Prevalence of HIV and Its Co-Infection with Hepatitis B/C Virus Among Chronic Liver Disease Patients in Ethiopia

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Background: The efficient use of antiretroviral drugs has significantly reduced AIDS-related morbidities and mortalities; however, mortality due to non-AIDS-related end-stage liver diseases is escalating in those living with HIV.

Objective: The study was designed to determine the prevalence of HIV and its co-infection with HBV and HCV among chronic liver disease (CLD) patients in Ethiopia.

Methods: Three hundred and forty-five CLD patients were included in this study in two groups: Hepatocellular carcinoma (HCC) (n=128) and non-HCC (n=217) patients. The non-HCC group comprised patients with advanced liver disease (n=98) and chronic hepatitis (n=119). Enzyme immunoassays were used to determine HBV and HCV infection markers. In addition, a serial rapid HIV testing algorithm was employed to screen HIV infection.

Results: Regardless of the stage of liver disease, the overall frequency of HIV was 4.3% (15/345), with a 2% (7/345) and 0.3% (1/67) among HCC and non-HCC (n=128) and non-HCC (n=217) patients. The non-HCC group comprised patients with advanced liver disease (n=98) and chronic hepatitis (n=119). Enzyme immunoassays were used to determine HBV and HCV infection markers. In addition, a serial rapid HIV testing algorithm was employed to screen HIV infection.

Conclusion: The frequency of HIV and its co-infections with HBV/HCV exhibited an increasing pattern with the severity of the liver disease. Thus, screening all HIV-positive patients for HBV and HCV infection and all CLD patients for HIV infection and taking necessary preventive measures would be an essential strategy to prevent the progression of CLD and death related to liver disease in people living with HIV.

Keywords: HIV, HBV, HCV, non-AIDS liver diseases

Introduction

Human immunodeficiency virus (HIV), one of the most common blood-borne viruses, has overlapping modes of transmission with the two hepatotropic viruses, hepatitis B virus (HBV) and hepatitis C virus (HCV). These two viruses (HBV & HCV) are the most common viral etiologic agents of chronic liver disease (CLD).1 AIDS-related illnesses have been responsible for the death of 36.3 million people since the HIV epidemic started (https://aidsinfo.unaids.org).2 In
In comparison, hepatitis B and C infections are accountable for 90% of viral hepatitis-related deaths (1.4 million per annum) globally.

In 2015, a total of 36.7 million people were infected with HIV, of whom 2.7 and 2.3 million people were co-infected with HBV and HCV, respectively. The reason for such a high co-infection rate could be the shared mode of transmission of these viruses, that is, contact with infected blood and body fluid through parenteral and sexual activities. The coexistence of HIV with hepatitis B or C viruses increases the chronicity, early progression, and high fatality of liver diseases compared to HBV or HCV mono-infection. Although deaths related to HIV infection have considerably dropped after large-scale use of highly active antiretroviral therapy (HAART), mortalities due to non-acquired immunodeficiency syndrome (AIDS) causes, such as viral and non-viral chronic liver diseases, remain prominent and challenging among HIV-infected individuals.

More than 70% of HIV cases in the world reside in sub-Saharan Africa, where HBV is prevalent and responsible for most viral-related chronic liver disease conditions. Consequently, about 71% of HIV/HBV co-infected individuals reside in sub-Saharan Africa. Generally, HIV/HBV co-infection prevalence is predominant compared to HIV/HCV co-infection in the region.

Chronic hepatitis B (CHB) and C (CHC) infections are the leading factors that complicate and accelerate liver disease progression to advanced stages (Cirrhosis and HCC) in those living with HIV/AIDS. Equally, HIV infection noticeably affects the natural process of hepatitis B and C infections. In most cases, depleting immune response due to HIV infection favors hepatitis B or C replication in the hepatocytes, subsequently enhancing chronic liver disease sequels, such as liver cirrhosis and fibrosis.

There are several mechanisms for the pathogenesis and injuries to the liver among HIV/AIDS patients, including immune-mediated injury, oxidative stress, mitochondrial injury, lipotoxicity, cytotoxicity, toxic metabolite accumulation, gut microbial translocation, and systemic inflammation.

Although chronic hepatitis B, C, and alcoholism-related CLD and HIV infection are common in Ethiopia, no scientific study has been conducted to evaluate the prevalence of HIV and its co-infection with HBV or HCV among CLD patients in Ethiopia. Thus, we aimed to assess the prevalence of HIV and its co-infection with HBV and HCV among CLD patients in Ethiopia.

Materials and Methods

Study Settings and Patients

A cross-sectional study involving 345 CLD patients was conducted between Dec. 2018 and Mar. 2019. The patients were recruited at the gastroenterology (GI) clinics of four selected referral hospitals (Tikur Anbessa Specialized Hospital, St. Paul’s Hospital, Armed Forces Hospital, and MyungSung Christian Medical Center /Korean Hospital/), two specialized private health care institutions (Adera Medical center and Yanet specialized clinic) during routine clinical practices. The four Hospitals and Adera Medical Center are located in Addis Ababa, the capital of Ethiopia, whereas Yanet specialized clinic is found in Hawassa, in southern Ethiopia. The CLD patients were included in the study in two groups: HCC (n=128) and non-HCC (n=217) patients. The non-HCC groups comprised patients with advanced liver disease (AdLD) (n=98) and chronic hepatitis (CH) (n=119).

Patient Selection

Chronic liver disease patients (patients with repeated and prolonged liver inflammation accompanied by gradual deterioration of liver functions) were recruited by the assigned gastroenterologist based on clinical, pathological, and biochemical analysis and imaging modalities during routine clinical practice at the GI unit of each study site. All HCC cases were confirmed with the standard diagnostic tests (Magnetic resonance imaging, MRI/ Computerized tomography, CT), biopsy, or Ultrasound (US) plus elevated serum alpha-fetoprotein (AFP) >400 ng/mL.
Inclusion and Exclusion Criteria

Inclusion Criteria
Chronic liver disease patients aged ≥18 years old, attending the gastroenterology units of all study sites, but mentally competent and willing to participate in the study.

Exclusion Criteria
Chronic liver disease patients with metastasized liver cancer, clinically confirmed chronic hepatic schistosomiasis, and a critical health condition/ hepatic coma were excluded from the study.

Data Collection
At each study site, the pre-assigned trained nurse collected all relevant information related to the sociodemographic data (age, gender, marital status, educational status, occupation, and residence) of the patients and their exposure to risk factors of HIV, HBV, and HCV infection (history of blood transfusion, tattooing, sexual practices, dental extraction, body piercing, abortion, and invasive medical procedures, and habit of smoking and alcoholic beverage drinking) using a pre-structured questionnaire in a face-to-face interview.

Specimen Collection and Processing
Ten milliliters (10 mL) of venous blood were taken from each study participant at each site and was dispensed into an ethylene diamine tetra-acetic acid (EDTA) tube and transported on dry ice to Armauer Hansen research institute (AHRI), where the plasma was separated via centrifugation at 3500 rpm/ 5min, transferred into cryotubes, and stored at - 20 °C until used for HIV, HBV, and HCV serology.

Detection of HIV1/2 Antibody
Plasma samples were screened for antibodies against HIV1/2 following the national serial rapid HIV testing algorithm and the WHO’s guidelines on HIV testing and counseling strategy. Accordingly, three commercially available rapid tests were used in the following order: Wantai (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., China) (screening test), Uni-Gold™ (Trinity Biotech PLC, Bray, Ireland) (confirmatory test), and Vikia (bioMérieux SA, France) (tiebreaker test). According to the HIV testing algorithm, to declare a person HIV positive, he/she should be positive with at least two tests. The sensitivity and specificity of each rapid test are summarized in the Supplementary Table 1.

Briefly, plasma samples that tested positive with the first test (Wantai) were subjected to the second test (Uni-Gold), and those found positive with the second test too considered positive for HIV1/2 antibodies. Likewise, those samples positive with the first test and negative with the second test (discordant result) were tested with the third test (Vikia), and samples that turned positive were considered positive for HIV1/2 antibodies. Results from each test were interpreted as per the manufacturer’s instruction.

Serodiagnosis of Chronic Hepatitis B and C Infection
The diagnosis of hepatitis B and C virus infection was established through serological assays and additional molecular assay for HCV RNA (RT-PCR). All the assays were employed, adhering to the WHO’s recommendation for diagnosing hepatitis B and C infection in developing and low-income countries.

Detection of HBsAg and HBcAb
Different 3rd generation enzyme-linked immunosorbent assay (ELISA) kits from Bio-Rad Company were used to screen plasma samples for evidence of HBV infection: HBsAg (Monolisa™ HBs Ag ULTRA) and Anti-HBc (IgM and IgG) (Monolisa Anti-HBc PLUS). Patients with positive HBsAg and HBcAb tests (HBsAg+ HBcAb+) were considered for HBV co-infection. All the assays were performed as per the manufacturer’s instruction.
Detection of Anti-HCV
Hepatitis C infection was determined based on anti-HCV and capsid antigens positivity using a 3rd generation enzyme-linked immunosorbent assay (Monolisa™ HCV Ag-Ab ULTRA V2 ELISA kits, Bio-Rad). All samples with detectable anti-HCV in the first assay were retested by the ARCHITECT Anti-HCV assay (Abbott). Both assays were performed according to the manufacturer’s instructions.

Detection of HCV RNA
The Abbott RealTime HCV assay (Abbott Molecular, Des Plaines, IL, USA) was employed to confirm further anti-HCV positive samples. Steps related to HCV RNA extraction, concentration, amplification, and detection of the target region were performed automatically via the Abbott m2000rt instrument. The protocol for the assay was strictly followed as per the manufacturer’s instructions.

Statistical Analysis
Chi-square ($X^2$) or Fisher’s exact tests were used to compare categorical variables, which were summarized as frequencies, based on the Statistical Package for the Social Sciences (SPSS) version 20 (IBM Corporation, Armonk, NY, USA). A $p$-value $\leq 0.05$ is considered statistically significant.

Results
Sociodemographic and Clinical Characteristics
In this study, 345 CLD patients were enrolled in two groups: HCC (n=128) and non-HCC (n=217) patients. Male participants were dominant, 64.6% (223/345), with a 1.8:1 male to female ratio. The mean ± SD age was 43.6 ± 14.7 ranging from 18–84 years old. Nearly half of the study participants, 49% (169/345), were from Addis Ababa, 78.6% (271/345) were married, and 71% (245/345) were employed. The mean ± SD age of HCC and non-HCC patients was 50.3± 14.5 (range: 20–84 years old) and 39.6± 13.4 (range: 18–77 years old). The study participants’ baseline demographic and clinical characteristics are summarized in Table 1.

Serology of HIV ½ and HIV/HBV and HIV/HCV Co-Infection
Plasma samples from all CLD patients were screened for HIV1/2, HBV, and HCV infection markers. The overall frequency of HIV infection was 4.3% (15/345), with a 2% (7/345) and 0.3% (1/345) of HIV/HBV and HIV/HCV co-infection rate. The overall and subgroup prevalence of HIV, HBV, and HCV mono- and HIV/HBV and HIV/HCV co-infection is summarized in Table 2.

Of all HIV-positive patients (n=15), 46.7% and 6.7% co-infected with HBV (HBsAg HBcAb+) and HCV (anti-HCV+ HCV-RNA+), respectively, and 86.7% exhibited a marker of HBV exposure (total hepatitis B core antibody, HBcAb+). Broadly, our results implied a considerably higher distribution of HIV infection among subjects with past or present HBV infection.

Overall, the frequency of HIV and its co-infection with HBV or HCV exhibited an increasing pattern with the severity of liver disease (Figure 1 and Table 1). Hence, the overall frequency of HIV and HIV/HBV co-infection was marked among HCC patients compared to non-HCC patients (8.6% vs 1.8%) and (3.9% vs 0.9%). In addition, the only HIV/HCV co-infection was detected among HCC patients (Tables 1, 2 and Figure 1). Also, the frequency of HIV mono-infection was relatively higher among HCC than non-HCC patients (3.9% Vs 0.9%), but the difference was not significant. Conversely, the frequency of HBV mono-infection was significantly higher in non-HCC patients than in their counterparts ($p<0.001$) (Table 2); because most of the chronic hepatitis patients in the none-HCC group were referred to the GI clinic from different outpatient clinics based on their HBV status during the routine clinical practice.

Factors Associated with HIV and HIV/HBV Co-Infection and HCC
Among the risk factors, age (38–47 years old, $p=0.003$) (Figure 2), gender (females, $p=0.05$), tattoo ($p=0.004$), marital status (divorced, $p=0.03$), province (Amhara region, $p=0.01$), clinical status (HCC, $p=0.005$), and chronic consumption of
alcoholic beverages \((p=0.01)\) significantly associated with the rate of HIV infection \((\text{Tables 1 and 3})\). Of the risk factors, only age \((38–47 \text{ years old})\) \((p=0.006)\) exhibited a significant association with HIV/HBV co-infection frequency. About 55\% \((189/345)\) and 14\% \((48/345)\) of chronic liver disease patients were chronic consumers of alcohol and cigarette smokers, respectively. Consequently, chronic alcohol consumption and smoking showed a marginal association with HCC than non-HCC cases \((61.7\% \text{ vs } 50.7\%, p=0.05\) and \(18.8\% \text{ vs } 11.1\%, p=0.05)\). Compared with non-HCC, older age \((\geq 58 \text{ years old})\) exhibited a significant association with HCC cases \((39.1\% \text{ vs } 16.1\%, p<0.001)\).
Table 2 Frequency of HIV, HBV, and HCV Mono- and HIV/HBV and HIV/HCV Co-Infection Among CLD Patients

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>Mono Infection</th>
<th>Co-Infection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HIV N (%)</td>
<td>P-value</td>
</tr>
<tr>
<td>HCC (n=128)</td>
<td>5 (3.9)</td>
<td>0.1</td>
</tr>
<tr>
<td>Non-HCC (n=217)</td>
<td>2 (0.9)</td>
<td>144 (66.4)</td>
</tr>
<tr>
<td>Total (n=345)</td>
<td>7 (2.0)</td>
<td></td>
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</tbody>
</table>

Abbreviations: HCC, hepatocellular carcinoma; Non-HCC, non-hepatocellular carcinoma; CLD, chronic liver disease.

Discussion

This study has revealed the prevalence of HIV and its co-infection with HBV and HCV and potential associated infection risk factors among hospitalized patients with chronic liver disease in Ethiopia.

Irrespective of the liver disease stage, the overall prevalence of HIV among the study group was 4.3%, slightly higher than rates of community-based prevalence that ranged from 3.0% to 3.7%, reported in Ethiopia. The prevalence of HIV infection...
### Table 3 Factors Associated with Human Immunodeficiency Virus Infection

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>HIV Status</th>
<th>P-value</th>
<th>X²</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Overall N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative N (%)</td>
<td>Positive N (%)</td>
<td></td>
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<tr>
<td></td>
<td>N (%)</td>
<td>(%)</td>
<td></td>
</tr>
<tr>
<td>Tattoo</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Received blood/blood products</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Body piercing</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Visit dental clinic</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Invasive medical procedure</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Alcoholic beverage</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Sex partners</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Smoking</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Abortion</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
<tr>
<td>Condom use</td>
<td>330 (95.7)</td>
<td>15 (4.3)</td>
<td>345 (100.0)</td>
</tr>
</tbody>
</table>

Notes: The symbol $^\text{a}$ represents only female subjects. All values in bold are statistically significant values.

Abbreviation: CLD, chronic liver disease.

In specific groups and geographic regions is known to vary. For instance, a systematic review indicated a 5.74% pooled prevalence of HIV among pregnant women with some regional variation (4.8% in Addis Ababa, 4.48% in Oromia, and 2.14 in SNNPR). Furthermore, others have shown a strikingly high frequency of HIV in patients who took anti-TB treatment in northern Ethiopia and among female sex workers in Addis Ababa (29.3% and 24%). The difference in the prevalence of HIV

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**Notes:**
- **Tattoo**
- **Received blood/blood products**
- **Body piercing**
- **Visit dental clinic**
- **Invasive medical procedure**
- **Alcoholic beverage**
- **Sex partners**
- **Smoking**
- **Abortion**
- **Condom use**

**Abbreviation:** CLD, chronic liver disease.
reported in this study from previous reports is due to study population differences (hospitalized CLD patients) and factors including sample size, HIV risk status, and reporting period due to the changing nature of the HIV epidemic.

Due to their shared mode of transmission and risk factors, detecting a considerable amount of HBV and HCV infection among people living with HIV (PLWH) is not unusual. Accordingly, the frequency of HIV/HBV and HIV/HCV co-infection in the present study was 46.7% (7/15) and 6.7% (1/15). The prevalence of HIV/HBV co-infection in this study is higher than the previous reports from Ethiopia, which ranged from 3.0% to 11.7%,23,33–40 and in other African countries, including Nigeria (7.8%), Kenya (5.8%), Uganda (16.9%), and Sudan (11.7%).31–44 Similarly, previous studies from Southern, Central, North-west, and Northeast Ethiopia have reported a higher anti-HCV prevalence than our report, ranging from 1.3% to 5.2% among PLWH.34,35,38–40 Other related studies in Ethiopia, on the other hand, reported a comparable45 and even higher46 frequency of HIV/HCV co-infection with our study. This variation in the frequency of HIV co-infection with HBV/HCV could be explained by a difference in geography, risk factor, transmission mode, and age of infection, plus the possible reasons mentioned above for the difference in HIV prevalence between the present and previous reports. This is also a likely explanation for the differences in HIV prevalence in this study, 4.3%, compared to previous reports among CLD patients from Southern Tamil Nadu, 5.3%; Addis Ababa, 9.8%; and Nigeria, 18.2%.57–49

Chronic liver disease is Ethiopia’s 7th leading cause of death50 and Chronic hepatitis B and C infections and chronic consumption of alcoholic beverages are the principal etiologic agents of CLD.18,51,52 Today, the number of deaths related to AIDS markedly reduced due to the launching of antiretroviral treatment (ART) programs in low and middle-income regions, such as sub-Saharan Africa, where both HBV and HIV are highly prevalent.11 However, viral hepatitis B and C-related liver disease are common among people living with HIV and are responsible for the death of co-infected individuals due to non-AIDS causes.53 In parallel, reports from the above studies and our study evidenced the concurrent existence of the two-hepatotropic viruses (HBV or HCV) among HIV-positive individuals in Ethiopia and their potential to cause CLD and subsequent non-AIDS end-stage liver diseases related death.

The overall frequency of HIV and its co-infection with HBV or HCV showed an increasing pattern with the severity of liver diseases. Notably, the frequency of HIV and HIV/HBV co-infection rate was augmented among HCC than non-HCC patients (8.6% vs 1.8%, \( p=0.005 \)) and (3.9% vs 0.9%, \( p=0.1 \)). Besides, the only HIV/HCV co-infection was detected among HCC patients. However, the exact reason for such disproportionate prevalence of HIV and co-HIV/HBV infection among HCC patients is not apparent.

A consistent report by Otedo et al from western Kenya showed a significantly higher frequency of HIV infection among patients with HCC than those without HCC.54 Thus, the present and the Kenyan study findings imply that HIV patients are more prone to HCC than HIV-negative patients. This might be due to the marked decline of most AIDS-related opportunistic infections following extensive and effective use of ARTs; alternatively, infections accompanying HBV or HCV and ART-associated (hepatotoxicity) liver diseases have emerged as fundamental causes of morbidity and mortality among HIV/AIDS patients. Robbins et al showed liver cancer as the second non-AIDS defining cancer with a high incidence rate over time among people living with HIV/AIDS in the United States since the introduction of ART (1996).55 Hence, these might partly be the reason for the high frequency of HIV-Ab among HCC patients in the current study. However, the exact correlation between HIV infection and HCC is unclear and needs further study.

Moreover, 86.7% (13/15) of HIV-positive patients exhibited a marker for HBV exposure (hepatitis B core antibody, HBCAb); the coexistence of these two viruses (HIV and HBV) promotes the faster progression of liver disease to the advanced stage (Cirrhosis and HCC) in those living with HIV/AIDS.56 According to the study of Nina et al among HIV/HBV co-infected individuals, HBV-DNA (>2000IU/mL) or detectable viremia of both HBV and HIV, or undetectable HIV with detectable HBV viremia showed a strong correlation with HCC apart from heavy alcohol drinking and HCV infection.56

In addition to HIV and co-HIV/HBV infections in the present study, older age (≥ 58 years), alcohol consumption, and cigarette smoking showed a significant correlation with HCC.56 In addition, different related studies have shown an increased risk of HCC among chronic alcoholic beverage consumers and cigarette smokers.57,58 Moreover, the incidence of HCC increases with advanced age, which is more pronounced in those who chronically consume alcohol and smoke.56,59,60

We have also demonstrated a significantly higher prevalence of HIV infection in those aged (38–47 years old), women, and divorced, which is in line with the Ethiopian national report. As per the central statistical agency report, the peak prevalence of HIV was between 40–49 years old for men and 40–44 years old for women.29 Likewise, as the 2011
and 2016 DHS national HIV data showed, the second-highest group next to widowed individuals with a high frequency of HIV infection was divorced. In addition, the high prevalence of HIV was demonstrated among patients from the Amhara region; however, it is difficult to conclude with such a small sample size and study design. Nonetheless, reports from two national surveys have suggested that areas in the Amhara region, bordering the Tigray and Afar regions, are critical hotspot cluster areas for HIV transmission. Likewise, the association of tattooing and alcohol drinking habits with HIV infection observed in this study was also mentioned previously.

**Conclusion and Recommendations**

Our study revealed an increasing pattern of HIV and HIV/HBV and HIV/HCV infection rates with the severity of the liver disease. Thus, screening all HIV-positive individuals for HBV and HCV infection and vice versa and taking all the necessary preventive measures, including immunization against HBV, is vital for prevention, early detection, and proper management of CLD and reduce CLD-related death in people living with HIV in Ethiopia.

**Limitation of the Study**

Since the HBV serology results did not accompanied by data from the HBV DNA test, we might miss occult hepatitis B infections.

**Abbreviations**

AdLD, advanced liver disease; AHRI, Armauer Hansen Research Institute; AIDS, acquired immunodeficiency syndrome; ART, antiretroviral therapy; CLD, chronic liver disease; DHS, Demographic & Health Survey; ELISA, Enzyme-linked immunosorbent assay; HAART, Highly Active Antiretroviral Therapy; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; PLWH, People living with HIV; SNNPR, southern nation and nationalities people region.

**Data Sharing Statement**

All data supporting our report are incorporated in the manuscript.

**Ethical Consideration**

The Institutional Review Boards (IRBs) of College of Medicine and Health Sciences, Hawassa University (Ref. no: IRB/099/08) and College of Health Sciences, Addis Ababa University (Ref. no: 056/16/DMIP), and Armauer Hansen Research Institute (AHRI)/All Africa Leprosy and Tuberculosis Rehabilitation and Training Center (ALERT) ethics review committee (AAERC) (Ref. no: P025/16) approved the protocol of this study. In addition, all eligible participants were informed about the purpose of the study, in accordance with the Declaration of Helsinki.

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**Disclosure**

The authors report no conflicts of interest in this work.

**References**

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