Effect of Antiretroviral Therapy on Immunological Response to Hepatitis B Vaccine in HIV infected Children, Addis Ababa, Ethiopia

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Abbreviations and Acronyms

AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunno Deficiency Syndrome
ALERT	All African Leprosy Rehabilitation and Training center
ART	Antiretroviral Therapy
BCR	B cell Receptor
cccDNA	covalently closed circular DNA
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FACS	Fluorescence-activated Cell Sorting
HAART	Highly Active Antiretroviral Therapy
HBsAb	Hepatitis B surface Antibody
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B virus
HCC	Hepato Cellular Carcinoma
HIV	Human Immunno Deficiency Virus
Ig	Immunoglobulin
ITB	Immature Transitional B cells
MUAC	Mid Upper Arm Circumference
PBMC	Peripheral Blood Mononuclear Cell
pg DNA	pregenomic RNA
rc DNA	relaxed circular DNA
RNA	Ribo Nuclic Acid

sg RNA	subgenomic RNA
SIV	Simian Immunodeficiency Virus
UNICEF	United Nations Children's Fund
WHO	World Health Organization

List of Tables

List of Figures

Abstract

Introduction: Hepatitis B virus (HBV) infection causes worldwide problem and the complication varies from acute liver failure to hepatocellular carcinoma. A vaccine is available to prevent HBV infection, however reduced vaccine response were reported among HIV infected individuals. The information on effect of ART on childhood response to HBV vaccine is fragmented and there is no different vaccination schedules for HIV positive children.

Objective: To assess the impact of HIV-1 infection and effect of antiretroviral drug therapy on the immune response to Hepatitis B vaccine and phenotypic property of B cells in children in Addis Ababa, Ethiopia

Methods: Longitudinal Cohort study was conducted from January, 2015 to June, 2015. Children, 5-9 years of age, from the Pediatric department of ALERT and Zewuditu Memorial Hospital, in Addis Ababa, Ethiopia were screened for HBV infection and standard hepatitis B vaccine was given to those children with no HBV infection at 0, 4 and 8 weeks. The vaccine response was evaluated by measuring Hepatitis B surface antibody(HBsAb) by ELISA and B cells phenotypic characterization using flowcytometry before and after vaccination.

Results: All healthy controls and 94 % of HIV positive children responded to hepatitis B vaccine. Plasma level of hepatitis B surface antibody was significantly reduced in HIV-1 positive children than healthy controls ($p= 0.0001$). Higher vaccine response was observed in early ART treated children ($r=-0.3497, p=0.0046$). The frequency of Resting Memory B cells showed no increment in HIV positive children ($p>0.05$) while significantly increased in healthy controls ($p<0.0001$), and the frequency of Activated Memory B cells decreased in HIV positive children($p<0.001$) and increased in healthy controls ($p<0.05$) after vaccination.

Conclusion and Recommendation: Our results showed reduced plasma antibody level and low frequency of RM B cells in HIV positive children. Therefore, regular monitoring of plasma antibody level to HBV vaccine should be integrated for HIV infected children and booster dose of HBV vaccine would be planned, irrespective of ART status.

Key Words: HBV, antibody response, HBsAb, B cells, ART

Contents

page

1. Introduction

1.1. Back ground

Hepatitis B Virus (HBV) infection is a global health problem (1). Ethiopia is one of the high hepatitis B prevalence countries: the overall prevalence of HBV in Ethiopia varies from 4.7-16.8% for Hepatitis B surface antigen (HBsAg) and 70-76.38% for at least one marker positive (2).

Hepatitis B virus, a hepadnavirus, is partially double stranded DNA virus, circular DNA genome enveloped with an icosahedral capsid replicating via reverse transcription (3-8). The virus is predominantly characterized by a liver cell (hepatocyte) tropism, but to a lesser extent the virus can replicate in the kidneys, pancreas, bone marrow, and spleen (6, 9).

Up on infection enhanced viral replication leading to a huge immune response that may lead to massive liver injury resulting in fulminate hepatic failure spontaneously. The severity of disease is mainly related to various host factors (age, gender, duration of infection, immune response) and viral factors (10, 11).

Hepatitis B virus infection is a vaccine preventable disease where more than 95% healthy individuals respond to hepatitis B vaccine after 3 standard doses (12).

Co-infection with HIV is associated with a 15-17 fold increase in mortality when compared with mono-infection with HBV (13, 14). Therefore, HBV vaccination among HIV infected individuals plays an important role as a primary protection of these individuals from HBV infection (15).

Although studies dealt on HIV positive individuals indicates that the immune response to hepatitis B virus vaccine may reduce 17.5%up to 60% (12, 13, 15, 16), there has not been any study done on the antibody response to HBV vaccine among HIV positive individuals in Ethiopia. Moreover, there is no special attention given to HIV positive children regarding to the dose as well as schedule of HBV vaccine. This study, therefore, evaluated the antibody response to Hepatitis B vaccine and characterized the B cell sub populations in HIV positive children in Addis Ababa, Ethiopia.

1.2. Statement of the Problem

Hepatitis B infection is the 10th leading cause of death worldwide. Currently, ten times the number of people with HIV, across the globe have chronic HBV infection. Approximately 15-40% of patients with chronic HBV will develop cirrhosis, end-stage liver failure or hepatocellular carcinoma (HCC) in their lifetime (17). Children are the most vulnerable group in acquiring chronic hepatitis B infection: 80–90% of infants and 30–50% of children less than 6 years develop chronic infections (18). HBV sero-prevalence among HIV+ patients in many African countries including Ethiopia approaches 50% where the prevalence of chronic active hepatitis B is 5-10% (19).

Hepatitis B virus present in very high numbers in the blood of infected individuals, up to one billion variant per milliliter of blood, in addition, the virus can also stay infectious outside the human body for at least seven days (20), which makes hepatitis B virus more than 100 times transmissible than HIV (21).

Routes of transmissions for Hepatitis B and HIV infections are almost the same which leads to an increased rate of co-infection (22). HIV infection diminishes the rate of HBV clearance, compromise prognosis of HBV and increases the chance of re-infection (14, 23).

Vaccines administered by injection like HBV, results in the enhancement of antigen processing by dendritic cells and presentation to T and B cells, which in turn initiate cascades of cellular and humoral immune responses (24). Long-term protection against clinically significant advance HBV infection and chronic carriage depends on persistence of immunological memory (25).

Despite successful antiretroviral therapy (ART), impaired responses to childhood vaccinations have been reported in HIV-1 infected children (26, 27); moreover, some studies reported that duration of sero-protection of vaccines is short in HIV infected individuals (28). In 2005, WHO and UNICEF launched a global plan to further reduce measles mortality in years 2006-2010. Despite of these joint efforts, an increased number of large and deadly outbreaks of measles on the African continent were reported, with the most severe outbreaks in Chad, Nigeria and Zimbabwe, countries with high HIV-1 prevalence (29).

Although HBV infection is a potential risk in children with HIV, the effect of HIV 1 infection on hepatitis B vaccine response is not fully addressed. In addition, the role of ART in improving immune responses in HIV positive children to HBV vaccine has not been assessed. The present study, therefore, fills these gaps.

1.3. Significance of the Study

The efficacy of the current HBV vaccine and mechanism(s) of possible abnormal responses to HBV vaccines among HIV-1 infected children, are not well established. Moreover, the effect of antiretroviral drugs on the immune response of these children is not known and no special immunization dose and schedules have been provided for these patients so far. We, therefore, studied the impact of HIV-1 and the effect of ART to the HBV vaccine and B cells to elucidate the mechanism of the presumed defect in response to the HBV vaccine in children. This study provides a framework for improved HBV vaccination in schedule or dose in HIV-1 infected children and generates new knowledge useful in the field of childhood vaccines and ART in children: one of the major public health issues. Besides, the information generated from the study may lay a ground for further related studies.

2. Literature Review

2.1.Hepatitis B virus

2.1.1. Epidemiology of Hepatitis B virus Infection

Globally, an estimated two billion people (one-third of the global population) have been infected with HBV at some point in their life; of these, more than 350 million suffer from chronic HBV infection, resulting, over 730,000 mortality annually (600,000 deaths mainly from cirrhosis or liver cancer and 130 000 deaths due to acute liver infection) (20, 30, 31).

The prevalence of HBV infection varies markedly throughout regions of the world. Hepatitis B is highly endemic in developing regions (20) with large population such as South East Asia, China, sub-Saharan Africa and the Amazon Basin where 70–95% of the population shows past or present serological evidence of HBV infection, In these areas, at least 8% of the population are HBV chronic carrier (30, 32).

Chronic HBV infection divides the world into three areas based on the prevalence (Fig 1): high (>8%), intermediate (2-8%), and low (<2%); sub-Saharan Africa falls under the high prevalence category (4). In 1994, community-based seroepidemiological survey of hepatitis B virus was conducted in Addis Ababa, HBsAg prevalence was 7% (11). Among pregnant women in Jimma (33), prevalence of HBsAg was 3.7%. The overall prevalence of HBsAg among chronic liver disease patients was 35.8% in Addis Ababa, Ethiopia (34).

Overall HBV seroprevalence (any marker), increases steadily with age (35) to over 70% in 40–49 year olds, indicating significant childhood and adult transmission (30). Estimated instantaneous incidence was 3–4/100 susceptible/year, higher in 0–4 year olds, and peaking in early childhood and young adults (11). The age at which 50% had evidence of infection was around 20 years (11, 34).

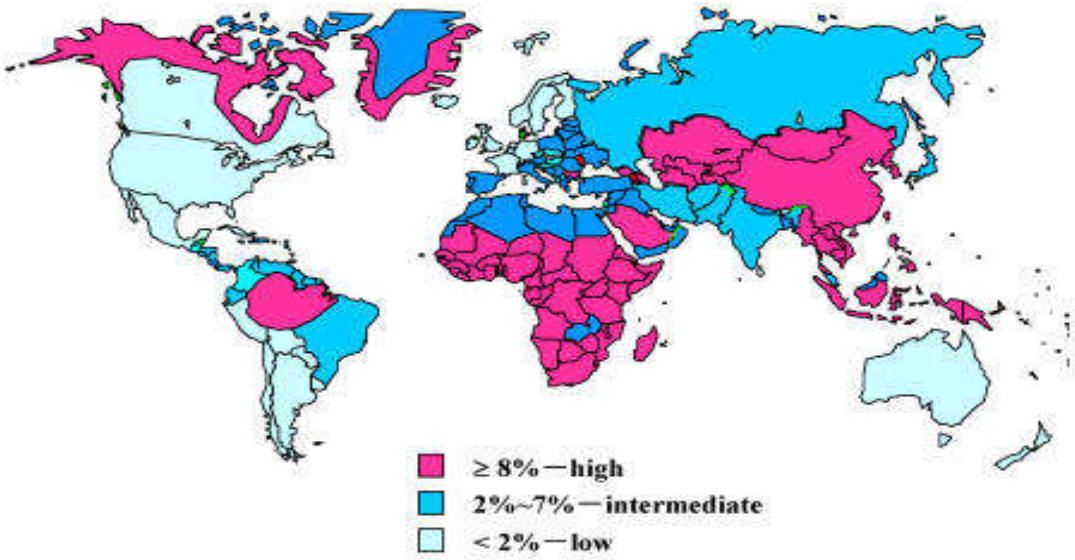

Figure 1: Chronic hepatitis B prevalence: categorizes countries in to three sub groups

source: Epidemiology and Prevention of Hepatitis B Virus Infection Int J Med Sci. 2005; 2(1): 50–57)

2.1.2. Mode of transmission

HBV can be transmitted through different ways, different scholars agreed that perinatal, sexual and parenteral modes of transmission are the three important way of transmission (36, 37). Adenkale *et al* and khan *et al* reported that it is not confirmed as HBV can be transmitted through air and it is not assured as feces cannot be one medium of HBV transmission. Contaminated food and water, vectors and insects are not means for HBV transmission (32, 37-39). Blood is an important body fluid in HBV transmission, in fact semen and saliva are also well involved in HBV transmission (32).

2.1.3. Molecular Virology

HBV infection was first identified in 1965 when Blumberg and co-workers discovered hepatitis B surface antigen (HBsAg), originally termed as *Australia antigen(10).* HBV is a small, enveloped, hepatotropic virus (6, 10) which is approximately 42 nM in diameter and comprised of ~3.2 kb partially double-stranded relaxed circular DNA (rcDNA) genome with in a nucleocapsid (core) that is surrounded by a lipid bilayer studded with complexes of viral glycoproteins. The

nucleocapsid is a 27 nm diameter icosahedron assembled from 240 viral capsid proteins and packages a single copy of viral genomic DNA and DNA polymerase that is covalently linked to the 5' end of minus strand DNA (6, 40).

2.1.4. Life Cycle

HBV enters the hepatocyte through sodium taurocholate co-transporting polypeptide (NTCP) receptor, gets uncoated in the cytoplasm, and the DNA is transported to the nucleus (41). Until few years back, the precise mechanism of viral entry had not been elucidated. Both, endocytosis and direct fusion of the viral envelope with the plasma membrane had been proposed as potential pathways (42). Recently, it was known that liver bile acids transporter, NTCP, is responsible for the majority of sodium-dependent bile salts uptake by hepatocytes. sodium taurocholate co-transporting polypeptide (NTCP) also functions as a cellular receptor for viral entry of hepatitis B virus (HBV) through a specific interaction between NTCP and the pre-S1 domain of HBV large envelope protein (43, 44).

After uncoating/release into the cytoplasm and transport of the nucleocapsid to the nucleus, the partially double-stranded viral relaxed circular DNA (rcDNA) is repaired by both viral and cellular enzymes to form covalently closed circular DNA (cccDNA) template (45). The cccDNA is transcribed into subgenomic RNA (sgRNA) and pregenomic RNA (pgRNA) (46). The pgRNA is encapsidated together with the P protein. Inside the nucleocapsid, the pgRNA is reverse transcribed into negative-strand DNA using viral reverse transcriptase. Relaxed circular DNA is generated by plus-strand synthesis from the negative-strand DNA (47). The nucleocapsids are either re-imported to the nucleus for cccDNA amplification or enveloped and released via the endoplasmic reticulum (ER) (48). This leads to huge viral replication that leads to a vigorous and extensive immune response which may lead to massive liver injury resulting in fulminate hepatic failure spontaneously, and consequently to liver cirrhosis and hepatocellular carcinoma (Fig 2) (8, 9).

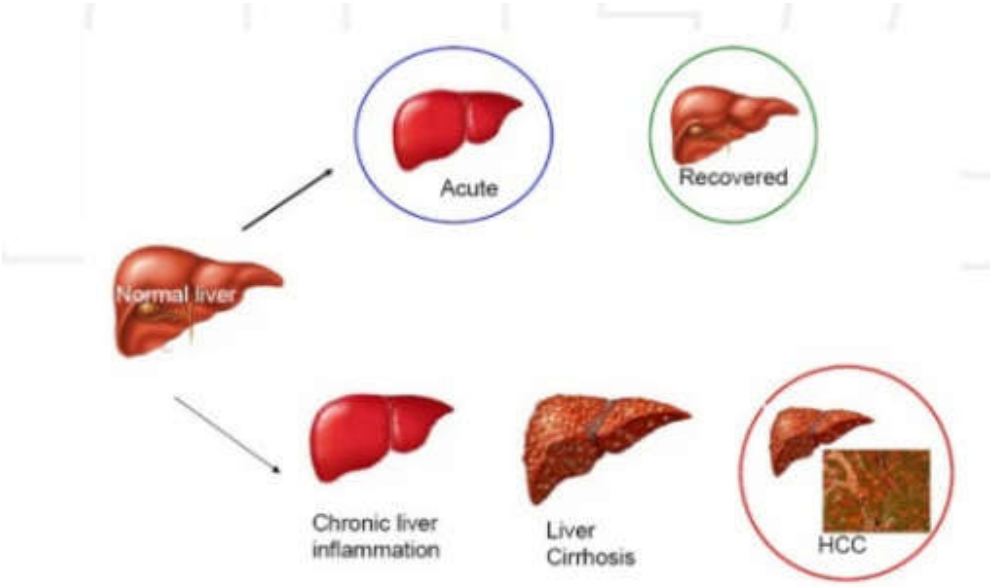

Figure 2: Hepatitis B induced liver damage,

source: Hepatitis B Virus Genetic Diversity: Disease Pathogenesis: INTECH Open Access Publisher; 2013.

2.1.5. Immunization

Hepatitis B is a vaccine preventable infection (1, 49), the vaccine against hepatitis B has been available since 1982 (1). After full dose vaccination, 95% infants, children and young adults develop protective antibody which protect from future hepatitis B infection and consequent chronic liver disease and liver cancer. Protection lasts for at least 20 years (50, 51) and is probably lifelong (1). In Ethiopia, Hepatitis B vaccination is given as a pentavalent on the 6^{th}, 10^{th}, and 14^{th} week after birth since 2007 (52).

2.2. Hepatitis B and Human Immunodeficiency Virus Co-infection

Co-infection with human immunodeficiency virus-1 (HIV) and hepatitis B virus (HBV) is common (53) worldwide, where an estimated 10% of HIV-infected persons have chronic hepatitis B(14). In SanFrancisco, 6.3% of HIV infected individuals were HBsAg positive (54). A cohort study at seven military medical centers in USA showed that 14% of HIV infected individuals had chronic HBV infection (55). In Brazil the prevalence of HBV exposure (anti-HBc

+ve) and HBsAg positivity reaches 40.0% up to 71% and 3.7% up to 24 %, respectively (23). The prevalence of HIV/HBV co-infection was 7.7% among Nigerian HIV infected children (56). HBsAg prevalence among HIV positive individuals ranges from 5% up to 17% in south Africa (57). In sub Saharan Africa, as many as 20% of the population are co-infected with the two viruses (22). According to a study conducted in Rwanda (7%), HIV infected children had active HBV infection (58). Another study in Uganda showed that 16.9% of HIV positive individuals are HBsAg positive (59). A study conducted in north Shewa, Ethiopia, showed that the cumulative prevalence of HBV in HIV-infected adults was 3.9% (60); however, there are no published data about HIV-HBV co- infection on children in Ethiopia.

2.2.1. The impact of HIV on Hepatitis B vaccine response

Since the start of the AIDS epidemics, around 78 million [71 million–87 million] people have become infected with HIV and 39 million [35 million–43 million] people died of AIDS-related illnesses. In 2013, there were 35 million [33.2 million–37.2 million] people living with HIV globally, and among this, 2.1 million [1.9 million–2.4 million] people were newly infected (61).

Globally, 2.9 million (2.6-3.2 million) children were living with HIV, out of whom, 240 000 [210 000–280 000] children newly infected with HIV in 2013. In the same year, 1.5 million [1.4 million–1.7 million] people died from AIDS-related causes worldwide (62).

As of June 2014, 13.6 million people living with HIV have access to antiretroviral therapy: However, 38% [36%-40%] of all adults living with HIV are receiving treatment, and only 24% [22%-26%] of all children living with HIV are receiving ART (61).

Despite all efforts in the control of HIV including the availability of antiretroviral therapy, every 15 seconds a new person is infected with HIV and every 20 second another person dies as a result of AIDS or associated opportunistic infection in 2013 (61, 62).

Studies showed that the serological response to hepatitis B vaccine is lower in HIV-infected children and adults than in uninfected individuals (16). However there is no sufficient information describing the impact of HIV/AIDS on vaccine responses, in particular to Hepatitis B vaccine. In addition, the optimal strategies for the prevention and treatment of

chronic HBV infection (CHBV) in those with HIV are not well defined (55). A Study done in Iran showed that the response to hepatitis B vaccine may fall up to 52.7% in HIV infected individuals (12). The non-responsiveness to Hepatitis B vaccine was around 35. 8%,and 56%-65% in HIV infected adults in Kenya and USA, respectively (63, 64). Among the few studies in children, the study in Rwanda and Tanzania showed that the protective ability of Hepatitis B vaccine in HIV positive children is, 71% and 59.5%, respectively (58, 65). In an observational cohort study in HIV-infected individuals conducted by Landrum et al HBV vaccination was not associated with reduced risk of HBV infection (66).

Although the influence of active antiretroviral therapy (ART) upon hepatitis B virus (HBV) vaccine responses is not well studied, concurrent use of ART with HBV immunization was associated with increased probability of responding to the vaccine. In fact, the response rate was reported to be low in a study conducted in USA, 35% of HIV positive and 49% of these who were on ART responded for hepatitis B vaccine after last vaccination (67). In a study conducted by Patricia *et al*, higher baseline CD4+ T cell counts was an independently associated variable with improved vaccine response; however, use as well as type of ARV drugs was not significantly associated to improved vaccine response (68).

The reduced vaccine response is mostly related with, numerous perturbations of B-cell phenotypes and function by HIV (69, 70). Peripheral blood B cells of HIV-infected individuals composed of abnormal B cells (71), namely activated and exhausted memory subsets, which are largely absent in the blood of uninfected individuals (72). Besides the polyclonal B cell activation and hypergammaglobulinemia, B cells from HIV patients show impaired reactivity to immunization and in vitro activation signals. In addition, B lymphocytes from HIV-infected subjects are primed for apoptosis (70). A study done by Amu *et a*l showed an increased frequencies of activated and tissue-like memory B cells occurring during HIV-1 infection which are corrected by prolonged ART therapy; however, the reduced number of resting memory B cells in both young and aged HIV-1 infected patients could not be reversed by prolonged treatment (73). Another study conducted on Malawian adults revealed that the ART treated (for a median of 33 months) adults had similar number of B cells except memory B cells that remained significantly lower than the healthy controls, whereas, both naive B cells and memory B cells were reduced in HIV positive ART naive participants (74).

In Ethiopia, so far, no study has been done on immune response to Hepatitis B vaccination in HIV infected children. Therefore, in this study, we assessed the responses of HIV infected children to HBV vaccination.

3. Objective

3.1. General Objective

To investigate the effect of antiretroviral therapy on the immune response to Hepatitis B vaccine in HIV infection.

3.2. Specific Objective

- To assess the effect of HIV-1 infection on Hepatitis B specific antibody response.
- To evaluate the effect of Antiretroviral therapy on HBV vaccine response.
- To evaluate the effect of HIV-1 infection on phenotypic properties of B cells in HBV vaccinated children.
- To evaluate the effect of antiretroviral drug therapy on phenotypic properties of B cells.

4. Materials and Methods

4.1. Study Area and Period

The study was conducted at Zewuditu Memorial Hospital and Pediatric department of ALERT hospital, in Addis Ababa, Ethiopia. Zewditu Hospital is one of the largest HIV clinic in Ethiopia, where more than 1630 children are registered and 1104 are receiving ART service in the hospital. ALERT hospital is the nearest hospital for sample recruitment to AHRI, at which a total of 1531 children are registered out of whom 1014 are on ART. The study period was from January to June, 2015.

4.2. Study Design

Case reference longitudinal cohort study design was conducted

4.3. Populations

4.3.1. Source population

Children aged between 5 and 9 years old, and all HIV infected children who were seeking HIV/AIDS care and ART service in Addis Ababa, Ethiopia at ALERT and Zewuditu hospitals.

4.3.2. Study population

HIV infected children aged between 5 and 9 years old who came to ART clinics of the selected hospitals during the study period were recruited for the study. The HIV infected children were subdivided into two groups: treatment naïve and those receiving ART. Healthy controls were enrolled by age and sex- matching.

4.3.3. Sample size

A total of 170 children (94 HIV negative/ controls and 76 HIV positive children) were recruited for the study. One HIV positive child was HBsAg positive, who was then referred to ALERT pediatric department for follow up and three HIV negative children were HBcAb positive indicating previous HBV infection; therefore, these subjects were excluded from vaccination.

4.3.4. Inclusion and exclusion criteria

Inclusion criteria

- Children aged 5 to 9 years.
- Both HIV infected and uninfected
- Children without active hepatitis B infection

Exclusion criterion

- Children on oral steroid or immunosuppressive drugs
- Lack of consent to participate in the study by parent or legal guardian.
- Children with confirmed laboratory evidence of previous HBV infection
- Children with acute febrile illness

4.4. Sampling technique

Purposive sampling for health facilities and convenient sampling techniques for study cohorts were used. Consecutive subjects meeting inclusion and exclusion criteria were recruited at participating health facilities.

4.5. Study variables

Dependent Variables

- ✓ Hepatitis B vaccine response
- ✓ B cell and B cell sub populations

Independent Variables

- ✓ Sex
- ✓ ART status
- ✓ ART initiation period
- ✓ CD4 count
- ✓ Mid-Upper Arm Circumference (MUAC).

4.6. Hepatitis B vaccine (rDNA)

The vaccine (rDNA, serum institute of India, India) is a non-infectious recombinant DNA Hepatitis B vaccine. It contains purified surface antigen of the virus obtained by culturing genetically-engineered *Hansenula polymorpha* yeast cells having the surface antigen gene of the Hepatitis B virus. The Hepatitis B surface antigen (HBsAg) expressed in the cells of *Hansenula polymorpha* is purified through several chemical steps and formulated as a suspension of the antigen adsorbed on Aluminum hydroxide and thiomersal is added as preservative. The vaccine contains the same HBV component as that available in pentavalent form with DPT (Diphtheria, Tetanus and Pertussis) and Hib (Haemophilus influenza type B) vaccines for national immunization program of infant in the country which is normally administered at 6, 10 and 14 weeks of age.

The rDNA vaccine was well tolerated, there was no any severe adverse event reported by the guardians and children. However, most of the study participants had major complaint of pain at injection site for a day. Only one healthy control had spontaneous vomiting after each shot but it was not serious.

4.7. Data and Sample Collection Procedures

4.7.1. Questionnaire:

A structured questionnaire was used to collect socio-demographic, anthropometric and clinical information by trained nurses (Annex X-XIII).

4.7.2. Sample Collection:

From eligible participants, 3 to 5 ml of blood was collected aseptically by experienced nurses and/or laboratory technologists/phlebotomists using EDTA anti-coagulated tubes. Recruitment for HIV positive children was timed with their regular visits in the health institutes for monitoring of their CD4 count and to collect antiretroviral therapy; written consent was obtained from parents and guardians, base line blood collected from children. Then, active and previous HBV infection was screened using Hepatitis B surface antigen rapid test kit and ELISA, respectively. The first dose of HBV vaccine (rDNA, serum institute of India, India) was given to HBsAg and

HBcAb negative children, parents were advised to bring their children for the 2nd and 3rd doses as well as follow up visits, and appointment card with date were provided. For HIV negative children, consent was obtained from parents or guardians, blood was collected for HBV serology, and HBsAg and HBcAb negative children scheduled for vaccination and blood sample collection. Parents or guardians were advised to bring their children to the health institution for vaccination and follow-up sample collection.

4.8. Laboratory analysis

4.8.1. Screening for HBV and HIV

All children were screened for hepatitis B active infection prior to vaccination using rapid Hepatitis B surface antigen test kit (Helagen, One Step Rapid Test, China), which determines whether a person currently has active infection. HIV testing for HIV-1 negative children subjects was performed with standard national procedures: Rapid test for HIV (1/2) antibody from whole blood was performed by serial testing algorithm **(75)**. The first screening test was performed using KHB (Shanghai kehua Bioengineering CO-Ltd, Shanghai, China) and if positive a second test was performed using STAT-PAK™ (Chembio HIV1/2, Medford, New York, USA), if positive the patient was considered positive for HIV but if negative, the third test which served as tie breaker was carried out using Uni-Gold TM (Trinity Biotech PLC, Bray, Ireland) **(76)**.

4.8.2. HBsAb Enzyme Linked Immuno Sorbent Assay(ELISA)

The hepatitis B surface antibody (HBsAb) was measured in plasma samples using HBsAb ELISA kit (Monilisa Anti-HBs PLUS, BIO-RAD, France). Briefly, plasma samples from patients and healthy controls were diluted in 3:4 ratio using specimen diluent and added to a plate with micro-wells coated with HBV antigen and incubated for 1hr at 37^{o}C. Plates/micro-wells were washed with 1x wash buffer provided with the kit and 100 µl conjugate working solution was added and incubated for 60±5 minute at 37^{o}C. Micro-wells were washed and 100 µl substrate solution was added to each micro-well and incubated for 30 ± 5 minute in the dark at room temperature. Finally, the reaction was stopped by adding 100 µl 1M H_2SO_4. The intensity of the color (optical density) was read at 450/620 and 405/605 nm using ELISA reader. The OD values were

converted to concentrations using Microplate manager version 6 (BIO-RAD, California, USA) and analyzed using graph pad prism version 6 (La Jolla, CA, USA).

4.8.3. HBcAb Enzyme Linked Immuno Sorbent Assay (ELISA)

Hepatitis B core antibody (HBcAb) was measured in plasma samples of HBsAb positive samples at base line using (Hepanostika anti-HBc Uni-Form, BIOMERIEUX, France). Plasma samples and assay controls were added to a wells of microelisa strip coated with HBcAb, 50µl conjugate was added, and washed with phosphate buffer after incubation for 90±5 minute at 37 ^{o}C, then TMB substrate was added and the reaction was stopped using 2N H_2SO_4 after 30 ± 2 minute incubation at room temperature in dark. Finally, the optical density was read at 450/620 nm and 405/605 nm. The antibody concentration were calculated using (Microplate manager version 6(BIO-RAD, California, USA) and data were analyzed using graph pad prism version 6 (La Jolla, CA, USA).

4.8.4. Flow Cytometry

Phenotypic characterization of B cell population was performed before and 1 month after vaccination using 8-color flow cytometry, (FACS canto- II BD, Biosciences; USA). Briefly, 100 µl of whole blood transferred to FACS tube, and lysed using 1X FACS lysing solution (BD Bioscience, Belgium) by incubating for 20 minute in the dark at room temperature. The lysed whole blood was washed using FACS buffer (0.1% BSA/FCS. 1mM EDTA in PBS). Appropriate volume of fluorochrome-conjugated monoclonal antibodies PerCp Cy5.5 anti- CD19(HIB 19), FITC anti-CD 10 (HI10), BV421 anti-CD21(B-ly4), APC anti-CD27(M-T271), PE-cy 7 anti- CD 95 (DX2), PE anti- CD2 (MIH 4) (all from BD bioscience) and infra-red APC-cy 7 live dead cell stain (Molecular Probes, Eugene, OR, USA) were added to each FACS tube. Following incubation for 30 min at +4 ^{o}C in the dark, cells were washed with FACS buffer. After decantation and resuspension, cells were fixed with 200 µl of 2% paraformaldehyde and stored at 2-8 ^{o}C until analyzed. Matched isotype control antibodies were included for gating strategy and compensation controls were run in each experiment to adjust spectral overlap.

4.8.5. Viral Load Assay

Viral load test was done within 2 months of sampling using an automated m2000 Abbot real time HIV-1 assay system following manufacturers protocol (Abbot laboratories, Abbot Park, Illions). Briefly, internal control RNA was added to 200 µl of plasma and loaded on to the m200sp instrument for RNA extraction. The limits of detection ranged between 40 and 10,000,000 HIV - 1 RNA copies. Undetectable VL was reported as 39 copies while; 10,000,000 copies served as the reportable upper limit.

4.9. Quality control

Standard operational procedures and manufacturer instructions were strictly followed throughout the procedures. Translation of the subject questionnaire to Amharic and back translation to English was done to assure consistency. Training for data collectors were done to maintain the quality of the data collection process. Data were checked daily after sample collection and lab procedures and needed amendments were done promptly. Data cleaning and cross validation was done prior to subsequent analysis. All reagents for each experiment were stored in appropriate storage conditions as recommended by the manufacturer.

In flow cytometry, leucogate (FITC-anti-CD45/PE- anti-CD14), isotype controls and unstained control cells were used during machine set-up to identify optimal gates: Compensation beads for each utilized flurochrome were run to define adequate compensation to avoid spectral overlap.

For quality control of ELISA, we perform negative controls, and attentively observed the expected chromogen induced reaction. Freshly reconstituted and prepared chromogen reagents were used.

4.10. Data analysis and interpretation

Demographical data were entered and cleaned using Microsoft Office Excel. Flow cytometry B cell data acquisition was done with FACS Canto II Diva software, exported to FlowJo V9.8 (Tree Star Inc., Ashland, Oregon, USA). Antibody concentration was calculated using micro plate manager version 6 (BIO-RAD, California, USA). Finally statistical analysis was computed using

SPSS version 19 (IBM Corp, Armonk, NY, USA) and Graph pad prism6 (La Jolla, CA, USA). The immunological data were quoted as median plus the interquartile range and non-parametric tests were used to compare different groups. Paired (Wilcoxon matched-pairs signed rank test) and unpaired t-tests (Mann Whitney test) were used to assess differences in the group. Spearman correlation was also used to demonstrate the relationship between two continuous variables. P value < 0.05 was taken as statistically significant.

4.11. Ethical consideration

The project proposal was reviewed and approved by the ethical review committees of AHRI/ALERT, College of Health Science Addis Ababa University, Addis Ababa Health Bureau Ethics Board and National Ethics Review Committee. Permission was obtained from respective hospitals and institutes before conducting the research. Written informed consent was obtained from parents/guardians of study participants following a clear explanation of study purpose, benefit and possible discomfort. The privacy was maintained and information obtained throughout the study was kept confidential.

4.12. Plan for dissemination of study findings

The finding of this study will be submitted and presented at Addis Ababa University, College of Health Science, School of Medicine, Department of Pharmacology and Armauer Hansen Research Institute. The clinical findings will be notified to study participants, health institutions from which participants are recruited and to the two charity organizations where healthy controls were recruited. Furthermore, the finding will be presented in different seminars, workshops, conferences, and symposiums, and will also be communicated to responsible governmental bodies including ministry of health. Finally, the manuscript will be published in a peer review journals.

4.13. General Outline of the work flow

Children with HBsAg and HBcAb negative were vaccinated and after 2 weeks of vaccination, blood was taken to measure anti-HBs and to characterized B. HIV counseling and screening were done for all healthy control children recruited.

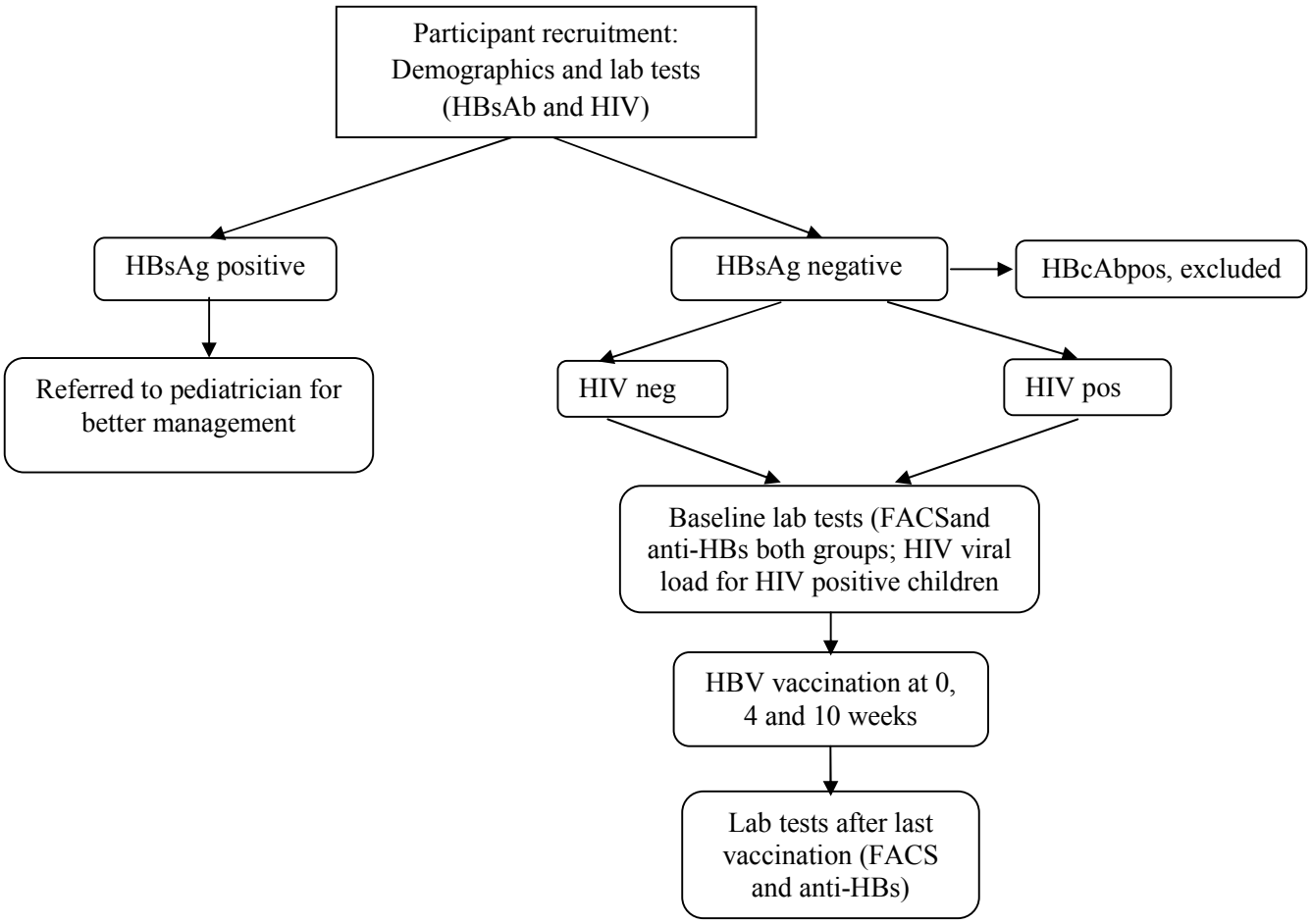

Figure 3: Diagram showing the overall work flow of the study from January-June, 2015

5. Results

5.1. Demographic characteristics of study participants

A total of 170 children: 94 HIV negative/controls and 76 HIV positive were recruited. We lost a total of 8 (4.7%) children, 1 healthy control and 7 from HIV positive during follow-up due to various reasons. One HBsAg positive child and 3 Anti-HBc positive children were excluded from the study, from HIV positive and healthy control, respectively. The study participants were age and sex matched. Children ranged in height from 85cm up to 132 cm and weight from 13 kg up to 28.5kg (Table 1*). The malnutrition status of children was categorized based on WHO: Guideline for Integrated Approach to the National Care of HIV Infected Children (6 months – 14 years), 2008.

Table 1: Demographical characterization of study participants completed follow up

Variables	Health Control	HIV positive	ART	Pre-ART
Age (mean, years)	6.52 (5-8)	6.81 (5-9)	6.87 (5-9)	6.54 (5-9)
Sex				
Female	34 (37.8%)	34 (50.0%)	26 (47.3%)	8 (61.5%)
Male	56 (62.2%)	34 (50.0%)	29 (52.7%)	5 (38.5%)
Height (mean, m)	116.68 (97-132)	113.4 (92-130)	113.25 (93-130)	113.92 (92-130)
Weight (mean, Kg)	19.95 (14.5-28.5)	19.86 (13-28)	19.81 (13-28)	20.06 (14-27)
MUAC				
<13.5 cm	0	5 (7.4%)	5 (9.1%)	0
13.5-14.5 cm	8 (8.9%)	15 (22.1%)	12 (21.8%	3 (23.1%)
>14.5 cm	82 (91.1%)	48 (70.6%)	38 (69.1%)	10 (76.9%)

"*" the table contains study participants who finished the follow up.

5.2. Clinical characterization of HIV infected children

The CD 4 count in 81% of the study participants was found to be in the range between 350 to 1500 cells/micro liter, and 60% of participants were at WHO stage I (Table 2*). Sixty one (81.4%) children were on ART, among this 90% were on first line regimen and 3TC, the safest antiretroviral (77), was the only nucleoside reverse transcriptase drug incorporated in all ART regimens. Among HIV positive children, 14 (18.6%) had consistently higher CD4 counts, no history of opportunistic infection and they were naive for ART (Table 3*).

Table 2: Clinical characteristics of HIV infected children completed follow up.

Variables	HIV pos Freq (%)	ART Freq (%)	Pre-ART Freq (%)
CD4 count (mean range)	812.5 (195-2744)	943 (195-2744)	658 (245-2103)
CD4 count, grp			
< 350 cells/ul	7 (10.3%)	5 (9.1%)	2 (15.4%)
350-750 cells/ul	23 (33.8%)	16 (29.1%)	7 (53.8%)
750-1500 cells/ul	32 (47.1%)	29 (52.7%)	3 (23.1%)
>1500 cells/ul	6 (8.8%)	5 (9.1%)	1 (7.7%)
Viral load			
UVL	33 (48.5%)	31 (56.4%)	2 (15.4%)
39-400	13 (19.1%)	11 (20.0%)	2 (15.4%)
≥400	22 (32.4%)	13 (23.6%)	9 (69.2%)
WHO stage			
I	39 (57.4%)	31 (56.4%)	8 (61.5%)
II	21 (30.9%)	16 (29.1%)	5 (38.5%)
III	5 (7.3%)	5 (9.1%)	0
IV	3 (4.4%)	3 (5.5%)	0

"*" the table contains study participants who finished the follow up

Table 3*: Antiretroviral drug regimens administered to study participants completed follow up

ART regimen	Frequency (%)
First line	
AZT + 3TC + NVP	43(78.2%)
AZT + 3TC + EFV	4(7.3%)
D4T + 3TC + NVP	1(1.8%)
ABC + 3TC + EFV	1(1.8%)
ABC + 3TC + NVP	1(1.8%)
Second line	
AZT + 3TC + LPV/r	4(7.3%)
TDF + 3TC + LPV/r	1(1.8%)
Changed regimen	
D4T + 3TC + NVP	22 (88%)
AZT + 3TC + NVP	2 (8%)
D4T + 3TC + LPV/r	1 (4%)

"*" the table contains study participants who finished the follow up

5.3. Decreased plasma level of Anti-Hepatitis B surface in HIV-1 positive children

At baseline 131 (77.5%) children had levels below 10mIU/ml of plasma HBsAb; among HIV positive children 67 (89.3%) and healthy controls 64 (68.1%) had plasma HBsAb levels below 10 mIU/ml. Eight (10.7%) of HIV positive and 20 (21.3%) of healthy controls had HBsAb levels between 10-100 mIU/ml, and only 10 (10.6%) of healthy controls had above 100 mIU/ml HBsAb. All children with HBsAb level $\geq$ 10mIU/ml were screened for Hepatitis B core antibody (HBcAb) and 3 healthy controls were positive which indicates resolved infection since they were negative for HBsAg.

After vaccination 2 (2.2%) healthy controls were hypo responsive, and 88 (97.8%) responded to hepatitis B vaccine with the median plasma HBsAb level of 4530 mIU/ml (IQR: 684.7-10000). Among HIV positive children 4 (5.9%) HIV positive children were unresponsive, 12 (17.6%) were hypo-response and 52 (76.5%) were responsive to hepatitis B vaccine with a median level of 318.8 mIU/ml (IQR: 224.5-1224).The reduction in HBsAb titers in vaccinated HIV infected children compared with healthy controls was highly significant ($p<0.0001$). The median level of HBsAb in plasma significantly increased after vaccination from nil to 318 mIU/ml (IQR: 224.5-

1224, p<0.001 and nil to 4530 mIU/ml (IQR: 684.7-10000, P<0.0001) in HIV positive and controls groups, respectively (Fig 4). The plasma HBsAb level of ART treated children was not significantly different from ART naive children (Fig 5).

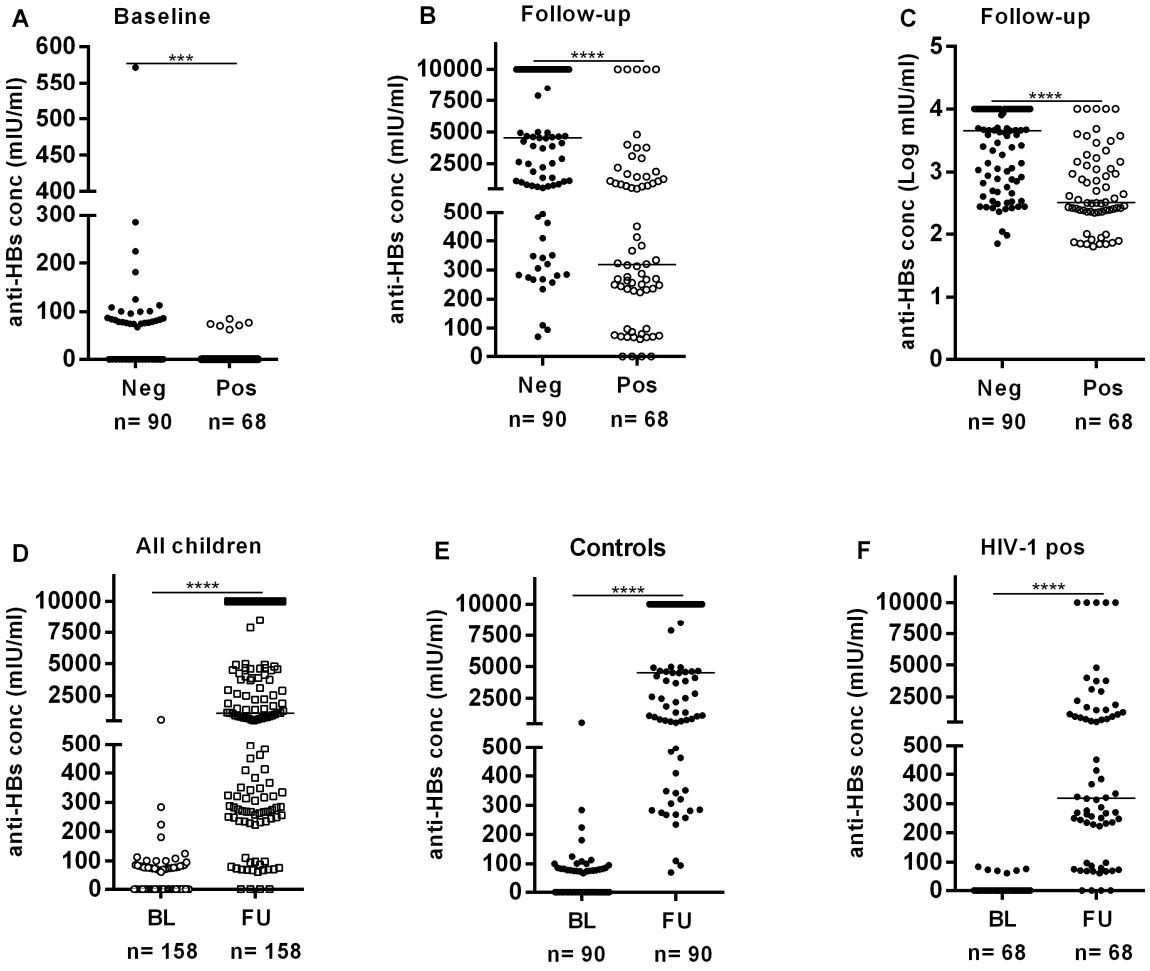

Figure 4: Hepatitis B virus surface antibody level in plasma of HIV positive children and Controls.

before vaccination (A), after vaccination (B and C); Level of anti-HBs before and after vaccination in all study participants (D), Controls (E) and HIV positive (F). Neg: healthy controls, Pos; HIV positive anti-HBS; hepatitis B surface antibody concentration, BL: baseline, and FU: follow up.***p < 0.001; **** p < 0.0001.

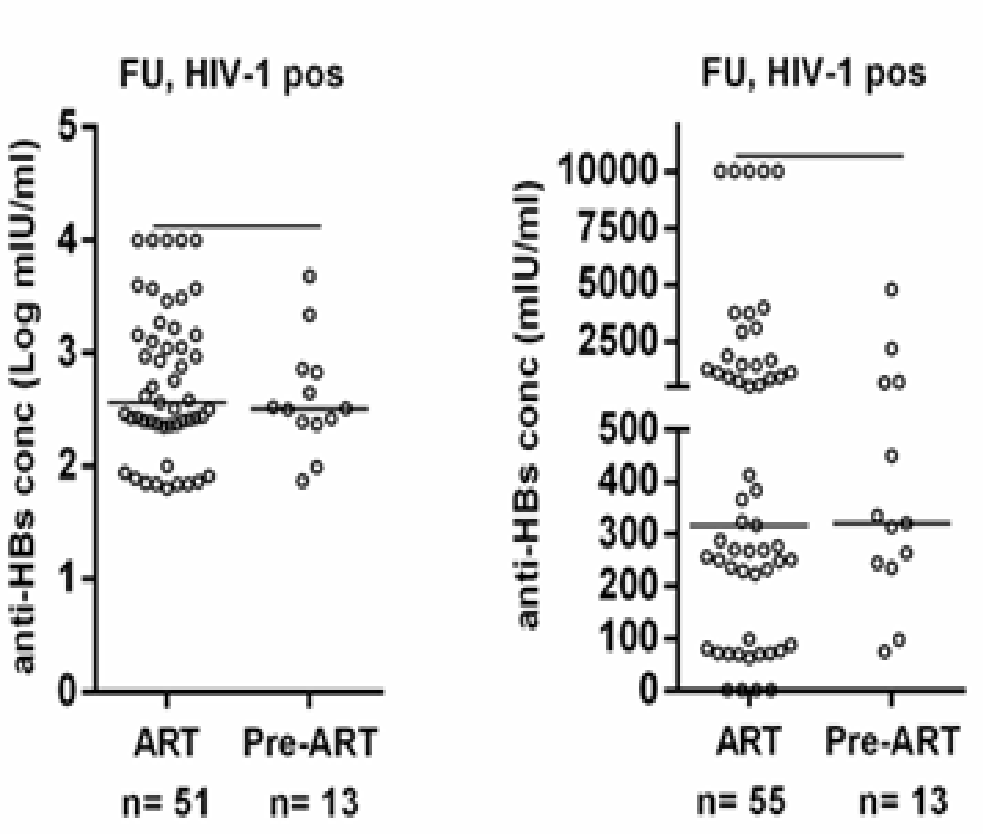

Figure 5: Hepatitis B virus surface antibody level in plasma of HIV positive ART treated and ART naive children

5.4. Booster doses of HBV vaccine increases plasma hepatitis B surface antibody

The plasma level of HBsAb were significantly higher in children receiving booster dose than in children with primary vaccination in both HIV positive ($p<0.01$) and healthy controls ($p<0.0001$); however, there was no significant difference in between revaccinated HIV positive and healthy controls ($p>0.05$) (Fig 6).

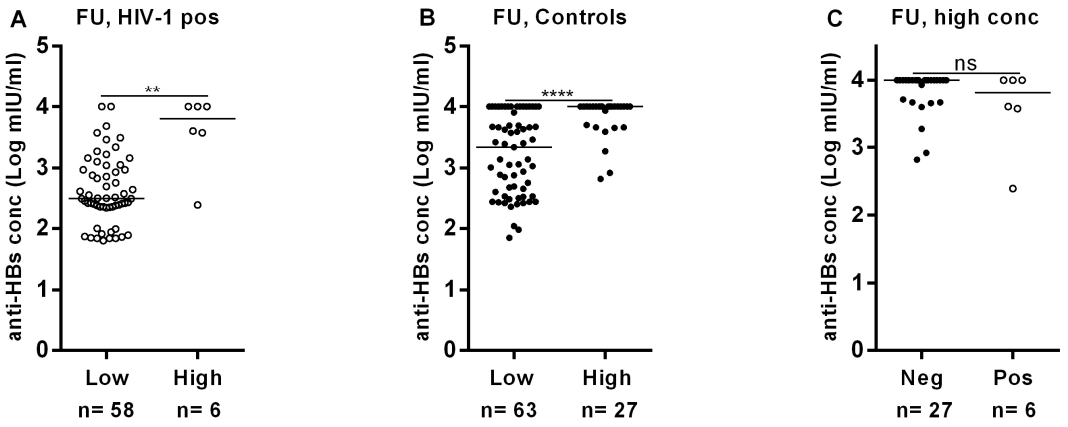

Figure 6: Antibody response in revaccinated children.

Antibody level in primary vaccinated and revaccinated children HIV positive (A), controls (B), and revaccinated HIV positive and controls (C). FU- follow up; **p < 0.01; **** p < 0.0001; ns-not significant difference

5.5. Early ART initiation improves antibody response

HIV infected children received ART for a mean of 43.9 months, and most of them had a good adherence profile. As shown in Fig 7, children who started ART at earlier period had higher plasma HBsAb level than children who initiated late (r=-0.3497,p=0.0046). On the other hand, there was no significant association between plasma HBsAb level and length of treatment (r= 0.1733, p=0.1708).

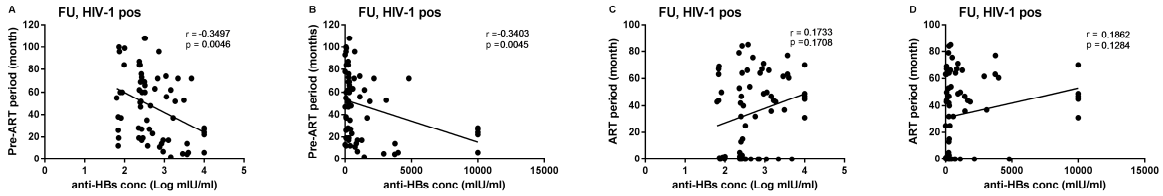

Figure 7: Correlation between ART time and vaccine response after vaccination.

Time before initiation of ART, Pre-ART period (A and B) and time after initiation of ART to sample collection, ART period (C and D).

5.6. HIV reduces the overall frequency of B-cells and alters the physiological distribution of B cell sub populations.

At base line, B cells were defined using anti-CD19 antibody and as shown in Fig 8 panel A lower frequencies were observed in HIV positive children ($p< 0.0001$). The frequency of immature B cells (CD19+CD10+) increased ($p<0.05$) while mature B (CD19+ CD10-) cells were decreased ($p<0.01$).

B cells subpopulations were further delineated (Fig 13) using anti-CD21 and anti-CD27 antibodies among gated CD19+CD10- B cells: Naïve cells were defined as ($CD19^+$ $CD10^-$ $CD21^+$ $CD27^-$), resting memory cells as ($CD19^+$ $CD10^-$ $CD21^+$ $CD27^+$), activated memory cells as ($CD19^+$ $CD10^-$ $CD21^-$ $CD27^+$) and tissue like memory cells as ($CD19^+$ $CD10^-$ $CD21^-$ $CD27^-$). In HIV positive subjects, naïve ($p<0.01$) and resting memory B cells ($p<0.01$) were significantly reduced, whereas activated memory ($p<0.001$) and tissue like memory ($p<0.0001$) B cell subsets significantly increased compared with HIV negative children (Fig 9).

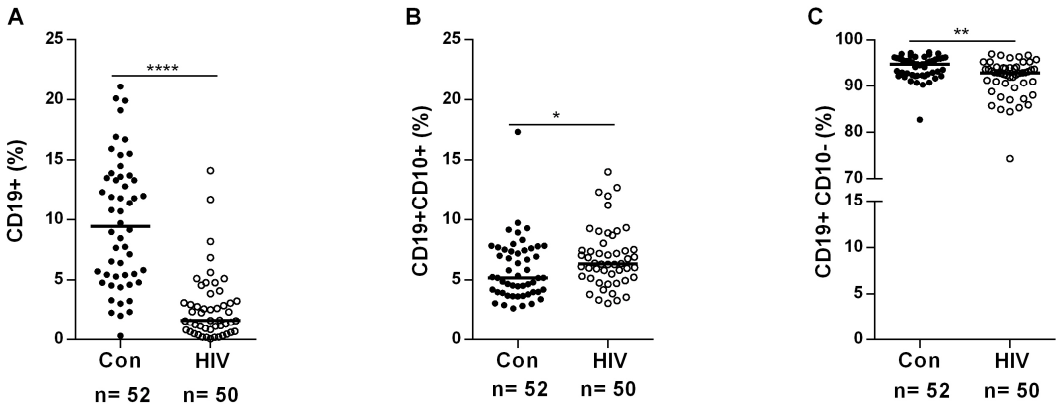

Figure 8: Frequency of B cells in control and HIV infected children.

The peripheral B cell population, CD19+, (A), Immature Transitional B cells, CD19+/CD10+, (B) and mature B cells, CD19+/CD10- *$p < 0.05$; **$p < 0.01$; **** $p < 0.0001$

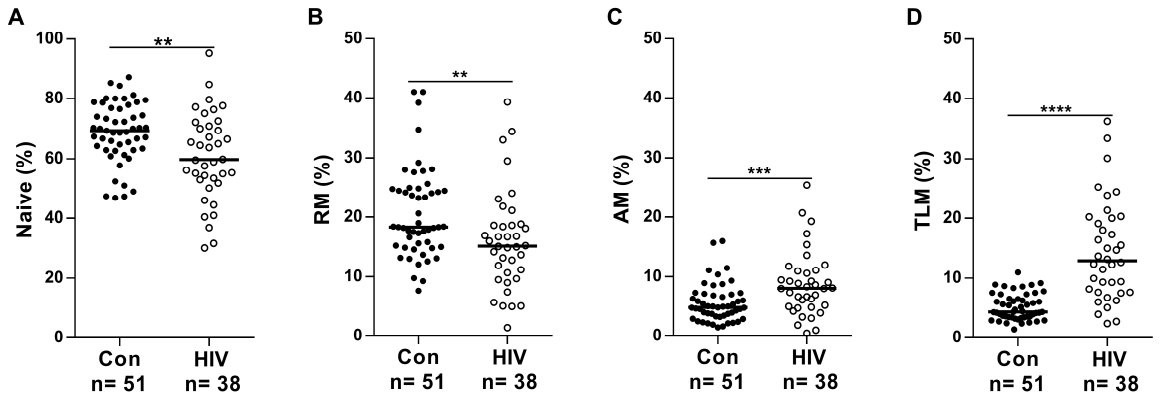

Figure 9: B cell subpopulation in HIV infected children and Controls.

B cell population (fig 5) were further delineated in to 4 B cell subsets: CD19+/CD10-/CD21+/CD27- (Naive: A), CD19+/CD10-/CD21+/CD27+ (Resting memory: RM), CD19+/CD10-/CD21-/CD27+ (Activated Memory: AM), and CD19+/CD10-/CD21-/CD27- (Tissue like memory: TLM). **p < 0.01; ***p<0.001; **** p < 0.0001.

5.7. B cell sub populations after Vaccination

The frequency of RM (p<0.0001) and AM B cells (p<0.01) increased; while the frequency of Naive B cells (p<0.0001) decreased in healthy controls after vaccination (Fig 10).

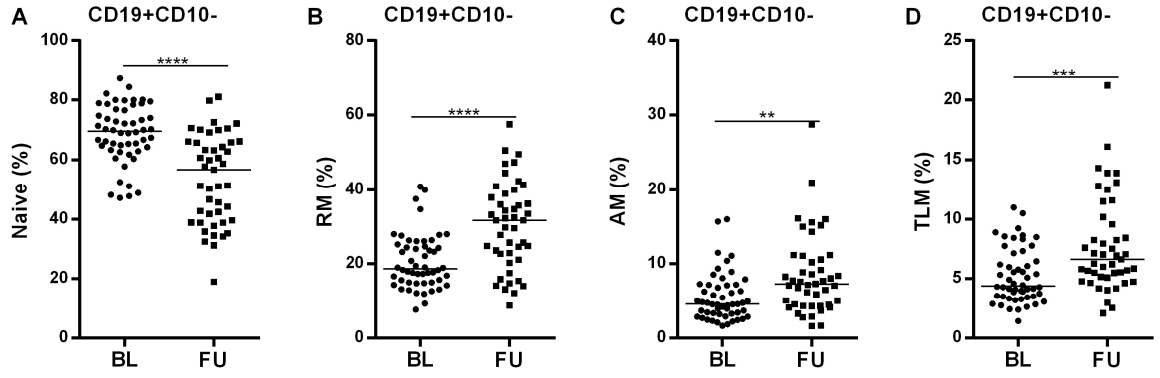

Figure 10: The distribution of B cell sub population in HIV negative children before and after vaccination. BL: base line, FU: follow up. **p < 0.01; *** p < 0.001; **** p < 0.0001.

In striking contrast, among HIV positive patients, the frequency of naive B cells increased ($p<0.05$) whereas the frequency of AM B cells decreased ($p<0.001$) and there was no significant change on the frequency of RMB cells, which is important for plasma cells differentiation and antibody production ($P>0.05$) (Fig 11). According to the present study, the frequency of CD 27+ cells, CD27++ cells , RM and AM B cells had positive correlation with plasma HBsAb level (Fig 12).

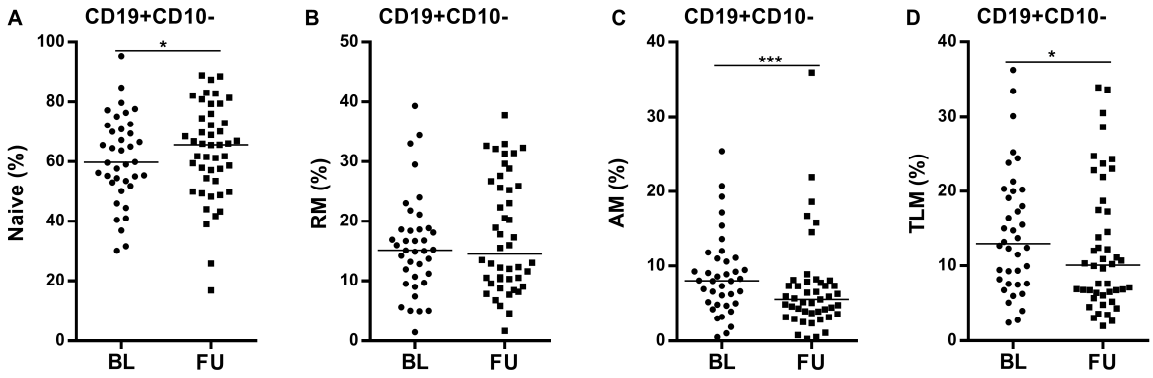

Figure 11: B cell sub population in HIV positive children, before and after vaccination.
BL: base line, FU: follow up;*p < 0.05; ***p < 0.001.

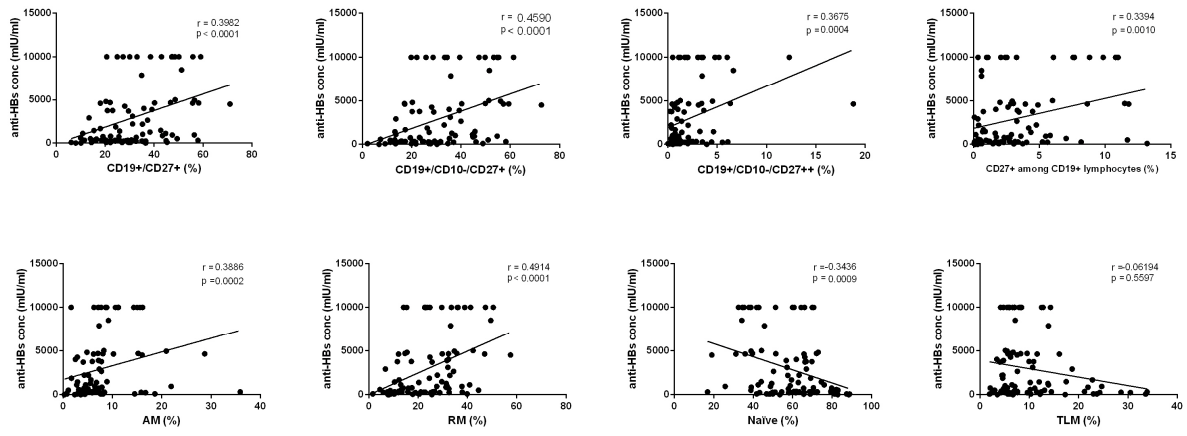

Figure 12: Correlation between plasma HBsAb level with B cells in all children

6. Discussion

Despite global attention on the HIV epidemic, particularly in developing countries, co-infection of HIV and hepatitis B, and the impact of HIV on the HBV vaccine response has received less attention and is an underappreciated problem (78). HBV infection rates in countries like Ethiopia have been reported to approach 50% among adults, so that co-infection of HIV and HBV is common, and HIV is well known to contribute to the chronic HBV as well as treatment complication(11, 33). Different reports showed that the HBV/ HIV co-infection is under reported (56, 79) and as there are limited data describing the epidemiology of HBV and HIV co-infection (54) at least in Ethiopia. The HBV control programs in endemic countries has focused on early childhood vaccination, but less emphasis has been given on following up efficacies of these administrated vaccines, or in efforts to vaccinate those individuals who missed vaccination in early child hood. Previous studies have suggested that HIV infection compromises HBV vaccination responses, but the mechanisms of hypo responsiveness remained to be defined. In our study, we assessed the response to HBV vaccine and the distribution of B cells sub population in HIV negative and HIV positive children who are naïve for treatment as well as on ART.

In this study, 94% of HIV infected children exhibited a positive vaccine response, defined as>10mIU/ml, which is higher than the previous reports of Patricia *et al* (68) and a study conducted in Thailand (80) in which 70% and 71% of HIV infected youths had response to hepatitis B vaccine, Engerix B 20 mcg, respectively. The higher response in our study could be related with the younger age of our study participants (5 up to 9 years old) compared with 12 up to 25 years old and 21 up to 45 years old at Patricia *et al* (68) and study in Thailand (80), respectively. In many other studies done on adults, younger age was associated with improved vaccine response (64, 81). Another reason for increased vaccine response in our study could be due to the accelerated vaccine schedule we used, the two studies mentioned above uses 0, 1, 6 month schedule which might allow plasma antibody titers to wane to a greater extent in the inter vaccination period.

We found that the vaccine induced plasma HBsAb level was lower in HIV positive children as compared with controls. This result is in line with studies conducted in Rwanda, Kenya and Tanzania (58, 63, 65). However, the median inter-quartile range for HIV positive children in our

study was 318 (224-1224) mIU/ml, whereas the reported median inter-quartile range for Rwanda children was151 mIU/ml (1.03–650) . The difference could be due to geographical variation and age, as most of the study participants in their study were greater than 9 years of age, which is the upper limit of our study participants. Previous study also showed that hepatitis B antibody titer was higher when vaccinated at early age (82, 83).

The low HBsAb plasma concentration in HIV positive children compared with the HIV negative children could be mainly due to depletion of CD4 cells (68), which plays an essential role in germinal center derived B cell proliferation and production of antibody secreting plasma cells (25). In addition, there is evidence of direct bound of HIV with CD 21 molecule of B cells (69, 84), which leads to destruction and decreased in number of B cells (69) that may consequently, leads to reduced plasma antibody level.

Another important finding of this study was that early age at which ART initiated was associated with higher vaccine responses. This may reflect the presence of better preserved number as well as function of CD4T cells enabling an effective responses by starting ART as early age as possible after HIV diagnosis or at infancy. Our finding agrees with those of the studies done in Zambia, Italy and a cohort on European countries (85-88).

A study conducted in Zambia showed initiation of ART in 2- to 5-year-old HIV-infected children resulted in reconstitution of RMB cell percentages to levels similar to control children and may help restore normal development and maintenance of B-cell immunity (85). The study in Italy reported that initiation of antiretroviral therapy (ART) within the first year of life in HIV-1 vertically infected children was able to preserve high numbers and functions of antigen specific memory B cells gained from child hood vaccination whereas reduced protective humoral and cellular immunity to routine childhood vaccines was observed in late-treated patients (86, 87). A study conducted by Violari *et al* indicated that earlier HIV diagnosis and antiretroviral therapy decreased infant mortality by 76% and HIV progression by 75% (88). A study in Iran also showed the correlation of immune response with the CD4 count and history of antiretroviral therapy (12).

In Ethiopia, ART for HIV positive children is initiated based on CD4 count and clinical criteria. The guideline for HIV/AIDS states that antiretroviral therapy is not an emergency intervention,

children need to be properly screened to ensure they meet eligibility criteria (89), This delay may lead to further impairment of the intact immune cells of the children, consequently, leading to incapability to fight opportunistic infections. In fact, the availability of ART through donation may not be enough to address all HIV infected children in Ethiopia. However, as currently released reports revealed that early ART initiation is important to reduce child mortality, disease progression and to preserve the immune system, it is important to give due attention how every HIV infected child can have access to ART as early as possible. It is also important to follow the adverse effects of ART, if any, followed by early initiation.

B-cells play a central role in humoral immunity, particularly against extracellular pathogens, and have a key role in viral infection too (90). HIV-1 infection causes numerous phenotypic and functional perturbation of B cells (87). During normal immune system development, naïve B cells constitute the highest proportion of B-cell populations, followed by RM B cells; whereas ITB cells, AM B and TLM B cells constitute a small portion (69, 91).

According to our study, at baseline the frequency of CD19+ B cell, naive B cells and RMB cells decreased in HIV positive children, whereas the frequency of AM B cells, IT B cells and T L M B cells increased. The reduction of B cells could be due to antigen dependent or independent (92) hyper activation and differentiation of B cells to antibody secreting plasma cells. Destruction of B cells due to direct interaction of HIV with B cells could be considered to be another plausible way of reducing the frequency of B cells in HIV positive children (93). An increased homing of B lymphocytes to lymphatic tissue due to an increased expression of homing receptors on lymphocytes, could also lead to the reduced frequency of B cells in peripheral blood (94, 95).

After vaccination the frequency of naive B cells increased; while the frequency of AM B cells decreased and the frequency of RMB remains almost unchanged in HIV positive children. Even though there is activation of CD27 + expressing lymphocytes, the activation of B cells to AM B cells is not enough to mount adequate antibody production of B cells in response to the vaccine. This implies that even though the maturation of Immature transitional B cells to naive B cells increased in the immune system of HIV positive children, the CD 4 T cell dependent germinal center formation and consequent cellular activation is impaired.

An increased immature transitional B (ITB) cells observed in this study is in line with a study conducted by Cagigi *et al* on HIV infected viremic children (96, 97). An increment of non-functional, more sensitive to spontaneous apoptosis (98) ITB may be associated with maintaining homeostasis between the (naive and RM B cells with TLM and AMB cells) wing of the B cell subset compartment in the immune system. A study conducted by Malaspina *et al,* showed as there was a direct correlation between serum level of IL-7 and IT B cells, which modulates the maturity of B cells to balance the homeostatic effect on lymphopenia in HIV (99). In fact, the frequency of IT B-cell is physiologically high in children and slowly decreases with age (100). In contrary to our study, frequency of IT B cells was similar among HIV positive and healthy controls in Malawi (101, 102). This difference could be associated with the sample size, age, genetics and geographical variation of the study sites and study participants.

Naive B cells represent a large proportion of B cells, and are the precursors for the primary humoral immune response, and germinal center formation, which is important for consequent activation for the production of rapid, high magnitude, and long lasting seconday responses (24). According to our investigation, these cells are low in frequency in HIV infected children and this finding is similar with the study by Amu *et al (73)* and a review by Moir (69).The possible mechanism for the reduction of Naive B cells inHIV positive children could be due to hyperactivation and differentiation of these cells to a short lived plasma cells (71). One study also noted defects in cellular proliferation of naive B cells in HIV infected individuals especially in viremic patients (103).

We also observed reduced frequency of Resting Memory B cells in HIV positive children in comparison with healthy controls. RMB cells are important B cell subsets for maintenance of long-term humoral responses against HIV, vaccination antigens and pathogens (93, 104, 105). Therefore, the reduction of the frequency of these cells may lead to reduced durability of humoral memory to vaccines or antigens. There is a similar study conducted by Pensieroso *et al* that showed the reduction of Resting memory B cells despite initiation of ART (106). Thus, longitudinal studies are needed to evaluate both the levels of these cells and associated vaccine specific antibodies, the importance being that booster doses of vaccine may be required at much shorter intervals in HIV patients compared with HIV negative individuals.

Activated memory B cells, which are known to be prone to apoptosis, were significantly elevated in HIV positive children in our study. This result is in agreement with previous studies done in HIV positive children and adults (70). In healthy individuals, most B cells in the peripheral blood are either naive B cells or resting memory B cells that express either switched (IgG, IgE and IgA) or unswitched (IgM and IgD) antibody isotypes, respectively (24). In HIV-infected individuals, several additional B-cell subpopulations (which are not normally present to any substantial degree in the peripheral blood of uninfected individuals) can make up considerable fractions of the total B-cell population in the peripheral blood. One of the additionally present B cell sub populations in HIV infected individuals are activated memory B cells (69). Activated memory B cells have short life span , they are used for immediate fight against antigens, and are not used for sustained long term memory, the increased frequency of these cells and the reduced frequency of RMB cells, implicates that the longevity for vaccine response could be compromised.

Another B cell sub population that was found higher in frequency in HIV positive study participants in our study are Tissue like Memory B cells. These cells expressed patterns of homing and inhibitory receptors similar to those described for antigen-specific T cell exhaustion (72). These cells proliferate poorly in response to B cell stimuli, which is consistent with high-level expression of multiple inhibitory receptors. Immunoglobulin diversities and replication histories were lower in TLM B cells, compared with classical, memory B cells, which is consistent with premature exhaustion (107). Therefore, these cells are unresponsive to antigens and they may not have a role in vaccine response. The increment of the frequency of ineffective and un responsive B cell subsets like Immature transitional B cells, Activated memory B cells, and Tissue Like Memory B cells may be the reason for the reduced HBsAb plasma level in HIV positive children.

In the study, we observed that while a large fraction of HIV-positive children responded to HBV vaccine, the antibody titre after two weeks were substantially lower than control HIV negative individuals. The reduced vaccine response was associated with abnormalities in the levels of B cell subsets, suggesting that B cell defects may account for at least part of the suboptimal response. These data suggest that studies evaluating the durability of vaccine responses should be undertaken, and may lead to additional periodic booster vaccination for optimal protection. In addition, we observed that HIV positive children who were initiated on ART more promptly after

diagnosis mounted better vaccine responses, implying that, for optimal preservation of immune function, HIV positive children should be initiated on ART as early as possible.

7. Limitation of the Study

Due to time and logistics constraints, this study addresses only two time frames: before and 2 weeks after vaccination. It should have been better to follow study participants for long period of time in order to look on the immune response longevity.

8. Conclusion

The present study revealed that Hepatitis B vaccine response was 100% in healthy controls and 94.1% in HIV positives, but the plasma HBsAb level was significantly reduced in HIV positive children.

Naive B cells important for germinal center formation, and RM B cells essential for maintaining long lasting memory were reduced in those HIV positive children. In contrary, antigen non-responding TLM B-cells, apoptosis prone AMB cells, and non-functional Immature transitional B cells were increased, where as in healthy controls naive B cells, RMB cells increased, AM B, TLM, ITB cells reduced.

We also found that early ART initiation had a positive correlation with plasma HBsAb level. However, there was no significant difference in vaccine response between ART treated and ART naive children.

9. Recommendation

- The decreased plasma level of HBsAb and frequency of RM B cell sub population in HIV positive children entails reduced vaccine response and there may be compromised longevity of vaccine response, therefore we recommend follow up assessment of vaccinated children to see the durability of the immune response against HBV.
- The schedule and booster dose of hepatitis B vaccine in HIV positive children, requires due attention.
- Early ART initiation with regular monitory of immune response and viral control should be started for better immune response and to prevent early wane of childhood vaccination.
- National survey to assess hepatitis B vaccine response to childhood vaccination should be emplace especially for children with HIV infection.

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Annex

Annex-I: Whole blood Surface staining laboratory protocols

1. Reagents and Equipment Required
   - ✓ K_3 EDTA blood collection tubes
   - ✓ Falcon disposable 12 x 75-mm capped polystyrene test tubes
   - ✓ 1000ul, 200ul and 20ul pipette man and tips
   - ✓ BD fluorochrome-conjugated monoclonal antibodies to human cell surface antigens
   - ✓ Vortex mixer
   - ✓ FACS Lysing Solution (10X)
   - ✓ Bench Centrifuge.
   - ✓ Wash buffer (FACS buffer)
   - ✓ 4% paraformaldehyde
   - ✓ FACS Canto^{II} flow cytometer
2. Procedure

   2.1.Specimen Collection and Preparation

   Collect blood aseptically by venipuncture into a sterile K3 EDTA vacutainer blood collection tube. Store anticoagulated blood at room temperature (20° to 25°C) until ready for staining and lysing.

   2.2. WARNING: All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.
3. Lysing and Staining
   1. Mix blood gently before doing the next step
   2. Transfer 100 µl of whole blood into 2 facs tube
   3. Add 1 mL of 1X FACS Lysing Solution. (1ml per 100ul blood)
   4. Vortex gently and incubate for 15 minutes in the dark at room temperature. (shake during the incubation this increases the lyses of the red blood cells)

5. Centrifuge at 1400 RPM for 7 minutes. Remove the supernatant. (Careful in this step as the pellet that has red blood cells dose not sit properly at the bottom of the tube and you may lose your cells).
6. Add 4 of wash buffer and centrifuge at 1400 RPM for 7 minutes. Remove the supernatant.
7. Repeat the above step again
8. Add appropriate volume of fluorochrome-conjugated monoclonal antibody to lysed whole blood in a 12 x 75-mm tube for 30 min at +4 ^{o}C in the dark.
9. Wash the stained cells by adding 4 mL of wash buffer and centrifuge at 1400 RPM for 7 minutes. Remove the supernatant.
10. Repeat washing step 2 times.
11. Add 200 µl of 4% paraformaldehyde and 200 µl facs buffer solution and mix thoroughly. Store at 2° to 8°C until analyzed.
12. Analyze on a FACS Canto^{II} flow cytometer. Mix samples thoroughly before acquisition.

4. Monoclonal antibodies added to tubes

Panel 1: CD19 (PerCp Cy5.5), CD10 (FITC), CD21 (BV421), CD27 (APC), Vivid (APC-cy7), CD95 (PE-cy7) and PD1 (PE)
Panel 2: CD19 (PerCp Cy5.5), CD10 (FITC), CD21 (BV421), CD27 (APC), Vivid (APC-cy7), Isotype Ms IgG1 k (PE-cy7), Isotype Ms IgG1k (PE)

Panel 1	CD19 (PerCp Cy5.5)	CD10 (FITC)	CD21 (BV421)	CD27 (APC)	Vivid (APC-cy7)	CD95 (PE-cy7)	PD1 (PE)
Tube 1	2.5ul	20ul	5ul	10ul	0.15/50ul	2.5ul	20ul
Panel 2	CD19 (PerCp Cy5.5)	CD10 (FITC)	CD21 (BV421)	CD27 (APC)	Vivid (APC-cy7)	Isotype Ms IgG1 k (PE-cy7)	Isotype Ms IgG1 k (PE)
Tube 1	2.5ul	20ul	5ul	10ul	0.15/50ul	2.5ul	5ul

5. Compensation bears staining

1. Tube labeled for each fluorochrome-conjugated mouse antibody and add 1 drop of AbC™ capture beads (Component A) to each tube. In case of LIVE/DEAD, 1 drop of ArC™ reactive beads (Component A) added to a labeled tube.

2. Mix well (Make sure to deposit the antibody directly to the bead suspension.)
3. Incubate for 20 minutes at +4 ^{O}C, protected from light.
4. Wash two times (1400 RPM for 7 - 10 min)
5. Fixed accordingly (200ul 1% Formalin)
6. For compensations beads, 10 ul of AbC™ negative beads (Component B) added to each tube and for LIVE/DEAD, 10 ul of ArC™ negative beads (Component B) added to a labeled tube.
7. Run on a FACS brand flow cytometer.

PerCp Cy5.5	FITC	BV421	APC	APC-cy7	PE-cy7)	PE
1ul	1ul	1ul	1ul	0.15/50ul (1 ul)	2ul	5ul

Annex II: Gating strategy

The following gating strategies for characterization of B cell phenotypes were used: primarily lymphocytes were gated on forward and side scatter. Then live cells were gated (Vivid Vs FSC-A), followed by total B cells gate on Vivid-CD19+ lymphocytes. Total $CD19^+$ B cells were subdivide in to mature ($CD10^+$) and $CD10^-$ B cells. From $CD10^+$ cells, immature-transitional ($CD19^+$ CD10+ $CD27^-$) and Germinal center-like ($CD19^+$ $CD10^+$ $CD27^+$) B cells were gated. Then after, naïve ($CD19^+$ $CD10^-$ $CD21^+$ $CD27^-$), resting memory ($CD19^+$ $CD10^-$ $CD21^+$ $CD27^+$), activated memory ($CD19^+$ $CD10^-$ $CD21^-$ $CD27^+$) and tissue like memory ($CD19^+$ $CD10^-$ $CD21^-$ $CD27^-$) B cells identified from $CD10^-$ gated populations

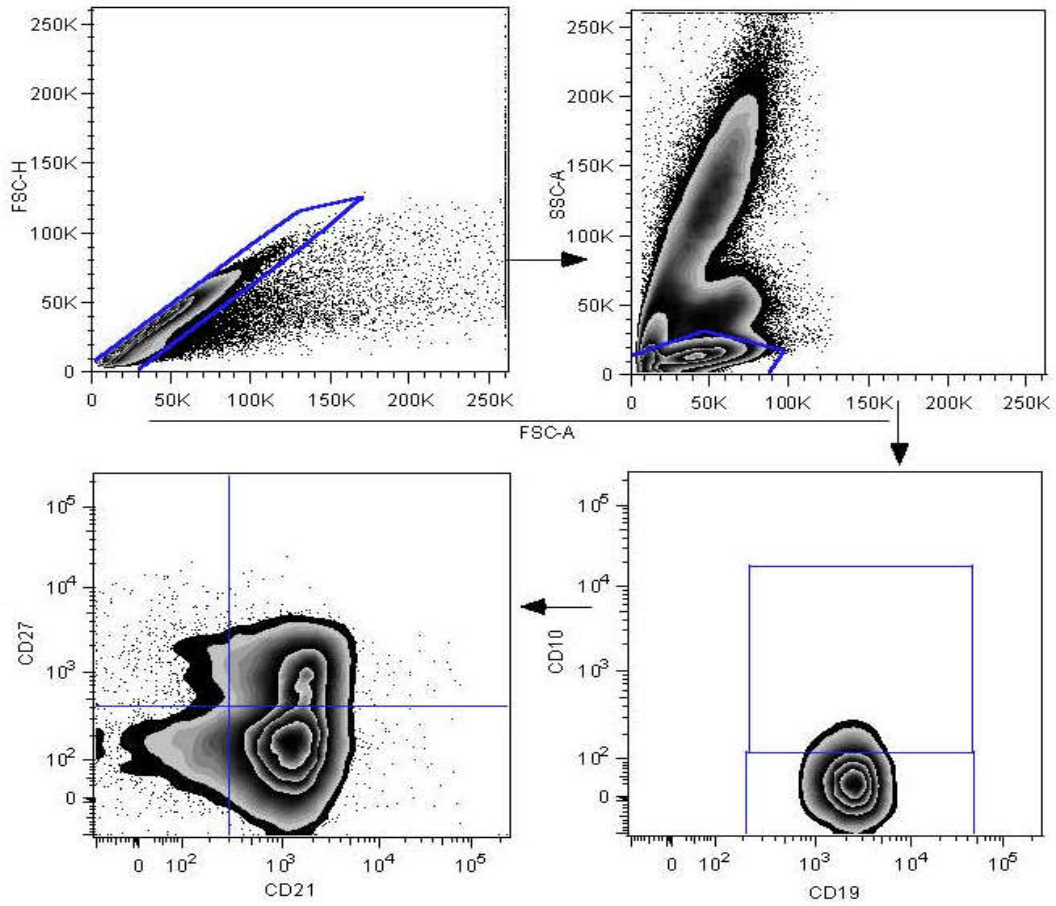

Figure 13: Gating strategy to identify B cell sub sets

Annex III: HBsAb Enzyme Linked Immuno Sorbent Assay (ELISA)

Reagents and Materials

Reagents provided by the manufacturer

- R1 MICROPLATE: 12 strips of 8 wells sensitized by a mixture of Hepatitis B surface antigen, subtype ad and ay (human origin)
- R2 CONCENTRATED WASHING SOLUTION (20X)
- C0 ANTI-HBS NEGATIVE CONTROL Buffer with fetal calf serum and protein stabilizers
- C1 10 mIU/ml CALIBRATOR Buffer with Anti-HBs antibodies of human origin, fetal calf serum , protein stabilizers and sample indicator dye
- C2 100 mIU/ml CALIBRATOR – POSITIVE CONTROL
- C3 400 mIU/ml CALIBRATOR
- C4 1000 mIU/ml CALIBRATOR
- R6 SPECIMEN DILUENT
- R7a CONCENTRATED CONJUGATE (11X)
- R7b CONJUGATE DILUENT
- R8 SUBSTRATE BUFFER
- R9 CHROMOGEN
- R10 STOPPING SOLUTION

Store the kit at 2-8°C. Bring all reagents except Conjugate Concentrate to room temperature (18-30°C) before use. Return reagents to 2-8°C immediately after use. Store all unused strips/plates in pouch and reseal. Do not remove desiccant. Store strip plates at 2-8°C.

MATERIAL REQUIRED BUT NOT PROVIDED

- Distilled water.
- Sodium hypochlorite (bleach) and sodium bicarbonate.
- Automatic or semi-automatic adjustable or fixed pipettes capable of delivering 10 µl to 200 µl, 1 ml, 5 ml and 10 ml.
- Graduated cylinders of 25 ml, 100 ml and 1000 ml capacity.

- Container for contaminated residues.
- Dry incubator, thermostatically set at 37°C ± 1°C.
- Micro plate washing system.
- Microplate reading device (equipped with 490, 450 and 620 nm filters).
- Absorbent paper.
- Disposable gloves.
- Clean polypropylene containers for TMB preparation.

ASSAY PROCEDURE

1. Strictly follow the protocol. Use negative and positive control sera for each test, in order to validatethe test quality. Apply good laboratory practice.
   Methods
2. Carefully define the sample distribution and identification plan.
3. Bring all of the reagents to room temperature before beginning the assay procedure.
4. Prepare Conjugate Working Solution (R7a + R7b), Working Diluted Substrate Solution (R8 + R9) and Diluted Washing Solution (diluted R2).
5. Remove the microplate frame and strips (R1) from their protective bag. Remove strips not needed for the assay and replace them with labeled Null Strips, as necessary.
6. Dilute specimens, calibrators and controls 3:4 in the Specimen Diluent R6
7. Add directly, without prior washing of the plate, and in succession for: Quantitative determination.
8. Cover, if it is possible, the wells with adhesive film by pressing over the whole surface to ensure tightness.
9. Incubate the plate for 60 ± 5 minutes at 37 ± 2°C.
10. Remove the adhesive film. Aspirate the contents of all wells into a liquid waste container and add immediately a minimum of 0.375 ml of Washing Solution to each well. Soak each well for 30 to 60 seconds between each wash cycle. Aspirate again. Repeat the washing step 4 times (minimum of5 washes). The residual volume must be lower than 10 µl (if necessary blot the plate by turning it upside down on absorbent paper).If an automatic washer is used, follow the same procedure.

11. Add quickly 100 µl of the Conjugate Working Solution (R7a + R7b) to each well. Cover, if it is possible, the wells with a new adhesive film and incubate for 60 ± 5 minutes at 37°C ± 1°C.

    NOTE: The conjugate is colored green. It is possible to verify the presence of conjugate in the wellsby spectrophotometric reading at 620 nm

12. Remove the adhesive film. Aspirate the contents of all wells into a liquid waste container and add immediately a minimum of 0.375 ml of Washing Solution to each well. Soak each well for 30 to 60 seconds between each wash cycle. Aspirate again. Repeat the washing step 4 times (minimum of 5 washes). The residual volume must be lower than 10 µl (if necessary blot the plate by turning it upside down on absorbent paper).If an automatic washer is used, follow the same procedure.
13. Add quickly 100 µl of the Working Diluted Substrate Solution (R8 + R9) to each well. Allow the reaction to develop in the dark for 30 ± 5 minutes at room temperature (18 - 30°C). Do not use adhesive film during this incubation.

    NOTE: The Working Diluted Substrate Solution is colored pink. It is possible to verify the conjugate in the wells by spectrophotometric reading at 490 nm

14. Add 100µl Stopping Solution (R10) by using the same sequence and rate of distribution as for the Working Diluted Substrate Solution. Homogenize the reaction mixture.
15. Carefully wipe the plate bottom. At least 4 minutes after stopping solution addition and within30 minutes of stopping the reaction, read at the optical density at 450/620-700 nm and 405/620- 700 nm using a plate reader.

Before recording the results, check the correspondence between the reading and the micro plate and sample distribution and identification plan.

Annex IV: Hepanostika anti-HBc ELISA

Materials and instrumentations required which are not provided by the manufacturer is almost similar with ELISA for HBsAb.

- Distilled water.
- Sodium hypochlorite (bleach) and sodium bicarbonate.
- Automatic or semi-automatic adjustable or fixed pipettes capable of delivering 10 µl to 200 µl, 1 ml, 5 ml and 10 ml.
- Disposable V shaped troughs
- Graduated cylinders of 25 ml, 100 ml and 1000 ml capacity.
- Container for contaminated residues.
- Dry incubator, thermostatically set at 37°C ± 1°C.
- Micro plate washing system.
- Microplate reading device (equipped with 490, 450 and 620 nm filters).
- Absorbent paper.
- Disposable gloves.
- Clean polypropylene containers for TMB preparation.

Assay Procedure

1. Fit the strip holder with the required number of microelisa strips
2. Pipette 100 µl (undiluted) sample or control into assigned wells. Include three negative controls (100 µl each) and three positive controls (100 µl each) in each strip holder. Pipette the controls after the samples.
3. Pipette 50 µl conjugate into each well with sample or control. Shake [e.g. using a micro shaker, speed approximately 15 Hz(= 900 revolutions per minute) for 15 seconds, or equivalent]. Incubate at 37^{o}Cfor 90 ± 5 minutes.
4. Wash and soak each well four times with phosphate buffer.

   - Aspirate the well contents completely into a waste flask. Then fill the wells completely with phosphate buffer avoiding over flow from one well to another and allow to soak for 30 to 60 seconds. Aspirate completely and repeat the wash and soak procedure three times for a total of four washes.

- It is preferable to wash the wells by filling them with a fluid volume greater than the well volume while simultaneously aspirating the excess volume in order to prevent over flowing.

- Remove any remaining fluid on the top and under the bottom of the microelisa strips and strip holder after the last aspiration; e.g., by blotting with absorbent tissue.

5. Pipette 100 µl TMB substrate into each well. Do not mix or shake, Discard any TMB substrate that has been stored beyond the indicated period of use.
6. Incubate the strips at 15 to 30 ^{o}C for 30 ± 2 minutes.
7. Stop the reaction by adding 100 µl 1mol/1 sulfuric acid to each well (maintain the same pipetting sequence and time intervals used for TMB substrate addition). Read plates within 15 minutes.
8. Blank the reader against air (without strip plate) and read the absorbance of the solution in each well at 450 nm (single wave length)

ANNEX V: Assay Protocol II: ABBOTT m2000sp INSTRUMENT AND M2000rt Instrument for HIV-1 Viral Load Determination

1. Thaw assay controls and IC at 15-30°C or at 2-8°C. Thaw calibrators at 15-30°C or at 2-8°C only if performing a calibration run;

   - Once thawed, assay controls, IC, and calibrators can be stored at 2-8°C for up to 24 hours before use, Vortex each assay calibrator and each control three times for 2-3 seconds before use. Ensure that the contents of each vial are at the bottom after vortexing by tapping the vials on the bench to bring liquid to the bottom of the vial.

2. Thaw amplification reagents at 15-30°C or at 2-8°C and store at 2-8°C until required for the amplification master mix procedure.
   - Once thawed the amplification reagents can be stored at 2-8°C for up to 24 hours if not used immediately. NOTE: Use one bottle of *m*Lysis Buffer, one vial of IC, and one RealTi*m*e HIV-1 Amplification Reagent Pack to support up to 24 reactions. Use a second set of reagents to support 25to 48 reactions. A maximum of 48 reactions can be performed per assay.
3. Gently invert the Abbott *m* Sample Preparation bottles to ensure a homogeneous solution. If crystals are observed in any of the reagent bottles upon opening, allow the reagent to equilibrate at room temperature until the crystals disappear. Do not use the reagents until the crystals have dissolved.
4. Vortex each IC three times for 2-3 seconds before use.
5. Use a calibrated precision PIPETTE DEDICATED FOR INTERNAL CONTROL USE ONLY to add 500 µL of IC to each bottle of *m*Lysis Buffer. Mix by gently inverting the container 5 to 10 times to minimize foaming.
6. A total of 48 samples can be processed in each run. A negative control, a low positive control, and a high positive control are included in each run, therefore allowing a maximum of 45 specimens to be processed per assay. If frozen, thaw specimens at 15-30°C or at 2-8°C.

   Once thawed, specimens can be stored at 2-8°C for up to 6 hours if not processed immediately.

   Vortex each specimen three times for 2-3 seconds before loading on the Abbott *m*2000*sp* worktable. Specimens showing particulate matter or turbidity should be clarified by

centrifugation at 2,000 g for 5 minutes prior to testing. Aliquot each specimen into clean tubes or vials if necessary. Refer to the Abbott *m*2000*sp* Operations Manual for tube sizes. Avoid touching the inside of the cap when opening tubes.

7. Place the low and high positive controls, the negative control, the calibrators, if applicable, and the patient specimens into the Abbott *m*2000*sp* sample rack.
8. Place the 5 mL Reaction Vessels into the *m*2000*sp* 1 mL subsystem carrier.
9. Load the Abbott *m* Sample Preparation System reagents and the Abbott 96 Deep-Well Plate on the Abbott *m*2000*sp* worktable
10. Enter calibrator (needed if a calibration curve has not been stored on the *m*2000*rt*) and control lot specific values in the Sample Extraction: Worktable Setup, Calibrator and Control fields.

• The Abbott *m*2000*sp* Master Mix Addition protocol (step 12) must be initiated within one hour after completion of Sample Preparation.

NOTE: Change gloves before handling the amplification reagents.

11. Load the amplification reagents and the master mix vial on the *m*2000*sp* worktable after sample preparation is completed.
    - Each Amplification Reagent Pack supports up to 24 reactions.
    - Prior to opening the amplification reagents, ensure that the contents are at the bottom of the vials by tapping the vials in an upright position on the bench.
    - Remove and discard the amplification vial caps.
    - A second Amplification Reagent Pack is required if performing 25 to 48 reactions.
12. From the Protocol screen, select the appropriate application file corresponding to the sample volume being tested. Initiate the Abbott *m*2000*sp* Master Mix Addition protocol. Follow the instructions as described in the Abbott *m*2000*sp* Operations Manual, Operating Instructions section.

    • The *m*2000*rt* protocol must be started within 40 minutes of the initiation of theMaster Mix Addition protocol.
13. Switch on and initialize the Abbott *m*2000*rt* instrument in the Amplification Area.
    - NOTE: The Abbott *m*2000*rt* requires 15 minutes to warm-up.
    - NOTE: Remove gloves before returning to the sample preparation area.

14. Seal the Abbott 96-Well Optical Reaction Plate after the Abbott *m*2000*sp* instrument has completed addition of samples and master mix according to the Abbott *m*2000*sp* Operations Manual, Operating Instructions section.
15. Place the sealed optical reaction plate into the Splash Free Support Base for transfer to the Abbott *m*2000*rt* instrument.
16. Place the Abbott 96-Well Optical Reaction Plate in the Abbott *m*2000*rt* instrument. From the Protocol screen, select the appropriate application file corresponding to the sample volume being tested. Initiate the Abbott RealTi*m*e HIV-1 protocol.

Annex VI: Information Sheet for Parents/Guardians of children 5 to 9 years old participants (English Version)

Research Title: The effect of antiretroviral drug therapy on the immune response to Hepatitis B vaccine in children in Addis Ababa, Ethiopia

Name of PI: Desalegn Yibeltal (B. PHARM)

Supervisor: Rawleigh Howe (MD, PhD, Senior Scientist, AHRI)

Sponsor: AHRI /ALERT, Karolinska Institute and Swedish medical research council and AAU

1. Background

Hepatitis B is a viral infection that attacks the liver and can cause both acute and chronic disease; seroprevalence in many African countries including Ethiopia approaches 50%, and prevalence of chronic active hepatitis B is 5-10%. HBV infection among HIV-1 seropositive patients is an underappreciated problem

HBV infection is vaccine preventable. However, the efficacy of the current HBV vaccine, as well as mechanism(s) of possible abnormal responses to HBV vaccines among HIV-1 infected infants, is not established. We therefore propose a study probing the impact of HIV-1 on the host response to the HBV vaccine and studies on B cells to elucidate the mechanism of the presumed defect in response to the HBV vaccine in HIV-1 infected children. These studies may provide a framework for improved HBV vaccination schedules in HIV-1 infected children and may generate new knowledge useful in the field of childhood vaccines for this group of children. Therefore, when research is conducted in children of 5 to 9 years old, his/her parents/guardian should be informed and asked for his/her permission to participate in the study. The parent/guardian has to agree independently before we begin the study in this age group. Before you decide, you can talk to anyone you feel comfortable with.

2. Research Objective

- To assess the effect of HIV-1 infection on Hepatitis B specific antibody response
- To evaluate the effect of HIV-1 infection on phenotypic properties of B cells in HBV vaccinated children.

- To evaluate the impact of Antiretroviral therapy on HBV vaccine response
- To evaluate the effect of antiretroviral drug therapy on phenotypic properties of B cells.

3. Study Procedure

To achieve the goals of this study, a total of 150 children of 5 to 9 years of age, 75 HIV positive and 75 HIV negative, will be recruited by pediatrician. Demographic information, other relevant clinical data and 3-5 ml blood samples will be collected at baseline. Samples will be screened for previous exposure to HBV or vaccine. Those children who are HIV positive among HIV suspected and HBsAg positive will be referred to the clinician for clinical management and follow-up. Only unexposed children will be enrolled in the study; participants will be vaccinated intradermally. And, at 1, 6 and 12 months blood sample will be collected again from the included children to measure response to HBV vaccine by attending nurses or laboratory technologists and then transported to the laboratory. Samples will be processed and lab analysis like HBV serological, Flow Cytometry and ELISpot assay will be done for each visit.

A subset of samples will be stored at AHRI for 10 years and will be used in the future for advanced techniques (ultra-sensitive methods) which may not available in Ethiopia. There is a possibility that these samples will be shipped to laboratories outside Ethiopia for this analysis, but the samples will only be used for this project. However, when the investigators wish to pursue this analysis abroad, ethical approval will be sought prior to any transfer of biological materials.

4. Risks and Discomfort

Your child participation in the study has minimum invasive procedure, he/she mayhave minor discomfort and pain during blood drawing and there may also be mild redness, or swelling on the site where the shot was given. But this will be minimized, as the procedure will be carried out by experienced health professionals in the health facility with a standard aseptic condition. Any vaccine related reaction or injury, or any psychological problem related to confirmation of positive HIV status and/or HBV infection will be dealt by pediatricians in each health institution and communicated with the children parents or guardian.

5. Benefits

You will not receive any direct benefit by participating in this research. However by letting your child take part, you will be assisting public heath bodies to gain an improved understanding of what vaccination schedule work best to secure protection against HBV for all children. At the same your child will get free HBV screening and a clinical assessment of his/her health condition. The vaccine should have been given along with other childhood vaccines and your children did not receive it. However, in this present study your child will receive the vaccine; the vaccine has the potential to protect the child from acute and chronic hepatitis B virus infection, protect from cirrhosis and liver cancer.

6. Participant's Role

If you are volunteered for your child's participation in this study, you will be asked different demographic and other health related questions. In addition, your child will be requested to give 3-5 ml (one tea spoon) of blood for screening HBV and after the result your children will receive HBV vaccine. Then, to bring your child again at 1, 6 and 12 month of final booster of the vaccine to quantify the number of total B cells and memory B cells by draw 3 to 5 ml of blood according to their body weight. The purpose of your revisiting the health institute of your child is to check the immunity response to the vaccine.

7. Participant's Right

It is your right to allow your child to participate in the study or not. Refusing to take part in the study will not result any penalty or loss of benefits or right to which your child might otherwise be entitled. Your child has the right to be treated by the examining paediatrician with appropriate management based on her/his results.

8. Incentives and Compensation

You will not be provided any incentives to take part in this research. However, after blood collected from your child nutritional supplement will be given and you will be reimbursed 60 birr per visit as a compensation for your time lost and travel expense during participation in the project.

9. Confidentiality

The information that we collect for this research project will be kept confidential. Information about your children that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. Information of your child will be used only for above mentioned purpose. No information obtained from this research will be disclosed to anyone other than those conducting the study. No personal identifiers will be included in the results of the study which may be published for scientific purposes.

10. Right to refuse or withdraw:

Your child has full right to refuse from participating in this research if he/she does not wish to do so; and this will not affect his/her treatment or health services at this health center in any way. He/she has also full right to withdraw from this research at any time you wish to, without losing any of your rights as a patient in the health institute.

11. Communication

In case you might have any questions, unclear ideas and doubt about the study please feel free to contact the following individuals through their addresses:

- Desalegn Yibeltal : Email: desalegy@gmail.com Mobile: +251918220996
- Yonas Bekele: Email: adugalab@gmail.com Mobile: +251911639810
- Rawleigh Howe (MD, PhD, Senior Scientist, AHRI) Mobile: +251910865382
- The Secretary of AHRI/ALERT Ethical Review Committee. Tel: +251113-481285
- National Ethical Review committee Tel: +251111-562155

Thank you very much!!

Annex-VII: እድሚያቸው 5 እስከ 9 ዓመትለሆኑየጥናቱተሳታፊወላጅ/አሳዳጊየሚሰጥመረጃ (በአማርኛየተዘጋጀ)

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ዋናተመራማሪ፡ ደሳለኝ ይበልለጣል

ዋናአማካሪ፡ ዶ/ር ራውሊ ሐው

የምርምሩ ወጪ የሚሸፈነዉ፡- አህሪ/አለርት፣ካሮሊኒስካ ኢንስቲቲዩት እና ሲዊዲሽ ሜዲካል ሴንተር ፣ አአዩ

1. የጥናቱ ጽንሰ ሀሳብ

ሄፐታይተስ ቢ በአጭር ወይም ከረዥም ጊዜ በኋላ የጉበት ችግር የሚያመጣ የቫይረስ ኢንፌክሽን ሲሆን በአብዛኛው የአፍሪካ ሀገራት ላይ ወደ 50% እየተጠጋ ሲሆን በዚሁ ቫይረስ የተጎዱ ሰዎች ብዛት ከ5-10% ይደርሳል፡፡ ሄፐታይተስቢ ቫይረስ ኢንፌክሽን የኤችአይቪ ቫይረስ በደማቸው ውስጥ ባለባቸው አፍሪካዊያን ሰዎች ላይ ደግሞ ችግሩ የተንሰራፋ ነው፡ የሄፐታይተስ ቢ ቫይረስ ኢንፌክሽን ክትባት ያለው ሲሆን ነገርግን የክትባቱ ተጓዳኝ የሰውነት ምላሾች በኤች. አይ. ቪ. ቫይረስ በተያዙ ህጻናት ላይ የሚያሳይ በቂ መረጃ የለም፡፡ስለዚህ ይህ ጥናት ኤች. አይ ቪ ቫይረስ በሄፐታይተስ ቢ ክትባት ላይ ያለውን ተጽዕኖ ለማወቅ ና የሄፐታይተስ ቢ ክትባት ከተሰጠ በኋላ የበሽታ ተከላካይ ደምህዋሳት መጠን ና ጥራት የኤች አይ ቪ ቫይረስ በደማቸው ባለባቸው እና በሌለበቸው ተሳታፊዎች መካከል በማነጻጸር መረጃ ይሰበስባል፡፡የዚህ ጥናት ውጤት ወደፊት የሄፐታይተስ ቢ ክትባት አሰጣጥ ኤች አይ ቪ ቫይረስ በደማቸው ውስጥ ላለባቸው ህጻናት እንዲስተካከል እናም ተጨማሪ አዳዲስ ጠቃሚ መረጃዎችንም ያስገኛል ብለን እናምናለን፡፡ ስለዚህ ጥናቱ ከ8 ዓመት በታች ያሉ ህጻናትን የሚያካትት ስለሆነ የህጻኑ ቤተሰብ ወይም አሳዳጊ እርስዎ ሙሉፍቃድ ያስፈልጋል፡፡ ስለሆነም የጥናቱን አላማ እና ሌሎች ሁኔታዎችን ከተረዱ ና ልጅዎ እንዲሳተፍ/እንድትሳተፍ ከተስማሙ በኋላ ጥናቱን እንጀምራለን፡፡ከመወሰነዎ በፊት ጊዜ ወስደው ያስቡበት ወይም ከሚፈልጉት ጋር ይወያዩ፡፡

2. የጥናቱዓላማ

ይህ ጥናታዊ ምርምር የሚከተሉት ዓላማዎች ይኖሩታል፡፡አንደኛው ኤች አይ ቪ ቫይረስ በሄፐታይተስ ቢ ክትባት ላይ ያለው ተጽዕኖ ይጠናል፡፡ሁለተኛው ከሄፐታይተስ ቢ ክትባት በኋላ የሰውነት በሽታ ተከላካይ ህዋሳት በተለይ ቢ ሴል የተባሉትን መጠን ና ጥራት ኤች አይ ቪ ቫይረስ በደማቸው ባለባቸው ና በሌለበቸው መካከል ይነጻጸራል፡፡

3. የጥናቱሂደት

ይህን ጥናት ለማካሄድ ከ5 – 9 ዓመት የሆኑ ኤች አይ ቪ ቫይረስ በደማቸው ያለባቸው 75 የሌለባቸው 75 በድምሩ 150 ህፃናትበጥናት ውስጥ ያካተታሉ፡፡ ከጤናና ከጤና ጋር ተዛማጅነት ያላቸው የግል መረጃዎች ከተሰበሰቡ በኃላ ከልጅዎ 3 -5 ሲሲ (1 የሻይ ማንኪያ) የደም ናሙና ይወሰዳል፡፡ ከደም ናሙናው ውጤት የሄፐታይተስ ቢ ቫይረስ ወይም ኤችአይቪ ቫይረስ (ኤችአይቪ ቫይረስ ነፃ ከሆኑት ውስጥ) ቢገኝ በቀጥታ ወደ ሐኪሙ/ሚ ለተጨማሪ ህክምና፣ ምርመራ እንዲሁም

ክትትል ይላካሉ፡፡ ሄፐታይተስ ቢ ቫይረስ ነፃ ከሆነ/ች ሂፐታይተስ ቢ ክትባት ይሰጣል፡፡ ክትባቱን ከወሰደ/ች ፣ 6 እና 12 ወር በኋላ 3-5 ሲሲ የደም ናሙና በድጋሚ ተወስዶ የተለያየ ምርምራ በአህሪ ላቦራቶሪ ይካሄድበታል፡፡

የተወሰነ ናሙና በአህሪ ላቦራቶሪ ለ10 ዓመት ብቻ ይቀመጣል፡፡ ወደፊት የተሻሉ ነገር ግን አገራችን ውስጥ የሌሉ የላቦራቶሪ ምርምር ዘዴዎች ሲገኙ ናሙናው ወደ ውጭ ይላካል፤ ናሙናው ተያያዥነት ላለው ምርምር ብቻ እንዲውል ይደረጋል፡፡ ነገር ግን ናሙናው ወደ ውጭ ከመላኩ በፊት ከስነ ምግባር ኮሚቴው ፍቃድ ይጠይቃል፡፡

4. ከጥናቱ ጋር የተያያዘ ጉዳት/ አለመመቸት

የደም ናሙናውን የሚወስደው ባለሙያው ብቁ የሆኑና ንፁህ በሆኑ የህክምና ዕቃዎች ስለሆነ ልጅዎ በዚህ ጥናት ውስጥ በመሳተፉ/ፏ ለከፋ ጉዳት የሚያጋልጥበት ሁኔታ አይኖርም፡፡ ደም ከልጅዎ በሚወሰድበት ወቅት አነስተኛ ህመም ሊሰማው/ት ይችላል፡፡ እንዲሁም የመቅላት፣ እና የማበጥ ሁኔታ ከትባት በተሰጠበት ቦታ ላይ ሊታይ ይችላል፡፡ ነገር ግን እነዚህ ጉዳቶች የከፋ ጉዳት የሚያስከትሉ አይደሉም፡፡ ማንኛውም አይነት ከክትባቱ እና ከጥናቱ ጋር የተያያዘ ችግሮች በህፃናት ሐኪም ይታያል፡፡ የኤችአይቪ ቫይረስ እና የሄፐታይተስ ቢ ቫይረስ ምርመራ ወጤትን በተመለከተ የህጻኑ ቤተሰብ ወይም አሳዳጊ ብቻ በሚከታተላቸው ሐኪም አማካኝነት ይነገራል፡፡

5. በጥናቱ የመሳተፍ ጥቅም

ልጅዎ በዚህ ጥናት ውስጥ በመሳተፉ/ፏ የሄፐታይተስ ቫይረስ በደሙ/ሚ መኖር አለመኖር የሚያሳይ ምርመራ ይካሂዳል፣ ልጅዎ ከሄፐታይተስ ቢ ቫይረስ ነፃ ከሆነ/ች የሄፐታይተስ ቫይረስ ክትባት በነጻ ያገኛል/ታገኛለች በተጨማሪም ሙሉ የጤና ምርመራ ይደረጋል፡፡ ልጆዎ እንደማንኛውም ክትባት ሄፐታይተስ ቢ ክትባት አልወሰድ/ችም፤ ይህ ክትባት ደግሞ የጉበት በሽታን በተለይም የጉበት ካንሰርን ይከላከላል፡፡ ልጅዎ እንዳይሳተፍ ማድረግ ይችላሉ፤ ይህንንም በማድረጎ የሚደርስቦ ምንም ነገር የለም፡፡ እንዲሁም ከትባቱን በማንኛውም ተቋም ማግኘት ይችላሉ፡፡

6. የጥናቱ ተሳታፊ ድርሻ

በዚህ ጥናት ልጅዎ እንዲሳተፍ ፍቃደኛ ከሆኑ ከጤና ሁኔታ ጋር የተያያዙና ሌሎች የግልመረጃዎችን እንዲሰጡ ይጠየቃሉ፡፡ በመቀጠልም ከልጅዎ ከ3-5 ሲሲ መጠን ያለው የደም ናሙና ለተጠቀሰዉ ዓላማ እንድንወስድ ይጠየቃሉ፤ በውጤቱም መሰረት ሄፐታይተስ ቫይረስ ቢ ክትባት ለልጅዎ ይሰጣል፡፡ ከክትባት በኃላ ከ፣ 6 እና 12 ወር በኃላ እንደህፃኑ/ንዋ ክብደት 3-5 ሲሲ የደም ናሙና በድጋሚ ተወስዶ የተለያዩ ምርምራዎች ይደረጋሉ፡፡ በተደጋጋሚ የደም ናሙና መወሰዱ ክትባቱ በደም ውሰጥ ምን ይህል እንደሰራ ለማየት ነው፡፡

7. የጥናት ተሳታፊዉ መብት

የልጅዎ ተሳትፎ በእርስዎ ወይም በእረስዎና በልጅዎ ሙሉ ፈቃደኝነት ላይ ብቻ የተመሰረተ ነው፡፡ ስለሆነም በጥናቱ ለመሳተፍ ባይስማሙ ምንም አይነት ቅጣት የማያስከትል ሲሆን ማንኛውም ልጅዎ ሊያገኝ የሚገባውን ህክምናና ተያያዥ መብት የማያሳጣ ሲሆን በውጤቱም መሰረት የልጅዎ የመታከም መብት የተጠበቀ ነው፡፡

8. ከጥናቱ ጋር የተያያዘ ክፍያ/ጥቅማጥቅም ይኖር የሆን

ልጅዎ በጥናቱ ውስጥ በመሳተፉ/ፏ ወይም እንዲሳተፍ/እንድትሳተፍ ለማበረታታት የሚሰጥ ምንም ዓይነት ክፍያ አይኖርም፡፡ ከልጀዎ ደም ከተወሰደ በኃላ ተጨማሪ ምግቦች እና ለትራንስፖርት ወጪዎና ላጠፉት ጊዜ ማካካሻ በየመጡበት ቀን ብር 60 (ሰድሳ) ብር ይሰጥዎታል ፡፡

9. የጥናት መረጃዎች ምስጢራዊነት

ከዚህ ምርምር የምንሰበስበው መረጃ በጥናቱ ወቅትም ሆነ ከዚያ በኃላ ባሉት ጊዚያት ከጥናቱ የተገኘው መረጃ ሚስጥራዊነት የሚጠበቅ ሲሆን መረጃዎቹም የሚያዙት በስም ሳይሆን በልዩ ኮድ ነው፡፡ ይኸው መረጃ በጥንቃቄ የሚያዝና የተፈቀደለት ተመራማሪ እና ለሃኪሙ ብቻ ይህም እጅግ አስፈላጊ በሆነ ጊዚ ብቻ ካልሆነ በስተቀር ለሌላ በማንም ሰው አይሰጥም፡፡ ማንኛውም ከልጅዎ ጋር የተያያዘ መረጃ በልዩ ኮድ ብቻ የሚያዝ ሲሆን ውጤቱም ለሳይንሳዊ ዓላማ ብቻ ስም በማይገልፅ ሁኔታ እንዲታተም ይደረጋል፡፡

10. በጥናቱ ያለመሳተፍ ወይም አቋረጦ የመውጣት መብት

በዚህ ጥናት ልጅዎ እንዲሳተፍ/እንድትሳተፍ ካልፈለጉ አይገደዱም እንዲሁም መሳተፍ ከጀመረ/ች በኋላ በማንኛውም ጊዜ ከጥናቱ አቋርጦ/ጣ መውጣት ይችላል/ለች፡፡ ይህንን በማድረግዎ በማዕከሉ የሚደለግልዎትን ህክምና በማንም መልኩ አያስተጓጉልም፡፡

11. ስለጥናቱ መረጃ ማግኘት ቢፈልጉ፡

ይህ የጥናት ዕቅድ በአህሪ/አለርት የስነ-ምግባር ቅኝት ኮሚቴ ተገምግሞ ፀድቋል፡፡ ጥናቱትን በተመለከተ ግልጽ ያልሆነ ማንኛዉንም ጥየቄ ካለዎት ነፃ ሆነው ከዚህ በታች ባሉት አድራሻዎች መጠየቅ ይችላሉ፡፡

- ደሳለኝ ይበልጣል ስልክ ቁጥር፡ 0918220996
- ዮናስ በቀለ ስልክ ቁጥር: 251 911639810
- ዶ/ር ራውሊ ሐው ስልክ ቁጥር:251 910865382
- የአህሪ/አለርት የምርምር ስነ ምግባር ኮሚቴ ፀሃፊ ስልክ ቁጥር: 251 113481285
- በኢትዮጵያ ሳይንስና ቴክኖሎጂ ኮሚሽን ስልክ ቁጥር: 251 111562155

በጣም እናመሰግናለን!!

Annex-VIII: Consent form for Parents/ Guardians of study participants 5 to 9 years old (English Version)

Study title: The effect of antiretroviral drug therapy on the immune response to Hepatitis B vaccine in children in Addis Ababa, Ethiopia

Principal Investigator: Desalegn Yibeltal

The Armauer Hansen Research Institute (AHRI) in collaboration with Karolinska Institutet (KI) will conduct research on HBV vaccination in HIV infected and uninfected children. The aim of the study is to evaluate the serological response to HBV vaccine in HIV positive and negative children, 5 to 9 years old, with no prior exposure to HBV vaccine or infection. And also, to evaluating functional and phenotypic properties of memory B cells to HBV vaccine within the aforementioned cohorts. I am asked my child participation in this study. I am informed that my child will be vaccinated for HBV and 3 to 5 ml blood will be taken before vaccination and during each visit after screening for HBV. I am informed that the subset of the sample will be stored for 10 years. I have also been informed that the risks during vaccination and sample collection are minimal, except for minor pain while drawing blood and mild redness, or skin swelling during vaccination on the site where the shot was given. I understand that my child's personal information will be kept confidential and all information regarding his/her result will be communicated directly with me and treating physician. I also informed that there is no incentive for taking part in the study.

I have read the information sheet attached with this consent form, or it has been read to me. I have had the opportunity to ask questions and satisfied with the answers given by the investigators. I also informed that my refusal or withdraw to participate in the study, it will not affect my child treatment or services in the health institute. With full understanding of the importance of the study, I agreed voluntarily to give information about my child, and allow the investigators to give vaccination and take the required blood sample (3-5 ml or one tea spoon) from my child. Therefore, I hereby give my consent on behalf of my child to participate in this study.

_____	_____	____/____/___
Name of Parent/ Guardian	Signature of Parent/ Guardian	Day/month/year

Witness for Illiterate participants:

_____	_____	____/____/___
Name of Witness	Signature of Witness	Day/month/year
_____	_____	____/____/___
Name of the researcher/physician	Signature of researcher/physician	Day/month/year

Annex-IX: እድሚያቸው 5 – 9 ዓመት ለሆኑ የጥናት ተሳታፊዎች በወላጅ/አሳዳጊ የሚሰጥ የፈቃደኝነት ማረጋገጫ ቅፅ (በአማርኛ የተዘጋጀ)

<u>የጥናቱ ርዕስ:</u>የሰውነት ምላሽ ለሄፐታይተስ ቢ ክትባት በህፃናት ላይ፤ የኤችአይቪ ቫይረስመድሃኒት በሄፐታይተስ ቢ ክትባት ላይ የሚያመጣው ተፅዕኖ በአዲስ አበባ ኢትዮጵያ

<u>ዋና ተመራማሪ፡</u> ደሳለኝ ይበልጣል

ይህ ጥናት አህሪ በስዊዲን ሀገር ከሚገኘው ከካሮሊንስካ ኢንስቲትውት ጋር በመተባበር የሄፐታይተስ ቢ ኢንፌክሽን በ ኤችአይቪ ቫይረስ በተያዙና ባልተያዙ ህፃናት ላይ የሚደረግ ነው፡፡የጥናቱ ዓላማ እድሜያቸው ከ5 –9 ዓመት በሆኑ ህፃናት ላይ ኤችአይቪ ቫይረስ በሄፐታይተስ ቢ ክትባት ላይ ያለውን ተጽኖ እና ሄፐታይተስ ቢ ክትባት የሰውነት በሽታ ተከላካይ ህዋሳት በተለይ ቢ ሴል የተባሉትን መጠንና ጥራት ኤችአይቪ ቫይረስ በደማቸው ካለባቸው እና ከሌለበቸው ጋር ለማወዳደር ነው፡፡ ልጄ በዚህ ጥናት ውስጥ እንዲሳተፍ/እንድትሳተፍ ፍቃደኝነቴን ተጠይቂያለው፡፡ በጥናቱ ወቅትም ከልጄ የደም ናሙና እንደሚወሰድ ከልጄ 3-5 ሲሲ ከክትባት በፊት ከዚያም ሄፐታይተስ ቢ ክትባት እንደሚሰጠው አውቂያለው፤ ከ ክትባት ፣ 6 እና 12 ወር በኋላ በድጋሚ ከልጄ 3-5 ሲሲ እንደሚወሰድ ተነግሮኛል፡፡ የተወሰደው ናሙና በከፊል በአህሪ ላቦራቶሪ ለ10 ዓመት ብቻ ተነግሮኛል፡፡ የልጄ ፍቃደኝነትም ተጠይቆ ፍላጎቱ እንደሚከበርለት ተረድቻለው፡፡ በጥናቱ ወቅትም ደም ሲወሰድ እንዲሁም በ ክትባት ሲሰጠው መጠነኛ ህመም በስተቀር ምንም አይነት የከፋ ችግር እንደሌለዉ ተገነዝቢያለሁ፡፡ የልጂ እና እኔ የምሰጠው እንዲሁም ከጥናቱ የሚገኘው መረጃ ሚስጥራዊነት እንደሚጠበቅ፤ የልጂም ወጤት ለእኔ ብቻ እንደሚነገረኝ ተነግሮኛል፡፡የዚህ ጥናት ውጤትም ለሚመለከተው አካል ብቻ እንደሚገለፅ አወቄአለሁ፤ ልጄ መሳተፉ/ፏ የሚሰጥ ምንም ማባበያ አይኖርም፡፡

ስለጥናቱ የሚገልፀውን መረጃ አንብቤአለው/ተነቦልኛል፡፡ ስለመረጃውም ጥያቄዎችን እንድጠይቅ እድል ተሰጥቶኛል፡፡ የጠየቅሁትም ጥያቄ በበቂ ሁኔታ ተመልሶልኛል፡፡ በጥናቱ አለመሳተፍ ከዚህ ተቋም በማገኘው አገልግሎት ላይ ምንም አይነት ተፅእኖ አይኖረውም፡፡ በመሆኑም የጥናቱ ዓላማና ጥቅም በሚገባ ስለተገነዘብኩ፤ ስለልጄ ማንኛውንም መረጃ ለመስጠት፣ ክትባት እንደአስፈላጊነቱ እንዲሰጠው/እንዲሰጣት አንዲሁም ከልጄ 3-5 ሲሲ (እስከ አንድ የሻይ ማንኪያ) የደም ናሙና እንዲወሰድ በሙሉ ፍቃደኝነት መስማሙቴን በፊርማዬ አረጋግጣለሁ፡፡

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የወላጅ/አሳዳጊ ስም የወላጅ/አሳዳጊ ፊርማ ቀን /ወር/ዓ.ም

<u>ማንበብና መፃፍ ለማይችሉ የጥናት ተሳታፊዎች፤ ምስክር</u>

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የምስክር ስም የምስክር ፊርማ ቀን /ወር/ዓ.ም

---------- ---------- -------/---------/-------

የተመራማሪው/የሐኪሙ ስም የተመራማሪው/ሐኪም ፊርማ ቀን /ወር/ዓ.ም

Annex-X: Demographical and Clinical data collection questionnaire for HIV positive children

1. Name of Health facility _____Study code No_____
2. Patient Identification: Patient card No: _____ ART code:_____
3. Address:

   Sub city_____ Woreda_____ Kebele_____ House .No_____

   Home Tel_____ Mobile_____ Alt. phone No:_____
4. Date of Birth: _____/ _____/ _____(DD/MM/YEAR)
5. Sex: 1. Male ☐ 2. Female ☐
6. Height: _____cm Weight _____kg Mid Upper arm circumference_____ cm
7. Do you know HIV status of your child? 1. Yes ☐ 2. No ☐

   If Yes: Date of diagnosis (from chart)_____

   CD4 count (from chart) _____

   Viral load (from chart) _____

   WHO HIV staging: Stage 1 ☐ Stage 2 ☐ Stage 3 ☐ Stage 4 ☐
8. Did your child take prophylaxis before HIV test? 1. Yes ☐ 2 No ☐

   If yes, drugs used for prophylaxis: NVP ☐ AZT ☐ Combination ☐

   Others (specify)_____
9. Is your child on ART? 1. Yes ☐ 2. No ☐ If yes: Date of ART start_____(dd/mm/year)

   Drug regimen_____

   1ST line: ABC + 3TC+NEV ☐ ABC+3TC+ EFV ☐ AZT+3TC+NEV ☐ AZT+3TC+EFV ☐

   2nd line: ABC+3TC+L/R ☐ DDI+3TC+L/R ☐ Adherence_____%

9.1. Is there any history of drug regimen change: Yes ☐ No ☐ if yes….,

Changed regimen (select from 1st or 2nd line regimen) _____

Reason for drug change_____, Date of regimen change_____

10. Is there any history of known chronic medical conditions for the last 3 months?

    1. Yes ☐ 2. No ☐ if yes please specify _____

11. Is there any history of Acute or Sub acute medical condition in the past two weeks?
    1. Yes ☐ 2. No ☐ if yes please specify_____

Vaccination status

1. Have your child vaccinated previously? (Please check child immunization card)? 1. Yes ☐ 2 No ☐
2. If Yes, tick the vaccines taken by the child;***(****please write the vaccination date /*DD/MM/YEAR/*at the space provided from vaccination card****)***

☐ Vaccine BCG/OPV_____ ☐ Rota_____

☐ DPT/HepB/ OPV/Hib1_____ ☐ Measles_____

☐ DPT/HepB/ OPV/Hib2_____ ☐ Vitamin A supplement_____

☐ DPT/HepB/ OPV/Hib3_____ ☐ Zinc supplementation_____

☐ PCV_____

Blood will be stored for further analysis Agree ☐ Disagree ☐

NB. Please attach the copy of lab result sheet for Immunological Factors, Hematological parameter, Organ function tests.

Annex XI: በኤ ች አይ ቪ ቫይረስ ለተያዙ ህጻናት የ ማህበራዊ እና ጤና ነክ መረጃዎች መጠይቅ

1. የጤና ተቋሙ ስም _____ የምስጢር ቁጥር _____
2. የህክምና ካርድ ቁጥር _____የመድሐኒት ቁጥር_____
3. አድራሻ፡ክ/ከተማ_____ ወረዳ _____ ቀበሌ _____ የቤት ስልክ_____ሞባይል _____አማራጭ ስልክ_____
4. ጾታ፡ ወንድ ☐ ሴት ☐
5. የትዉልድ ዘመን_____ ቀን/ ወር/ ዓም.
6. ቁመት _____ ሳ.ሜ. ክብደት _____ ኪ.ግ. የክንድ ዙሪያ_____ ሳሜ
7. የልጁን/የልጇን ከኤች አይ ቪ ጋር መኖር እና ሁኔታ ያዉቃሉ?

   የተመረመረበት ቀን (ከካርድ) _____

   የቅርብ ቀን CD 4 ቁጥር (ከካርድ) _____

   በዓለም የጤና ድርጅት መስፈርት መሰረት የ ህመሙ ደረጃ

   ደረጃ 1 ☐ ደረጃ 2 ☐ ደረጃ 3 ☐ ደረጃ 4 ☐
8. ልጅዎ የኤች አይ ቪ ምርመራ ከማድረጉ በፊት የመከላከያ መድሐኒት ወስዶ ነበር አለ የለምመልስዎ አለ ከሆነ፡ የሚወስደዉን መድሐኒት ቢነግሩን

   NVP ☐ AZT ☐ ከአንድ በላይ ☐ ሌሎች _____
9. ልጅዎ የኤች አይ ቪ መደሀኒት እየወሰደ ነዉ? አለ ☐ አይደለም ☐

   መልስዎ አለ ከሆነ፡

   መደሀኒቱን መዉሰድ የጀመረበት ቀን (ከካርድ)_____

   የመድሀኒቱ አይነት(ከካርድ)የመጀመሪያ ተመራጭ ABC + 3TC+NEV☐ ABC+3TC+ EFV ☐ AZT+3TC+NEV☐ AZT+3TC+EFV☐

   ሁለተኛ ተመራጭ: ABC+3TC+L/R☐ DDI+3TC+L/R☐ ሌሎች_____%
10. የተለወጠ መድሀኒት አለ? መልስዎ አለ ከሆነ

    የተተወዉ መድሀኒት_____ የተተወበት ምክንያት_____
11. ባለፉት 3 ወራት ውስጥ የተከሰተ ለብዙ ጊዜ የሚቆይ አይነት የህመም ችግር አጋጥሞት ነበር?

    አለ ☐ የለም☐ መልስዎ አለ ከሆነ የ ህመሙ ዓይነት_____
12. በዚህ ሁለት ሳምንት ዉስጥ የተከሰተ አጣዳፊ ህመም ነበር?

    አለ ☐ የለም ☐ መልስዎ አለ ከሆነ የ ህመሙ ዓይነት_____

የክትባት ሁኔታ

13. ልጁ የወሰዳቸዉ የ ክትባት አይነቶች እና የተከተበበት ጊዜ (የክትባት ካርዱን ይመልከቱ

☐ ቢ.ሲ. ጅ./አ. ፒ. ቪ_____ ☐ ሮታ_____

☐ ዲ.ፒ.ቲ./ሄፓ.ቢ/ አ. ፒ. ቪ/ሒብ1_____ ☐ ሚዝል_____

☐ ዲ.ፒ.ቲ./ሄፓ.ቢ/ አ. ፒ. ቪ/ሒብ2_____

☐ ዲ.ፒ.ቲ./ሄፓ.ቢ/ አ. ፒ. ቪ/ሒብ3_____ ☐ ቪታሚን ኤ ሰፕልመንት_____

☐ ፒ. ሲ. ቪ._____ ☐ ዚንክ ሰፕልመንት_____

የልጅዎት ደም ለተጨማሪ ምርመራ በ አግባቡ ይቀመጣል፡ እስማማለሁ ☐ አልስማማም ☐

Annex-XII: Demographical and Clinical data collection forms for HIV negative children

1. Name of Health facility _____Study code No_____
2. Address:

Sub city_____ Woreda_____ Kebele_____ House .No_____

Home Tel_____ Mobile_____ Alt. phone No:_____

3. Date of Birth: _____/ _____/ _____(DD/MM/YEAR)
4. Sex: Male ☐ Female ☐
5. Height: _____cm Weight _____kg Upper arm circumference_____ cm
6. Is there any history of known chronic medical conditions for the last 3 months?

   Yes ☐ No ☐ if yes please specify _____

7. Is there any history of Acute or Sub acute medical condition in the past two weeks?

   Yes ☐ No ☐ if yes please specify_____

   Vaccination status

8. Have your child vaccinated previously? (Please check child immunization card)?

   Yes ☐ No ☐

9. If Yes, tick the vaccines taken by the child;***(****please write the vaccination date /*DD/MM/YEAR/*at the space provided from vaccination card****)***

Vaccine BCG/OPV_____ DPT/HepB/ OPV/Hib1_____

DPT/HepB/ OPV/Hib2_____ DPT/HepB/ OPV/Hib3_____

PCV_____ Rota_____

Measles_____ Vitamin A supplement_____

Zinc supplementation_____

Part of blood will be stored for further analysis. Agree ☐ Disagree ☐

Annex XIII፡ ኤች አይ ቪ ቫይረስ በደማቸዉ ውስጥ ለሌለ ልጆች የተዘጋጀ መጠይቅ

1. የጤና ተቋሙ ስም _____ የምስጢር ቁጥር _____
2. አድራሻ

ክ/ከተማ_____ ወረዳ _____ ቀበሌ _____

ስልክ የቤት_____ ሞባይል _____አማራጭ _____

3. ጾታ፡ ወንድ ☐ ሴት ☐
4. የትዉልድ ዘመን _____ ቀን/ ወር/ ዓም.
5. ቁመት _____ ሳሜ. ክብደት _____ ኪግ. የክንድ ዙሪያ_____ ሳሜ
6. ባለፉት 3 ወራት ውስጥ የተከሰተ ለብዙ ጊዜ የሚቆይ አይነት የህመም ችግር አጋጥሞት ነበር

አወ ☐ የለም ☐ መልስዎ አወ ከሆነ የ ህመሙ ዓይነት_____

7. በዚህ ሁለት ሳምንት ዉስጥ የተከሰተ አጣዳፊ ህመም ነበር

አወ ☐ የለም ☐ መልስዎ አወ ከሆነ የ ህመሙ ዓይነት_____

የክትባት ሁኔታ

8. ልጁ የወሰዳቸዉ የ ክትባት አይነቶች እና የተከተበበት ጊዜ (የክትባት ካርዱን ይመልከቱ)

☐ ቢ.ሲ. ጅ./አ. ፒ. ቪ_____ ☐ ሮታ_____

☐ ዲ.ፒ.ቲ./ሄፓ.ቢ/ አ. ፒ. ቪ/ሒብ1_____ ☐ ሚዝል_____

☐ ዲ.ፒ.ቲ./ሄፓ.ቢ/ አ. ፒ. ቪ/ሒብ2_____ ☐ ቪ.ታሚን ኤ ሰፕልመንት_____

☐ ዲ.ፒ.ቲ./ሄፓ.ቢ/ አ. ፒ. ቪ/ሒብ3_____ ☐ ዚንክ ሰፕልመንት_____

☐ ፒ. ሲ. ቪ._____

9. የልጅዎት ደም ለተጨማሪ ምርመራ በ አግባቡ ይቀመጣል፡፡

እስማማለሁ ☐ አልስማማም ☐

Annex XIV: Declaration

I, the undersigned, declare that this MSc. thesis is my original work, has not been presented for a degree in this or any other University and that all sources of materials used for the thesis have been duly acknowledged.

MSc. candidate: Desalegn Yibeltal (BPharm.)

Signature: _____ Date of submission: _____

This thesis has been submitted with our approval by Advisors:

Abiy Habtewold (PhD; Addis Ababa University)

Signature: _____ Date_____ Place: Addis Ababa, Ethiopia

Prof. Eyasu Makonnen (PhD), AAU

Signature: _____ Date_____ Place: Addis Ababa, Ethiopia.

Rawleigh Howe (MD, PhD), Senior Scientist, AHRI

Signature: _____ Date_____ Place: Addis Ababa, Ethiopia.