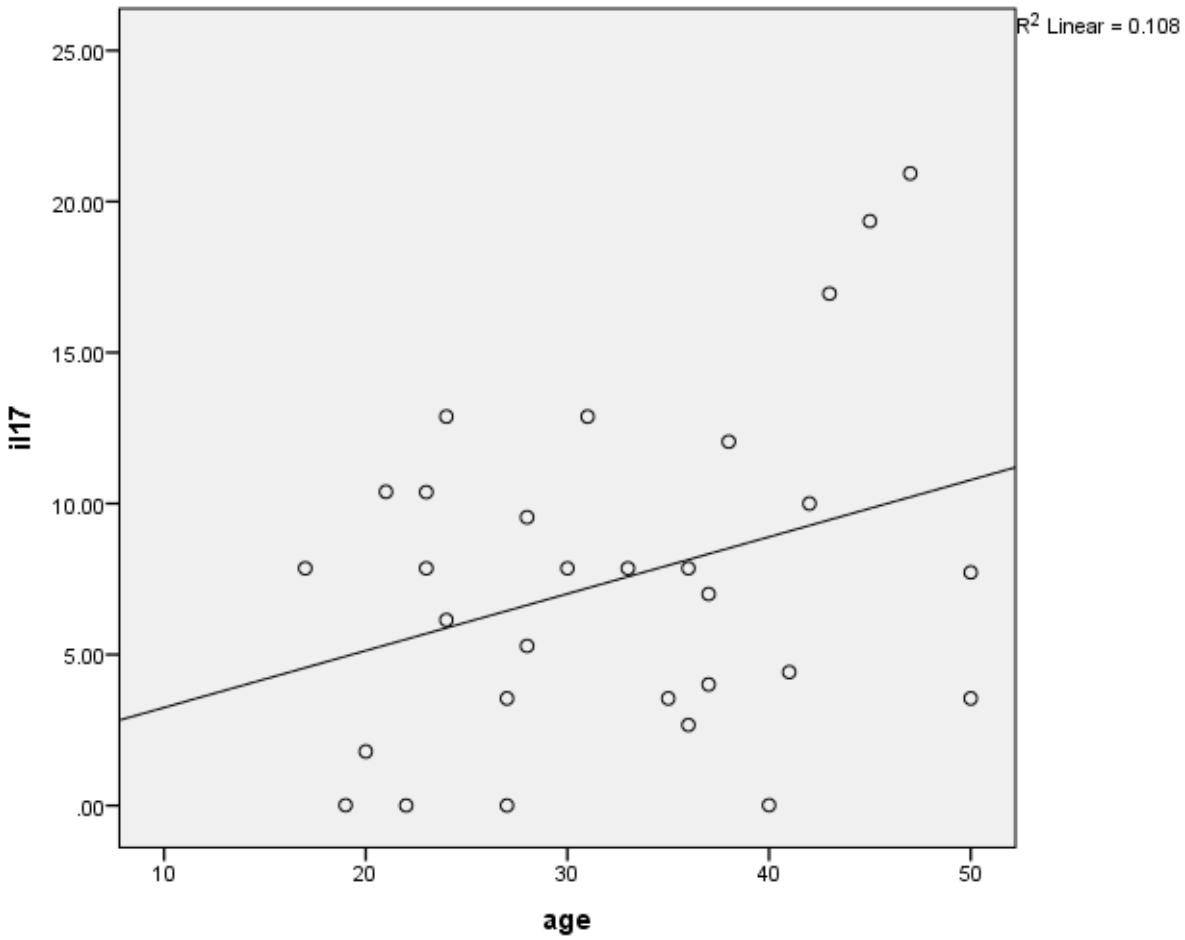
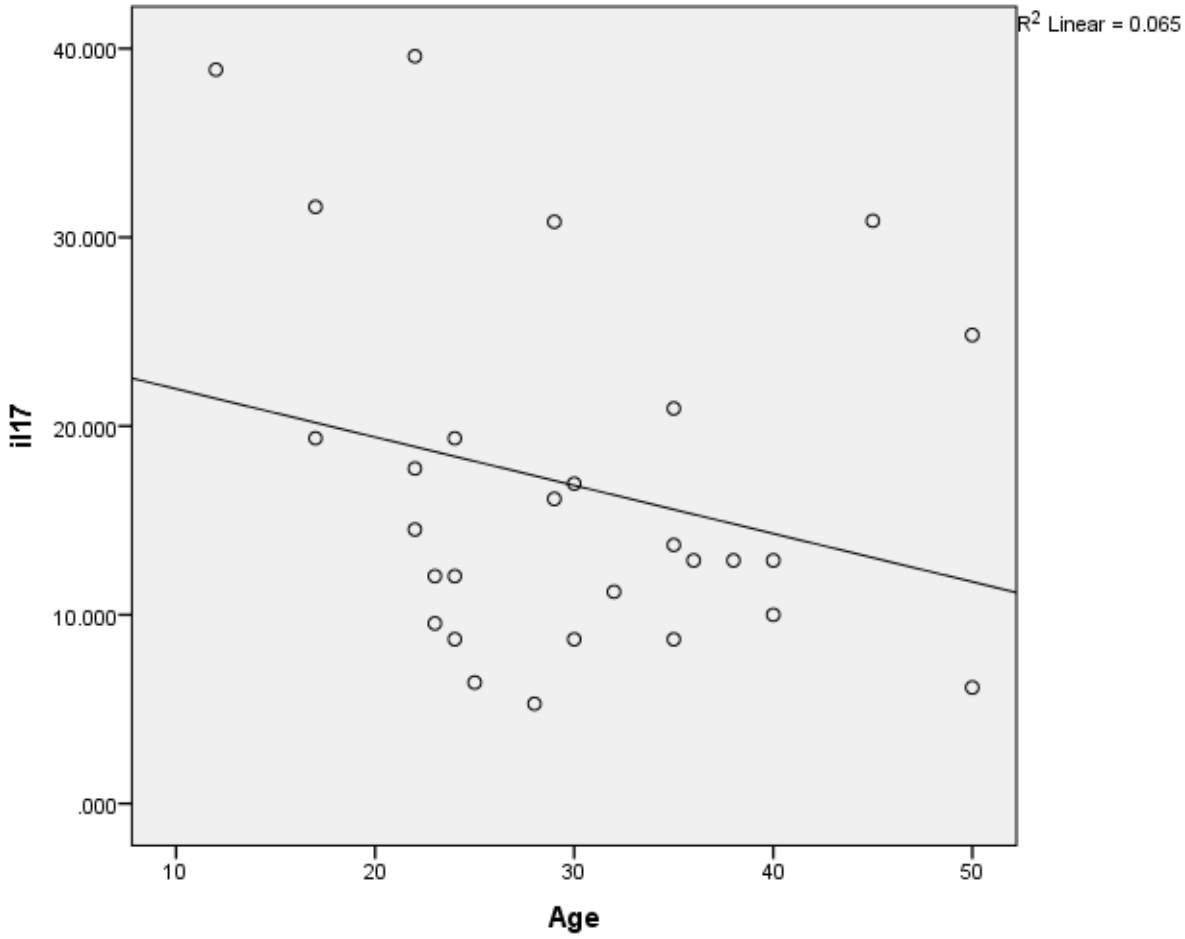


Figure (4-2): Mean level of IL-17 concentration in hepatitis B patients and healthy subjects group according to sex.



R=0.108
*p.value*0.076

Figure (4-3):Correlation between IL-17 level and age of healthy subjects.



R=0.065
p.value 0.190

Figure (4-4):Correlation between IL-17 level and age of hepatitis B patients.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Chapter five
5.Discussion , Conclusions and Recommendations

5.1.Discussion

IL-17 is pro-inflammatory cytokines that has been determined as an important mediator in autoimmune diseases and immune reaction against some specific pathogens , although the mechanism is unclear .(Metanat *et al*., 2019).

In the present study plasma level of IL-17(pg/ml) is significantly higher in hepatitis B virus patients compared to healthy control group *(p value* 0.00) , This finding was supported by Yang *et al* who demonstrate that significantly elevated of serum level of IL-17 protein in CHB patients compared to healthy control (*p.value* <0.05) (Yang *et al*., 2018) , Song *et al* showed that significant increases of IL-17 in CHB patients compared with healthy control group (*P.value* <0.05) (Song *et al*., 2016) , Cia and his colleagues who demonstrate that IL-17 level in HbsAg clearance group strikingly increased compared with CHB (*P.value* <0.05) (Cia *et al*., 2016) and Song *et al* who demonstrate that Compared with the healthy control group IL-17 level in CHB patients was significantly increased (*p.value* <0.05) (Song *et al*., 2016).

The study revealed that no statistical correlation between IL-17 and sex in hepatitis B virus patients and healthy control group (*p.value* 0.328 , 0.075 respectively). This results was supported by Kazemi *et al* who demonstrate no significant difference between sex and IL-17 (Kazemi *et al*., 2010) and Yang *et al* who demonstrate that no statistical difference between sex and IL-17 (Yang *et at*., 2018). Our result also demonstrate that no statistical difference in IL-17 level between who received blood transfusion and didn't receive (*P.value* 0.061) on the contrary , Salih *et al* who demonstrate that they was significant statistically difference between blood transfusion and IL-17(*P.value* 0.012) (Salih *et al*., 2018), may be this difference due to small sample size in our study and etical background of the studies population. Also the study demonstrate that no statistical difference between IL-17 and Jaundice (*P.value* 0.854) and no statistical difference between IL-17 and treatment (*P.value* 0.673).

One way ANOVA test showed insignificant correlation between age group and IL-17 (*P.value* 0.061) on the contrary Kleiner *et al* showed that IL-17 level increasing

trend , correlated with the age (Kleiner *et al*., 2012) may be this difference due to levels of exposure to potential risks environment and sample size.

Study revealed that no statistical correlation between IL-17 levels and age in hepatitis B virus patients group (*P.value* 0.190) and healthy control group (*P.value* 0.076).

5.2.Conclusions

-Plasma level of IL-17 was compared in hepatitis B patients group and healthy subjects group with statistically significant difference (*P.value* 0.00).

-There was no correlation between IL-17 level with age and gender .

-IL-17 can be considered useful as prognostic marker for hepatitis B virus infection .

5.3.Recommendations

1. Regular measurement of IL-17 level in Sudanese hepatitis B patients.
2. Large sample size (chronic carriers , patients on antiviral therapy , patients has not starting antiviral treatment yet , patients with liver cirrhosis and hepatocellular carcinoma) is likely to enhance our understanding of IL-17 roles and biological activities.
3. Taking this work into the next levels the genomic levels and estimating the effects of different mutation and gene polymorphisms using a number of molecular and bioinformatics tools will expanse our knowledge about the role of IL-17 in the pathogenesis of hepatitis B virus infection and so many other diseases.

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APPENDICES

Appendix (1)

Sudan university of sciences and technology

Evaluation of Interleukin 17 among Sudanese Hepatitis B patients

Date / 2019

ID Number:……..

Age:……Years

Gender:

Male ( ) Female ( )

Duration of disease:

Less than six months ( ) More than six months ( )

Treatment:

Yes ( ) No ( )

Blood transfusion:

Yes ( ) No ( )

Jaundice:

Yes ( ) No ( )

Any chronic disease:

Yes ( ) No ( )

Appendix(2)

ELISA Kits

Appendix(3)

Human IL-17A ELISA MAX™ Deluxe Set

Certificate of Analysis

Product Name: Human IL-17A ELISA MAX™ Deluxe Set
Product Cat. No: 433914 (5 plates) / 433915 (10 plates) / 433916 (20 plates)
Lot No: B228340
Expiration Date: 30-NOV-2018

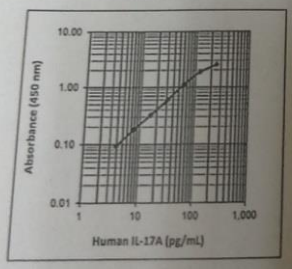
Contents Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
Human IL-17A ELISA MAX™ Capture Antibody (200X)	1 vial	300 µL	79665	B226808
Human IL-17A ELISA MAX™ Detection Antibody (200X)	1 vial	300 µL	79667	B226809
Human IL-17A Standard	2 vials	12 ng	79055	B228118
Avidin-HRP (1,000X)	1 vial	60 µL	79004	B224077
Substrate Solution A	1 bottle	30 mL	78570	B224256
Substrate Solution B	1 bottle	30 mL	78571	B224257
Coating Buffer A (5X)	1 bottle	30 mL	79008	B225257
Assay Diluent A (5X)	1 bottle	60 mL	78888	B227532
Nunc™ MaxiSorp™ ELISA Plates, Uncoated	5 plates	-	423501	-

Storage Conditions

1. Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.
2. Opened or reconstituted components:
   2.1. Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
   2.2 Other components: Store opened reagents between 2°C and 8°C and use within one month.

Note: Precipitation of Assay Diluent A (5X) may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.

Lot #:B22834D



This standard curve is for demonstrative purposes only.
A standard curve must be run with each assay.

This is to certify that the product was manufactured under stringent process controls to ensure lot to lot consistency and complete lot traceability. The product has been tested and meets quality control specifications.

Signature: _____ (Quality Control) Date: 11/17/16

BioLegend is ISO 9001:2008 and ISO 13485:2003 Certified

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ELISA MAX™ Deluxe Set Protocol

Materials to be Provided by the End-User

1. Phosphate-Buffered Saline (PBS): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.
2. Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
3. Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, *e.g.* 2N H_2SO_4.
4. Plate Sealers: BioLegend Cat. No. 423601 is recommended.

Reagent Preparation

Reagents Description	Dilute with	Dilution for 1 plate
Coating Buffer A (5X)	Deionized Water	2.4 mL in 9.6 mL DI H_2O
Capture Antibody (200X)	1X Coating Buffer A	60 µL in 12 mL Buffer
Assay Diluent A (5X)	PBS	12 mL in 48 mL PBS
Detection Antibody (200X)	1X Assay Diluent A	60 µL in 12 mL Buffer
Avidin-HRP (1,000X)	1X Assay Diluent A	12 µL in 12 mL Buffer

Standard reconstitution : Reconstitute the lyophilized Human IL-17A Standard by adding 0.2 mL of 1X Assay Diluent A to make the 60 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

Prepare 1,000 µL of the top standard at 250 pg/mL by adding 4.2 µL of reconstituted standard stock solution to 995.8 µL 1X Assay Diluent A Perform six two-fold serial dilutions of the 250 pg/mL top standard with 1X Assay Diluent A in separate tubes. 1X Assay Diluent A serves as the zero standard (0

Samples: For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. Serum or plasma samples should be tested initially without any dilution. If dilution is required, samples should be diluted in 1X Assay Diluent A before adding to the wells.

TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

ELISA Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, seal the plate and incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times*, block the plate by adding 200 µL 1X Assay Diluent A to each well, seal the plate and incubate at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly.
2. Wash plate 4 times*, add 100 µL diluted standards and samples to the appropriate wells.
3. Seal the plate and incubate at room temperature for 2 hours with shaking.
4. Wash plate 4 times* , add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
5. Wash plate 4 times*, add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
6. Wash plate 5 times*, soaking for 30 seconds to 1 minute per wash. Add 100 µL of freshly mixed TMB Substrate Solution to each well and incubate in the dark for 30 minutes.
7. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

***Plate Washing:** Wash step is crucial to assay precision. Wash the plate with at least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper.

For more detailed set information, please refer to the online manual at:
www.biolegend.com/media_assets/pro_detail/datasheets/433914.pdf

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Part No. 79671_002

Appendix(4)

ELISA Washer

Appendix(5)

ELISA micro plate

Appendix(6)

ELISA Reader