From the department of Gastroenterology, Hepatology and Infectious Diseases

Effect of *H. pylori* infection on the immune response and clinical parameters: Prospective hospital-based interventional study

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Summary

Understanding the immunomodulatory effect of microbiota could have vast clinical implications for common diseases like allergies and autoimmune diseases. Recently, Dr. Feldt et al could show that chronic infection with *H. pylori* was associated with decreased immune activation and favourable clinical outcomes in HIV-infected patients, but apparently also in HIV negative individuals.

As it was a cross-sectional study, determining causality was not possible. Moreover, the number of HIV negative participants was relatively low. As a result, we were interested to extend the previous study implementing a longitudinal interventional study design with a larger sample size of HIV negative individuals. In this study T cell profiles, complete blood cells count and liver enzymes were assessed before and after administration of H*. pylori* eradication therapy.

There was a high prevalence of *H. pylori* infection among HIV positive (78.7%) and HIV negative (75.1%) asymptomatic individuals in Asella teaching and referral hospital, Ethiopia. This prevalence was more than four times higher than the prevalence reported from the five years trend of *H. pylori* infection (15.2%) in the hospital. We have shown that the test kit used by the hospital was of lower quality and *H. pylori* might have been under-diagnosed in the hospital. This could explain the low prevalence of *H. pylori* infection reported from the hospital laboratory. The eradication rate after treatment with the standard eradication therapy was much lower among HIV positive patients (37.5%, 9/24) compared to HIV negative individuals (62.5%, 15/24).

There was a statistically significant increase in hemoglobin level at three and six months after eradication of *H. pylori* among HIV negative individuals. Eradication of *H. pylori* was also associated with increased RBC indices and percentage of lymphocytes. Nevertheless, an increase in RBC indices was also observed among the non-eradicated group. Significantly lower of T cell activation marker (HLA-DR+CD38+CD4+), exhaustion markers (PD1+CD8+, TIM3+CD4+, TIM3+CD8+) markers and higher T regulatory cell markers (CD25+Foxp3+CD4+) were observed among *H. pylori*-infected individuals in comparison to *H. pylori*-negative individuals. Eradication of *H. pylori* was associated with increased proliferation and decreased expression of markers for regulatory T cells. Moreover, a significant decrease in T regulatory cells was also observed among not successfully eradicated HIV negative individuals.

Some changes in the T cell profile and clinical parameters after intervention were not exclusively related to *H. pylori* eradication. The lack of causality in this study could be due to the small sample size of eradicated groups, which made causality analysis difficult and limits the interpretation of the results. Therefore, having the findings of this prospective study as a baseline, conducting further studies with a larger sample size could solve the limitations observed in this study.

Zusammenfassung

Das Verständnis der immunmodulatorischen Wirkung von Mikroorganismen könnte enorme klinische Auswirkungen auf häufige Krankheiten wie Allergien und Autoimmunerkrankungen haben. Kürzlich konnten Dr. Feldt et al. zeigen, dass eine chronische Infektion mit H. pylori bei HIV-infizierten Patienten, aber offenbar auch bei HIV-negativen Personen, mit einer verminderten Immunaktivierung und besseren klinischen Ergebnissen einhergeht.

Da es sich um eine Querschnittsstudie handelte, war die Bestimmung der Kausalität nicht möglich. Zudem war die Zahl der HIV-negativen Teilnehmer relativ gering. Daher waren wir daran interessiert, die vorherige Studie, die ein längsschnittliches, interventionelles Studiendesign umsetzte, mit einer größeren Stichprobengröße von HIV-negativen Personen zu erweitern. In dieser Studie wurden die T-Zellprofile, die Anzahl der vollständigen Blutzellen und die Leberenzyme vor und nach der Behandlung mit der *H. pylori*-Therapie untersucht.Es gab eine hohe Prävalenz der *H. pylori*-Infektion unter HIV-positiven (78,7%) und HIV-negativen (75,1%) asymptomatischen Personen im Asella krankenhaus, Äthiopien. Diese Prävalenz war mehr als viermal so hoch wie die Prävalenz, die aus dem Fünf-Jahres-Trend der H. pylori-Infektion (15,2%) im Krankenhaus berichtet wurde. Wir haben gezeigt, dass das vom Krankenhaus verwendete Testkit von geringerer Qualität war und *H. pylori* im Krankenhaus möglicherweise unterdiagnostiziert wurde. Dies könnte die geringe Prävalenz der aus dem Krankenhauslabor gemeldeten *H. pylori*-Infektion erklären. Die Eradikationsrate nach der Behandlung mit der Standard-Eradikationstherapie war bei HIV-positiven Patienten (37,5%, 9/24) im Vergleich zu HIV-negativen Personen (62,5%, 15/24) wesentlich geringer.

Drei und sechs Monate nach der Eradikation von *H. pylori* war bei HIV-negativen Personen ein statistisch signifikanter Anstieg des Hämoglobinspiegels zu verzeichnen. Die Eradikation von *H. pylori* war auch mit erhöhten RBC-Indizes und einem erhöhten Prozentsatz von Lymphozyten assoziiert. Dennoch wurde auch bei der nicht eliminierten Gruppe ein Anstieg der RBC-Indizes beobachtet. Signifikant niedrigere T-Zell-Aktivierungsmarker (HLA-DR$^+$CD38$^+$CD4$^+$), Erschöpfungsmarker (PD1$^+$CD8$^+$, TIM3$^+$CD4$^+$, TIM3$^+$CD8$^+$) und höhere T-Regulationszellmarker (CD25$^+$Foxp3$^+$CD4$^+$) wurden bei *H. pylori*-Infizierten im Vergleich zu *H. pylori*-negativen Individuen beobachtet. Die Eradikation von H. pylori war mit einer erhöhten Proliferation und einer verminderten Expression von Markern für regulatorische T-Zellen assoziiert. Darüber hinaus wurde ein signifikanter Rückgang der T-Regulationszellen auch bei nicht erfolgreich eliminierten HIV-negativen Individuen beobachtet.

Einige Veränderungen im T-Zell-Profil und in den klinischen Parametern nach der Intervention standen nicht ausschließlich im Zusammenhang mit der Eradikation von *H. pylori*. Der Mangel an Kausalität in dieser Studie könnte auf die kleine Stichprobengröße der ausgemerzten Gruppen zurückzuführen sein, was die Kausalitätsanalyse erschwerte und die Interpretation der Ergebnisse einschränkt. Wenn man die Ergebnisse dieser prospektiven Studie als Grundlage nimmt, könnte die Durchführung weiterer Studien mit größeren Stichproben die in dieser Studie beobachteten Einschränkungen lösen.

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List of Abbreviations and Acronyms

ALP-Alkaline phosphatase
ALT-Alanine Aminotransferase
ART- Antiretroviral treatment
AST- Aspartate Aminotransferase
ATRH-Asella teaching and referral hospital
BSA -*bovine serum albumin*
CagA- cytotoxin-associated gene A
CCR6- C-C-Chemokine receptor Type 6
CD- cluster of differentiation number
CFU- colony forming unit
CRP-C-reactive protein
DCs - dendritic cells
DMSO- Dimethylsulfoxid
DNA- Deoxyribonucleic acid
EDTA- Ethylenediaminetetraacetic acid
ELISA- enzyme-linked immunosorbent assay
FACS- Fluorescence-activated cell sorting
FCS -*fetal calf serum*
FITC-Fluorescein isothiocyanate
Foxp3- Forkhead Box P3
H.pylori –Helicobacter pylori
HbsAg- Hepatitis B surface Antigen
HBV- Hepatitis B virus
HCV- Hepatitis C virus
HITM- Hirsch Institute of Tropical Medicine
HIV- human immunodeficiency virus
HLA-DR- Human Leukocyte Antigen – DR isotype
HRP- horseradish
ICA- immunochromatography
IDA- iron deficiency anaemia
IFNGR1- interferon-γ receptor gene1
IFN-γ- interferon-γ
IL- Interleukin
ITP- immunologic thrombocytopenic purpur
Ki67- Kiel 67
LPS- lipopolysaccharide
MALT- mucosa-associated lymphoid tissue
MCHC-Mean cell hemoglobin concentration
MCH-Mean cell hemoglobin
MCV-Mean cell volume
MPV-Mean platelet volume
OD- optical density
PBMCs- peripheral blood mononuclear cells
PCR -polymerase chain reaction
PD1- programmed death-1
PDW-Platelet distribution width
PE- Phycoerythrin
PerCp- Peridinin Chlorophyll Protein Complex
PPI- proton pump inhibitor
PPs -Peyer's patches
PRR-pattern recognition receptors
PUD- Peptic Ulcer Disease
RBC-red blood cells
RCF-relative centrifugal force
RDW-Red cell distribution width
RPM- Revolution per minute
RPMI- Roswell Park Memorial Institute
RUT - Rapid Urease Test
SATs -Stool antigen tests
TB- Tuberculosis
TGF-β1- transforming growth factor β1
Th1- T helper 1
TIM3- T-cell immunoglobulin and mucin-domain containing-3
TLRs- toll-like receptors
TNF-α -tumor necrosis factor-α
Treg- T regulatory cells
UBT- Urea Breath Test,
VacA- vacuolating cytotoxin A
VCT- Voluntary Counselling and Testing
WBC-white blood cells

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1. Introduction

1.1. *H. pylori i*nfection

Helicobacter pylori (*H. pylori*) is a helical gram negative microaerophilic bacteria which belongs to the family Helicobacteraceae. The name, Helicobacter pylori, is derived from its helical shape and its predominant habitat in human body, pylorus. The bacteria measures about 3.5 μm long and 0.5 to 1 μm wide. It is flagellated bacteria in which 6-8 flagella are found at one end of the bacteria. Those flagella are vital for its existence as they help the bacteria to rapidly reach in the mucus layer and burrow itself into the less acidic stomach lining. The helical shape of Helicobacter also supports a screw like movement which facilitates motility within the viscous mucus layer and enhances colonization efficiency. It ensures its existence in the naturally harsh stomach environment by neutralizing the acid in its surroundings by producing large amounts of the enzyme urease (Konieczna, Żarnowiec et al. 2012). It adheres to the epithelial cells by producing adhesins, which bind to lipids and carbohydrates in the epithelial cell membrane. *H. pylori* is usually found in the mucus, on the inner surface of the epithelium, and occasionally inside the epithelial cells themselves (Petersen and Krogfelt 2003).

The spiral-shaped bacteria in the stomach lining could weaken the mucus lining and allows acid to enter to the sensitive coating of the stomach. The acid and the bacteria irritate the lining and cause an ulcer. Following initial acute inflammation and associated changes in gastric permeability, continuous exposure to antigen results in the generation of *H. pylori* specific T cell and B cell responses. Evidence from murine studies suggests that T cell responses against *H. pylori* play a role in the induction of the gastric mucosal damage (Zuckerman 2015). The intragastric distribution and severity of this chronic inflammatory process depend on a variety of factors, such as *H. pylori* virulence factors (CagA and VacA), host genetics and immune response, diet, and the level of acid production (Thye, Burchard et al. 2003, Campbell, Pearce et al. 2004).

Gastric cancer is the most severe consequence of an *H. pylori* infection. *H. pylori* was found to increase the risk of gastric cancer 2-6 times. Among patients with *H. pylori* infection, the estimated lifetime risk of developing Peptic ulcer disease is 10-20% and gastric cancer is 1-2% (Kusters, van Vliet et al. 2006). It is not clearly known why many individuals infected with *H.pylori* remain asymptomatic and some develop serious complications (Mohammadi, Czinn et al. 1996). Despite the high prevalence of virulent *H. pylori* strains in many African countries, low incidence of peptic ulcer and gastric cancer has been observed (Walker, Segal et al. 2000, Segal, Ally et al. 2001). This could suggest a different natural history from that seen in developed countries or could mean that peptic ulcer and gastric cancers are under diagnosed.

H. pylori infection is also associated with esophageal cancer (Xie, Zhang et al. 2013), idiopathic thrombocytopenic purpura, nonalcoholic fatty liver disease (Yu, Cai et al. 2018), increased liver enzyme such as Alkaline Phosphatase (Gong, Wei et al. 2015) and iron-deficiency anemia probably due to chronic internal bleeding and impairment of iron absorption (Kusters, van Vliet et al. 2006, Ierardi, Goni et al. 2014). Eradication of *H. pylori* was associated with increased Hemoglobin level, platelete count and lymphocyte count (Gong, Wei et al. 2015, Nagata, Sekiguchi et al. 2015, Mwafy and Afana 2018).

1.2 Epidemiology of *H. pylori* infection and co-infection with HIV

1.2.1 Transmission and distribution of *H. pylori* infection

Since the first description of *H. pylori* by Warren and Marshall in 1983, bacterium has been thought to be one of the most common human infections worldwide. *H.pylori* is more prevalent among community members with low economic status and poor hygiene which favor the bacteria's common oral- oral and feco-oral routes of transmissions. In developing countries, a combination of untreated water, crowded conditions, and poor hygiene contributes to higher *H. pylori* prevalence. The prevalence ranges up to 70–90 % in developing countries and approximately 25–50 % in developed countries (Cover and Blaser 2009).

The person-to-person mode of transmission is supported by the higher incidence of infection among institutionalized children and adults and the clustering of *H. pylori* infection within families. Infection occurs mainly as childhood and parents as well as siblings are considered to have a key role in transmission (Zuckerman 2015). Two Japanese studies used multilocus sequence typing and rapid amplified polymorphic DNA to show the strain transmission in families. Of 35 index pediatric patients, the allele match was found between the index child and the mother for 25 (60%) and between the index child and both father and mother for 9 (25.7%) (Yokota, Konno et al. 2015). In the other study, 19 isolates from five families were analyzed. Mother-to-child transmission was documented in four of the five families, father to child in two families and sibling to sibling in one family (Osaki, Konno et al. 2015).

As far as the transmission of *H. pylori* is considered, iatrogenic transmission of *H. pylori* following endoscopy is also one of the most known modes as individuals may carry an estimated of 10^4 to 10^7 *H. pylori* colony-forming unit (CFU) per gram of gastric mucus (Atherton, Tham et al. 1996). The detection of *H. pylori* DNA in vomitus, saliva, dental plaque, gastric juice, and feces could support the concept of oral-oral transmission of the bacteria (Al Sayed, Anand et al. 2014). The organism has also the ability to become a coccoid form. This may represent a persistent form in which *H. pylori* can exist in the environment and it has also been suggested that some cocci can revert to their original spiral shape (Andersen, Elliott et al. 1997). Waterborne transmission, probably due to fecal contamination, may be also an important source of infection, especially in parts of the world in which untreated water is common. It is also believed that, housefly can serve as reservoir to transmit *H. pylori* (Brown 2000).

In Ethiopia, the prevalence of *H. pylori* among dyspeptic patients ranges from 52.4% (Kibru, Gelaw et al. 2014) to 85.6% (Moges, Kassu et al. 2006) as it is summarized in table 1. A retrospective study done on the trend of sero prevalence of *H. pylori* infection in Gondar University Hospital among dyspeptic patients showed a declining trend (86.5% in 2009 to 61.3% in 2011) (Mathewos 2013). A similar declining trend

was reported from Bahir Dar Felege Hiwot Referral Hospital where *H.pylori* sero prevalence was declined from 44.5% in the year 2009 to 30.2% in the year 2013 (Workineh and Andargie 2016).

Table 1 Prevalence of *H. pylori* infection in adults reported in studies performed in different part of Ethiopia

SN	City/Town	Population studied	Year	Test Method	Number of Participants	Prevalence (%)	Reference
1	Yirga Alem	Patients with ulcer	1992-1995	Rapid urease	174	92.5	(Henriksen, Nysaeter et al. 1999)
2	Gondar	dyspeptic patients	2003	Serology	215	85.6	(Moges, Kassu et al. 2006)
3	Gondar	dyspeptic patients	2009-2011	Serology	1388	65.7	(Mathewos 2013)
4	Bahir Dar	dyspeptic patients	2009-2013	Serology	2,733	41.6	(Workineh and Andargie 2016)
5	Addis Ababa	dyspeptic patients	2010-2011	Serology	212	57.08	(Teka 2016)
6	Hawassa	symptomatic patients	2012-2013	Serology	408	83.3	(Tadesse 2014)
7	Debretabore	dyspeptic patients	2013	Serology	209	72.2	(Abebaw 2014)
8	Butajira	dyspeptic patients	2013	SAT	401	52.4	(Kibru, Gelaw et al. 2014)
9	Mekanasalem	Symptomatic adult patients	2015-2016	Serology	363	70.25	(Seid 2018)
10	Jigjiga	dyspeptic patients	2016	Serology	145	71.0	(Alebie and Kaba 2016)

1.2.2 *H. pylori* and HIV co-infection

HIV infection is a pandemic infection which resulted in an estimated 770,000 deaths in 2018. It was estimated that 66% of the HIV infected individuals in 2017 were from sub-Saharan Africa (WHO 2019). The prevalence of HIV infection and clinical outcomes following HIV infection depends on many different factors. One of the factors which influence the outcome of the infection is co-infection with gastrointestinal bacteria. Studies showed that there is an association between HIV and *H. pylori* infection. It was also shown that HIV infection could be associated with a reduced eradication rate of triple *H. pylori* therapy (Nkuize, De Wit et al. 2015).

There are ongoing discussions concerning the association between HIV and *H. pylori* infection. Some epidemiological studies have shown that *H. pylori* infection is high among HIV infected individuals (Abdollahi, Shoar et al. 2014, Nevin, Morgan et al. 2014, Teka 2016). Other Studies have shown that *H. pylori* prevalence is low among HIV infected patients, especially among those with low $CD4^+$ T cells (Fialho, Braga-Neto et al. 2011, Hestvik, Tylleskar et al. 2011, Sarfo, Eberhardt et al. 2015). No differences in the prevalence of *H. pylori* infection between HIV negative and positive individuals were also reported (Moges, Kassu et al. 2006, Hossein Samadi Kafil, Fatemeh Farahi Jahromi et al. 2011).

Both *H. pylori* and HIV infection are common in Ethiopia. According to the WHO 2018 report, there were nearly 1.2 million people living with HIV/AIDS in Ethiopia. The adult prevalence rate was 2.4% and the incidence rate was 0.29% (WHO 2018). Despite their co-existence, the association between both medically important infections is not well studied in Ethiopia. This study was designed to provide data on the difference in the prevalence of *H. pylori* infection based on the HIV status of participants in Asella, Ethiopia.

1.3 Routine laboratory diagnostic techniques of *H. pylori* infection

There are different clinical and laboratory based diagnostic options to detect *H. pylori* infection. The bacterium can be detected in gastric mucosa samples, saliva, breath, blood, feces and even in urine. The means of detection include direct endoscopy (Miftahussurur and Yamaoka 2016), Culture (Anderson and Wadstrom 2001), Rapid Urease Test (Uotani and Graham 2015), Histology (Lee and Kim 2015), Urea Breath Test (Berger 2002), Stool antigen tests (Korkmaz, Kesli et al. 2013), IgG Antibody blood test (Kato, Furuyama et al. 1999), and Polymerase Chain Reaction (Wang, Kuo et al. 2015).

H. pylori stool antigen tests are the most convenient and effective diagnostic method in the developing world. Two types of stool antigen tests exist for the diagnosis of *H. pylori* infection, one based on enzyme-linked immunosorbent assay (ELISA) and

another on immunochromatography (ICA). Although both types of tests are highly sensitive and specific, ICA-based tests are shown to provide less reliable results than ELISA-based tests (Korkmaz, Kesli et al. 2013). It is also reported that there was a variety on the quality of test kits produced by different companies (Andrews, Marsden et al. 2003, Erzin, Altun et al. 2004, Khalifehgholi, Shamsipour et al. 2013, Hong, Chung et al. 2015).

Utilization of low quality test kits could result in under or over diagnosis of *H. pylori* infection. Controlling the outcome of treatment would also be not accurate. Therefore, it is mandatory to evaluate the quality of test kits utilized in health facilities to ensure accurate diagnosis of *H. pylori* infection and to follow outcome of eradication therapy. No evaluation of *H. pylori* test kit was done in Asella teaching and referral hospital. Therefore, the quality of the test kit was not locally tested. Evaluating the test kit utilized by the hospital was the other aim of this PhD project.

1.4 *H. pylori eradication therapy and drug resistance*

Unlike other gastrointestinal bacteria, it is difficult to eradicate *H. pylori* with monotherapy as it has developed resistance to many antibiotics. Therefore, two or more antibiotics are usually given together with a proton pump inhibitor (PPI) and/or bismuth containing compounds to eradicate the bacterium. The routine clinical treatments are usually triple or quadruple antibiotic therapies (Tian, Yang et al. 2015). Selection of the drugs for *H. pylori* infection regimens are also influenced by diverse reasons involving efficacy, patient tolerance, existing antibiotic resistance, and cost of the drugs.

According to the classic triple eradication therapy 500 mg of clarithromycin, 1000 mg of amoxicillin or 500 mg metronidazole and standard dose of PPI are given twice a day for 2 weeks (Paul, Adimoolam et al. 2017). For patients allergic to penicillin, amoxicillin is replaced by metronidazole. The PPI prevents acid production which is helpful in the healing of damage in gastroesophageal tissue (Fass 2012). Triple

therapy regimens have shown better eradication rates than dual therapy and longer length of treatment (14 days versus 10 days) resulted in better eradication rates (Sánchez-Delgado, García-Iglesias et al. 2012). Similarly, the quadruple therapy comprising bismuth substrate, PPI, metronidazole and tetracycline had better eradication rate than the standard triple therapy (Adjéka Stanislas Doffou, Koffi Alain Attia et al. 2015).

Reports have been showing low efficacy of the triple eradication therapy and some countries have already adopted quadruple therapy to overcome the resistance (Jaka, Rhee et al. 2018, Savoldi, Carrara et al. 2018). These low cure rates have been linked to the rise in *H. pylori* primary resistance to clarithromycin (Graham and Fischbach 2010). High Clarithromycin resistance has been observed in Japan and Italy (30%), China (50%) and Turkey (40%) (Thung, Aramin et al. 2016). In West Africa three patterns of first-line 7-day triple therapy based on combining a PPI, and 3 types of antibiotics: omeprazole, amoxicillin, clarythromycin and metronidazole were tested in a randomized clinical trial and the overall *H. pylori* eradication rate was 22.3% (Adjéka Stanislas Doffou, Koffi Alain Attia et al. 2015). It was also found that *H. pylori* eradication is lower among HIV co-infected patients compared to HIV negative individuals (Nagata, Sekiguchi et al. 2015, Nkuize, De Wit et al. 2015).

According to the Ethiopian standard treatment guideline for primary hospitals, all patients with *H. pylori* associated peptic ulcer disease should be treated with the standard eradication therapy (Amoxicillin 1g, Clarithromycin 500mg, Omeprazole 20mg P.O. BID for 7 - 14 days)(Drug Administration and Control Authority of Ethiopia 2010). The combination containing metronidazole instead of Amoxicillin is also recommended by the national treatment guideline for *H. pylori* eradication as second option. The efficacy of the triple study is understudied in Ethiopia. An old report by Asrat et al showed that *H. pylori* isolated from patients were resistant to both metronidazole and amoxicillin but not to clarithromycin (Asrat, Kassa et al. 2004). A recent study by Gebeyehu et al showed more than 90 % efficacy of the standard triple therapy (Gebeyehu, Nigatu et al. 2019).

Drug resistance depends on variety of factors and the distribution of resistant bacteria is different in different localities. Therefore it is mandatory that the status of the resistance is assessed at a community level to be able to assess the exiting situation and choose the most effective regime of treatment accordingly. Even though Asella teaching and referral hospital serves for more than 3 million inhabitants in the catchment area and many patients are treated for *H. pylori* every day, there was no assessment done to evacuate the efficacy of the triple therapy. The current study has tried to provide data on the efficacy of the triple therapy in patients from the hospital.

1.5 Effect of *H. pylori* infection on complete blood cells count and liver enzymes

Complete blood cell count (CBC) includes red blood cells and white blood count, percentage of lymphocyte and other leukocyte sub populations, hemoglobin and hematocrit value, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume, red cell distribution width, mean platelet volume and platelet distribution width. CBC count is one of the most common laboratory investigations prescribed to differentially diagnose infections. The combination of RBC indices could indicate bleeding and infections targeting red blood cells or red blood cell contents, in which plasmodium infection is best example (White 2018). Whereas the WBC related test results could help to differentiate among bacterial (higher percentage of neutrophils) (Kobayashi, Malachowa et al. 2018), viral (higher percentage of lymphocytes) (Riley and Rupert 2015) and parasitic infection (higher percentage of Eosinophils) (Huang and Appleton 2016).

The lack of standard normal range values and the variability of the test results due to many personal, environmental and type of instrument used to measure the parameters made interpretations of the results difficult. As a result of this fact, CBC count is relatively underutilized and most results are overlooked by physicians (ICSH 1993, Peck Palmer 2013, Wang, Huang et al. 2018). In case of *H. pylori* infection, hemoglobin level and platelet count are routinely utilized test parameters, as the effect of *H. pylori* infection on those parameters is better studied compared to the

other CBC count parameters. The effect of *H. pylori* infection on those hematological parameters is still less understood, as a result there are controversial results on this regard.

It has been shown that *H. pylori* infection is associated with low hemoglobin level (Monzón, Forné et al. 2013, Kibru, Gelaw et al. 2014, Sato, Yoneyama et al. 2015, Taye, Enquselassie et al. 2015, Hudak, Jaraisy et al. 2017, Xu, Cao et al. 2017). Successful eradication of *H. pylori* was also found to improve the value of hemoglobin (Fagan, Dunaway et al. 2009, Huang, Qu et al. 2010, Sapmaz, Basyigit et al. 2016). *H. pylori* infection could result in reduced ascorbic acid secretion and reduced intestinal iron absorption, hypochlorhydria, occult blood loss due to chronic erosive gastritis, and sequestration and utilization of iron by the bacteria (DuBois and Kearney 2005). Sequestration of Iron is achieved indirectly due to the production of Hepcidin. Hepcidin is an iron metabolism controlling hormone mainly produced in the liver in response to inflammation (Kroot, Tjalsma et al. 2011). Hepcidin level is elevated following *H. pylori* infection and the raised hepcidin level prevents absorption of iron by the enterocytes and release of iron from macrophages. This scenario ends up with Sequestration of iron. Normalization of the iron level was achieved by successful eradication of *H. pylori* (Azab and Esh 2013).

In other hand, there are reports which argued that there is no association between *H. pylori* infection and anemia. It was also reported that eradication of *H. pylori* was not able to improve the hemoglobin level (John, Baltodano et al. 2018, Mozhgan Zahmatkeshan, Mehran karimi et al. 2019).Those controversial study findings call up on further studies to understand the effect of *H. pylori* on hemoglobin and other hematological parameters.

Although the mechanism is not yet clearly understood, *H. pylori* eradication was associated with improved thrombocytopenia in a patient with immunologic thrombocytopenic purpura (ITP) (Tag, Lee et al. 2010, Rocha, Botelho et al. 2014, Aljarad, Alhamid et al. 2018). As a result *H. pylori* eradication is considered as a good option for a nonimmunosuppressive treatment in some ITP patients (Emilia, Longo et

al. 2001). A cross sectional study on Ethiopian school children showed that *H. pylori* infection is associated with low platelet count (Baxendell, Walelign et al. 2019).

It was reported that *H. pylori* infection increases neutrophil and monocyte counts. Eradication of the bacteria were also associated with decrease in both parameters (Karttunen, Niemela et al. 1996, Kondo, Joh et al. 2004). This is supported with the fact that the inflammatory response observed in active gastritis is characterized by the infiltration of polymorphonuclear leukocytes (Dixon, Genta et al. 1996). *H. pylori* infection was found to be inversely related to esophageal eosinophilia and *H. pylori* exposure decreased eosinophilic esophagitis (Dellon, Peery et al. 2011, Shah, Tepler et al. 2019). But it was also reported that symptomatic eosinophilic gastritis was cured with *H. pylori* eradication (Papadopoulos, Tzathas et al. 2005). Eradication of *H. pylori* was also associated with decreased liver enzymes among patients with hypertransaminasemia (Shah, Tepler et al. 2019).

Despite the fact that there are some studies done to show the association of *H. pylori* infection with complete blood cell count and liver enzymes among patients in Ethiopia (Lindkvist, Enquselassie et al. 1999, Kibru, Gelaw et al. 2014, Taye, Enquselassie et al. 2015, Baxendell, Walelign et al. 2019), there is no study done to investigate the effect of eradication on complete blood cell count and liver enzymes. One of the aims of this PhD project was to fill this information gap.

1.6 Immunomodulatory effect of *H. pylori* infection

The immune response to *H. pylori* is a versatile group of mechanisms involving responses that are both protective and damaging to the host. The immune response starts with the prevention of microbial invasion through the epithelial tight junctions and through the production of mucus and various antibacterial proteins such as lysozyme. Successful colonization of the stomach lining is achieved if the bacteria is able to evade those innate host protections (Hatayama, Shimohata et al. 2018).
Furthermore, the innate immune response in the gastrointestinal tract can be triggered by pattern recognition receptors (PRR). These receptors recognize

conserved microbial constituents such as flagellin, peptidoglycan and lipopolysaccharide (LPS). PRR such as Toll-like and Nod-like receptors are expressed on epithelial cells as well as on neutrophils (Peek, Fiske et al. 2010).

Although lipopolysaccharide (LPS) is the classical bacterial ligand for TLR4, *H. pylori*-derived LPS has been reported to signal through TLR-2 and has low binding affinity for the TLR4 (Rad, Ballhorn et al. 2009, Smith 2014). Recognition of the bacteria directly triggers the recruitment of neutrophils which are responsible to induce the inflammatory response to eliminate the foreign organism. The DCs activation by *H. pylori* is mediated largely by the TLRs (Rad, Ballhorn et al. 2009).

Local inflammatory processes in the gastric mucosa are followed by extensive immune cell infiltration of lymphocytes and monocytes, along with significantly increased expression of IL-1, IL-8, and IL-6 in the gastric antrum. *H. pylori* infection leads to a Th1-polarized response (Moyat and Velin 2014). Other cells that infiltrate the gastric mucosa were Th17 cells, which are $CD4^+$ T cells associated with infections and inflammation. Th17 are induced during both *H. pylori* infection and gastric cancer in the inflammatory process of gastric stroma and may be an important link between inflammation and carcinogenesis (Liu, Zhang et al. 2016). Despite the induction of a comprehensive inflammatory reaction, the immune response is not sufficient to clear the infection (Aviles-Jimenez, Reyes-Leon et al. 2012).

H. pylori positive and *H. pylori* free stomachs are immunologically different, including a far greater population of regulatory T-cells, that is largely absent in *H. pylori* negative subjects (Lundgren, Stromberg et al. 2005a, Goll, Gruber et al. 2007). Treg cells are activated to prevent hyper inflammatory conditions and infection-induced immunopathology. In turn, they may also increase the load of infection and prolong pathogen persistence by suppressing protective immune responses. Treg cells modulate the gastric inflammation induced by *H. pylori* infection, as demonstrated in both experimentally infected mice and humans with natural infection. They may attenuate immunopathology in *H. pylori* infection, possibly by reducing the activation

of IFN-gamma producing $CD4^+$ T cells at the expense of a higher *H. pylori* load in the gastric mucosa (Kandulski, Malfertheiner et al. 2010).

H. pylori infected individuals are found to have higher transforming growth factor (TGF)-β1 and IL-10, which modulate the immune response, than *H. pylori* negative individuals (Bornschein et al., 2010). *H. pylori* positive patients showed lower IL-2 and IFNγ levels, and higher IL-4, IL-10 and TGF-β levels when compared with *H. pylori* negative patients (Dlugovitzky 2005). Prior depletion of Tregs through injection of anti-CD25 antibodies in mice promoted gastritis and reduced bacterial load (Rad, Brenner et al. 2006).

Feldt *et al* could show that *H. pylori* infection modulates the immune system with decreased T-cell activation in ART-naive HIV-positive patients and HIV negative individuals. Analyzing the peripheral blood mononuclear cells of 243 ART–naive patients, *H. pylori* infection was associated with decreased markers of $CD4^+$ T-cell activation ($HLA\text{-}DR^+CD38^+CD4^+$), cell proliferation (Ki67), and immune exhaustion (PD-1). *H. pylori* infection was also associated with higher CD4 cell counts and low viral load (Eberhardt, Sarfo et al. 2015, Sarfo, Eberhardt et al. 2015).

This study was planned to extend the study done by Feldt et al in Ghana implementing interventional cohort study design. Unlike the previous cross sectional study, this study could show the effect of eradication of *H. pylori* on the immune response and other clinical parameters by comparing the data before and after eradication.

1.7 Aims of the study

The immunomodulatory effect of bacterial microbiota, such as *H. pylori*, might potentially provide therapeutic principles for infections and inflammatory disorders. Given the fact that the majority of individuals worldwide are infected with *H. pylori*, such effects would have important implications and would also contribute to the understanding of immunopathology. The possible availability of a *H. pylori* vaccine in the near future further emphasizes the importance of investigating the effects of *H. pylori* infection.

A previous study by Dr. Feldt and the study team has shown that *H. pylori* infection was associated with decreased markers of immune activation and increased $CD4^+$ T cells count. But the study was done based on a cross sectional study design as a result investigation of causality was not possible. We were interested to extend the pervious study in such a way that cause and effect analysis could be possible. In this study the effect of *H. pylori* infection on the immune response and other clinical parameters such as complete blood cells count and liver enzymes was assesses by measuring the parameters before and after eradication of *H. pylori*.

The controversial reports concerning the prevalence of *H. pylori* infection based on the individuals' HIV status and the lack of data on the prevalence of *H. pylori* infection in Asella lead us to the second aim of this project. This study provides data on the difference in the prevalence of *H. pylori* infection among individuals visiting ATRH based on their HIV status. This aim could also provide the first data on the efficacy of *H. pylori* eradication therapy among patients in ATRH as there was no similar study done in the hospital.

Understanding the trend of a disease could provide important clue about the effectiveness of treatment and prevention strategies followed to control the disease. Unfortunately, the trend of *H. pylori* infection was not assessed in the ATRH. To fill this information gap, assessment of five years trend of *H. pylori* infection among dyspeptic patients in ATRH was done by collecting data from the hospital registration

books. To our surprise the positive results reported every year were extremely small and the total prevalence of *H. pylori* infection reported was not comparable with result from other hospitals in the country. We thought that *H. pylori* is under diagnosed in the hospital due to low quality test kits. As a result we have evaluated the quality of the test kit used by the hospital laboratory to insure improved hospital service.

2. Methods and Materials

2.1. Materials

Table 2 Blood collection, PBMC isolation and cryopreservation materials

Items	Specification	Manufacturer
EDTA test tube	366643	BD biosciences, USA
Serum separate tube	367855	BD biosciences, USA
0.2µl Filter	83.1826.001	Sarstedt AG & Co. KG, Germany
Fetal calf serum (FCS)		Gibco Life Technology, Germany
PBS Dulbecco Powder	L 182-50	Biochrom GmbH, Berlin, Germany
Ficoll-Paque Plus Density 1.077g/ml	GE 17-1440-03	GE Healthcare Life Sciences, USA
Dimethyl sulfoxide (DMSO)		Sigma-aldrich, USA
Trypan blue		Sigma-aldrich, USA
50 ml LeucoSep tube(with filter)	227920	Greiner Bio One Intern. GmbH, Austria
50ml Falcon tube (without filter)	227261	Greiner Bio One Intern. GmbH, Austria

Table 3 FACS analysis related materials

Items	Manufacturer
Phosphate-buffered saline (PBS)	Gibco Life Technology, Germany
Compensation beads	eBioscience, Germany
Foxp3 / Transcription Factor Staining Buffer Set	eBioscience, Germany
RPMI with Glutamine	Gibco Life Technology, Germany
FACS-Buffer	500 ml PBS (w/o Ca2+, w/o Mg2+)
	10 g BSA
	2ml EDTA
	500 µl Natriumazid (10 % stock solution)

Table 4 List of Antibodies

Antibodies	Fluorochrome	Clone	Manufacturer
ANTI-HU CD4	eFluor 450	OKT4	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD3	EF506	UCHT1	eBioscience, Frankfurt a. M., Germany
ANTI-HU HLA-DR	PE	L243	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD127	eFluor 660	EBIORDR5	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD25	PE	BC96	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD366 (TIM3)	PE	F38-2E2	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD38	APC-EF780	HIT2	eBioscience, Frankfurt a. M., Germany
ANTI-H FOXP3	PerCP-CYN5.5	PCH101	eBioscience, Frankfurt a. M., Germany
ANTI-H CD161	PerCP-CYN5.5	HP3G10	eBioscience, Frankfurt a. M., Germany
ANTI-M/R KI-67	PE-CYN7	SOLA15	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD279 (PD1)	PE-CYN7	J105	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD69	APC	FN50	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD57	EF660	TB01	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD8A	APC-EF780	SK1	eBioscience, Frankfurt a. M., Germany
ANTI-HU CD196 (CCR6)	PE	R6H1	eBioscience, Frankfurt a. M., Germany
Fixable Viability Dye	eFluor 520		eBioscience, Frankfurt a. M., Germany

Table 5 Clinical chemistry and serological test Kits

Items	Manufacturer
SGOT	Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany
SGPT	Human Gesellschaft für Biochemica und Diagnostica mbH
ALP	Human Gesellschaft für Biochemica und Diagnostica mbH
Urea	Human Gesellschaft für Biochemica und Diagnostica mbH
Createnin	Human Gesellschaft für Biochemica und Diagnostica mbH
Bilurubin_total	Human Gesellschaft für Biochemica und Diagnostica mbH
Bilurubin_direct	Human Gesellschaft für Biochemica und Diagnostica mbH
Hbsag rapid Test Kit	InTec Products Inc., Fujian, China
HCV ab rapid Test Kit	InTec Products Inc., Fujian, China
CRP latex test	Nal von minden GmbH, Germany

Table 6 list of materials for stool collection and processing

Items	Manufacturer
0.9 % Normal saline	Sanscheng Parmaceutical plc, Ethiopia
10% formalin	Uni chem chemical reagents, UK
Diethyl ether	Loba chemie pvt ltd, India
Helicobacter Antigen Quick stool antigen test	GA Generic Assays GmbH, Germany
Serazym *H. pylori* 2nd Gen test ELIASA kit	*VIROTECH* Diagnostics GmbH ,Germany
Wondofo one step *H. pylori* feces test	Guangzhou *Wondfo* Biotech Co., Ltd, China

Table 7 List of instruments

Items	Manufacturer
FACS Conto II	BD Bioscience, San Diego, USA
BD FACSCount™ Flow Cytometer	Becton Dickinson, NJ, USA
Multiskan Spectrum	Termo fischer Massachusetts, USA
BC-3000 Plus auto Hematology Analyzer	Shenzhen Mindray Bio-medical Electronics Co., Ltd., ShenZhen, China
Chemistry Analyzer semi-auto Photometer 5010	ROBERT RIELE GmbH & Co KG, Berlin, Germany
ROTANTA 460/460 R Centrifuge	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Light microscope	ZEISS international, Oberkochen, Germany
Cool-Cell LX	BioCision LLC, CA, USA
Ultra-Low Freezer	Termo fischer Massachusetts, USA
ZetaView multi parameter particle tracking analyzer	Particle Metrix GmbH, Meerbusch, Germany

2.2. Methods

2.2.1. Study area and design

The study was conducted in Asella teaching and referral hospital which is located 175 km southeast of Addis Ababa, the capital city. The hospital is a referral site for more than 3 million inhabitants of Arsi zone. It has more than 250 beds. Besides to the regular outpatient and inpatient departments, the hospital has a HIV clinic and a voluntary counseling and a HIV testing (VCT) clinic. There were more than 8000 HIV positive patients registered in the HIV clinic. The study participants were recruited from the HIV clinic and VCT clinics of the hospital. Sample collection and some part of the sample analysis were done in the Hirsch Institute of Tropical Medicine (HITM), a research institute established by Heinrich Heine University Düsseldorf in collaboration with Arsi University. Blood and stool samples were then exported to Germany and further laboratory analysis were done at the Heinrich Heine University Düsseldorf.

A Prospective, randomized interventional study design was implemented in such a way that HIV positive patients from the HIV clinic and HIV negative participants from

the VCT clinic were screened for *H.pylori* infection and randomly assigned to the eradication therapy. Participants were then followed for one year to investigate the effect of eradication on the immune response and other clinical parameters in a case control approach.

2.2.2. Sampling techniques

2.2.2.1. Sample size determination and sampling techniques

Since data on immune alterations after eradication of *H. pylori* infection was not available, sample size calculation were performed based on an assumed effect size d of 0.5, an α error of 0.1 and a power of 0.80, a total sample size of 27 eradicated participants in the HIV negative and the HIV positive group would be sufficient (G* Power Software).

To determine the difference in the prevalence of *H. pylori* infection between HIV positive and HIV negative individuals, 306 HIV positive and 201 HIV negative participants consecutively visited the HIV and VCT clinic, respectively, were tested for *H. pylori* infection using Helicobacter Antigen Quick stool antigen test (GA Generic Assays GmbH, Germany) in HITM. The sample collection period was from March 2017 to June 2017.

Twenty seven *H. pylori* positive participants from the HIV positive and twenty eight *H. pylori* positive participants from the HIV negative group were randomly selected and treated with standard eradication therapy. The eradication therapy is a combination of three drugs; metronidazole 500mg (Lagap Pharma, Switzerland) , clarithromycin 500mg (Bilim Pharmaceuticals, Turkey) and pantoprazole 40mg (1A Pharma GmbH) which were administered twice a day for 14 days. Two treated participants from both groups were excluded from the analysis due to confounding factors. Participants were then followed for one year. Physical examination, blood and stool sample analysis were done at baseline, three, six and twelve months after intervention as described in the study flow chart below (Fig. 1);

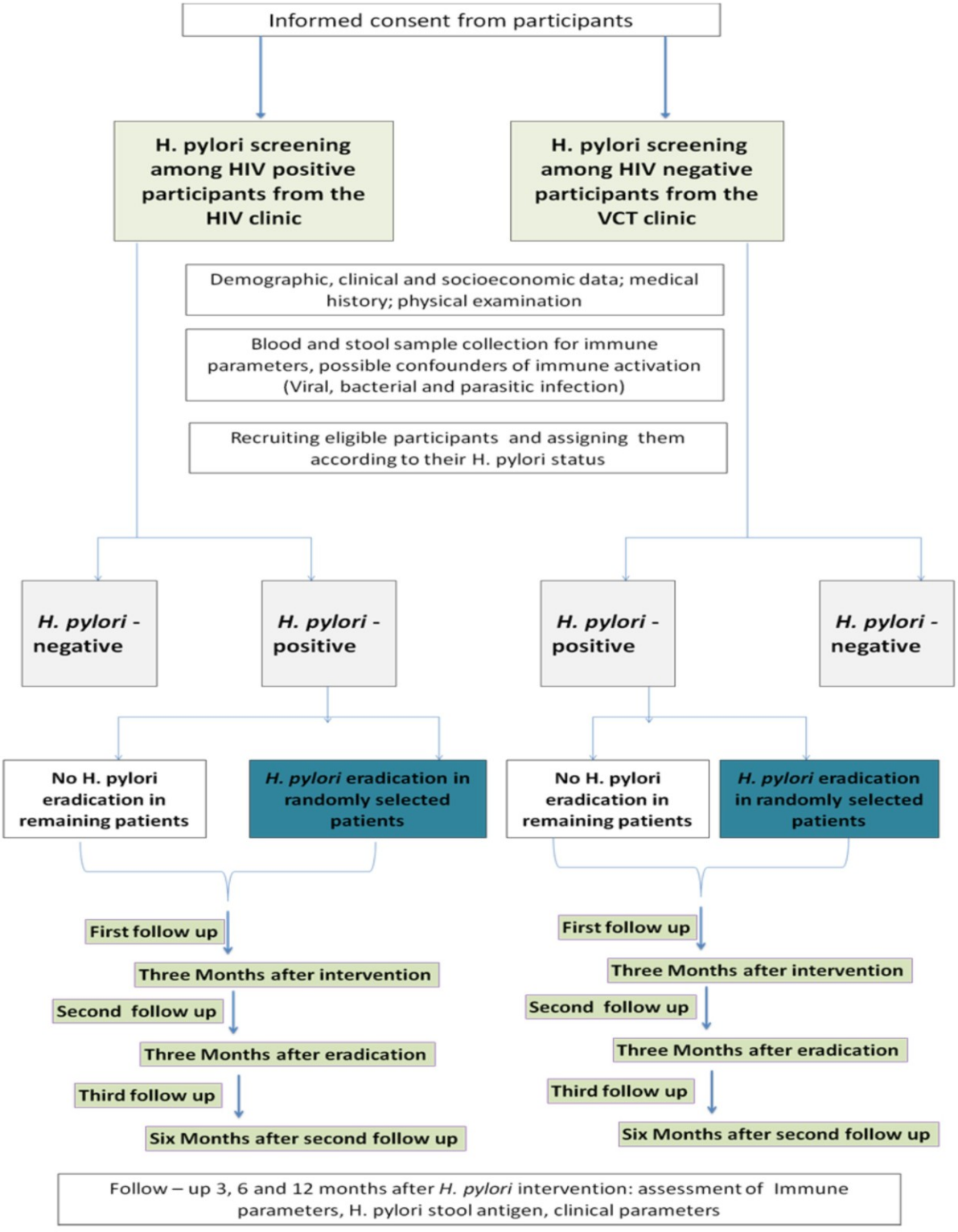


Fig. 1 Flow chart of the study protocol

2.2.2.2. Inclusion and exclusion criteria

A. Inclusion criteria

i. Able and willing to give informed written consent
ii. Age between 18 and 55 years
iii. In HIV-patients: CD4 cells >350/µl

B. Exclusion criteria:

i. Medication possibly influencing immune response (e. g., steroids)
ii. Previous malignancy (e. g. lymphoma)
iii. Known chronic infection (e. g. HBV, HCV, tuberculosis)
iv. CRP positive
v. Parasitic infection
vi. Anti-helminth or antibiotic treatment in the past 6 months
vii. Upper gastrointestinal symptoms (self-report)
viii. Acute infection (e. g. Pneumonia) in the past 6 months
ix. Anemia (Haemoglobin <10g/dl)
x. Pregnancy
xi. Not willing or able to comply with study procedures

2.2.3 Stool sample collection and laboratory analysis

2.2.3.1 Direct wet mount and concentration stool examination

About 5 gram of stool sample was collected from each participant using screw cupped stool containers in HITM. Stool sample was examined microscopically for parasitic infection using direct and formol ether concentrated wet mount techniques.

For the direct wet mount examination, small portion of fresh stool sample was homogenized in 2-3 drop of 0.9 % NaCl on a transparent slide and examined under 10X and 40X magnifications. The stool concentration technique was done following the formol ether concentration technique described by Monica Cheesbrough (Cheesbrough 2005). Shortly, about two gram of stool sample was dissolved in

formaldehyde and mixed with ether after being sieved to avoid large size debris. The formol ether mixture is spined at 3000 rpm for 1 minute. The concentrated sediment was examined under 10X and 40X magnifications. Portion of the stool specimen was preserved in -80 ^{o}C and exported to Germany for further analysis in Heinrich Heine University.

2.2.3.2 *H. pylori* rapid stool antigen test

All stool samples were screened for *H. pylori* antigen using a Helicobacter Antigen Quick stool antigen test (GA Generic Assays GmbH, Germany). The test was done according to the manufacturer's instruction. Like other immunochromatographic assay, this test works based on the principle that specific *H. pylori* antigen present on the stool bound with mounted monoclonal antibodies. This combination results in red band due to the reaction with colored conjugates. In this rapid test, 1ml of sample diluent was placed in 1.5 ml eppendorf tube and about 5mm diameter stool portion was dissolved in the diluents. The mixture was allowed to settle for 5 minutes after being shortly shacked. The supernatant were transferred to new eppendorf tube and the test strep was immersed to the supernatant. Result was read five minutes after immersion (GmbH 2014).

2.2.3.3 ELISA for *H. pylori* Ag test

The stool *H. pylori* antigen tests were confirmed using Serazym *H.pylori* 2nd Generation ELISA (Seramun Diagnostica GmbH, Germany) in Germany. Treatment outcomes of the triple therapy were also done by ELISA stool antigen test. For this purpose about 2-3 gram of fresh stool was preserved and stored in -80^{o}C and transported in dry ice. ELISA Rapid *H. pylori* test was done according to the manufacturer's instructions. The ELISA test works based on the principle that *H. pylori* antigen from the stool binds to the coated monoclonal antibody on the plate and the enzyme substrate complex will result in the conversion of colorless chromogenic substrate solution (HRP-conjugate) into a blue product until stopped by sulphuric

acid. The optical density (OD) of the solution read at 450 / ≥ 620 nm was directly proportional to the specifically bound amount of *Helicobacter pylori* antigen.

For the ELISA test 100mg of stool sample was diluted with 0.5 ml of diluents. 100µl of diluted sample and controls were pipetted into the plate and incubated for 60 minutes at room temperature. Plates were washed three times manually and 100 µl of CONJ HRP was pipetted in to the plates. Plates were washed three times after 30 minutes incubation period. 100µl of substrate was added and reaction stopped after 10 minutes of incubation period. The OD was read at 450 nm / ≥ 620 nm with a Multiskan Spectrum (Termo fischer Massachusetts, USA). Samples with OD value equal or higher than OD of negative control plus 0.10 were considered to be positive for *H. pylori* antigen(GmbH 2016).

2.2.3.4 Evaluating the quality of rapid *H. pylori* test

To evaluate the quality of the stool antigen test widely used in Ethiopia, Wondfo test kit (Guangzhou *Wondfo* Biotech Co., Ltd, China), randomly selected 137 stool samples were simultaneously test by Wondfo test kit and Serazym *H.pylori* 2nd Generation ELISA. Both tests were done according to the manufacturers' instructions. Sensitivity, specificity, positive predicative value and negative predicative values for the Wondfo test kit were calculated having the results from the ELISA test as a reference.

2.2.4 Blood sample collection and laboratory analysis

About 20 ml of venous blood (15 ml EDTA tube and 5ml serum separator tube) were collected from all recruited participants at baseline. Blood sample were collected during the follow up at three, six and twelve months intervals. Complete blood count was done and peripheral blood mononuclear cells (PBMCs) were isolated from the EDTA blood. Isolated PBMCs and plasma samples were stored in -80 ^{0}C until transported to Germany for further analysis. Liver enzymes, serological screening tests for HBSag, HCV, C-reactive protein were done form the serum sample.

The parasitological stool examination, serological tests and clinical chemistry analysis were basically intended to rollout confounding factors such as co-infections and chronic diseases which can influence the immune response.

2.2.4.1 Complete blood count

After the written consent was obtained and the socio-demographic data was collected, 15 ml of venous blood was taken from 280 participants. Hematological parameters such as WBC count, differential white cell count, RBC count, Hemoglobin, Hematoctit, MCV, MCH, MCHC, and platelet count were determined using an automated haematology analyzer Mindray BC300 (Mindray Biomedical Electronic Corporation limited, China). The laboratory analysis was done following the manufacturer's instruction and the laboratory standard operational procedure.

2.2.4.2 Liver enzymes test

About 5ml of venous blood were collected with serum separator tube in addition to the 15ml EDTA blood. Serum was separated by spinning for 5 minutes at 500 rcf speed. Liver enzymes and liver products such as Aspartate Aminotransferase, Alkaline phosphatase, Alanine Aminotransferase, urea, creatinine and bilirubin were analyzed from the separated serum using Chemistry Analyzer semi-auto Photometer 5010 (ROBERT RIELE GmbH & Co KG, Berlin, Germany) following the standard protocol specified by the manufacturer. Preparation of samples for analysis was made based on the insertion kit from Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany.

2.2.4.3 Serological tests for HBSag, HCV and CRP

Rapid serological tests were performed to screen participants for HBsAg, anti HCV antibodies and CRP. Hepatites B virus surface antigen test and anti Hepatitis C virus antibodies tests were done using chromatography rapid test (InTec Products Inc.,

Fujian, China) following the manufacturer's instructions. For the HBsAg test 100µl of serum was added in to the sample well in the test kit and result was read after 15 minutes. For the anti HCV antibody test 10µl of serum was added in to the sample well in the test kit and four drop of diluents was added in to the buffer well. The result was read after 15 minutes. Test kits with two bands were reported as positive.

The CPR test was done based on agglutination principle (Nal von minden GmbH, Germany). The latex reagent was shaken gently and one drop of it was mixed with a drop of serum on the slide and mixed with a mixer. The slide was rocked forth and back for two minutes for observing macroagglutination. Titration test were done from serum which showed agglutination to determine the strength of the reaction(Sorsa 2018).

2.2.4.4 $CD4^+$ T cells count

CD4+ T cell count was done from 70 HIV positive patients' fresh whole blood sample using a FACSCalibur® flow cytometer (Becton Dickinson, USA) in Asella teaching and referral Hospital, Ethiopia. The manufacturer's instruction and the standard operational protocol of the hospital laboratory were strictly followed.

2.2.4.5 PBMC isolation and cry preservation

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood using the Ficoll centrifugation method (Boujtita 2015, Corkum, Ings et al. 2015). EDTA blood was centrifuged for 15 minutes at 1200 rcf at 20 ^{o}C. The plasma was transferred into cryovials and stored at -80 ^{0}C. The remaining blood was resuspend with PBS in a 50ml falcon tube until a total volume of 35 ml. The resuspended blood was transferred into a LeucoSep tube with 15ml Ficoll and centrifuged for 10min at 1200 rcf at 20 ^{o}C without break. All the supernatant with the ring of PBMC over the filter was transfered into a new 50ml falcon tube and washed two times with PBS and centrifuged for 7min at 1000 rcf speed at 20 ^{o}C centrifugation with break. Finally the PBMCs were cryopreserved in 20% of DMSO freezing media (100 µl of DMSO in

400 µl of FSC). Cryovials were initially stored for 24h in a Cool-cell at -80 °C to ensure steady freezing of the PBMCs. Cryo-preserved PBMC samples were transported to Germany in dry ice.

2.2.4.6 Flow cytometry analysis for T cell profile

For the flow cytometry analysis, frozen PBMC were shortly thawed in a 37°C water bath. Cells were washed with 10ml of warm RPMI and PBS two times by spinning for 5 min at 500 rcf at 20 ^{o}C (Nazarpour, Zabihi et al. 2012). The pellets were resuspend with 500 µl PBS and transferred into a 1.5 ml eppendorf tube. 100 µl of the suspension was transferred into a new eppendorf tube as an unstained control. The cells for the unstained control were once more washed with PBS and finally resuspended with 300 ml FACS buffer. The remaining 400 ml PBMC suspension was filled with 600ml of PBS for viability staining.

PBMCs were stained for extracellular and intracellular markers. Staining protocol was adapted from Thermo Fisher Scientific protocols(Scientific 2015). To avoid dead cells from the analysis a viability staining dye was included to each panel. 1 µL of FVD was added into 1 mL of cell suspension and shortly vortexed. Cells were washed 2 times with FACS buffer after a 30 minute incubation at 2–8°C and ready for extracellular staining.

After the second wash, cells were resuspended with 400 µl FACS-Buffer and aliquoted into four eppendorf tubes each with 100 µl volume of suspension. Each aliquot represents one panel (Activated T cells, Exhausted T cells, Treg cells and Th17 cells) according to the table below.

Table 8 Panels for flow cytometry analysis

	405 Laser Violet		488 Laser Blue				633 Laser	
	eFluor 450	eFluor 506	eFluo 520	PE	PerCP-Cy5.5	PE-Cy7	eFluor 660	APC-eFluor 780
T cell Profile (Panels)	450/25	510/50	530/15	585/42	670LP	780/60	660/20	780/60
Panel 1- Activated T cells	CD4	CD3	Fixable Viability Dye eFluor™ 520	HLA-DR	CD8	Ki67	CD69	CD38
Panel 2- T cell exhaustion	CD4	CD3	Fixable Viability Dye eFluor™ 520	TIM3	CD8	PD1	CD57	CD38
Panel 3- T regulatory cells	CD4	CD3	Fixable Viability Dye eFluor™ 520	CD25	Foxp3	PD1	CD127	CD38
Panel 4- Th17	CD4	CD3	Fixable Viability Dye eFluor™ 520	CCR6	CD161	PD1	CD69	CD38

1 µl of antibodies for extracellular markers according to the list of antibodies listed in table 8 were added to each tube and incubate for 20 min at 4°C. Cells were washed with PBS and incubated for 30 minutes at room temperature after resuspending with 200 µL of fixation/permeabilization. Cells from panel 2 and 4 were finally washed and resuspended with 300 µL FACS buffer and ready for FACS analysis.

Cells in panel 1 and 3 were further resuspended with 100 µL of 1X Permeabilization buffer and incubated for 30 minutes at room temperature after adding 1 µl of directly conjugated primary antibody for detection of intracellular antigens according to the table 8. Cells were washed and finally resuspend with 300 µL of FACS buffer for the flow cytometry analysis.

Flow cytometric data were acquired using Canto II (BD Bioscience, San Diego, USA). Acquisition was set to 300,000 cells per sample for all panels. Compensation was

conducted with UltraComp compensation beads (eBioscience, Germany), stained separately with the individual fluorochrome-conjugated monoclonal antibodies used in all samples. The flow data was analyzed in Flowjo version 10.1. Fluorochrome minus one (FMO) was used to gate the T cell subpopulations (Fig. 2 and 3).

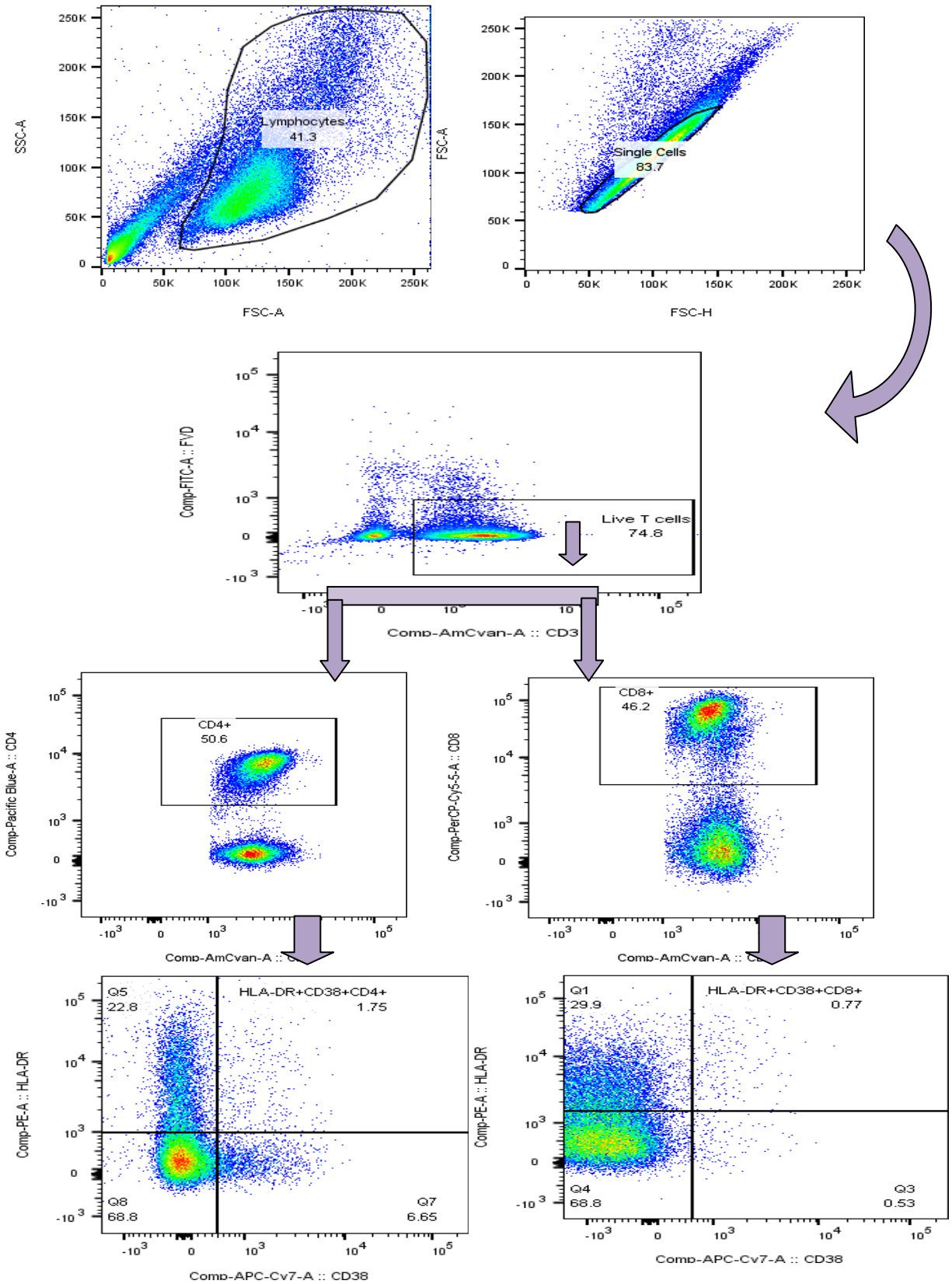


Fig. 2 Gating strategies and flowjo analysis for HLA-DR^{+}CD38^{+} sub-population
(Legend see page 31)

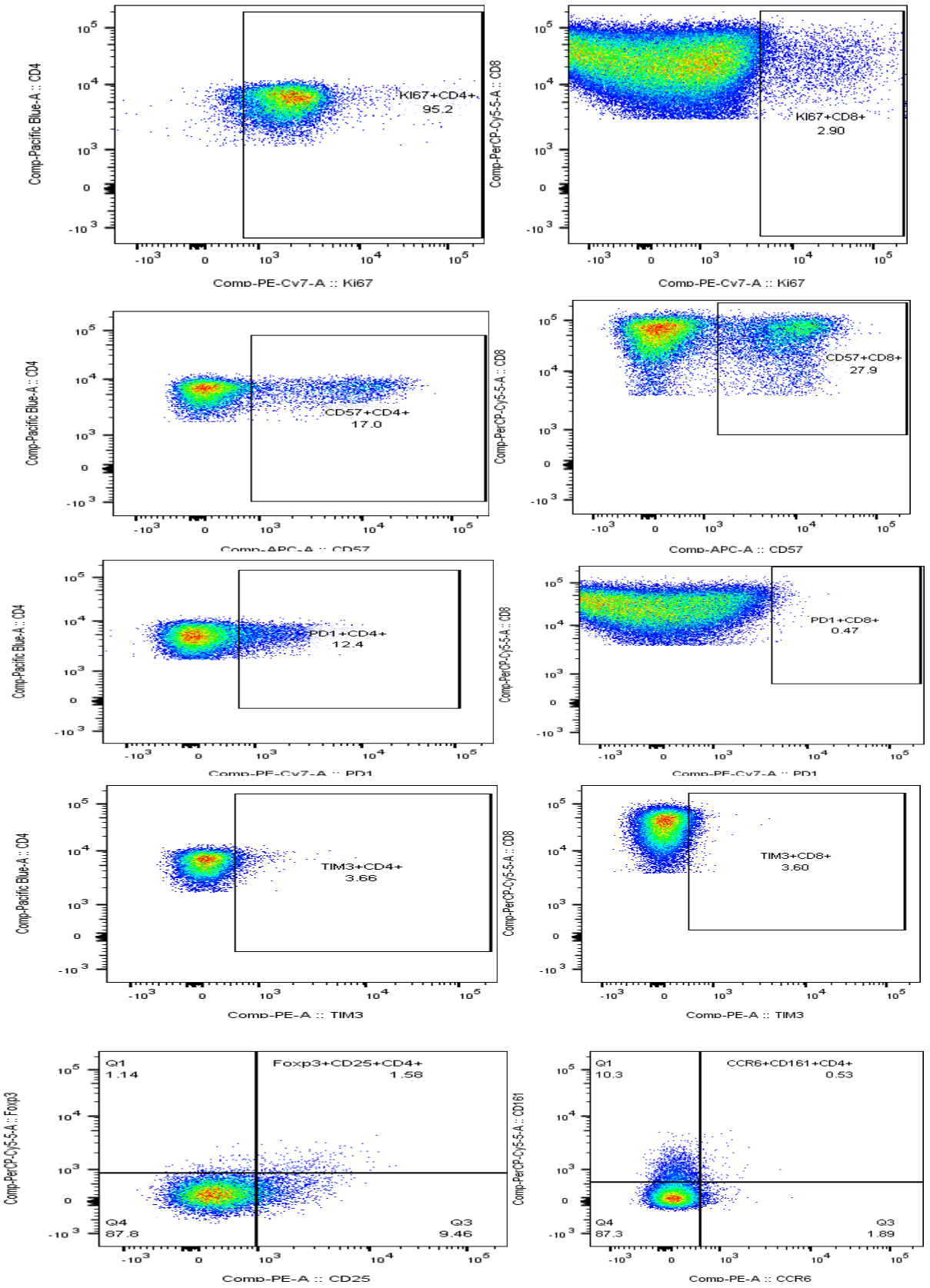


Fig. 3 Gating for T cell subpopulations of interest (Legend see below)

Fig. 2: lymphocytes were gated in an SSC A and FSC A dot plot. Consequently, singlets were gated in FSC A and FSC H dot plot. From the singlets the live T cells were gated using the live-dead staining (FITC- Fixable viability dye) and anti CD3 antibody (Amcyan). Further sub populations were then gated according to the four panels in table 8. In all panels $CD4^+$ T cells were gated from the live T cell population using the pacific blue fluorochrome conjugated anti CD4 and anti CD3 antibodies (Amcyan). From the T cell activation panel $HLA\text{-}DR^+CD38^+CD4^+$ were gated from the $CD4^+$ T cell population using anti HLA-DR (PE) and anti CD38 (APC-Cy7) antibodies. In similar way $HLA\text{-}DR^+CD38^+CD8^+$ were gated from $CD8^+$ (PerCP-Cy5) T cell population.

Fig. 3: From the activation panel $Ki67^+CD4^+$ and $Ki67^+CD8^+$ were gated using anti Ki67 antibody (PE-Cy7) from $CD4^+$ and $CD8^+$ T cell population, respectively. From the exhaustion panel; $CD4^+CD57^+$ (APC), $CD4^+PD1^+$ (PE-Cy7) and $CD4^+TIM3^+$ (PE) T cells were gated from the $CD4^+$ T cell population. Likewise $CD8^+CD57^+$ (APC), $CD8^+PD1^+$ (PE-Cy7) and $CD8^+TIM3^+$ (PE) were gated from the $CD8^+$ T cell population. From the Treg panel $CD25^+Foxp3^+CD4^+$ were gated from the $CD4^+$ T cell population using anti CD25 (PE) and anti Foxp3 (PerCp-Cy5) antibodies. From the Th17 panel $CCR6^+CD161^+CD4^+$ T cells were gated from the $CD4^+$ T cell population using anti CCR6 (PE) and anti CD161 (PerCp-Cy5) antibodies.

2.2.4.7 Microvesicle analysis

Size and concentration of Microvesicles were measured using ZetaView multi parameter particle tracking analyzer (Particle Metrix, Germany). The analysis was done as described by Castoldi et al (Castoldi, Kordes et al. 2016). In short; the plasma isolated from whole blood was centrifuged at 10,000 rcf for 30 min at 4°C to exclude bigger particles and the resulting supernatant was analyzed. 0.5µl of the isolated plasma was diluted with 14ml of ddH_2O and injected in to the analyzer. Number of vesicles was adjusted to be 100 to 300 vehicles per visual field. Those samples with high concentrations were further diluted and concentration of vesicles was calculated based on the dilution factor.

2.2.5 Retrospective data collection for trend analysis

As stated under 2.2.1, retrospective data were collected to assess the trend of *H. pylori* infection among dyspeptic patients from January 1, 2013 until October 30, 2017 in Asella teaching and referral hospital. Data such as *H. pylori* test result, age, sex and the department ordering the *H. pylori* test were collected from the log book of the parasitology laboratory. Laboratory data which were not fully documented were excluded. *H. pylori* test has been done based on the detection of *H. pylori* derived antigens from stool sample using wondofo one step *H. pylori* feces test (Guangzhou *Wondfo* Biotech Co., Ltd, China).

2.2.6 Physical examination and socio-demographic data collection

Physical examination was done for recruited participants at baseline and every follow up by experienced health professionals following a standard protocol (Annex 1) to evaluate the clinical status of the participant. Socio-demographic data was collected by semi-structured questionnaire which was translated in to the local languages, Afan Oromo and Amharic (Annex 2).

2.2.7 Statistical Analysis

Data was analysed by SPSS version 21. Simple frequencies and description analysis were done to summarize mean values and prevalence. Association between variables were analysed by chi-square test. Continuous variables were compared by Wilcoxon signed rank test as the data was not normally distributed. Specificity, sensitively, PPV and NPV of rapid diagnostic test kits were analysed by chi-square. A p-value <0.05 was considered statistically significant. Flow cytometry data were analysed by Flowjo version 10 (Tree Star, Inc. Ashland, OR, USA).

2.2.8 Ethical considerations

Ethical clearances were obtained from the review board of the college of Health Sciences of Arsi University (Reference number-A/U/H/S/C/87/6392), National Research Ethics Review Committee (NRERC) (Reference number-3.10/271/2017) and research and ethical commission of Medical faculty of Heinrich Heine University (Studiennummer-5728). Personnel proficient with the local dialects (Amharic and Afan Oromo) has provided information about aims, purpose and conduct of the study to the participants (Annex 3). Written/thumb printed informed consent (Annex 4) were obtained from participants and was a prerequisite for recruitment.

A unique study identification number were assigned to recruit participants. All data were analyzed with respect to these study numbers and all information was treated confidentially. Only members of the research team had access to these files. Pathological results of the stool and blood tests were reported to the attending physician for proper evaluation of clinical relevance and management according to clinical indication. Treatment costs were covered by HITM. Transport and meal cost of those participants who came for the follow up were covered by HITM.

3. Results

3.1 Prevalence of *H. pylori* infection and associated risk factors

Three hundred six HIV positive and 201 HIV negative participants were tested for *H. pylori* infection a Helicobacter Antigen Quick stool antigen test (GA Generic Assays GmbH, Germany). Two hundred forty one (78.7%) of the HIV positive and 151 (75.1%) of the HIV negative participants were positive for *H. pylori* antigen. There was no statistically significant difference in the prevalence of *H. pylori* infection between HIV positive and HIV negative individuals (78.7 vs 75.1% p= 0.386). The difference in the prevalence of *H. pylori* infection between male and female participants was also not statistically significant (p=0.657). Prevalence of *H. pylori* was higher among participants who were ≤20 years old compared to other age groups. The result of *H. pylori* infection prevalence among different factors is presented in table 9.

Table 9 Prevalence of *H. pylori* among different variables, Asella Teaching Hospital, Ethiopia

Factors		*H. pylori* status		P-value
		Positive N (%)	Negative N (%)	
Sex				0.657
	Male	140 (78.7)	38 (21.3)	
	Female	252 (76.6)	77 (23.4)	
Age group				**0.043***
	≤20	22 (84.6)	4 (15.4)	
	21-30	108 (81.2)	25 (18.8)	
	31-40	134 (70.5)	56 (29.5)	
	>40	128 (81.0)	30 (19.0)	
HIV status				0.386
	HIV positive	241 (78.8)	65 (21.2)	
	HIV negative	151 (75.1)	50 (24.9)	

* Statistically significant

3.2 Socio-demographic data of participants interviewed

One hundred forty individuals from each HIV positive and HIV negative groups were interviewed for socio-demographic data including sex, age, area of residence, educational status, occupation, family size, accessibility of latrine facility and source of water. Majority of the participants 83.6% live in urban area of the region. Above 30% of the participants have a family size more than four which ranges up to ten persons per family. More than 90% of participants have a latrine in their compound. The distribution of *H. pylori* infection among different socio-demographic factors is presented in the table below (Table 10).

Table 10 Distribution of *H. pylori* infection among different socio-demographic factors

Factors		***H. pylori***	
		H. pylori negative N (%)	*H. pylori* positive N (%)
Sex	**Male**	38 (38.8)	62 (62.0)
	Female	71 (39.4)	109 (60.6)
Age group	**≤20**	3 (21.4)	11 (78.6)
	21-30	24 (35.3)	44 (64.7)
	31-40	53 (46.5)	61 (53.5)
	>40	29 (34.5)	55 (65.5)
Residence	**Urban**	95 (40.6)	139 (69.4)
	Rural	14 (30.4)	32 (69.6)
Educational status			
	Illiterate	16 (44.4)	20 (55.6)
	Elementary School	37 (38.5)	59 (61.5)
	Secondary school and above	56 (37.8)	92 (62.2)
Occupation			
	Governmental Employed	31 (32.6)	64 (67.4)
	Self Employed	31 (41.9)	43 (58.1)
	Farmer	14 (37.8)	23 (62.2)
	House wife	17 (45.9)	20 (54.1)
	Student	11 (47.8)	12 (52.2)
	Other	5 (35.7)	9 (64.3)
Family size	**≤ 4**	70 (37.2)	118 (62.8)
	>4	39 (42.4)	53 (57.6)
Having latrine facility			
	Yes	109 (39.1)	170 (60.9)
	No	0 (0.0)	1 (100.0)
Source of drinking water			
	Tap water	108 (38.8)	170 (61.2)
	Other source	1 (50.0)	1 (50.0)

3.3 Efficacy of *H. pylori* eradication therapy

A total of 25 HIV positive patients and 26 HIV negative participants infected with *H. pylori* were randomly selected from the participants tested for *H. pylori* infection and treated with the standard triple therapy. A combination of three drugs; metronidazole 500mg (Lagap Pharma, Switzerland) , clarithromycin 500mg (Bilim Pharmaceuticals, Turkey) and pantoprazole 40mg (1A Pharma GmbH) was administered twice a day for 14 days. *H. pylori* test was done after three months to evaluate the efficacy of the eradication therapy. One participant from the HIV positive group and two participants from the HIV negative group were lost during the follow-up. Fifteen (62.5%) out of the twenty four followed HIV negative participants and nine (37.5%) out of those HIV positive participants were successfully treated from *H. pylori*. The overall eradication rate was 50% (24/48).

In the second follow up, which is three months after the eradication, two participants from HIV positive and one participant from HIV negative participants who were successfully eradicated were positive for *H. pylori*. This could be either re-infection or recurrence. There was no new positive case reported in the last follow up, which is six months after the second follow up. Results of the follow up study are summarized in the following flow chart (Fig. 4)

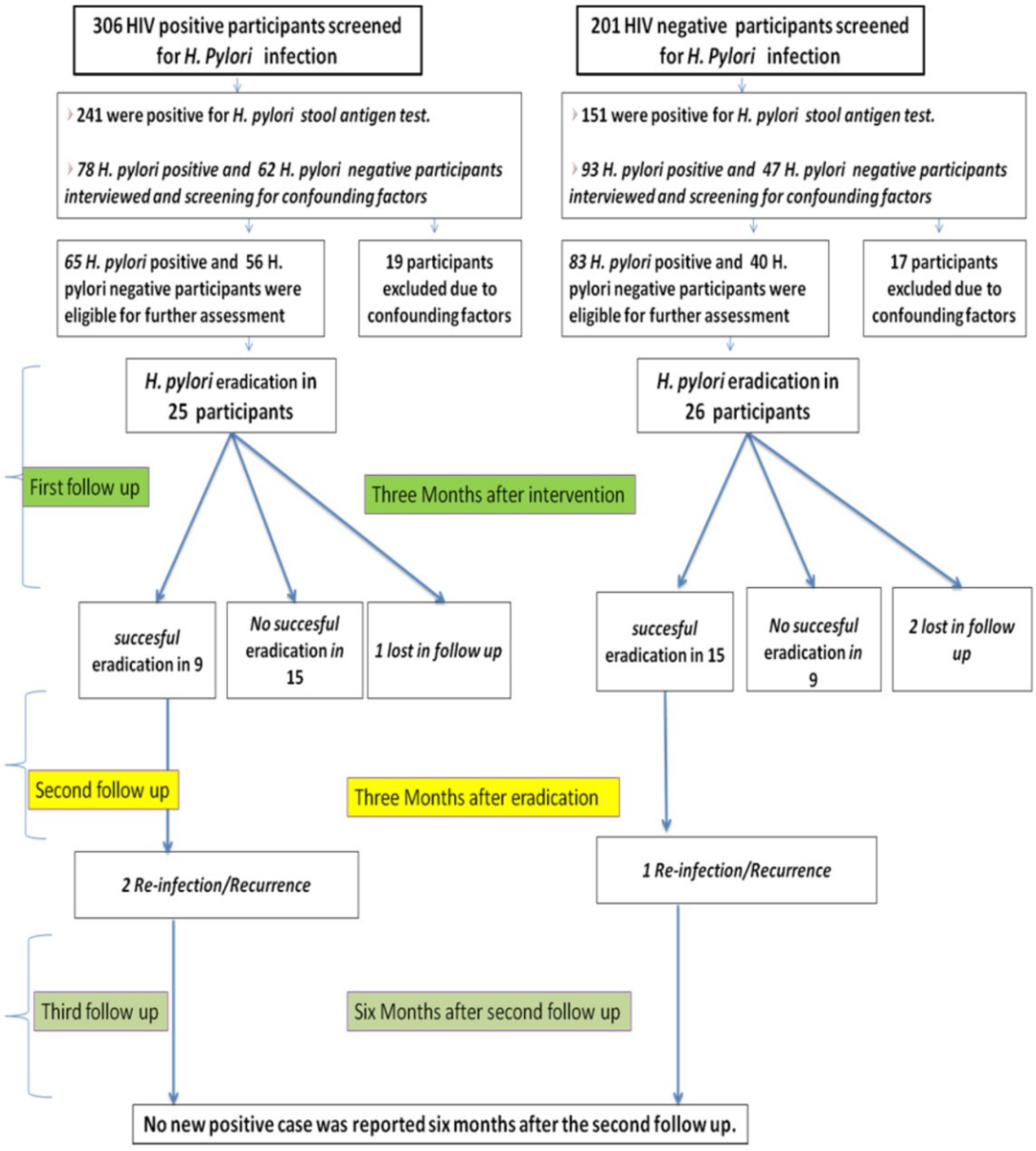


Fig. 4 Flow chart of one year follow up

306 HIV positive individuals who visited the HIV clinic and 201 HIV negative individuals who visited the VCT clinic during the sample collection period were willing to participate in the study and to provide stool sample for *H. pylori* screening. 140 participants from HIV positive group (78 H. positive and 62 *H. pylori* negative participants) and 140 participants from HIV negative group (93 *H. pylori* positive and 47 *H. pylori* negative) were interviewed and screened for confounding factors. 19/140

HIV positive and 17/140 HIV negative participants were excluded due to confounding factors such as infection with parasitic infection, Hepatitis or positive for CRP. 27 HIV positive and 28 HIV negative participants infected with *H. pylori* were randomly selected and treated with standard triple eradication therapy. Two treated participants from each group were excluded due to confounding factors.

3.4 Analysis of complete blood count and liver enzymes

3.4.1 Screening for confounding factors

Stool examination for intestinal parasitic infection, serological screening test for hepatitis infection and C-reactive protein (CRP) were done on 280 participants. Nineteen (6.8%) of the participants were positive for HbsAg and none of the participants was infected with HCV. Seventeen (6.1%) of the participants were reactive for CRP with titer ranging from 6 mg/L up to 192 mg/L. Four (1.4%) participants were infected with intestinal parasites. In which three of them were infected with *Entamoeba histolytica/ Dispar* and one with Taenia species. Participants infected with helminthic parasite, Hepatitis virus and reactive for CRP were excluded from the follow up study to minimize confounding factors.

3.4.2 Baseline analysis of complete blood cells count and liver enzymes

EDTA blood was used to analyze the profile of complete blood cell (CBC) count from 280 participants. Analysis was done on automated hematology analyzer. The analysis includes WBC and percentage of WBC subgroups such as lymphocytes; RBC and related parameters such as hemoglobin, hematocrit, MCH. Platelet count is also part of the CBC count. Liver enzymes and liver products such as AST, ALT, ALP, urea, creatinine and bilirubin were measured with semi-automated spectrophotometer from serum samples.

The mean RBC count, Hgb and hematocrit level, serum AST, ALT, Urea and creatinine concentration of HIV positive female participants were significant lower than their counter part male participants ($p<0.001$) (Table 11).

Table 11 Difference on the range and mean value of complete blood count between male and female HIV positive participants

Parameter	Male N=51		Female N=89		P value
	Range	Mean (±SD)	Range	Mean (±SD)	
White blood cells ($x10^3$/µl)	3.1-9.6	5.7 (± 1.6)	2.2-10.5	5.6 (± 1.8)	0.678
Lymphocyte (%)	16.5-71.4	38.4 (± 12.1)	15.3-64.2	39.4 (± 12.0)	0.618
Mid [Eosinophils, Basophils and Monocytes] (%)	4.8-16.5	10.5 (± 2.7)	4.5-70.2	10.2 (± 6.9)	0.777
Granulocytes (Neutrophils) (%)	21.6-75.4	51.0 (± 12.3)	25.4-76.7	51.0 (± 12.4)	0.982
RBC ($x10^6$/ µl)	3.1-5.4	4.5 (± 0.4)	3.1-5.1	4.0 (± 0.4)	**<0.001****
Hemoglobin (g/dl)	13.2-17.9	15.5 (± 1.0)	9.2-16.7	13.9 (± 1.1)	**<0.001****
Hematocrit (%)	40.4-53.6	47.1 (± 2.9)	31.1-50.6	42.6 (± 3.1)	**<0.001****
MCV (fl)	90.0-138.7	104.4 (± 10.2)	12.9-126.1	103.2 (± 17.6)	0.660
MCH (pg)	28.9-44.6	34.4 (± 3.6)	19.9-42.6	34.4 (± 4.1)	0.964
MCHC (g/dl)	31.6-34.3	33.0 (± 0.6)	28.2-34.6	32.5 (± 0.8)	**0.001***
RDW-CV (%)	12.1-16.4	13.5 (± 0.6)	12.3-121.9	15.0 (± 11.5)	0.359
Platelet ($x10^3$/µl)	130-417	253.6 (± 58.8)	114-570	298.2 (± 79.7)	**0.001***
MPV (fl)	6.9-10.3	7.9 (± 0.7)	6.3-10.5	7.9 (± 0.8)	0.737
PDW	14.8-16.1	15.3 (± 0.2)	14.0-16.2	15.2 (± 0.3)	0.208
PCT (%)	0.11-0.29	0.19 (± 0.04)	0.09-0.46	0.23 (± 0.05)	**<0.001****
AST (IU/L)	11-94	28.6 (± 15.3)	9-43	21.5 (± 6.5)	**<0.001****
ALT (IU/L)	11-94	31.6 (± 17.0)	9-58	21.2 (± 8.3)	**<0.001****
ALP (IU/L)	142-379	215.5 (± 58.0)	99-540	207.7 (± 71.7)	0.511
Urea	13-47	27.7 (± 7.2)	8.2-37.0	22.9 (± 5.9)	**<0.001****
Creatinine	0.5-1.1	0.8 (± 0.1)	0.5-1.2	0.6 (± 0.1)	**<0.001****
Total Bilirubin	0.2-0.8	0.4 (± 0.1)	0.2-1.5	0.4 (± 0.1)	0.090
Direct Bilirubin	0.1-0.4	0.1 (± 0.07)	0.1-0.7	0.14 (± 0.08)	0.108

AST- Aspartate Aminotransferase, ALP= Alkaline phosphatase, ALT= Alanine Aminotransferase, MCH= Mean cell hemoglobin, MCHC= Mean cell hemoglobin concentration, MCV=Mean cell volume, MPV= Mean platelet volume, PDW= Platelet distribution width, RBC=red blood cells, RDW= Red cell distribution width , WBC=white blood cells

* Statistically significance, ** Strong statistically significance

Similarly, HIV negative female participants had significantly lower RBC count, Hgb level and liver enzymes in comparison with their male counter parts (Table 12).

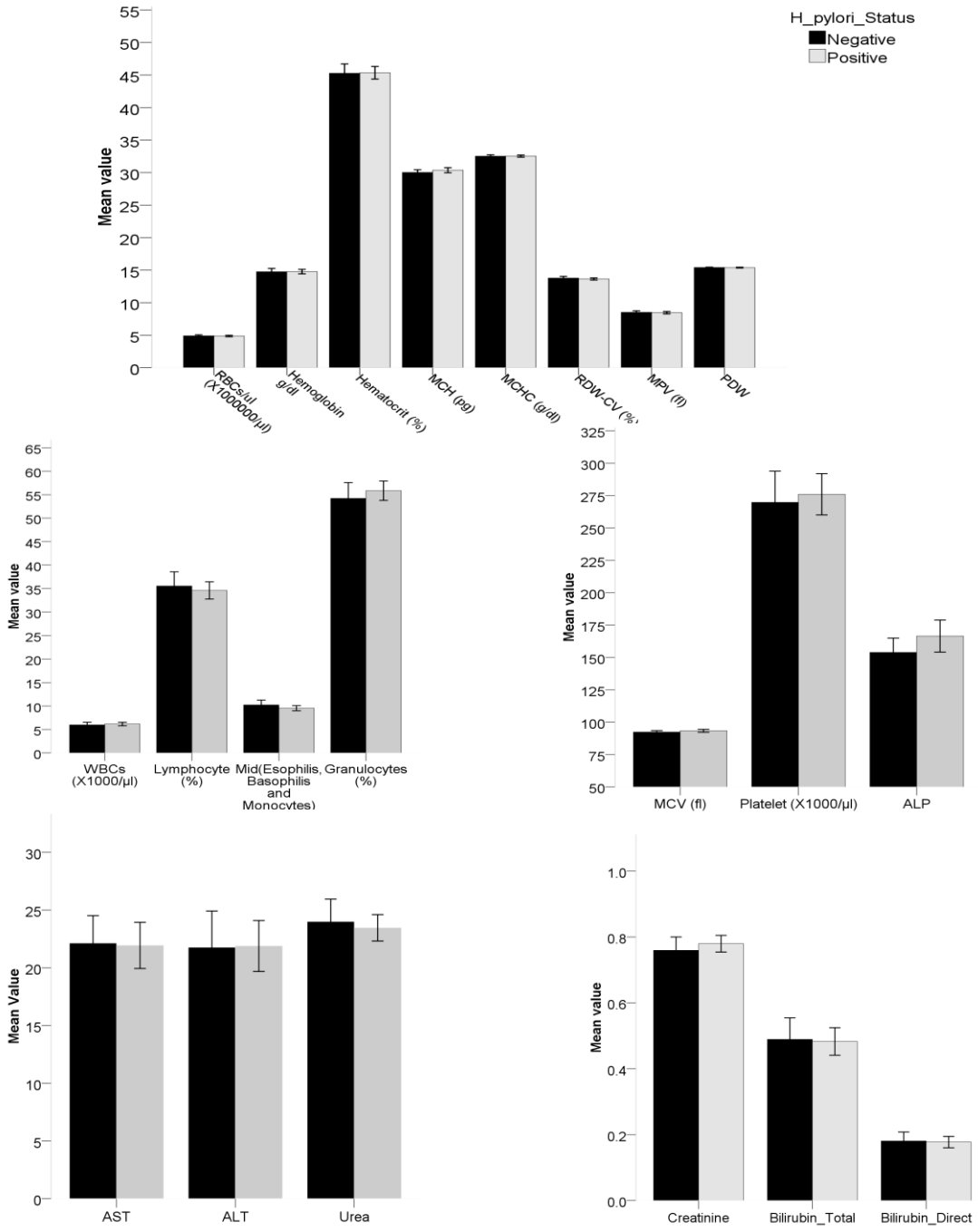
Table 12 Difference on the range and mean value of complete blood count between male and female HIV negative participants

Parameter	Male N=49		Female N=91		P value
	Range	Mean (±SD)	Range	Mean (±SD)	
WBC ($x10^3$/µl)	2.8-12.8	6.01 (± 2.01)	2.1-10.1	6.1 (± 1.7)	0.687
Lymphocyte (%)	12.7-53.2	33.2 (± 8.8)	17.0-57.4	35.8 (± 9.5)	0.128
Mid (%)	4.7-27.4	10.0 (± 3.5)	4.2-18.8	9.6 (± 2.5)	0.424
Neutrophils(%)	36.1-82.1	56.6 (± 10.3)	30.9-74.2	54.5 (± 10.5)	0.258
RBC ($x10^6$/ µl)	3.1-6.2	5.2 (± 0.5)	3.5-5.5	4.6 (± 0.3)	**<0.001****
Hemoglobin (g/dl)	12.3-19.3	16.0 (± 1.5)	10.1-17.5	14.0 (± 1.2)	**<0.001****
Hematocrit (%)	38.9-59.7	49.2 (± 4.0)	33.5-53.7	43.2 (± 3.5)	**<0.001****
MCV (fl)	83.9-125.5	94.1 (± 6.0)	82.1-101.2	92.4 (± 4.1)	0.054
MCH (pg)	26.3-39.6	30.6 (± 1.9)	24.6-33.4	30.0 (± 1.5)	**0.041***
MCHC (g/dl)	30.6-35.3	32.6 (± 0.8)	30.1-34.1	32.4 (± 0.7)	0.205
RDW-CV (%)	12.6-17.2	13.6 (± 0.8)	12.3-16.7	13.6 (± 0.8)	0.907
Platelet ($x10^3$/µl)	143-466	255 (± 58.5)	84-654	284.2 (± 86.3)	**0.036***
MPV (fl)	6.9-9.9	8.5 (± 0.6)	6.6-11.6	8.4 (± 0.9)	0.797
PDW	15.0-16.1	15.5 (± 0.2)	14.6-16.6	15.3 (± 0.2)	**0.001***
PCT (%)	0.13-0.38	0.21 (± 0.04)	0.03-0.45	0.23 (± 0.06)	0.068
AST (IU/L)	3-88	26.0 (± 12.9)	12-41	19.8 (± 5.1)	**<0.001****
ALT (IU/L)	11-92	26.8 (± 13.9)	8-42	19.1 (± 7.1)	**<0.001****
ALP (IU/L)	81-255	159.6 (± 39.3)	53-436	163.7 (± 60.2)	0.673
Urea	14.0-44.0	26.1 (± 6.5)	10.0-38.0	22.3 (± 5.0)	**<0.001****
Creatinine	0.5-1.2	0.8 (± 0.1)	0.5-1.0	0.7 (± 0.1)	**<0.001****
Total Bilirubin	0.2-1.2	0.5 (± 0.2)	0.2-1.2	0.4 (± 0.1)	**<0.001****
Direct Bilirubin	0.1-0.4	0.2 (± 0.07)	0.1-0.6	0.1 (± 0.08)	**0.010***

AST- Aspartate Aminotransferase, ALP= Alkaline phosphatase, ALT= Alanine Aminotransferase, MCH= Mean cell hemoglobin, MCHC= Mean cell hemoglobin concentration, MCV=Mean cell volume, MPV= Mean platelet volume, PDW= Platelet distribution width, RDW= Red cell distribution width , WBC=white blood cells , * Statistically significance, ** Strong statistically significance

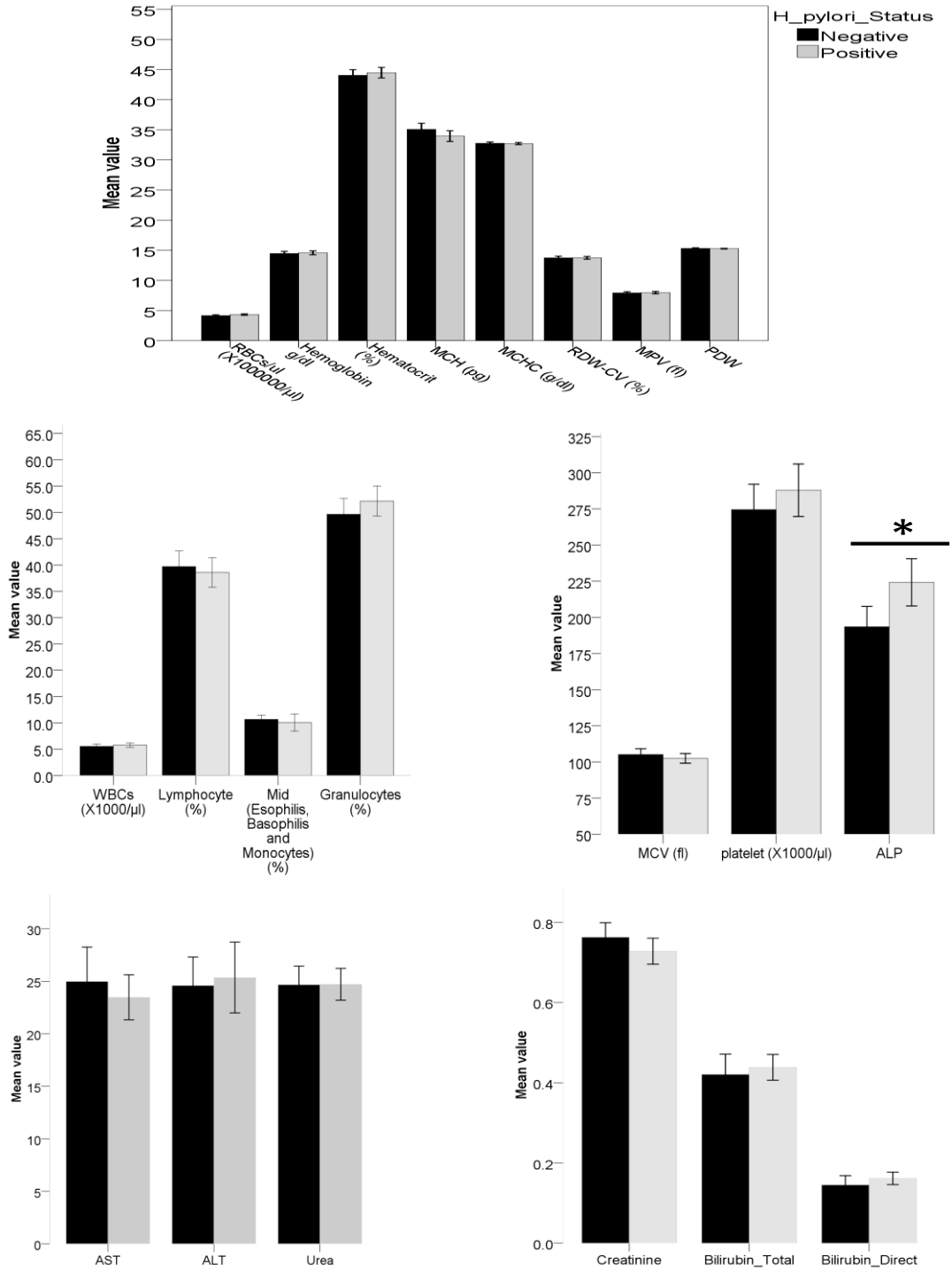
The haematological reference range was calculated from clinically healthy 123 HIV negative participants. The median value and IQR of CBC count were; WBC [6.1 $x10^3$ cells/µl (4.7-7.2 $x10^3$)], absolute lymphocytes [34.4% (29.4-41.7)], mixed cells [9.5% (8.2-10.9)], neutrophils [56.0% (47.7-61.9)], RBC [4.8 $x10^6$ cells/µl (4.5-5.2)], hemaoglobin [14.6 g/dl (13.7-15.9)], hematocrit [44.8 %(42.3-48.3)], MCV [92.4 fl (90.4-96.5)], MCH [30.2 pg (29,2-31.2)], MCHC [32.5 g/dl(32.0-33.1)],RDW [13.6 %g/dl(13.1-14.1)], platelet [267 $x10^3$ (236-313 $x10^3$)], MPV [8.4 fl(7.8-8.9)], PDW [15.4(15.2-15.6)] and PCT [0.22(0.19-0.25)]. The mean and SD value of liver products were; AST [21.3±8.8 U/L], ALT [20.8±9.8 U/L], ALP [161.5±52.1 U/L], urea [23.6±6.0 mg/dl], creatinine [0.76±0.12 mg/dl], total bilirubin [0.48±0.2 mg/dl], direct bilirubin [0.17±0.07 mg/dl].

Association of *H. pylori* infection with complete blood cells count as well as liver enzymes were analyzed for HIV positive and negative groups separately to avoid the confounding effect of HIV infection. The mean WBC count, percentile of neutrophils, platelet count and ALP value were consistently higher among *H. pylori* positive participants both in the HIV positive and HIV negative groups. On the contrary, the mean value of lymphocytes and eosinophils were smaller among *H. pylori* positive participants in comparison to their counterparts in both groups. However the only statistically significant difference was seen on ALP value among the HIV co-infected group (Fig. 5 and 6).



AST- Aspartate Aminotransferase, ALP= Alkaline phosphatase, ALT= Alanine Aminotransferase, MCH= Mean cell hemoglobin, MCHC= Mean cell hemoglobin concentration, MCV=Mean cell volume, MPV= Mean platelet volume, PDW= Platelet distribution width, RBC=red blood cells, RDW= Red cell distribution width , WBC=white blood cells

Fig. 5 Difference in mean values of complete blood cell count and liver enzymes among HIV negative participants based on their *H. pylori* infection status (n=123, 40 *H. pylori* negative and 83 *H. pylori* positive)



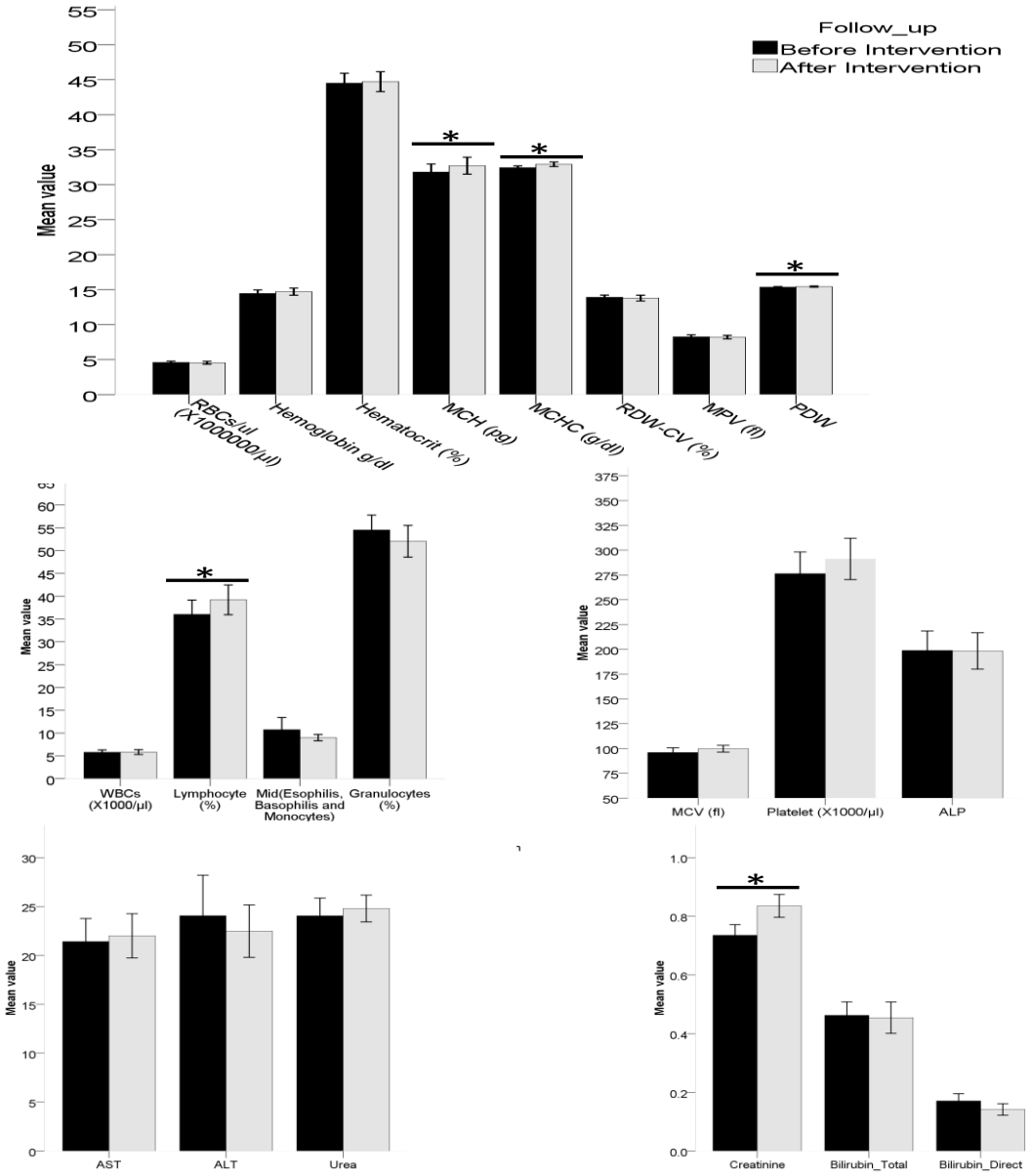
AST- Aspartate Aminotransferase, ALP= Alkaline phosphatase, ALT= Alanine Aminotransferase, MCH= Mean cell hemoglobin, MCHC= Mean cell hemoglobin concentration, MCV=Mean cell volume, MPV= Mean platelet volume, PDW= Platelet distribution width, RBC=red blood cells, RDW= Red cell distribution width , WBC=white blood cells

* Statistically significance (P<0.05)

Fig. 6 Difference in mean values of complete blood cell count and liver enzymes among HIV positive participants based on their *H. pylori* infection status (n=121, 56 *H. pylori* negative and 65 *H. pylori* positive)

3.4.3 Effect of *H. pylori* eradication on complete blood count and liver enzymes

The differences on the mean values of CBC count and liver enzymes before and after the eradication therapy were analyzed to evaluate the effect of intervention. Difference in the participants HIV status and outcome of the eradication therapy were not considered. The mean MCH, MCHC, PDW, lymphocyte and creatinine values were significantly higher ($p < 0.005$) after intervention (Fig. 7).



AST- Aspartate Aminotransferase, ALP= Alkaline phosphatase, ALT= Alanine Aminotransferase, MCH= Mean cell hemoglobin, MCHC= Mean cell hemoglobin concentration, MCV=Mean cell volume, MPV= Mean platelet volume, PDW= Platelet distribution width, RBC=red blood cells, RDW= Red cell distribution width , WBC=white blood cells
* Statistically significance P <0.05)

Fig. 7 Difference in mean values of complete blood cells count and liver enzymes among before and after intervention (n=48, 24 *H. pylori* negative and 24 *H. pylori* positive)

The effect of *H. pylori* eradication on CBC count and liver enzymes was calculated by comparing the median value of the parameters before and after eradication. Comparison was done for HIV positive and HIV negative individuals separately. There was a significant increase in MCV, MCH, MCHC, RDW, PDW values after eradication of *H. pylori* infection among HIV negative individuals (Table 13). Among the HIV co-infected individuals there was a significant increase on the lymphocyte and creatinine value after eradication of *H. pylori* (Table 14). There was a significant increase in the MCH value after intervention among HIV negative individuals not successfully eradicated from *H. pylori* (Table 15). Significant increase in the MCHC, platelete, PCT, creatinine and bilirubin values was seen after intervention among HIV positive individuals not successfully eradicated from *H. pylori* (Table 16).

Table 13 Difference on the complete blood cell count before and after *H. pylori* eradication among HIV negative participants successfully eradicated from *H. pylori*

Parameter	Successfully eradicated HIV negative participants N=15		P value
	Before eradication Median (IQR)	After eradication Median (IQR)	
White blood cells (x10^3/µl)	5.9 (4.6-7.0)	5.4 (4.5-7.6)	0.460
Lymphocyte (%)	35.5 (29.5-42.1)	36.3 (33.2-42.4)	0.201
Mid [Eosinophils, Basophils and Monocytes] (%)	10.0 (8.5-12.2)	9.0 (8.2-10.9)	0.147
Granulocytes (Neutrophils) (%)	51.2 (46.0-62.3)	55.2 (44.6-58.5)	0.776
RBC (x10^6/ µl)	4.9 (4.4-5.1)	5.0 (4.2-5.4)	0.712
Hemoglobin (g/dl)	14.3 (13.0-16.5)	15.0 (13.3-16.7)	0.055
Hematocrit (%)	45.3 (40.7-50.0)	46.5 (39.4-50.9)	0.513
MCV (fl)	93.5 (90.8-96.9)	95.7 (91.5-98.3)	**0.005***
MCH (pg)	30.6 (28.9-31.3)	31.1 (30.0-32.6)	**0.002***
MCHC (g/dl)	32.3 (31.9-32.8)	33.1 (32.2-33.7)	**0.013***
RDW-CV (%)	13.7 (13.2-14.1)	13.1 (12.9-13.7)	**0.038***
Platelet (x10^3/µl)	291.0 (267.0-326.0)	259.0 (252.0-315.0)	0.944
MPV (fl)	8.2 (7.6-8.9)	8.2 (7.7-9.3)	0.888
PDW	15.4 (15.1-15.6)	15.5 (15.2-15.7)	**0.031***
PCT (%)	0.24 (0.23-0.26)	0.23 (0.18-0.26)	0.820
Aspartate Aminotransferase (IU/L)	22.0 (17.0-26.0)	19.0 (15.0-25.0)	0.219
Alanine Aminotransferase (IU/L)	22.0 (16.0-26.0)	19.0 (15.0-25.0)	0.571
Alkaline phosphatase (IU/L)	170.0 (136.0-205.0)	180.0 (128.0-230.0)	0.300
Urea	20.0 (17.0-30.0)	25.0 (20.0-28.0)	0.490
Creatinine	0.8 (0.8-0.9)	0.8 (0.8-1.0)	0.107
Total Bilirubin	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.903
Direct Bilirubin	0.2 (0.1-0.2)	0.1 (0.1-0.2)	0.084

* Statistically significance

Table 14 Difference on the complete blood cell count before and after *H. pylori* eradication among HIV positive participants successfully eradicated from *H. pylori*

Parameter	Successfully eradicated HIV positive participants N=9		P value
	Before eradication Median (IQR)	After eradication Median (IQR)	
White blood cells ($x10^3$/µl)	5.1 (3.7-8.0)	5.8 (3.5-8.0)	0.726
Lymphocyte (%)	35.4 (24.7-44.9)	46.1 (31.8-55.2)	**0.044***
Mid [Eosinophils, Basophils and Monocytes] (%)	9.7 (7.8-10.4)	8.9 (7.9-11.4)	0.678
Granulocytes (Neutrophils) (%)	55.8 (45.7-67.1)	45.9 (33.2-57.8)	0.086
RBC ($x10^6$/ µl)	4.4 (4.0-4.6)	4.1 (3.9-4.9)	0.906
Hemoglobin (g/dl)	14.3 (13.0-15.9)	14.2 (13.2-15.9)	0.514
Hematocrit (%)	43.9 (40.7-47.7)	43.0 (40.8-48.1)	0.953
MCV (fl)	101.7 (94.7-112.4)	103.1 (98.2-112.0)	0.325
MCH (pg)	32.4 (30.5-37.3)	33.6 (32.0-36.9)	0.343
MCHC (g/dl)	32.2 (31.8-32.6)	33.0 (31.7-33.1)	0.314
RDW-CV (%)	14.0 (13.6-15.5)	13.5 (13.2-14.4)	0.109
Platelet ($x10^3$/µl)	262.0 (215.0-341.0)	304.0 (253.5-366.5)	0.515
MPV (fl)	7.8 (7.4-8.4)	7.9 (7.2-8.6)	0.438
PDW	15.4 (15.2-15.7)	15.5 (15.2-15.6)	0.857
PCT (%)	0.19 (0.17-0.26)	0.23 (0.18-0.27)	0.441
Aspartate Aminotransferase (IU/L)	22.0 (19.0-27.5)	28.0 (20.5-35.0)	0.213
Alanine Aminotransferase (IU/L)	19.0 (18.0-38.5)	26.0 (17.0-37.0)	0.674
Alkaline phosphatase (IU/L)	256.0 (205.0-300.0)	220.0 (195.0-310.5)	0.138
Urea	21.0 (19.3-29.3)	25.0 (20.0-30.5)	0.108
Creatinine	0.7 (0.6-0.7)	0.8 (0.7-1.0)	**0.026***
Total Bilirubin	0.3 (0.3-0.5)	0.4 (0.2-0.4)	0.483
Direct Bilirubin	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.564

* Statistically significance

Table 15 Difference on the complete blood cell count before and after *H. pylori* eradication among HIV negative participants not successfully eradicated from *H. pylori*

Parameter	Not successfully eradicated HIV negative participants N=9		P value
	Before eradication Median (IQR)	After eradication Median (IQR)	
White blood cells ($x10^3$/µl)	5.8 (4.5-7.7)	5.7 (4.1-6.8)	0.064
Lymphocyte (%)	36.0 (24.3-41.5)	36.2 (24.4-49.9)	0.767
Mid [Eosinophils, Basophils and Monocytes] (%)	11.1 (8.0-11.9)	8.8 (7.3-11.2)	0.293
Granulocytes (Neutrophils) (%)	52.9 (47.7-66.5)	50.6 (41.5-66.1)	0.722
RBC ($x10^6$/ µl)	5.0 (4.4-5.2)	4.7 (4.6-5.2)	0.859
Hemoglobin (g/dl)	15.1 (13.6-15.8)	14.7 (13.9-16.0)	0.236
Hematocrit (%)	46.7 (42.0-48.5)	44.8 (42.4-48.1)	0.767
MCV (fl)	91.7 (90.7-93.6)	93.4 (91.3-96.5)	0.086
MCH (pg)	29.6 (29.1-30.8)	31.0 (29.9-31.3)	**0.018***
MCHC (g/dl)	32.3 (32.1-33.0)	32.8 (32.4-33.1)	0.373
RDW-CV (%)	13.5 (13.0-14.0)	13.8 (13.2-14.2)	0.343
Platelet ($x10^3$/µl)	266.0 (246.5-306.5)	281.0 (249.0-315.0)	0.722
MPV (fl)	8.3 (7.7-8.9)	8.0 (7.4-8.9)	0.137
PDW	15.4 (15.0-15.5)	15.3 (15.0-15.6)	0.582
PCT (%)	0.20 (0.20-0.24)	0.22 (0.19-0.24)	0.813
Aspartate Aminotransferase (IU/L)	18.0 (14.5-21.0)	18.0 (16.0-22.0)	0.549
Alanine Aminotransferase (IU/L)	19.0 (15.0-24.0)	21.0 (14.5-22.5)	0.632
Alkaline phosphatase (IU/L)	171.0 (123.5-199.5)	174.0 (131.0-200.5)	0.441
Urea	20.0 (18.0-26.5)	25.0 (21.0-28.5)	0.208
Creatinine	0.8 (0.7-0.8)	0.8 (0.7-0.9)	0.518
Total Bilirubin	0.6 (0.35-0.7)	0.50 (0.41-0.65)	0.491
Direct Bilirubin	0.2 (0.1-0.3)	0.2 (0.1-0.25)	0.863

* Statistically significant

Table 16 Difference on the complete blood cell count before and after *H. pylori* eradication among HIV positive participants not successfully eradicated from *H. pylori*

Parameter	Not successfully eradicated HIV positive participants N=15		P value
	Before eradication Median (IQR)	After eradication Median (IQR)	
White blood cells ($x10^3$/µl)	5.3 (4.2-6.5)	6.1 (5.2-6.6)	0.245
Lymphocyte (%)	37.0 (32.2-45.1)	38.6 (29.6-43.9)	0.629
Mid [Eosinophils, Basophils and Monocytes] (%)	8.2 (6.5-10.2)	8.0 (7.1-8.6)	0.932
Granulocytes (Neutrophils) (%)	53.3 (47.4-60.7)	52.8 (46.9-62.4)	0.649
RBC ($x10^6$/ µl)	4.4 (3.6-4.7)	4.31 (3.51-4.83)	0.470
Hemoglobin (g/dl)	12.3 (13.7-46.6)	14.4 (13.2-15.4)	0.222
Hematocrit (%)	43.3 (42.0-45.1)	43.1 (39.9-46.9)	0.551
MCV (fl)	100.8 (95.3-107.2)	104.7 (39.3-115.9)	0.061
MCH (pg)	33.2 (31.3-37.1)	34.6 (31.4-39.3)	0.047
MCHC (g/dl)	32.7 (32.4-33.0)	33.0 (32.4-33.8)	**0.008***
RDW-CV (%)	13.6 (13.5-14.0)	13.5 (13.0-14.0)	0.378
Platelet ($x10^3$/µl)	262.0 (193.0-313.0)	279.0 (253.0-352.0)	**0.016***
MPV (fl)	8.2 (7.7-9.2)	8.0 (7.7-8.9)	0.587
PDW	15.4 (15.1-15.6)	15.4 (15.2-15.6)	0.186
PCT (%)	0.19 (0.16-0.27)	0.23 (0.20-0.29)	**0.013***
Aspartate Aminotransferase (IU/L)	19.0 (13.0-26.0)	22.0 (18.0-27.0)	0.310
Alanine Aminotransferase (IU/L)	22.0 (16.0-28.0)	20.0 (17.0-32.0)	0.727
Alkaline phosphatase (IU/L)	180.0 (160.0-215.0)	198.0 (149.0-219.0)	0.551
Urea	25.0 (19.0-33.0)	24.0 (21.0-28.0)	0.382
Creatinine	0.7 (0.6-0.7)	0.8 (0.7-0.9)	**0.005***
Total Bilirubin	0.4 (0.3-0.5)	0.3 (0.3-0.5)	0.142
Direct Bilirubin	0.1 (0.1-0.2)	0.1 (0.1-0.1)	**0.020***

* Statistically significant

The hemoglobin levels of successfully treated HIV negative individuals were analyzed at baseline, three, six and twelve months after eradication (Fig. 8). There was a

statistically significant increase in hemoglobin value three and six months after eradication of *H. pylori* (Fig. 9).

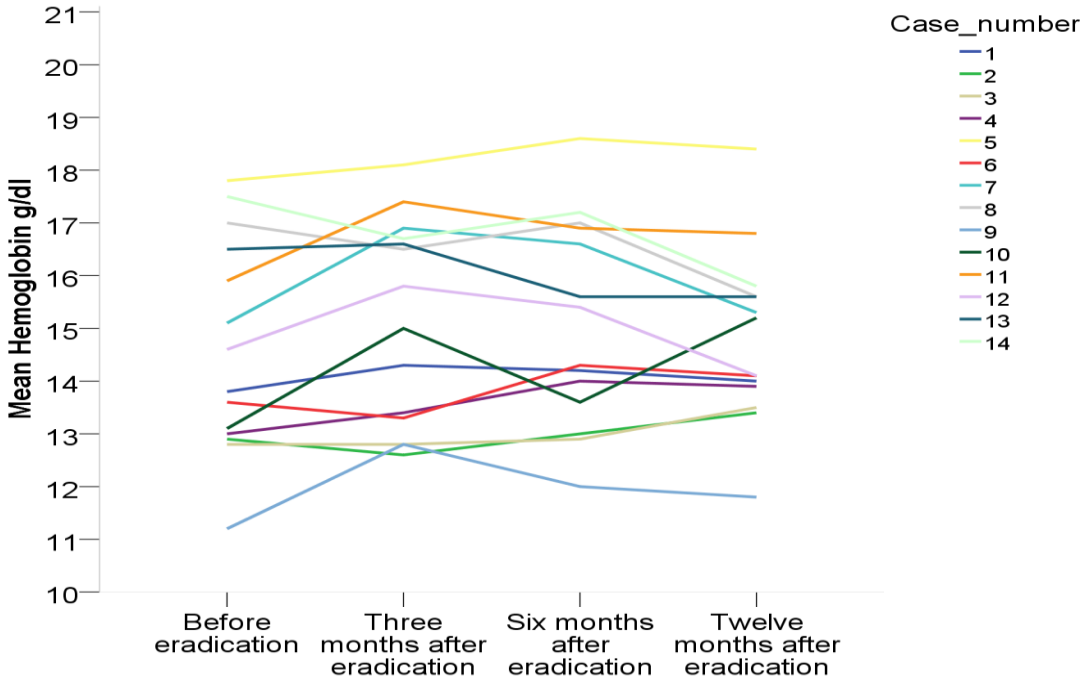
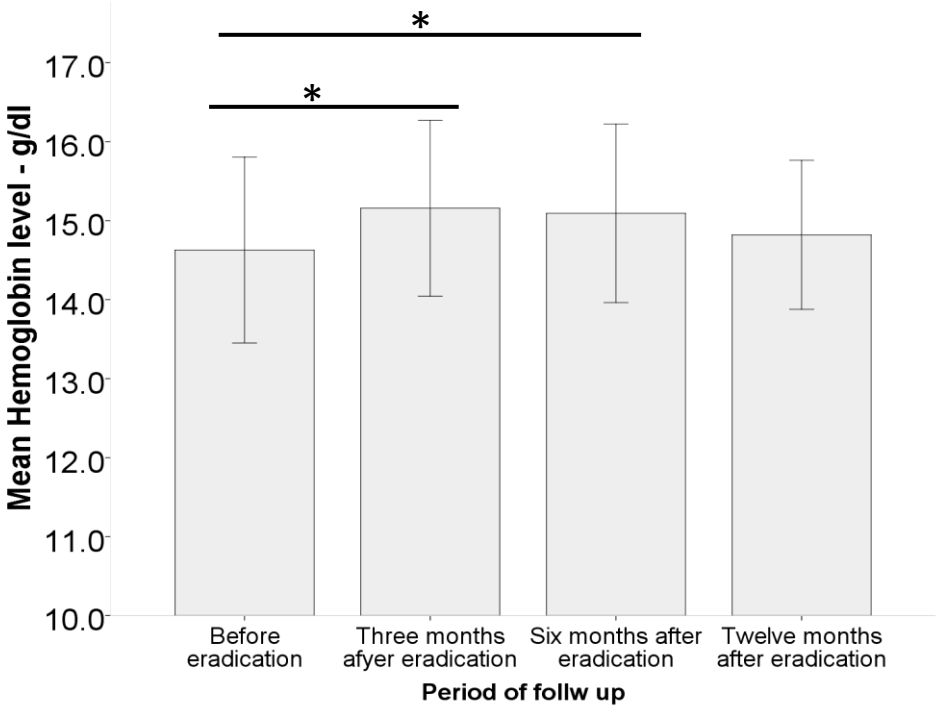


Fig. 8 Trend of Hemoglobin among HIV negative individuals



* Statistically significance (P< 0.05)

Fig. 9 Mean hemoglobin value of repeated measures

3.5 T cell profile analysis

3.5.1 Differences in T cell profiles based on *H. pylori* infection status

The association between *H. pylori* infection and T cell profiles were analyzed by comparing the median values of T cell activation, exhaustion, Treg and Th17 cells markers among *H. pylori* positive and negative groups. The analysis was done for HIV negative and HIV positive groups separately to avoid the confounding effect of HIV on those parameters. In HIV negative participants, there were significantly lower values of T cell activation (HLA-DR$^+$CD38$^+$CD4$^+$), T cell exhaustion markers (PD1$^+$CD4$^+$, PD1$^+$CD8$^+$, TIM3$^+$CD4$^+$, TIM3$^+$CD8$^+$) and higher value T regulatory cells marker (CD25$^+$Foxp3$^+$CD4$^+$) among *H. pylori* infected participants compared to those *H. pylori* negative individuals (Table 17). Similarly, significantly higher value of T regulatory cells marker (CD25$^+$Foxp3$^+$CD4$^+$) was measured among *H. pylori* infected participants compared to those *H. pylori* negative individuals co-infected with HIV (Table 18).

Table 17 Summary of immunological parameters among HIV negative participants according to *H. pylori* infection Status

Variables	**HIV Negative, N= 123**		
	H. pylori Negative N=40 Median (IQR**)	*H. pylori* Positive N=83 Median (IQR)	P-vaue
HLA-DR$^+$CD38$^+$CD4$^+$	2.24 (1.46-4.15)	1.52 (0.87-2.75)	**0.003***
HLA-DR$^+$CD38$^+$CD8$^+$	0.53 (0.29-1.13)	0.47 (0.24-1.01)	0.437
Ki67$^+$CD4$^+$	86.8 (78.25-94.92)	88.8 (72.9-97.2)	0.551
Ki67$^+$CD8$^+$	6.50 (2.86-69.13)	4.87 (2.35-9.17)	0.426
CD57$^+$CD4$^+$	7.43 (3.92-15.02)	7.91 (3.78-11.90)	0.942
CD57$^+$CD8$^+$	38.20 (33.10-49.35)	40.60 (31.80-53.90)	0.680
PD1$^+$CD4$^+$	16.05 (13.15-24.22)	12.70 (8.22-20-.80)	**0.030***

PD1$^+$CD8$^+$	1.12 (0.44-2.88)	0.49 (0.19-1.28)	**0.001***
TIM3$^+$CD4$^+$	2.39 (1.30-4.99)	1.28 (0.66-2.35)	**0.002***
TIM3$^+$CD8$^+$	5.61 (2.71-10.80)	3.13 (1.58-5.72)	**0.007***
CD25$^+$Foxp3$^+$CD4$^+$	1.08 (0.83-1.65)	2.00 (1.23-3.15)	**<0.001***
CCR6$^+$CD161$^+$	0.34 (0.12-1.26)	0.27 (0.06-0.71)	0.136

*Statistically significant association
** interquartile range

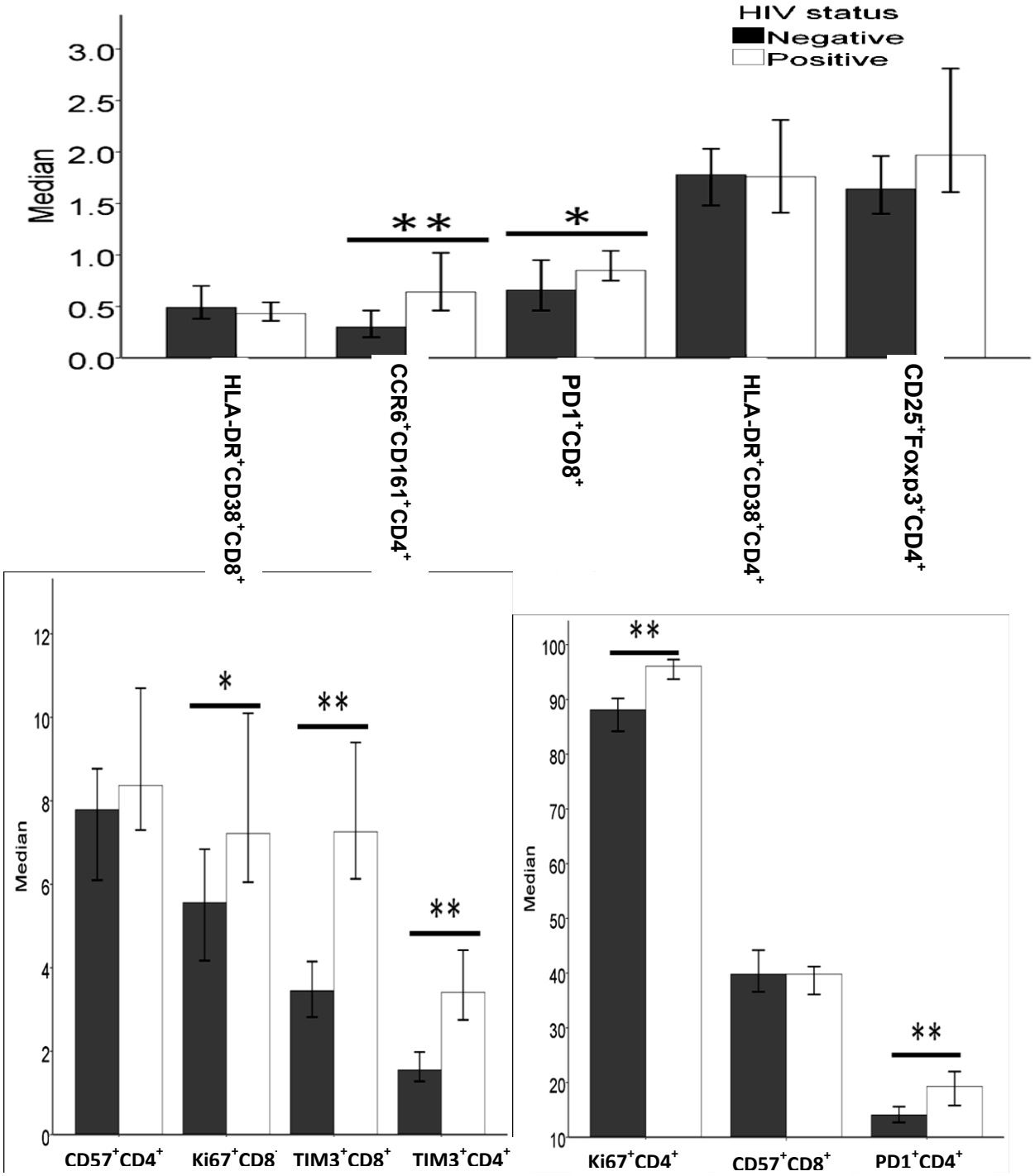
Table 18 Summary of immunological parameters among HIV positive participants according to *H. pylori* infection Status

Variables	**HIV positive, N= 121**		
	H. pylori Negative N=56 Median (IQR)	*H. pylori* Positive N=65 Median (IQR)	P-value
HLA-DR$^+$CD38$^+$CD4$^+$	2.09 (1.08-3.42)	1.66 (0.91-3.44)	0.394
HLA-DR$^+$CD38$^+$CD8$^+$	0.47 (0.14-0.93)	0.39 (0.13-0.95)	0.676
Ki67$^+$CD4$^+$	95.7 (84.42-98.25)	96.5 (85.1-98.45)	0.416
Ki67$^+$CD8$^+$	7.44 (3.59-17.20)	6.79 (4.0-18.6)	0.767
CD57$^+$CD4$^+$	9.13 (5.21-21.20)	7.48 (4.43-15.95)	0.094
CD57$^+$CD8$^+$	39.05 (26.07-45.35)	40.10 (31.35-46.90)	0.631
PD1$^+$CD4$^+$	22.30 (12.65-30.87)	18.60 (13.35-22.80)	0.128
PD1$^+$CD8$^+$	0.84(0.64-1.64)	0.90 (0.55-1.42)	0.919
TIM3$^+$CD4$^+$	3.71 (1.31-7.54)	2.99 (1.72-6.12))	0.631
TIM3$^+$CD8$^+$	8.34 (3.72-13.10)	7.03 (4.38-11.05)	0.423
CD25$^+$Foxp3$^+$CD4$^+$	1.62 (0.78-2.82)	2.90 (1.35-4.52)	**0.009***
CCR6$^+$CD161$^+$	0.83 (0.26-1.54)	0.56 (0.2-1.57)	0.618

*Statistically significant association

Different T cell markers were compared between HIV positive and HIV negative participants irrespective of their *H. pylori* infection status. The proportions of

proliferation markers ($Ki67^+CD4^+$ and $Ki67^+CD8^+$), exhaustion markers ($PD1^+CD4^+$, $PD1^+CD8^+$, $TIM3^+CD4^+$, $TIM3^+CD8^+$) and Th17 cells marker ($CCR6^+CD161^+CD4^+$) were higher among HIV positive participants compared to HIV negative participants (Fig. 10).



* Statistically significant (P<0.05), ** Strong statistically significant (P<0.001)

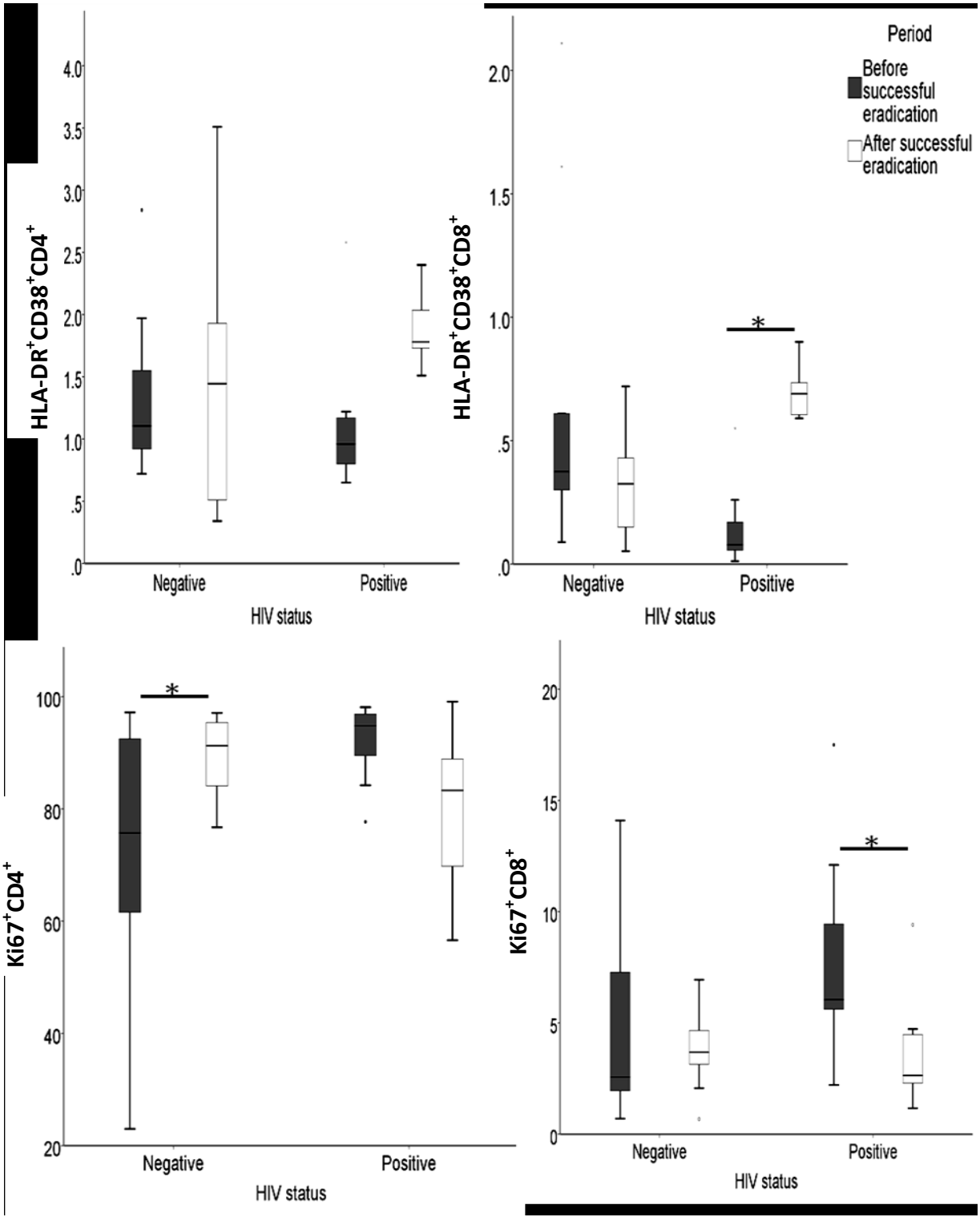
Fig. 10 Difference in T cell sub-population among participants based on their HIV status. (n=244, 123 HIV negative and 121 HIV positive)

3.5.2 Effect of *H. pylori* eradication on T cell profile

$CD4^+$ T cells count was done on seventy HIV positive participants and the mean $CD4^+$ T cell count was 662 ± 205 cells/µl. There was no significant difference in the mean $CD4^+$ cells count between male (N=22) and female (N=48) participants (687± 234 vs 651± 192, p=0.500). $CD4^+$ T cell count was compared between baseline and six months after eradication of *H. pylori* to assess the effect of eradication of $CD4^+$ T cell count among HIV positive patients. Only seven participants who were successfully treated were eligible for the follow up analysis. Even though there was a decreasing trend of $CD4^+$ T cells count after eradication, the difference in mean $CD4^+$ cell count was not statistically significant (702±367 cells/µl vs 668± 302 cells/µl, P value= 0.733).

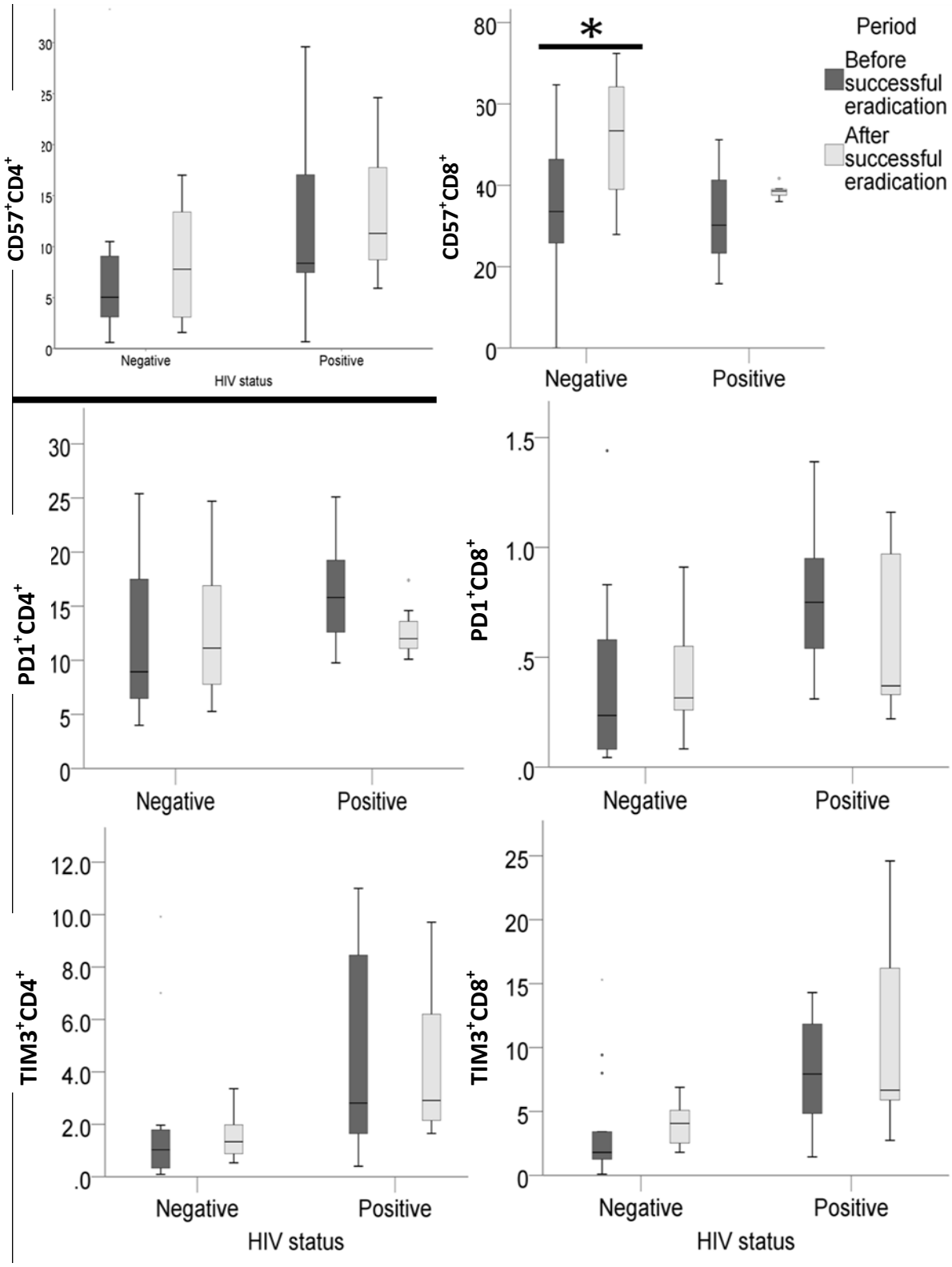
The effect of *H. pylori* eradication on T cell profile was assessed by comparing the values before and after successful eradication. Data from HIV positive and HIV negative groups were analyzed separately. Data from not successfully eradicated participants were also considered to be evaluated if the change in T cell profile would be exclusively dependent on *H. pylori* eradication.

In those groups where *H. pylori* was successfully eradicated, there was a statistically significant increase in $HLA\text{-}DR^+CD38^+CD8^+$cells and reduction of $Ki67^+CD8^+$ cells among HIV positive individuals after eradication (Fig. 11). Increased median values of $Ki67^+$ $CD4^+$ and $CD57^+CD8^+$ cells were seen after *H. pylori* eradication among HIV negative participants (Fig. 11 and 12). There was a statistically significant decrease in Treg cells after eradication in both HIV positive and HIV negative groups (Fig. 13).
In those groups where *H. pylori* was not successfully eradicated, statistically significant decrease in median values of $Ki67^+CD8^+$, $PD1^+CD4^+$ cells and $PD1^+CD8^+$ cells as well as increased value of $CD57^+CD4^+$ cells (Fig. 15 and 16) were observed among HIV positive individuals after intervention. There was a statistically significant decrease in T regs after intervention among HIV negative individuals (Fig. 17). No difference in Th17 cells marker was observed in both groups (Fig. 14 and 18).



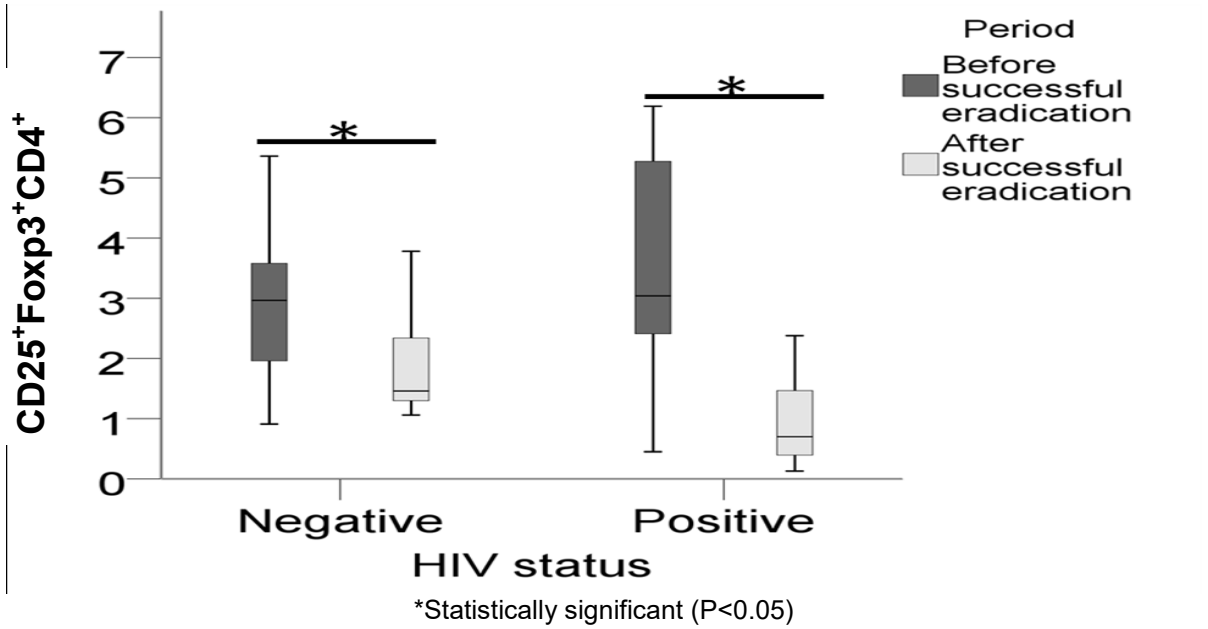
* Statistically significance

Fig. 11 Comparison of proportion of T cell activation markers before and after eradication among participants successfully eradicated from *H. pylori.* (n=21)



* Statistically significance (P< 0.05)

Fig. 12 Comparison of T cells exhaustion markers proportion before and after eradication among participants successfully eradicated from *H. pylori* (n=21)



*Statistically significant (P<0.05)

Fig. 13 Comparison of T regulatory cells proportion before and after eradication among participants successfully eradicated from *H. pylori* (n=21)

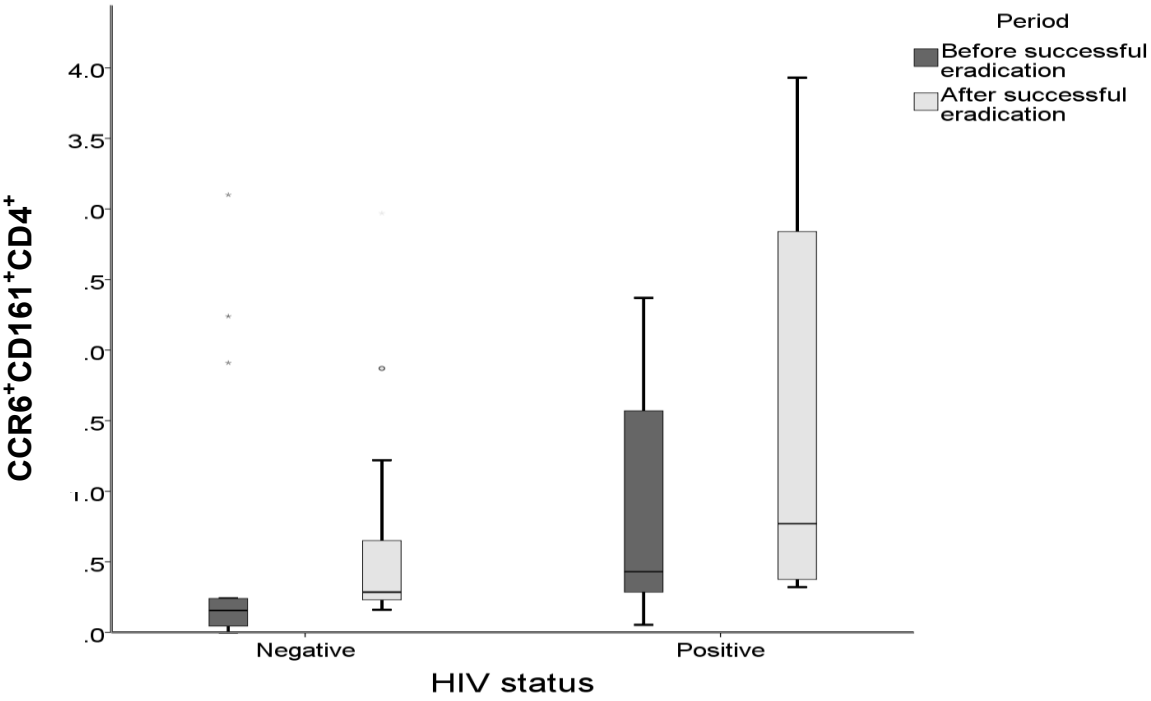


Fig. 14 Comparison of Th17 cells proportion before and after eradication among participants successfully eradicated from *H. pylori* (n=21)

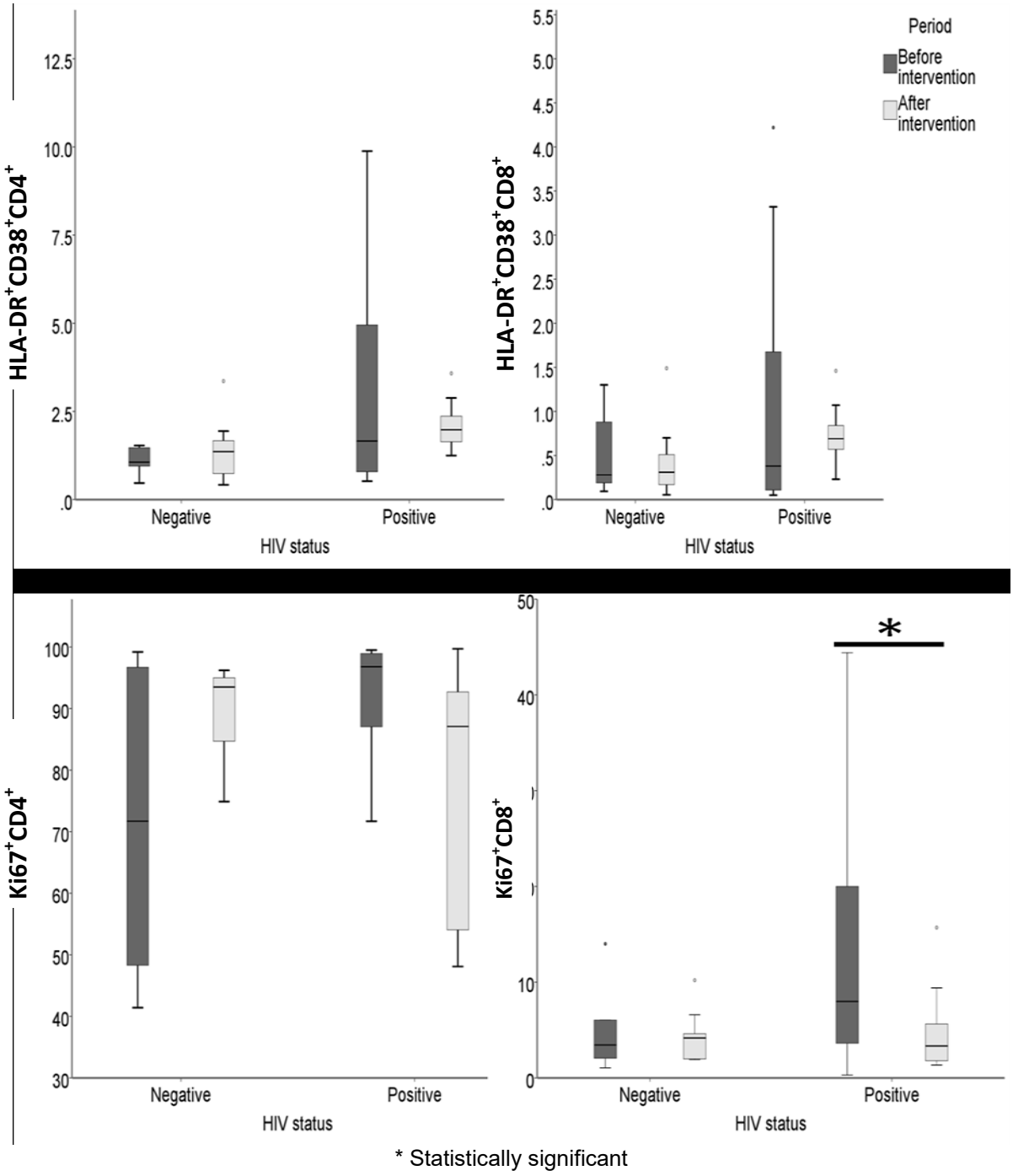
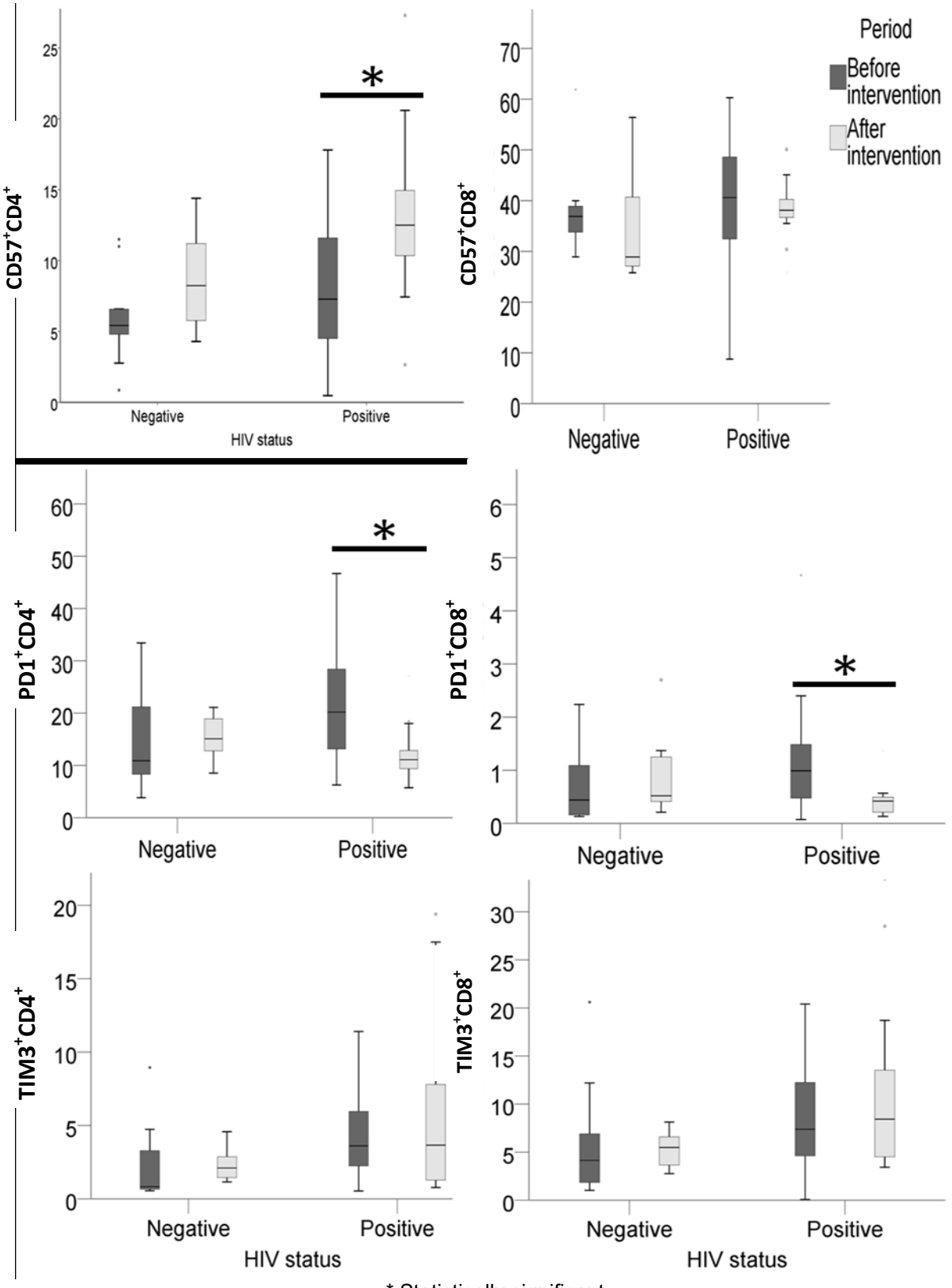


Fig. 15 Comparison of activated T cells proportion before and after intervention among participants not successfully eradicated from *H. pylori* (n=24)



* Statistically significant

Fig. 16 Comparison of T cells exhaustion markers proportion before and after intervention among participants not successfully eradicated from *H. pylori* (n=24)

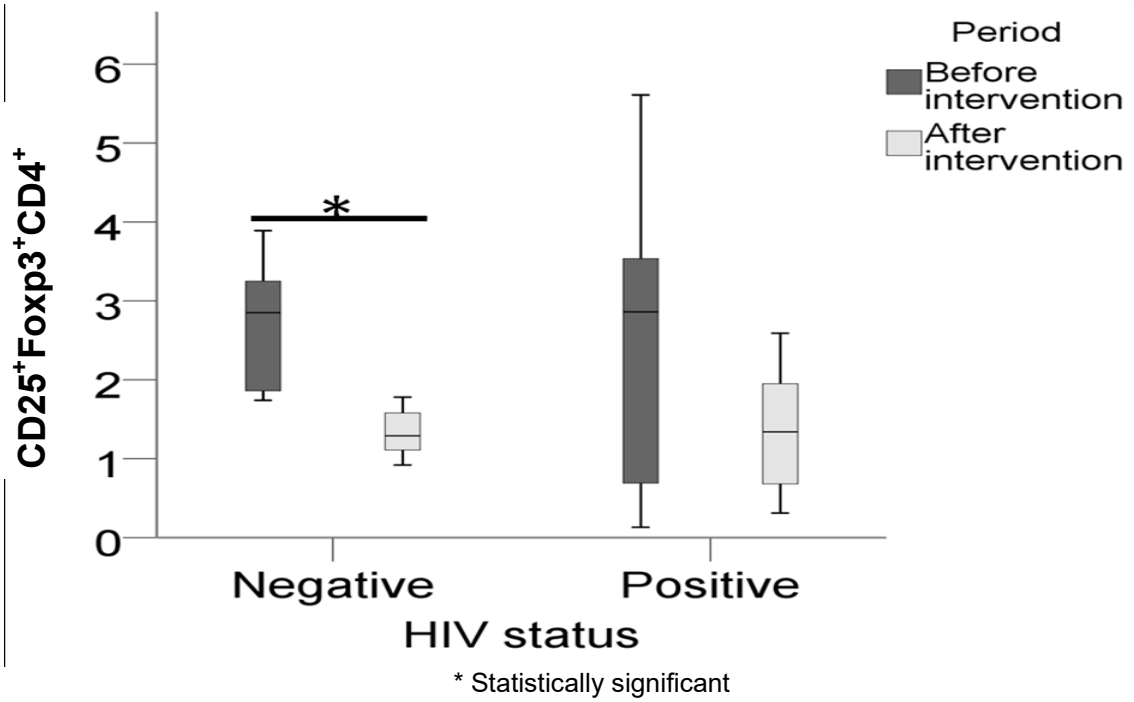


Fig. 17 Comparison of T regulatory cells proportion before and after intervention among participants not successfully eradicated from *H. pylori* (n=24)

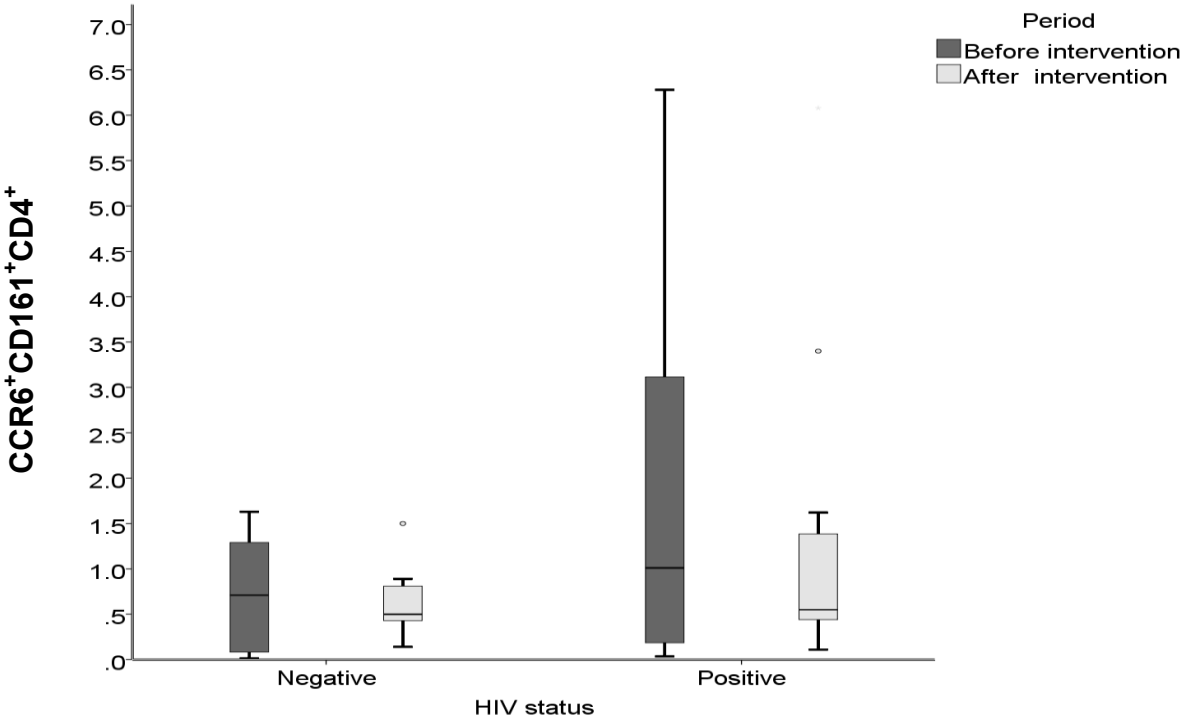


Fig. 18 Comparison of Th17 cells proportion before and after intervention among participants not successfully eradicated from *H. pylori* (n=24)

3.6 Difference in size and concentration of Microvescles among *H. pylori* infected and non infected individuals

Plasma samples from thirty *H. pylori* negative and 76 *H. pylori* positive participants were analysed for the size and concentration of extracellular vesicles. All the participants were HIV negative. There were no statistically significant differences in size and concentration of extracellular vesicles between *H. pylori* infected and non-infected individuals (Fig. 19 and 20). The median size and concentration of extracellular vesicles among *H. pylori* positive and negative individuals were 155.60 [IQR 144,05-163.05]nm Vs 158.75 [IQR 149,40 -169.45]nm (P=0.111); $1.30X10^{12}$ [IQR 8.7 $60X10^{11}$ -$1.60X10^{12}$] vs1.20 $X10^{12}$ [IQR $7.85X10^{11}$ -$1.72X10^{12}$] (P=0.541), respectively.

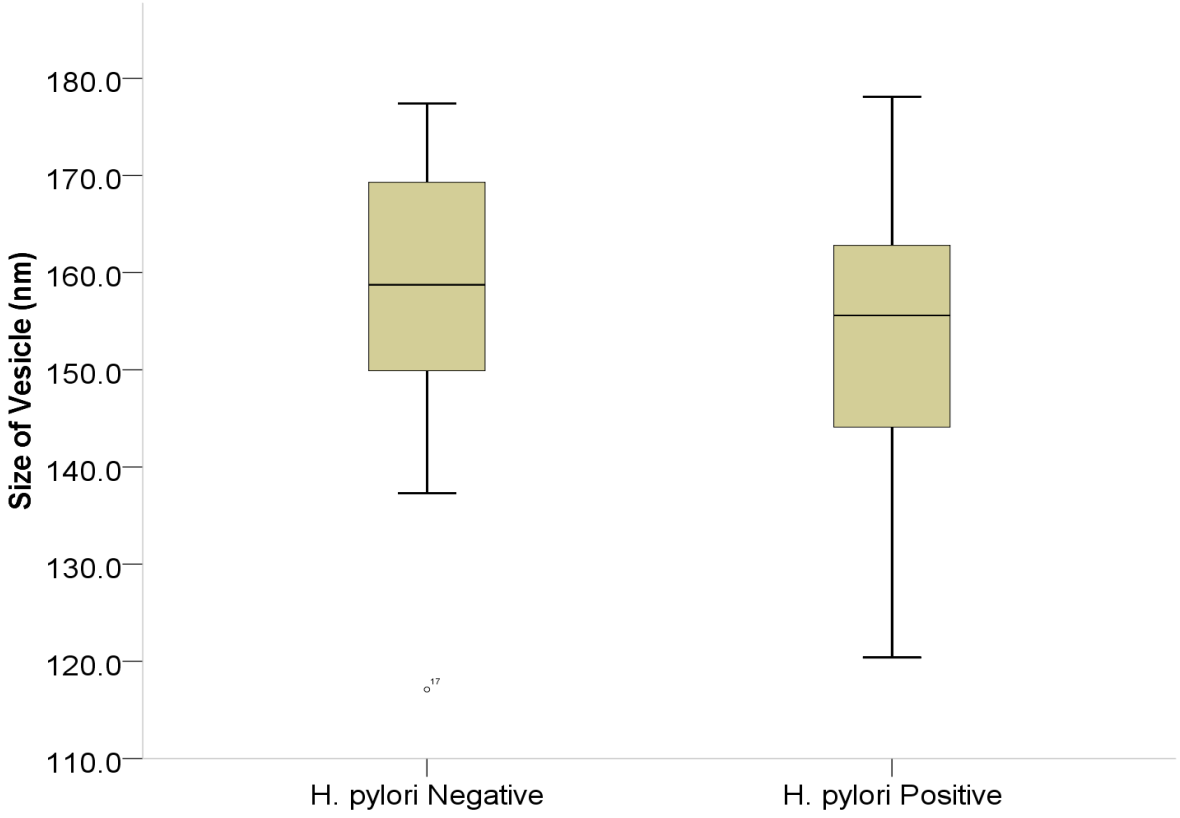


Fig. 19 Difference in size of extracellular vesicles between *H. pylori* positive and negative individuals (n=106)

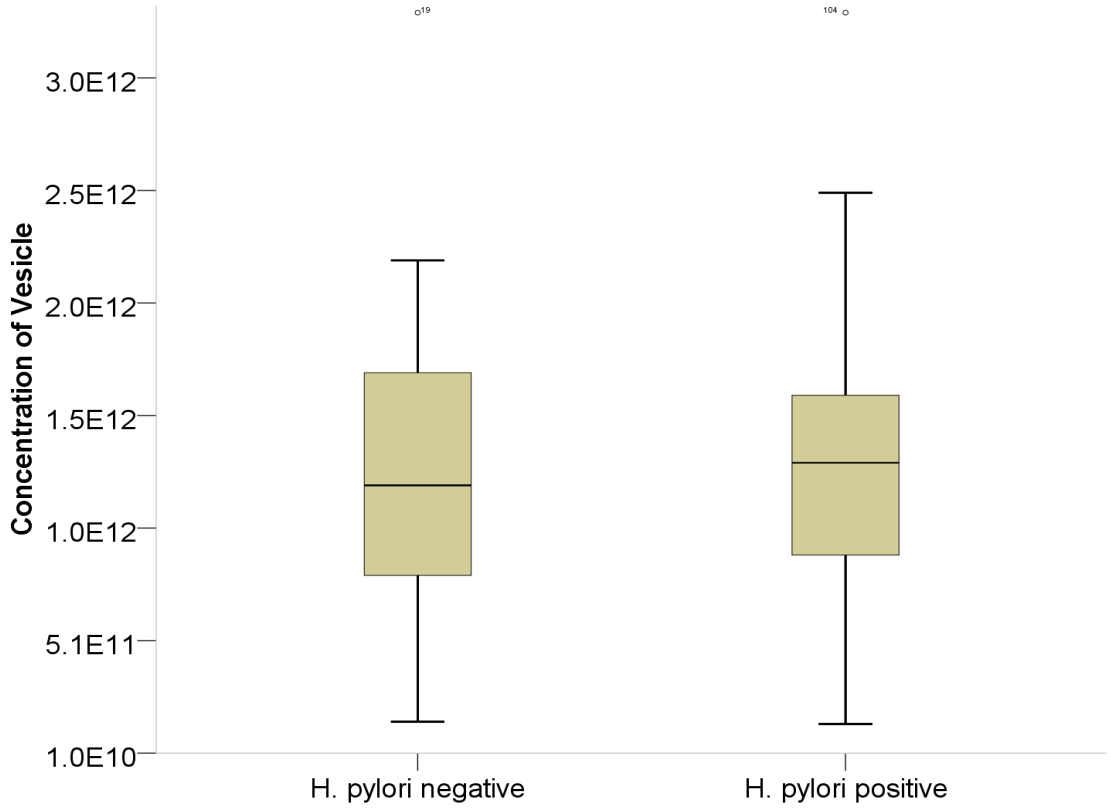


Fig. 20 Difference in concentration of extracellular vesicles between *H. pylori* positive and negative individuals (n=106)

3.7 Five years trend of *H. pylori* infection among dyspeptic patients in Asella teaching and referral hospital

(The data under the sub topic 3.7 is directly adopted from my published article with the permission of the publisher)(Mesfun, Teshome et al. 2018)

3.7.1 Demographic characteristics and prevalence of *H. pylori* among dyspeptic patients

A total of 9493 dyspeptic patients were tested for *H. pylori* infection in the last five years in Asella teaching and referral hospital. Among them, 5956 (62.7%) were females and 3537 (37.3%) were males. The mean age of the study subjects was 33.9 (SD±15.1) years with age ranging from 1 to 96 years. More than half of those dyspeptic patients 5151 (54.3%) were young adults under 30 years (Table 1).

3.7.2 *Prevalence of* H. pylori infection among dyspeptic patients

The total prevalence of *H. pylori* was 15.2% (1444/9493) in which 863 (59.8%) of them were females and 581 (40.2%) were males. There was higher *H. pylori* prevalence among male patients (16.4%) than female patients (14.5%) and this difference in *H. pylori* prevalence between male and female patients were statistically significant (p=0.012). Highest prevalence of *H. pylori* was recorded among dyspeptic patients with age less than or equal to 20 years (18.0%) and lowest prevalence of *H. pylori* infection was recorded among patients older than 60 years (10.9%) (Table19).

Table 19 Prevalence of *H. pylori* infection among dyspeptic patients in Asella teaching and referral Hospital, Ethiopia, 2017

Variable	*H. pylori*		P-Value
	Positive N (%)	Negative N (%)	
Sex			0.012*
Female	863 (14.5)	5093 (85.5)	
Male	581 (16.4)	2956 (83.6)	
Age group			<0.001**
<=20	338 (18.0)	1540 (82.0)	
21-30	482 (14.7)	2791 (85.3)	
31-40	270 (14.9)	1546 (85.1)	
41-50	193 (16.2)	1002 (83.8)	
51-60	99 (13.0)	661 (87.0)	
>60	62 (10.9)	509 (89.1)	
Year			<0.001**
2013	133 (10.6)	1121 (89.4)	
2014	128 (11.1)	1023 (88.9)	
2015	37 (6.1)	574 (93.9)	
2016	634 (16.6)	3184 (83.4)	
2017	512 (19.3)	2147 (80.7)	
Department			<0.001**
Emergency OPD	332 (18.9)	1426 (81.1)	
Gyn OPD	10 (27.0)	27 (73.0)	

Medical OPD	986 (13.6)	6258 (86.4)
ART clinic	57 (30.8)	128 (69.2)
MCH	3 (75.0)	1 (25.0)
Pedatric ward	7 (26.9)	19 (73.1)
Medical ward	38 (20.7)	146 (79.3)
Gyn ward	11 (20.0)	44 (80.0)

* Statistically significance, ** Strong statistically significance

3.7.3 Five years trend of *H. pylori* infection among dyspeptic patients

There was a fluctuating trend of *H. pylori* infection in the last five years, with annual total cases of *H. pylori* ranged from 19.3% in 2017 to 6.1% in 2015. There was statistically significant inter-annual variation of malaria occurrence in the study area (p <0.001). There was a significant incline in the prevalence of *H. pylori* in 2017 compared to 2013 (p <0.012). There was successive increase in prevalence from 2013 onwards with an exceptional decline in 2015 (Fig. 21).

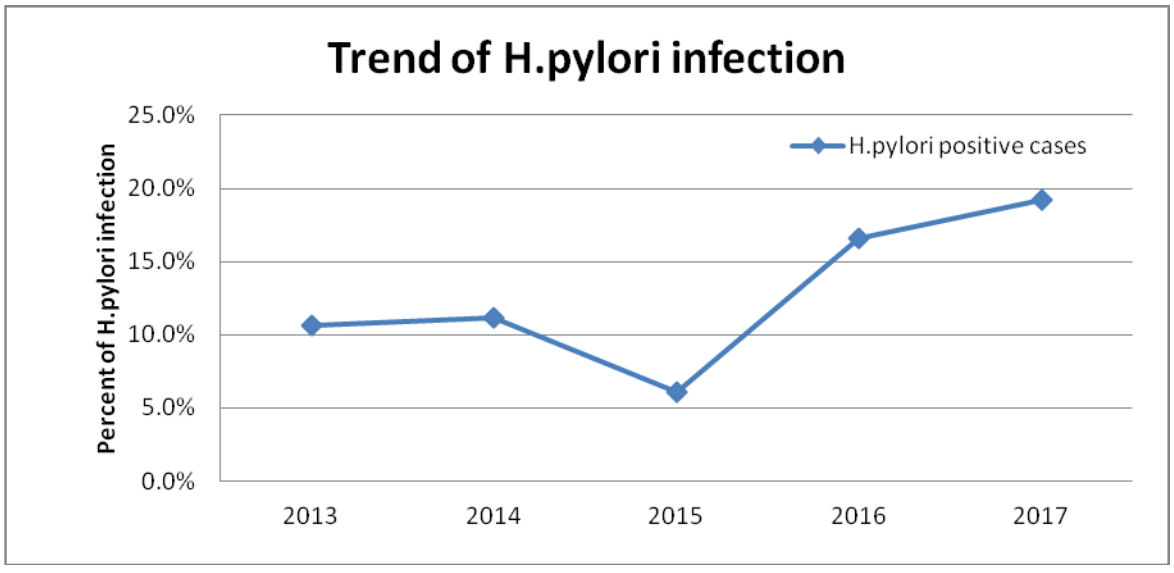


Fig. 21 Trend of *H. pylori* from 2013 to 2017 among dyspeptic patients in Asella teaching and referral hospital , Arsi University, Ethiopia

In each year the prevalence of *H. pylori* was higher among male than female patients (Fig. 22)

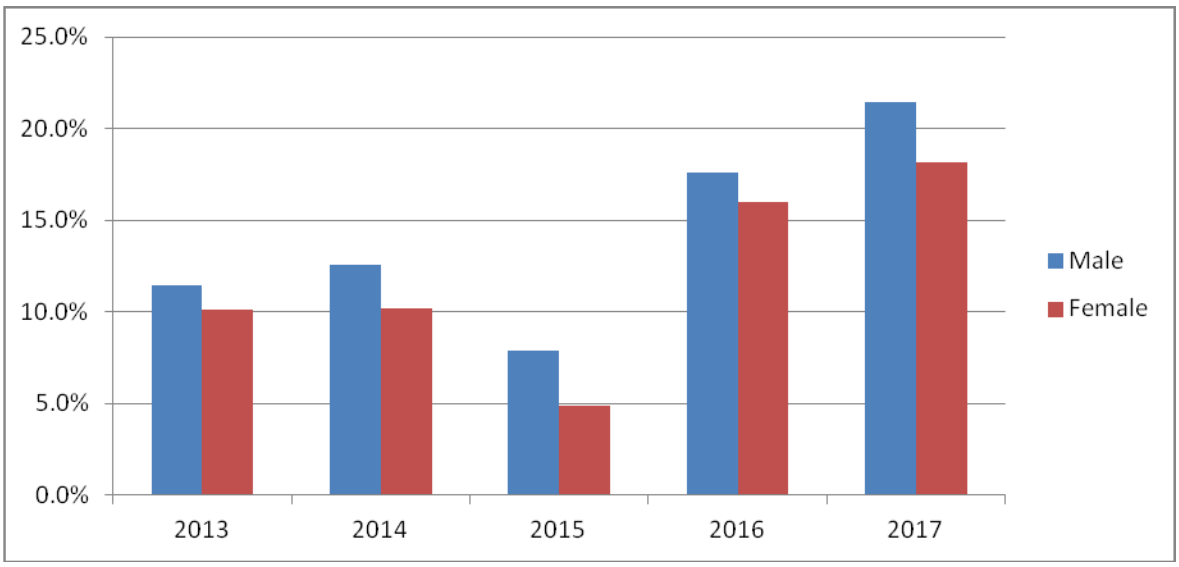


Fig. 22 Distribution of *H. pylori* infection among male and female dyspeptic patients from 2013 to 2017 in Asella teaching and referral hospital, Arsi University, Ethiopia

3.8 Evaluation of *H. pylori* test kits used by Asella Hospital

The prevalence of *H. pylori* in the prospective study was much higher (more than 78%) than the prevalence of *H. pylori* reported from the retrospective data collected from the hospital laboratory (15.5%). This difference could be either due to low quality of the test kit used by the hospital or technical errors of the hospital laboratory staffs. To evaluate the quality of the test kit, one hundred thirty seven stool samples were tested by wondofo one step *H. pylori* feces test (Guangzhou *Wondfo* Biotech Co., Ltd, China) and a stool antigen ELISA (Serazym Helicobacter pylori 2nd gen ELISA, *VIROTECH* Diagnostics GmbH , Germany) at the same time. The test results are summaries in the table below;

Table 20 Comparison between test results wondofo one step *H. pylori* feces test and Serazym Helicobacter pylori 2nd gen ELISA

		Wondfo rapid test		Total
		Negative	Positive	
Serazym Helicobacter pylori 2nd gen ELISA	Negative	23	14	37
	Positive	20	80	100
Total		43	94	137

Having the ELISA test result as a reference, the sensitivity, specificity, positive predicative and negative predicative values of the rapid *H. pylori* test used by the hospital were 80.0%, 62.2%, 85.1% and 53.5% , respectively.

4. Discussion

In a previous study among participants from Ghana, *H. pylori* infection was associated with low activation markers and higher $CD4^+$ cells count. The study was not able to show a cause effect relationship as it was a done by comparing those parameters between *H. pylori* positive and negative individuals cross-sectionally. This study was conducted by implementing interventional prospective study design to investigate the causality. This is the first longitudinal study done in Ethiopia to investigate the effect of *H. pylori* infection on the immune response and other clinical parameters. However the small sample size could limit further interpretation of some of the results from this study. Evaluating the efficacy of *H. pylori* eradication therapy and assessing quality of the *H. pylori* rapid test kits utilized in most of Ethiopian hospitals were also additional aims of this study.

4.1 There was high prevalence of *H. pylori* infection among asymptomatic individuals in Asella and the prevalence doedn't significantly varied based on the HIV status of participants

H. pylori infection is one of the most prevalent bacterial infection with up to 70–90 % and 25–50 % prevalence in developing and developed countries, respectively (Cover and Blaser 2009). The prevalence of the bacteria is mainly dependent on the hygiene conditions of the community. That is why it is highly prevalent in developing countries with contaminated environment (Andersen, Elliott et al. 1997). Like the other developing countries, the prevalence of *H. pylori* infection is high in Ethiopia. It was reported that the sero-prevalence of *H. pylori* infection among dyspeptic patients in Ethiopia ranges from 52.4% (Kibru, Gelaw et al. 2014) to 85.6% (Moges, Kassu et al. 2006). A recent systematic review and meta-analysis by Melese *et al* showed the pooled prevalence of *H. pylori* in the country to be 52.2% (Melese, Genet et al. 2019). The results from our study were similar to previous reports. In this study 78.7% (241/306) of the HIV positive and 75.1% (151/201) of the HIV negative participants were positive for *H. pylori* antigen.

Numbers of environmental and socio-demographic factors are known to influence the distribution of *H. pylori* infection. Studies done in Ethiopia showed that drinking unprotected surface water (Abebaw 2014, Seid 2018), not washing hands before a meal (Abebaw 2014), older greater(Moges, Kassu et al. 2006, Mathewos 2013) and alcohol consumption (Moges, Kassu et al. 2006, Melese, Genet et al. 2019) could be possible predators for *H. pylori* infection. From the factors assessed in this study only the age group had significant association to *H. pylori* infection. Unlike the previous studies this study showed that younger age is associated with *H. pylori* infection in comparison with older age. The difference could be the difference in grouping the age of participants and the difference in the range of participants' age. Like the other studies sex of participants was not significantly associated with *H. pylori* infection (Moges, Kassu et al. 2006, Abebaw 2014, Tadesse 2014).

The prevalence of *H. pylori* infection among HIV infected patients is controversial. It has been shown that *H. pylori* infection is high among HIV infected individuals (Abdollahi, Shoar et al. 2014, Nevin, Morgan et al. 2014, Teka 2016) which other studies have also shown contradictory results, suggesting that *H. pylori* prevalence is low among HIV infected patients, especially among those with low $CD4^+$ T cells. The possible justification for this negative correlation could that *H. pylori* requires intact immunity to establish infection and those with very low $CD4^+$ T cells count lack the required immune response (Fialho, Braga-Neto et al. 2011, Hestvik, Tylleskar et al. 2011, Sarfo, Eberhardt et al. 2015).

There are also studies which showed no differences in the prevalence of *H. pylori* infection based on HIV infection status (Moges, Kassu et al. 2006, Hossein Samadi Kafil, Fatemeh Farahi Jahromi et al. 2011). In this study, no statistically significant difference in the prevalence of *H. pylori* infection was found between HIV positive and negative individuals (p= 0.386) in Asella teaching and referral hospital, Ethiopia. Unlike the other studies stated above, this study was done on HIV positive individuals with $CD4^+$ T cell count greater than or equal to 350 cells/µl. This might have a contribution on the lack of the association between *H. pylori* and HIV infection.

4.2 *H. pylori* eradication therapy is less effective among HIV co-infected patients

The classic eradication therapy is a combination of clarithromycin, amoxicillin or metronidazole and PPI. A series of reports from different countries have shown that the standard triple therapy was not effective because of mainly due to clarithromycin resistance by *H. pylori* (Thung, Aramin et al. 2016, Jaka, Rhee et al. 2018, Savoldi, Carrara et al. 2018). In this study 25 HIV positive and 26 HIV negative participants infected with *H. pylori* were treated with a combination of three drugs; metronidazole 500mg, clarithromycin 500mg and pantoprazole 40mg for 14 days. Drugs were administered twice a day. We were able to follow only 48 of the treated participants there months after the intervention. 50 % (24/48) of the participants were successfully eradicated. The rate of eradication observed in the study is unexpected according to the international classification and requires call for further study and revision of the treatment guideline(Graham, Lu et al. 2007).

The efficacy of triple therapy reported from this study was more than twice the efficacy of triple therapy reported from a clinical trail from West Africa (22.3%) (Adjéka Stanislas Doffou, Koffi Alain Attia et al. 2015). The possible reason for this difference could be in the length of treatment. Unlike our study drugs treatment was administered only for seven days. This is supported the by the data of Sánchez-Delgado J et al, which have shown that longer treatment (14 days versus 10 days) resulted in better eradication rates (Sánchez-Delgado, García-Iglesias et al. 2012).

In contrast, the efficacy of the triple therapy was much lower than the one reported from a similar epidemiological study done in Bahr-dar, Ethiopia (90.3%) (Gebeyehu, Nigatu et al. 2019). One of the possible explanations for this considerably high difference in the efficacy of the triple therapy between both studies could be the difference in the drug combinations utilized. Unlike the study from Bahr-dar we have used metronidazole and not amoxicillin. There is no recent study done to show the resistance pattern of *H. pylori* for metronidazole and amoxicillin. But according to an

old report from Tikur Anbassa University Hospital, Ethiopia, 76% and 6% of the *H. pylori* isolates were resistant to metronidazole and amoxicillin, respectively. No résistance to clarithromycin was documented from the isolates (Asrat, Kassa et al. 2004).

Another possible explanation for this observation could be the differences in the character of both studies. The pervious study was done among dyspeptic patients who visited the outpatient department of Bahr-dar hospital with a possibly lower HIV positive to HIV negative proportion. But in our study all the participants were asymptomatic and half of them were HIV positive. Being asymptomatic and having HIV infection could be associated with less efficacy of therapy (Cai, Nesi et al. 2015, Chisholm, Campbell et al. 2018). Further detailed studies are required to assess the difference on the resistance pattern between isolates from symptomatic and asymptomatic *H. pylori* positive individuals.

To our surprise, only 37.5% (9/24) of the HIV co-infected patients were successfully treated. This was relatively low compared to the eradication rate among HIV negative participants 62.5% (15/24). Similar results were published by Marcel Nkuize *et al* from Brussels (Nkuize, De Wit et al. 2015). This could be due to the development of resistance as a result of high antibiotic consumption to prevent opportunistic infections (Megraud, Coenen et al. 2013).

4.3 Eradication of *H. pylori* infection increases hemoglobin level

Anemia is described by many studies as one of the extra intestinal complication due to *H. pylori* infection (Monzón, Forné et al. 2013, Kibru, Gelaw et al. 2014, Sato, Yoneyama et al. 2015, Taye, Enquselassie et al. 2015, Hudak, Jaraisy et al. 2017, Xu, Cao et al. 2017). Studies have shown controversial results on this regard. It has been repeatedly reported that *H. pylori* could result in intestinal bleeding which eventually leads to anemia. Increased production of hepcidin in response to *H. pylori* infection could also contribute to anemia (DuBois and Kearney 2005). Rise in hemoglobin level and depletion of hepcidin level were also achieved by eradicating *H.*

pylori (Fagan, Dunaway et al. 2009, Huang, Qu et al. 2010, Kroot, Tjalsma et al. 2011, Azab and Esh 2013, Sapmaz, Basyigit et al. 2016). But not all studies support this phenomenon as some studies couldn't show a positive association between *H. pylori* infection and anemia (John, Baltodano et al. 2018, Mozhgan Zahmatkeshan, Mehran karimi et al. 2019).

Having locally standardized reference ranges for clinical parameters such as CBC and liver enzymes as well as clear understanding about the effect of *H. pylori* infection on those clinical parameters could help physicians to better diagnose and handle *H. pylori* related complications including anemia. In this study CBC count and liver enzymes of 140 asymptomatic HIV negative and 140 asymptomatic HIV positive individuals were analyzed using automated hematology analyzer and semi-auto-method spectrophotometer, respectively. Significant differences between male and female participants were seen in some parameters. The mean RBC count, hemoglobin, hematocrit level, serum AST, ALT, urea and creatinine concentration of HIV positive female participants were significant lower than their male counterparts. Similarly, HIV negative female participants had significantly lower RBC count, Hgb level and liver enzymes in comparison with their male counterparts. Similar results were reported from studies done in different parts of Ethiopia (Yalew, Terefe et al. 2016, Mekonnen, Amuamuta et al. 2017, Mulu, Abera et al. 2017).

Possible confounding factors were excluded to determine the hematological reference ranges and to assess the association between *H. pylori* infection and clinical parameters. 280 participants were screened for confounding factors. The screening tests include stool examination for intestinal parasitic infection, serological test for hepatitis infection and C-reactive protein (CRP). According to those tests, 6.8% (19/280) of the participants were positive for HbsAg and none of the participants was infected with HCV; 6.1% (17/280) of the participants were reactive for CRP; 1.4% (4/280) participants were infected with intestinal parasites. Accordingly, data from 123 clinically healthy HIV negative and 121 HIV positive participants were eligible for further analysis. Data from 36 participants were excluded from further analysis.

Hematological reference ranges were analyzed from 123 clinically healthy HIV negative participants. Slightly higher WBC and RBC, platelet, AST, creatinine and bilirubin values and lower, hemoglobin, hematocrit, ALT and ALP values were reported from this study compared to the report of Gojjam Zones, Ethiopia. The MCV, MCH, MCHC values in both studies were similar (ICSH 1993). Higher WBC count and hemoglobin level were seen in this study compared to the report from blood donors in Gondar University hospital (Yalew, Terefe et al. 2016). Those relatively small differences seen between our findings from Asella, central Ethiopia, and other studies from Northwest Ethiopia could be due to difference in the topography or differences in the instruments utilized. As there was no significantly huge difference seen among those studies, reference ranges from one location of the country could be adopted by others. Utilizing local reference ranges could be also more helpful than using international text book based references.

Furthermore association of *H. pylori* infection with complete blood cell count and liver enzymes were analyzed for clinically healthy HIV negative and HIV positive groups separately to avoid the confounding effect of HIV infection. The mean WBC count, absolute neutrophil value, platelet count and ALP value were consistently higher among *H. pylori* positive participants both in the HIV positive and HIV negative groups. In contrast, the mean value of lymphocytes and eosinophils were smaller among *H. pylori* positive participants in comparison to their counterparts in both groups. However the only statistically significant difference was seen in ALP value among the HIV co-infected group.

Effects of eradication on those parameters were also analyzed comparing values before eradication and three months after eradication. The conclusions from the results of comparison are limited as the sample size was small. Moreover the rate of eradication was smaller. Having this limitation in consideration, there was a statistically significant increase in hemoglobin values among HIV negative participants three and six months after eradication of *H. pylori*. There were also significantly increased MCV, MCH, MCHC and PDW values after eradication of *H. pylori* infection among HIV negative individuals. This could support the findings from

other studies which showed rise in serum iron level and platelet count after *H. pylori* eradication (Emilia, Longo et al. 2001, Fagan, Dunaway et al. 2009). Among the HIV co-infected individuals there was a significant increase on the lymphocyte and creatinine value after eradication of *H. pylori*.

We observed also a significant increase in the MCH value among HIV negative participants and increase in the MCHC, platelet, PCT, creatinine and bilirubin values among HIV positive individuals not successfully eradicated from *H. pylori*. Based on this observation we could speculate that *H. pylori* infection was not the only responsible factor for those parameters. But this speculation is limited due to the small sample size. Therefore it is mandatory to further investigate this issue in large sample size cohorts.

4.4 *H. pylori* infection is associated with lower T cell activation markers and higher T regulatory cells

H. pylori is able to establish a chronic infection and withstand the harsh human environment, probably by modulating the immune response. This could explain the favorable conditions among individuals chronically infected with *H. pylori*. Epidemiological studies have shown that there is less asthma and other autoimmune diseases among *H. pylori* infected individuals. The high T regulatory cells among *H. pylori* co-infected HIV positive patients could attenuate immunopathology in *H. pylori* infection, possibly by reducing the activation of IFN-gamma producing $CD4^+$ T cells at the expense of a higher *H. pylori* load in the gastric mucosa (Kandulski, Malfertheiner et al. 2010).

In a previous study by Feldt et al *H. pylori* infection was found to be associated with decreased T-cell activation, cell proliferation and immune exhaustion markers and increased $CD4^+$ cell count (Eberhardt, Sarfo et al. 2015, Sarfo, Eberhardt et al. 2015). However the study was not able to show the causality. In this study, *H. pylori* infected patients were treated and followed to investigate the effect of eradication on the immune response. Unfortunately interpretation of the results from this study is limited

due to the small sample size and low eradication rate of the triple therapy which made a comparison difficult.

Significantly a higher value of T regulatory cell marker ($CD25^+Foxp3^+CD4^+$) was observed among *H. pylori* infected individuals irrespective of their HIV status. In HIV negative individuals, lower values of T cell activation marker ($HLA\text{-}DR^+CD38^+CD4^+$) and exhaustion markers ($PD1^+CD8^+$, $TIM3^+CD4^+$, $TIM3^+CD8^+$) were observed among *H. pylori* infected participants. This report is supported by previous studies (Lundgren, Stromberg et al. 2005a, Goll, Gruber et al. 2007, Eberhardt, Sarfo et al. 2015).

We asked ourselves if this association could be reversed after eradication of the bacteria. As a result the effect of *H. pylori* eradication on the T cell profile was assessed by comparing the values before and after successful eradication. Data from HIV positive and HIV negative groups were analyzed separately. Data from not successfully eradicated participants were also considered to be evaluated if the change in the T cell profile is exclusively dependent on *H. pylori* eradication.

$CD4^+$ T cell count was done on seventy HIV positive participants and the mean $CD4^+$ T cells count was 662 $\pm$ 205 cells/µl. $CD4^+$ T cells count was compared between baseline and six months after eradication of *H. pylori* to assess the effect of eradication of $CD4^+$ T cells among HIV positive patients. Only seven participants who were successfully treated were eligible for the follow up analysis. Even though there was a decreasing trend of $CD4^+$ T cells after eradication, the difference was not statistically significant (702$\pm$367 cells/µl vs 668$\pm$ 302 cells/µl, P value= 0.733). This doesn't support findings of a previous study which showed the positive association between *H. pylori* and $CD4^+$ T cells (Sarfo, Eberhardt et al. 2015). Hence, the small sample size could be an issue in our study.

H. pylori eradication was associated with a statistically significant increase on $HLA\text{-}DR^+CD38^+CD8^+$ cells and reduction of $Ki67^+CD8^+$ cells among HIV positive individuals. HIV negative individuals successfully eradicated from *H. pylori* infection showed increased median values of $Ki67^+$ $CD4^+$ and $CD57^+CD8^+$ cells. Reduction of

T regulator cell markers were also observed after eradication in both HIV positive and HIV negative groups. Those results could support the previous finding from Ghana.

Significant decrease of median values of $Ki67^{+}CD8^{+}$, $PD1^{+}CD4^{+}$ and $PD1^{+}CD8^{+}$ cells and increase of $CD57^{+}CD4^{+}$ cells were observed among not successfully eradicated HIV positive groups. There was a statistically significant decrease of T regulator cells after intervention among not successfully eradicated HIV negative groups. Those results could show that *H. pylori* is not the only reason for the immunomodulation as there was similar effects seen among those successfully eradicated and not eradicated groups. We couldn't clearly show the cause and effect relationship mainly due to the small sample size. Therefore having our findings as base line future studies with a large sample size could help to solve the inconsistencies in this study.

4.5 There is no difference in size and concentration of extracellular vesicles among *H. pylori* infected and non infected individuals

As in the case of other bacterial agents, *H. pylori* is also known to produce extracellular vesicles (EVs) which are useful for the intercellular communication between the bacteria and host cells (Kim, Lee et al. 2015). EVs exist in a wide range of sizes. The size as well as concentration of EVs could be correlated with disease condition (Eitan, Green et al. 2017). We were interested to analyze the difference in size and concentration of EVs between *H. pylori* positive and clinically healthy HIV negative individuals.

In this study Plasma samples from 106 HIV negative individuals were analyzed to measure the difference in the size and concentration of EVs based on their status of *H. pylori* infection. There were no statistically significant differences in size and concentration of extracellular vesicles between *H. pylori* infected and non-infected individuals. Further analysis with larger sample size and additional analysis such as analysis of microvesicle bound miRNA could provide important insight on the role of EVs in *H. pylori* infection.

4.6 Relatively low prevalence of *H. pylori* infection was reported from ATRH

(The data under the sub topic 4.6 is directly adopted from my published article with the permission from the publisher) (Mesfun, Teshome et al. 2018)

In the last five years 9,493 dyspeptic patients have been tested for *H. pylori* infection in Asella teaching and referral hospital. Even though half of the world‘s population was supposed to have *H. pylori* infection, this retrospective data showed that only 15% of the dyspeptic patients were positive for *H. pylori* infection. This prevalence was very low compared to other seroprevalence studies done in different hospitals (Mathewos 2013, Abebaw 2014, Watanabe, Ito et al. 2015, Workineh and Andargie 2016). This could be mainly due to the difference of tests utilized. The serological tests used in the current study were based on antigen detection and show active

infection while the other studies were based on antibody detection which could be positive even after eradication of the bacteria. But still this was very low prevalence compared to studies done with antigen detection test (Asrat, Nilsson et al. 2004) and this very low prevalence could be due to the difference in the quality of test kits.

Many studies showed that the prevalence of *H. pylori* was higher among older age groups (Moges, Kassu et al. 2006, Lim, Kwon et al. 2013, Mathewos, Moges et al. 2013) and this was different to the current study result in which prevalence of *H. pylori* infection was higher among young age participants ($p>0.001$). *H. pylori* infection was more frequent among male participants and this finding was similar to the finding from Korea (Lim, Kwon et al. 2013), Gonder, Ethiopia (Mathewos, Moges et al. 2013), and Bahr Dar, Ethiopia (Workineh and Andargie 2016).

The majority of the *H. pylori* positive patients 1388 (96.1%) were from the outpatient department and only 56 (3.9%) were admitted patients. It has been reported that the prevalence of *H. pylori* related complications are lower in developing countries than developed countries. Huge number of patients carry the bacteria but don't experience its consequences in developing countries (Segal, Walker et al. 2001). Our finding was in line with this fact, as most of those *H. pylori* positive patients had no further complication which requires admission.

There was an annual fluctuation of *H. pylori* prevalence with minimum (6.1%) and maximum (19.3%) prevalence in 2015 and 2017, respectively. There was statistically significant incline in the prevalence of *H. pylori* from 2015 to 2017 ($p=0.011$). This was a different finding compared to other similar studies done in Ethiopia (Mathewos 2013, Workineh and Andargie 2016).

4.7 Rapid *H. pylori* test kit utilized in Asella teaching and referral hospital has low sensitivity, specificity, PPV and NPV

The very low prevalence of *H. pylori* infection found from the hospital registry was the reason to ask about the quality of the test kit utilized by the hospital laboratory. To evaluate the quality of the test kit, one hundred thirty seven stool samples were tested by wondofo one step *H. pylori* feces test (Guangzhou *Wondfo* Biotech Co., Ltd, China) and a stool antigen ELISA (Serazym Helicobacter pylori 2nd gen ELISA, *VIROTECH* Diagnostics GmbH , Germany) at the same time. Having the ELISA test result as a reference, the sensitivity, specificity, positive predicative and negative predicative values of the rapid *H. pylori* test used by the hospital were 80.0%, 62.2%, 85.1% and 53.5%, respectively.

Even though it is difficult to compare this finding with other study findings as there was no similar study done to compare those specific test kits, our finding has clearly showed that the test kits were of lower quality in comparison to the ELISA test utilized in Germany. This supports the fact that ICA-based tests provide less reliable results than ELISA-based tests (Korkmaz, Kesli et al. 2013). Furthermore, we speculated that the low prevalence in *H. pylori* infection reported by the hospital laboratory could not be only due to the low quality of the test as the sensitivity of the test kits was high enough to detect about 80% of cases. Therefore personal technical errors by the laboratory technicians could also be other reason for under diagnosis of *H. pylori* infection in the hospital. But this needs further investigation.

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Annexes

Annex 1. Physical exam report

Examiners Name:_____ **Date of Exam:**_____

Study ID	Date of Birth	Age	Gender ☐Female ☐Male

Vital Signs: HR____ RR____ BP____ Temp.____ Growth: Weight _____ lbs. _____ %

Height: _____inches ____ %

Review and Description of Systems (Please note pertinent findings)

	NAS
General ☐fatigue ☐fever ☐weight loss ☐diaphoresis	☐
Skin ☐persistent rash/spots ☐acne ☐tattoo ☐ ulceration/lesions	☐
HEENT ☐headache ☐TMJ pain ☐visual/hearing problems ☐rhinitis ☐sore throat ☐frequent nosebleeds	☐
Neck ☐masses	☐
Chest ☐chronic cough ☐wheezing ☐DOE ☐chest pain ☐breast lumps/discharge	☐
CVS ☐murmurs ☐palpitations	☐
GI ☐vomiting ☐diarrhea/constipation ☐jaundice ☐food intolerance ☐abdominal pain where?_____	☐
GU ☐dysuria ☐discharge ☐scrotal masses ☐urinary frequency ☐incontinence ☐enuresis	☐
CNS ☐fainting ☐LOC ☐weakness ☐tremor ☐seizures	☐
Muscles-skeletal ☐scoliosis ☐joint aches/swelling	☐

Annex 2. Questionnaire

This Questionnaire designed to collect data for the research conducted in Asella teaching hospital to study the prevalence of *Helicobacter pylori*, risk factors for *Helicobacter pylori infection* and the eeffect of *Helicobacter pylori* infection on the immune response

General instructions- For all the close ended questions please give your responses by Check box (✓) containing your answer(s) and incase of the open ended questions please write your responses on the space provided. Data collectors are responsible to help illiterate participants by reading the questions and writing their responses.

Date	(*DD-MMM-YYYY)*	Site of data collection ☐ 1 = ART ☐ 2 = VCT	Category of Participant	
Participant Identification number				

Section I Demographic and Socioeconomic Information			
SN	**Questions and Filters**	**Response & Coding Categories**	**Skip**
101	Full Name		
102	Address		
	Wereda		
	Kebele name		
	Gote/sefer		
103	Residence	☐ 1 = Rural ☐ 2 = Urban	
104	Sex	☐ 1 = Male ☐ 2 = Female	
105	How old are you?(years)		
106	Which religion do you follow?	☐ 1 = Muslim☐ 2 = Orthodox☐ 3 = Protestant ☐ 4 = Catholic ☐ 5 = Other	
107	To which ethnic group do you belong	1) ☐ 1 = Oromo☐ 2 = Amhara 2) ☐ 3 = Gurage ☐ 4 = Other	
108	What is your family size (number)		
109	What is your major occupation currently? (Whatever you do to earn money)?	☐ 1 = Employed ☐ 2 = Businessman/women ☐ 3 = Farmer☐ 4 = Housewife ☐ 5 = Daily laborer☐ 6 = Student ☐ 7 = Have no Job ☐8 = Other specify _____	
110	What is the current educational status	☐ 1 = Illiterate☐ 2 = Read and write ☐ 3 = Literate _____(Exact grade)	
111	What is the monthly income (on average) of your household including your own?	Birr/month_____	
112	What is your current marital status?	☐1=Single ☐2= Married ☐3 = Divorced ☐4 = Widowed	

Section II Medical History			
201	Active or past history of gastric or duodenal ulcer	☐ 1 = Yes ☐ 2 = No	
202	Family history of gastric cancer	☐ 1 = Yes ☐ 2 = No	
203	Taking aspirin or non-steroidal anti-inflammatory drugs	☐ 1 = Yes ☐ 2 = No	
204	Do you suffer of any of the following major symptoms (**one** positive answer leads to immediate eradication)	☐ 1 = vomiting blood/coffee ground like material ☐ 2 = black tarny stool	
205	Do you suffer of any of the following minor symptoms (**three or more** positive answers lead to immediate eradication)	☐ 1 = nausea, upset stomach ☐ 2 = abdominal pain ☐ 3 = abdominal bloating ☐ 4 = loss of appetite ☐ 5 = burning between meals/night	
206	Do you take any medication?	☐ 1 = Yes ☐ 2 = No	
207	Do you have any allergies?	☐ 1 = Yes ☐ 2 = No	
Section IIIKnowledge, attitude and practice related information			
301	Do you know about gastritis?	☐ 1 = Yes ☐ 2 = No →	To 307
302	How do you think gastritis is caused?		
303	Do you think gastritis is infectious disease?	☐ 1 = Yes ☐ 2 = No →	To 305
304	How is gastritis transmitted from one individual to others?		
305	What conditions do you think can aggravate gastritis	☐ 1 = Spicy food ☐ 2 = Alcohol consumption ☐ 3 = Smoking ☐ 4 = Chewing chat ☐ 5 = Fasting for long time ☐ 6 = Other specify _____	
306	What do you do/recommend to prevent gastritis (You can give one or more response/s)	_____ _____ _____	
307	Have you ever heard about *Helicobacter pylori?*	☐ 1 = Yes ☐ 2 = No	
Section IV Information related to risk factors			
401	Have you ever had gastritis?	☐ 1 = Yes ☐ 2 = No	
402	At what age you have it for the first time?	_____	
403	Do you have the habit of drinking alcohol?	☐ 1 = Not at all ☐ 2 = Light/(equivalent to 0-5 bottle of beer per week) ☐ 3 = Moderate(equivalent to 6-20 bottle of beer per week) ☐ 4 = Heavy (equivalent to > 20 bottle of beer per week)	

404	Do you have the habit of smoking?	☐ 1 = Yes ☐ 2 = No	
405	Do you have the habit of chawing chat?	☐ 1 = Yes ☐ 2 = No	
406	How often do you eat spicy foods	☐ 1 = Not at all ☐ 2 = Occasional ☐ 3 = Many times☐ 4 = Always	
407	What is your source of water	☐ 1 = Tap☐ 2 = well/spring ☐ 3 = River/pond ☐ 4 = Others, specify_____	
408	Do you have toilet facility in your compound?	☐ 1 = Yes ☐ 2 = No	
409	Do you always use toile to defecate?	☐ 1 = Yes ☐ 2 = No	
410	Do you always wash your hand after defecation?	☐ 1 = Yes ☐ 2 = No	
411	Do you always your hand before feeding?	☐ 1 = Yes ☐ 2 = No	
412	Have you ever had family member with gastritis?	☐ 1 = Yes ☐ 2 = No	
413	Have you ever been care giver (socially, occupationally or any other reason) for individuals with gastritis?	☐ 1 = Yes ☐ 2 = No	
414	Do you have oral-to- oral contact (Kissing) with individual/s with gastritis or peptic ulcer?	☐ 1 = Yes ☐ 2 = No	
Thank you for your participation in the study!			
Contact address of the participant Phone no. 1 Phone no. 1 Other way of communication (if any?) _____			
Name of interviewer_____		Signature: _____	

Annex 3. Information Sheet

Information Sheet prepared for participants of study on the Effect of *H. pylori* infection on the immune response in HIV infected patients and HIV negative individuals: Prospective interventional study

All participants are expected to read/understand the whole content of the information sheet before being involved in the study

Name of the organization: Arsi University, Hirsch Institute of Tropical Medicine (HITM)

Title of the study: Effect of *H. pylori infection* on the immune response in HIV infected patients and HIV negative individuals: Prospective interventional study

Introduction: This information sheet is prepared for the PhD study on the effect of *H. pylori* infection on the immune response among HIV positive and negative participants in Asella, Ethiopia.

Aim of the study: The main aim of the study is to assess the prevalence of *H. pylori* infection and investigate the impact of *H. pylori* infection on the immune response in HIV positive and HIV negative patients in Asella. Risk factors for *H. pylori* infection, efficacy of the eradication treatment and the molecular factors responsible for the immunomodulation will be determined utilizing advanced diagnostic methods. Findings from this study will help to fill the knowledge gap about the effect of *H. pylori* on immune response and to revise the *H. pylori* treatment guideline. The study findings will be also a base for other further studies on therapeutic investigations for infections and inflammatory disorders.

Procedure: When you are willing to participate in the study, after being informed about the study procedure and potential risks associated with participating in the study, your demographic, clinical and socioeconomic data, as well as the medical history will be documented by the attending physicians or study nurses based on a standardized protocols. You will also be asked to provide a fresh stool sample (about 5gm) to be tested for gastrointestinal parasites and *H. pylori*. Venous blood sample (about 15ml) will be collected for the analysis of immune parameters. Blood and stool sample will also be preserved for further molecular analysis in Germany. If you are found to be infected with parasitic infections the institution will cover all your treatment expenses.

After baseline evaluation and *H. pylori* eradication, you will be followed up for 12 months clinically and immunologically. You will be asked to provide stool and blood sample in 3, 6 and 12 months after the baseline study. Immune parameters are tested 3, 6 and 12 months after *H. pylori* eradication, and clinical parameters are recorded by a standardized questionnaire. All *H. pylori* positive and untreated study subjects will be treated after the follow-up period.

Complications and risks: For collection of venous blood samples a peripheral vein will be punctured and results in pain. In rare cases this may lead to hematoma

formation. Whenever possible, blood collection for the study purposes shall be combined with routine diagnostics to avoid additional punctures. As this is a clinical basic science study no harm is expected to arise from the information gained.

Benefits: If pathogens are detected on stool examination this information will be passed to the treating physician. Depending on the signs and symptoms of the patients this may have therapeutic consequences. All your treatment expenses will be covered by the institution. By better understanding immunopathogenesis of chronic viral infections and their associated complications new therapeutic models may arise in future (e.g. by administration of modulatory substances or probiotics). However, no immediate new therapeutic approach is expected to arise.

Confidentiality and Anonymity: A unique study identification number will be assigned to your data. All data will be analyzed with respect to these study numbers and all information will be treated confidentially. Only members of the research team will have access to these files. Even though the data is published, your name will never be cited.

Voluntariness: It is only when you are willing that you will be involved in the study. In case if you are not willing to participate in the study, your non-voluntariness never hider you from getting the regular service of the hospital and the research institute. You have the full right to refuse from participating in this study. you can choose not to respond some or all of the questions from the questionnaire. You can withdraw from this study at any time you wish to, without consulting anyone.

Persons to contact: If you have any question you can ask the one who provide you the information sheet or contact the principal investigator, Million Getachew (0912106595).

Unka odeeffannoo

Dhukkubni *H. pylori*i madinummaa qaama keenya irratti rakkoo inni fidu hubachuudhaaf qorannoo kanarratti qophaa'aniif unka qophaa'e.

Hirmaattonni qorannoo kanarratti hirmaatan hunduu waa'ee unka kanaa dubbisuu qabu.

Bakka hojii: Yunivarsiitii Arsiitti Instituutii Hirsch Tropical medicine

Mata duree qorannichaa: dhiibbaa H.pyolori madinummaa qaama keenya irratti geessu hubachuudhaaf qorannoo adeemsifamu.

Seensa : unki kun kan qorannoo kanaa kan qophaa'ef qorannoo digirii sadaffaaf waa'ee *H. pylori* n madinummaa qaama keenya irratti geessu hubachuudhaaf qorannoo adeemsifamu.

Kaayyoo qorannichaa: kaayyoo qorannaa kanaa tatamsa'ina H.pyolori hubachuudhaafi dandeettii madinummaa qaama keenya hangam akkam ta'e beekuudhaaf ,waantota dhukkubaaf nama saaxilan, dandeettii fayyisuu qorichaa fi humna ittisa qaama keenyaa beekuudhaaf qorannoo adeemsifamu.

Bu'aa qoranichaa: hanqina odeeffannoo guutuudhaaf fi ittisuudhaaf nama gargaara.akkasumas qorannoon kun akka jalqabaatti ta'ee qorannoo biraadhaaf tajaajila.

Adeemsa: qorannicharratti hirmaachuudhaaf heyyemamaa ta'uu,qophaa'ina yaadaatii fi hawaasummaarratti yaada walitti qabuuf unka qophaa'e. sammuuda gatteechumaati fi dhiiga siisii kudha shan ta'u kennitan. Sammuuda gattechumaa irratti raammoon macuree qorachuudhaaf kan oolu yoo ta'u. sammuudichis gara biyya German demee hanga qoratamutti Instituticha keessa taa'a.

Raammoo macuree yoo argame institutichi kaffaltii yaalaa isin danada'a. Guyyaa qorannichaarratti hirmaattanirraa ji'a tokkoon booda hordoffii duraa, ji'a sadaffaa fi ji'a ja'affaa irratti hordoffii 2ffaa fi 3ffaa irratti hirmaachuudhaan sammuuda gattechummaa fi dhiiga kennitu.

Hirmaattonni hunduu jalqaba qorannichaa fi dhuma qorannichaa irratti yaaliin ni kennama.

Yaaddoo: dhiigni yeroo isinirraa fudhatamu dhukkubbiin isin irratti dhaga'amuu danda'a.iddoon waraanamtanis isin dhiita'uu danda'a.hanga danda'ametti dhiigni fudhatamu yeroo hospitaala dhuftanii fi hordoffiidhaaf yeroo addaa addaa ta'e gaaridha.

Bu'aa: bu'aan qorannoo sammuudaa ogeessa isin yaalutti kennamee akka isin yaalu taasifama.

Kaffaltiin yaalaaf kennamu Institutichaan deggerama.qorannoo kana booda bacteeriyichi ittisa qaamaa irratti dhiibbaa inni qabu addaan ba'ee erga beekamee booda yaada fooyya'aa baasuudhaafi qoricha fooyya'aa baasudhaaf nama gargaara.

Icciitii : eenyummaa keessan gara kamiinuu nama biraatiif akka ifa hin taane lakkoofsi bakka isin bu'u isniif kennama . qorannichi kan inni adda ba'u lakkoofsicha kanaan waan ta'eef icciittin keessan eenyuyyuu beekuu hin danda'u.

Eeyyemummaa : qorannicharratti kan hirmaachuu danda'u heyyemamaa yoo taatan qofa. Namni kamiyyuu fedhii keessaniin ala isin dirqisiisuu hin danda'u.
Yeroo kamiyyuu qorannicha keessaa ba'uu dandeessu.
Teessoo : gaaffii yoo qabaattan odeeffannoo argachuudhaaf qorataa olaanaa kan ta'an obbo Mliyoon Getaachoo ,lakkoofsa bilbilaa (+251912-10-65-95)' kanaan dubbisuu dandeessu.

የመረጃ ቅፅ

የሄሊኮባክተር ፓይሎ ኢንፌክሽን በሽታ መቋቋም ላይ ያለው ተፅእኖ ለመረዳት ለሚደረግ ጥናት ለተሳፊዎች የተዘጋጀ መረጃ። ሁሉም ተሳታፊዎች ጥናቱ ውስጥ ከመሳተፋቸው በፊት ይህንን የመረጃ ቅፅ ማንበብና መረዳት ይጠበቅባቸዋል።

የድርጅቱ ስም፡- አርሲ ዩኒቨርሲቲ ሂርሽ ኢንስቲቱት ኦፍ ትሮፒካል ሜዲስን

የጥናቱ ርእስ፡- ሄሊኮባክተር ፓይሎሪ የሰውነት በሽታን የመቋቋም ዓቅም ላይ ያለው ተፅኖ ማጥናት።

መግቢያ፡- ይህንን የመረጃ ቅፅ የተዘጋጀው የሦስተኛ ዲግሪ ጥናት ለሆነውና የሂሎባክተር ፓይሎሪ በሰውነት በሽታ የመቋቋም ዓቅም ላይ የሚያደርሰው ተፅእኖ ላይ ለሚያተኩረው ምርምር የተዘጋጀ ነው።

የጥናቱ ዓላማ፡- የዚህ ጥናት ዓላማ የሂሊኮ ባክተር ፓይሎሪ ስርጭት ለመዳሰስና ባክቴሪያው ህመም በመቋቋም ዓቅም ላይ ያለው ተፅኖ ለማጥናት ነው። ለህመሙ አጋላጭ የሆነ ጉዳዩች የመድኃኒቱ የማከም ዓቅም ባክቴሪያው በሰውነት የበሽታ የመቋቋም ዓቅም ተፅኖ የሚፈጥርባቸው መንገዶች ይጠናሉ። የዚህ ጥናት ግኝትም ያለውን የመረጃ ክፍተት ለመሙላትና የህክምናው መመሪያም ለመከለስ ይረዳል። ለሌሎች ምርምሮችም እንደ መነሻ ሃሳብ ሆኖ ያገለግላል።

ሂደት፡- ጥናቱ ላይ ለመሳተፍ ፋቃደኛ ሲሆኑ ስለግልና ማህበራዊ ጉዳይ መረጃ ተዘጋጅቶ መጠየቅ መሰረት ይሰበሰባል። የህክምና መረጃውም በሃኪም ወይም ነርስ ይሰበሰባል። የሲጋራናሙና 11 ሲሲ የሚሆን የደም ናሙና ይሰጣሉ። ሲጋራው ናሙና ለሆድ ትላትል ምርመራ የሚውል ሲሆንደሙ ደግሞ ለኢሞኖሎጂካል ምርመራ ይሞላል። ናሙናው ወደ ጀርመን ሀገር ተልኮ እስኪጠና ድረስም በኢንስቲቱዩት ይቀመጣል። የትላትል በሽታ ከተገኘም ድርጅቱ የህክምና ወጪውን ይችላል። ከመነሻ ምርመራ በኋላም ለ12 ወር ክትትል ይደረግለታል። በ3-6 እና 12 ሆኖ ተመሳሳይ ናሙናዎች ይሰጣሉ። ሁሉንም ባክቴሪያ የታመሙ ተሳታፊዎች በጥናቱ መጀመሪያ ወይም መጨረሻ ይታከማሉ።

ስጋት፡- ደም በሚሰበሰብበት ግዜ የደም ስርዎ የሚወጉ ጊዜ የህመም ስሜት ይሰማዎታል። አልፎ አልፎም በተወጋበት ቦታ እብጠት ሊያጋጥም ይችላል። በተቻለ መጠን ደም የሚወሰደው ከተለመደው የሆስፒታል የክትትል ናሙና መስጪያ ጊዜ ጋር አብሮ እንዲወሰድ ይደረጋል። በጥናቱ ምክኒያት ከሰጡት ናሙና የሚገኘው ውጤት እርሷ ላይ ምንም ዓይነት ጉዳት አያደርስም።

ጥቅም፡- በሲጋራ ናሙናው ምርመራው ውጤት ለሚከታተሎት ሐኪም ተሰጥቶ የሚገባውን ህክምና ይደረግሎታል። የህክምና ወጪዎትን ድርጅቱ ይሰጦታል። ከዚህ ጥናት የተነሳ ባክቴሪያው በህመም የመቋቋም አቅም ላይ ያለው ተፅእኖ ተለይቶ ከታወቀ የተሻለ የህክምና ሀሳብ ለመቅረጽና የተሻለ መድሃኒት ለማግኘት አጋዥ ሊሆን ይችላል። ቀጥተኛ የሆነ አዲስ መድሃኒት ግን ከዚህ ጥናት አይጠበቅም።

ሚስጥራዊነት፡- የእርሷ ማንነት በማይገልጽ መልኩ መረጃዎትን የሚወክል ቁጥር ይሰጦታል። መረጃዎ የሚተነተነው በዚህ ቁጥር ሲሆን መረጃዎ በሙሉ ለምርምር ይያዛል። የጥናቱ አባላት መረጃዎትን ማግኘት የሚችሉ ሲሆኑ የጥናቱ ውጤት ሲታተምም ስሞዎ አይገለፅም።

ፍቃደኝነት፡- በጥናቱ የሚሳተፉት በፍቃደኝነቶዎ ሲሆን ፍቃደኛ ባለመሆንዎ በሚያገኙት የሁስፒታሉ አገልግሎት ምንም ዓይነት ተፅእኖ አያሳድርም፡፡ የሚጠይቁት ጥያቄ በከፊልም ሆነ በሙሉ ያለመመለስ መብትዎ የተጠበቀ ነው፡፡ በየትኛውም ጊዜ ማንንም ሳያማክሩ ጥናቱን የማቋረጥ መብቶዎ አሎት፡፡
አድራሻ፡- ጥያቄ ካሎት መረጃ የሰጦት ሰው ወይም ዋናው ተመራማሪ አቶ ሚሊዮን ጌታቸው 0912106595 ማነጋገር ይችላሉ፡፡

Annex 4. Informed Consent English Version

Informed Consent Form for the study on Immune modulation by *Helicobacter pylori* infection

Participant Name _____

Participant ID _____

Consent of participant

I have got enough information about the purpose of study, the procedures, expected risks and benefits of the study. I am also informed that I have the rights to refuse from being part of the study or withdraw at any time without consulting anyone. I also know that being not volunteer to participate on the study does not affect the service I am supposed to get from the health facility. I also know that *H. pylori* positive and untreated study subjects will be treated after the follow-up period. Therefore, I am voluntarily participating in the study without any pressure. I have taken copy of this signed consent form.

Name of participant _____

Date _____

Signature/finger print _____

Name and signature of witness (If participants are illiterate)

_____ _____

Informed Consent Afan Oromo Version

Unkaa walii galtee qorannoo waa`ee jermii heelikoobaacte paayolorii qaama human dhukkuba ittisaa irratti qabuu.

Maqaa hirmaataa _____

Lakk. Hirmaataa _____

Ragaa hirmaatottaa

Ani oddeeffannoo guutuu waa`ee kaayyoo, akkataa, miidhaa fi faayidaa qorannoo kana argadheera. Dabalatas dhiibaa tokko malee hirmaachuu fi hirmachuu dhiisuufi mirga guutuu akkan qabu natti himameera. Qorannoo kana keessatti hirmaachuu dhiisuun koo tajaajjila fayyaa argadhu irratti dhiibbaa tokko illee akka hin qabne hubadheera. Kanaafuu fedhiidhaan dhiibbaa tokko malee qorannoo kana keessatti hirmaachuukootiif mallattookotiin naan mirakaneesa. Haftee tokkos fudhadheera.

Maqaa hirmaataa _____

Guyyaa _____

Mallattoo/ asharaa_____

Maqaa nama raga bahuu / yoo nama hinbarannee/

_____ _____

Informed Consent Amharic Version

ሄሊኮባክተር ፓይሎሪ(የጨጎራ በሽታ የሚያመጣ ጀርም) የሰውነት በሽታን የመቋቋም ዓቅም ላይ ያለው አስተዋፅኦ/ጉዳት ለማጥናት የተዘጋጀ የተሳታፊዎች የስምምነት ቅጽ

የተሳታፊው ሥም _____

የተሳታፊው መለያ ቁጥር _____

የተሳታፊው የስሜሜነት ቃል/ፈቃድ

የጥናቱ ዓላማ፣ ሂደት ፣ ከጥናቱ የሚገኙ ጥቅሞችና ሊከሰቱ የሚችሉ ጉዳቶች በተመለከተ በቂ መረጃ አግኝቼአለሁ። በጥናቱ ያለኝ ተሳትፎም ማንንም ሳላማክር ማቆም እንደምችልና ለመሳተፍ ፍቃደኛ ባልሆን ላገኘው የሚገባኝ አገልግሎት ላይ ምንም ዓይነት ተፅእኖ እንደሌለው አውቃለሁ። ስለዚህ በዚህ ጥናት ላይ የምሳተፈው ያለማንም ተፅእኖ በራሴ ፈቃድ ነው። የተፈረመው የስምምነት ቅጽ ቅጂ ወስጃለሁ።

የተሳታፊው ሥም _____

ቀን _____

ፊርማ/ የጣት አሻራ _____

የምስክሮች ሥምና ፊርማ(ተሳታፊዎች መፃፍና ማንበብ የማይችሉ ከሆኑ)

_____ _____

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