Catha edulis and chronic liver disease in eastern Ethiopia

by

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Abbreviations

AIH:	autoimmune hepatitis
ALT:	alanine aminotransferase
AMA:	anti-mitochondrial antibodies
ANA:	anti-nuclear antibodies
Anti-HCV:	hepatitis C virus antibody
APRI:	aspartate aminotransferase to platelet ratio index
CI:	confidence interval
CLD:	chronic liver disease
CYP2D6:	cytochrome P450 2D6
ELISA:	enzyme-linked immunosorbent assay
HBsAg:	hepatitis B surface antigen
HBV:	hepatitis B virus
HCV:	hepatitis C virus
HDV:	hepatitis D virus
HIV:	human immunodeficiency virus
IgG:	immunoglobulin G
IIF:	indirect immunofluorescence
NAFLD:	non-alcoholic fatty liver disease
NASH:	non-alcoholic steatohepatitis
OR:	odds ratio
PBC:	primary biliary cholangitis
RDT:	rapid diagnostic tests
SMA:	smooth muscle antibodies
VL:	visceral leishmaniasis
WHO:	World Health Organization

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I. Unexplained chronic liver disease in Ethiopia: a cross-sectional study.

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II. Khat chewing increases the risk for developing chronic liver disease: a hospital-based case-control study.

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III. High seroprevalence of autoantibodies typical of autoimmune liver disease in eastern Ethiopia – is chewing khat (*Catha edulis*) a triggering factor?

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1 Background

The liver is a pyramid-shaped organ sitting in the upper right quadrant of the abdominal cavity just below the diaphragm protected by the lower half of the right rib cage [1]. It is the largest solid organ in the body; median liver weight is 1800 grams in men and 1400 grams in women, comprising approximately 2% of the adult body weight [2].

The liver performs numerous vital functions, including (i) metabolism of carbohydrates, lipids, proteins, and vitamins; (ii) maintaining the acid-base and energy balance; (iii) storage of trace elements and vitamins; (iv) extramedullary haematopoiesis; (v) production of various substances, including carrier proteins, hormones, vitamins, cholesterol, bile acids, coagulation factors and signal substances; and, (vi) degradation and/or detoxification of superfluous and harmful endogenous or exogenous substances [3].

1.1 Chronic liver disease

In a clinical context, ‘chronic liver disease’ (CLD) is the term used to describe disordered liver function lasting for six months or more [4]. It results from a process of progressive destruction and regeneration of the liver parenchyma and encompasses a wide range of liver pathologies including chronic hepatitis and cirrhosis.

Cirrhosis is characterised by evidence of diffuse regeneration and nodular regrowth of normal liver parenchyma surrounded by widespread fibrosis with parenchymal destruction and collapsed liver structures [5]. However, the liver may continue to function well for several years and the CLD usually is asymptomatic until cirrhosis with clinical decompensation occurs. Hence, in a clinical context, decompensated cirrhosis is defined by the development of liver insufficiency (clinically: jaundice) and/or complications of portal hypertension (clinically: ascites, haematemesis due to bleeding oesophageal varices, and hepatic encephalopathy) [6].

Both compensated and decompensated cirrhosis may be subclassified further, and cirrhosis is not strictly the end stage of CLD but encompasses a pathological spectrum reflecting a dynamic process; increasing evidence suggests that with successful treatment cirrhosis may even reverse [7].

CLD is a major cause of morbidity and mortality and was responsible for an estimated 1.3 million deaths worldwide in 2015 [8]. The commonest causes of CLD are chronic infection with hepatitis B (HBV) or C (HCV) virus, alcohol misuse and non-alcoholic fatty liver disease (NAFLD) [5].

Chronic viral hepatitis is a major health problem with an estimated 257 million individuals chronically infected with HBV and 71 million with HCV worldwide [9]. In the absence of treatment, about a fifth of patients with chronic HBV and a similar proportion of patients with persistent HCV will progress to cirrhosis [10]. Between 1990 and 2013, global viral hepatitis deaths increased from 0.89 million to 1.45 million and disability-adjusted life years (DALYs) from 31.7 million to 42.5 million [11]. There is wide geographical variation in the prevalence of HBV and HCV; the highest rates are found in East Asia and Africa [12, 13].

Alcohol misuse is also a well-known risk factor for liver cirrhosis and a leading cause of death and disability [14]; in 2012 over three million deaths were attributed to alcohol consumption worldwide, corresponding to 5.9% of the global total or one in every twenty deaths [15]. Moreover, 5.1% of the global DALYs were attributable to alcohol consumption [15]. Persistent alcohol misuse results in the development of cirrhosis in about a fifth of misusers.

There is wide geographical variation in the proportion of alcohol-attributable deaths and DALYs worldwide. The highest alcohol-attributable fractions are reported in the World Health Organization (WHO) European Region [15]. In this region, alcoholic liver disease is the most frequent cause of advanced liver disease and the leading cause of death in adults with alcohol misuse [16].

NAFLD has become one of the most important causes of liver disease worldwide and is the leading cause of CLD in the USA and Europe [17]. A subgroup of NAFLD patients has non-alcoholic steatohepatitis (NASH), which is more progressive and associated with an increased risk of developing advanced liver disease, cirrhosis or hepatocellular carcinoma [18].

NASH-related cirrhosis is now the second most common cause of CLD among adults waiting for liver transplantation in the USA [19] and has been projected to become the leading indication for liver transplantation within the next decade [20]. Fuelled by the global epidemic of obesity, the prevalence rates of NAFLD are constantly increasing worldwide;

estimated global prevalence of NAFLD is 25.2%, with the highest rates in the Middle East (31.8%) and South America (30.5%), and the lowest rates in Africa (13.5%) [21]. However, the estimates differ widely depending on the definition used and the population studied, and the prevalence of NAFLD is less well described outside the Western world.

1.1.1 Chronic liver disease in Ethiopia

Ethiopia is a low-income country in East Africa with a population of around 100 million with an estimated life expectancy of 63 years for men and 67 years for women [22]. The prevalence of CLD in Ethiopia is largely unknown but is assumed to be high [23]. Likewise, the relative burden of CLD by aetiology is largely unknown although it has been suggested that more than 60% of CLD are attributed to chronic HBV or HCV infections [24].

The estimated seroprevalence of hepatitis B surface antigen (HBsAg) in Ethiopia is 6.0% [25] and of HCV-antibody (anti-HCV) 3.1% [24]. However, these data are mainly extracted from institution-based studies and may not be representative of the situation nationwide.

Alcohol-related disorders are less prevalent in Ethiopia (2.1%) compared to both the regional (WHO Africa: 3.3%) and the global estimates (4.1%) [15]. Likewise, the estimated alcohol-attributed fractions from all-cause deaths in Ethiopia were 3.2%, and thus less than the regional estimate (WHO Africa: 3.3%) and the global estimate of 5.9% [15]. As far as we know, there are no updated representative nationwide data on the prevalence of alcoholic liver disease in Ethiopia, but it is considered to be low [23]. A recent retrospective study of 117 patients presenting with CLD at St. Paul's Hospital Millennium Medical College in Addis Ababa between 2009 and 2014 reported a low proportion of alcohol-related CLD (1.7%) [26].

To the best of our knowledge, no prevalence studies on NAFLD or NASH in Ethiopia are available, however, since Ethiopia has some of the lowest prevalence rates of body mass index (BMI) $\geq$25.0 kg/m^2 worldwide [27], it is expected to be low.

Intestinal schistosomiasis caused by the trematode *Schistosoma mansoni* may trigger a granulomatous response in periportal regions in the liver leading to periportal fibrosis and a gradual occlusion of portal veins with subsequent portal hypertension [28]. *S. mansoni* is highly endemic in Ethiopia, although with wide geographical variations [29, 30].

Long-standing hepatosplenic schistosomiasis with high intensity of infection has been shown to be an important risk factor for CLD in Ethiopia [31].

The previous study exploring the aetiological spectrum of CLD in Ethiopia was undertaken over a three-year period in the late 1980s studying 334 hospitalized adults with CLD in a governmental reference hospital in Addis Ababa [23]. The diagnoses were based on clinical, laboratory and histological information; overall 4% had chronic hepatitis; 62% had cirrhosis and 34% hepatocellular carcinoma. Overall, one or more HBV markers were found in 84% of the patients; other causes of liver disease were identified only infrequently.

The patients were subjected to a comprehensive assessment, but prior to the discovery of HCV and with only limited diagnostic assays available. The study was conducted in the Shewa province in central Ethiopia but reported a substantial fraction of the CLD cases originating from the Harerghe province in eastern Ethiopia, where CLD for long has been reported as one of the most frequent reasons for hospital medical admission [32].

In recent years, community-based longitudinal studies have been undertaken in several rural areas of Ethiopia using a verbal autopsy method to assign causes of death [33-35]. CLD was the leading cause of death in the age group 15-49 years in Kersa in eastern Ethiopia (13.7%) [35] and in Butajira in central Ethiopia (11.3%) [33]. In contrast, CLD was the cause of death in only 3.5% of adults of the same age in Kilte Awlalo in northern Ethiopia [34].

One suggested explanation for this difference is the relative availability of khat (*Catha edulis*), an indigenous plant which is chewed for its psychotropic effects. Khat chewing has been associated with the development of CLD [36]; its use is widespread in eastern [37] and south-central Ethiopia [38] but much less so in northern parts of the country [39].

1.2 Khat - Catha edulis

1.2.1 History of khat use

During an ill-fated Danish expedition to Egypt and Yemen in 1761-63, the Swedish botanist Peter Forsskål (1736-63) identified an edible evergreen shrub with the common Yemeni name ‘qât’ (Arabic: قات) which he designated the Latin scientific name *Catha edulis*, also known as khat* (Figure 1) [40-42].

Fig. 1: A bundle of fresh khat leaves from the fields of Harar

Khat is indigenous to East Africa with a natural range extending from Eritrea, Ethiopia, and Somalia in the north, throughout to South Africa [43]; it has been introduced worldwide and is also found in Afghanistan, Turkmenistan, Syria, Israel, Saudi Arabia and Yemen [36, 41]. Khat grows naturally in humid mountainous environment, favouring altitudes of 1200-2500 metres depending on latitude.

* Khat is spelled in numerous ways: e.g. *kat*, *kaht*, *chat*, *tchat*, *gaad*, *quat*, *qat* (Arabic: قات; Amharic: ጫት). *‘Qât’* is probably the most correct transcription from Arabic; we will, however, use ‘*khat*’ because it is widely used in the current literature. Moreover, more than fifty names are attributable to khat, e.g. the Oromo ethnic group call it ‘*jimma*’, while in Kenya khat is known as ‘*miraa*’ and in Tanzania ‘*mairungi*’; khat is also known as *Abyssinian -* / *African - / Arabian -* or *Bushman’s tea* [41].

The primary areas of commercial cultivation have traditionally been in the Oromo and Harari regions in eastern Ethiopia, the Meru County in central Kenya and the slopes around the city Taiz in south-western Yemen [41]. Although the exact origin of khat is uncertain, most researchers claim it originates from Ethiopia [44] where the Harari region traditionally is thought of as the centre of origin of its use [45].

One of the earliest reliable historical references to the use of khat is in a contemporary account of the military campaigns of the emperor ‘Āmda Ṣeyon I, who reigned in Ethiopia from 1314 to 1344 [46]. The chronicle quotes the Sultan of Ifat, Sabr ad-Din, bragging about how he would conquer ‘Āmda Ṣeyon’s Christian Kingdom and plant khat in the capital “*because the Moslims love that plant*” [46, p. 55-56]. Another early account of khat is by the Arab historian Ibn Fadl Allâh al-‘Umari (1301-1349) describing the psychostimulatory effects of khat in his authoritative work *Masâlik al-absâr fi mamâlik al-amsâr* [47].

At harvest, the top shoots of khat are cut and packed into bundles. To keep the khat fresh it may be wrapped in banana leaves, wet clothes or plastic bags [44]. Khat is usually chewed fresh but occasionally the leaves are dried and brewed as tea, prepared as infusions or decoction [41], or more rarely smoked [43]. In the USA, a dried form of khat known as ‘graba’ has gained popularity, especially among Somali and Ethiopian nationals [48].

In Scotland, a study of young adults (16-25 years of age) attending rave parties found that khat was one of the drugs of choice [49]. Khat leaves were blended with water and lemon, filtered and the khat juice sold by the glass, or as 25 ml shots of a khat tincture. According to a marketing leaflet, khat produces “*feelings of euphoria, increased libido, talkativity, excitement, loads of energy, and a big khat smile*” [49, p. 169].

Although there are different modes of use, khat is traditionally consumed by chewing freshly picked khat leaves over several hours and the juice of the masticated leaves is swallowed. During a typical ‘khat chewing session’, 100-300 grams of fresh khat will be consumed [50]. Khat chewing is predominately a social habit and the fresh khat leaves are chewed for its stimulating effect and to attain a state of euphoria enhancing social interaction [51].

The effects of khat use have interpersonal variations; however, usually there are certain stages of intoxication associated with recreational khat chewing during a khat gathering: (i) the first part is dominated by an atmosphere of optimism, socially friendliness, high spirits and excited

mood that causes loquacity and a general sense of well being; (ii) after the initial phase, which usually lasts for 1-2 hours, tension arises as the discussion tend to focus on greater problems and verbal aggressiveness and emotional instability becomes apparent; (iii) following the loud and intense exchange of ideas, the khat chewers enters a quieter and introspective mood, characterized by increased imagination and creativity; (iv) the final stage is dominated by a depressive atmosphere filled with melancholia and great concern and a feeling of depletion as the khat session ends, usually after some hours [44, 52, 53].

In addition to its social and recreational use, farmers and manual labourers use khat before and during work in order to provide energy and increase performance, while many students chew khat to increase alertness and be wakeful especially during examination periods [54]. Khat chewing is still considered as a predominantly male habit [55], but women are increasingly practicing it, including during pregnancy and lactation [56, 57].

In traditional medicine, processed leaves and roots of khat have been used for a wide range various ailments, such as male impotence, gonorrhoea, headache, stomach ache, vomiting, asthma, cough, influenza, and malaria [41-43, 58]. Furthermore, khat chewing practises have a long tradition in a religious context during praying and worship sessions among Muslims, and some believe that khat use facilitates contact with Allah, hence khat is referred to as the Flower of Paradise [59] and the Leaf of Allah [60].

1.2.2 Epidemiology: khat going global

The habit of chewing khat is common in the Horn of Africa, Arabian Peninsula and the East Coast of Africa where it is considered as a part of the social and cultural heritage which has prevailed for centuries, probably as far back as the 13th century [41]. Khat usage has traditionally been confined to the Islamic religious and political elite and among people living in or adjacent to areas of cultivation; however, the khat user habits have changed dramatically the last five decades and now cuts through social levels, religion, and age groups [61].

Agricultural development and the introduction of motor and air transport have increased the availability and enlarged the area of consumption; consequently, the use of khat has mushroomed [62]. Over the last three decades, the Horn of Africa and the Middle East have been engulfed by war and instability, leading to mass migration and a wider spread of khat consumption within immigrant communities worldwide [63].

In addition, there is a growing interest in plant-derived and uncontrolled psychoactive substances among youth in Europe [64] and the USA [65]. The exact global prevalence is unknown, but estimates range up to 20 million daily users, which most probably is an underestimate [66].

1.2.3 Epidemiology: khat in Ethiopia

Since the turn of the new millennium, khat has become Ethiopia's second most important national export crop after coffee [67, 68]. Khat is freely available in Ethiopia and khat chewing is widespread, particularly in the eastern regions where most of the khat cultivation in the country takes place [60, 68]. The number of khat chewers in Ethiopia has increased rapidly and become popular in all segments of the population [61].

A house-to-house survey in a rural community in south-central Ethiopia, comprising 10 468 adults reported a prevalence of lifetime khat chewing 55.7% [38], whereas a survey conducted in different rural areas of Ethiopia reported a prevalence of 31.7% [57]. The 2011 Ethiopian Demographic and Health Survey, identified an overall national prevalence of khat chewing of 15.3% with vast regional variations; the highest prevalence was in the Harari region (53.2%) and the lowest in the Tigray region (1.1%) [39]. A regional study among 1890 secondary school students in Harar city found a lifetime khat chewing prevalence of 24.2% and 20.9% chewed khat daily [37].

All studies indicate that khat chewing is more common among Muslims than Christians and males more than females. However, a community-based cross-sectional study of 1678 pregnant women in rural communities in the Haramaya district in eastern Ethiopia reported a daily khat chewing prevalence of 34.6%, showing that khat chewing is highly prevalent in this area, even among pregnant women [56].

1.2.4 Pharmacology

Khat leaves contain a number of different chemical substances, including different kinds of alkaloids, flavonoids, tannins, steroids, terpenoids, vitamins and minerals [69]. The main alkaloids are phenylalkylamines comprising phenylpropylamines and phenylpentenylamines, and cathedulins, of which more than 60 different types have been characterized but not yet explored for their biological activities [70, 71].

The phenylpentenylamines are considered unlikely to possess any significant psychostimulatory property [72]. The three main phenylpropylamine types are cathinone (aminopropriophenone), cathine (norpseudoephedrine) and norephedrine. Cathinone is considered the main molecule associated with the state of alertness and euphoria associated with khat usage [66].

Cathinone is an unstable intermediate in the biosynthesis of cathine, and thus the highest concentration is found in the young shoots and sprouts, where the cathinone content comprises about half of the total [73]. However, the cathinone content depends on the plant quality, time of harvest and geographic origin; khat from Ethiopia or Kenya has significantly higher cathinone content than khat from Yemen [74].

A two-compartment model characterizes the khat absorption with the first compartment being the oral cavity and the second stomach and/or small intestines, which is responsible for the delayed phase of absorption [50]. The buccal mucosa plays a major role in the absorption of and most of the khat alkaloids are extracted during chewing [50].

Cathinone decomposes to cathine, mainly by light or upon wilting of the khat leaves, or to norephedrine, mainly through human metabolism (Figure 2) [71].

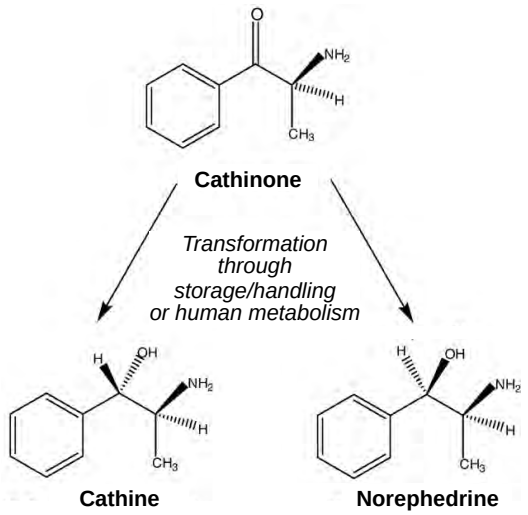

Fig. 2: Cathinone transforms into cathine or norephedrine. Modified from [71].

Cathinone is metabolized extensively in the liver via the cytochrome P450 enzyme 2D6 (CYP2D6) [75], and after a single dose of khat the peak plasma concentration (T_{max}) of cathinone is obtained after 1.5-3 hours [71]. Cathinone is undetectable in blood samples 24 hours after ingestion (Figure 3) [50].

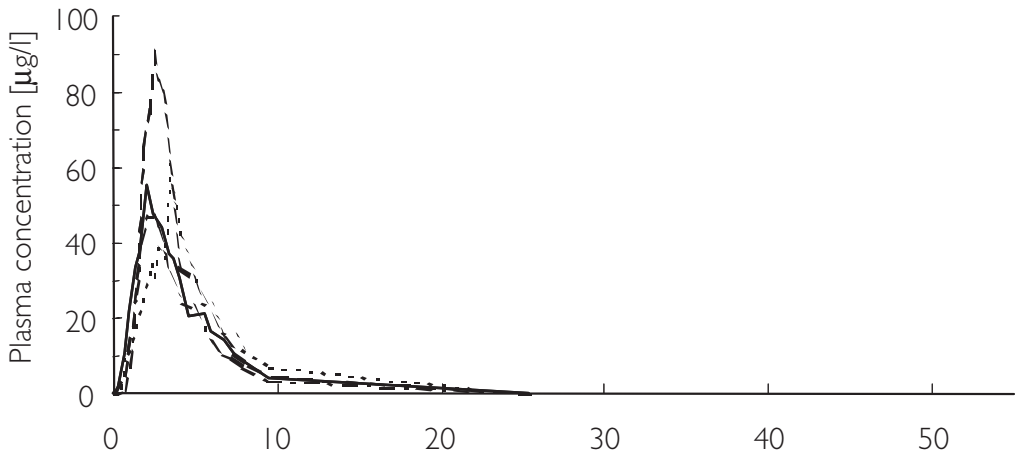

Fig. 3: Time profile of plasma cathinone. Modified from [50].

Less than 7% of the cathinone absorbed can be detected in urine by gas chromatography-mass spectrometry, whereas cathine and norephedrine are slowly absorbed and then excreted mainly in the unchanged form within approximately 24 hours [76].

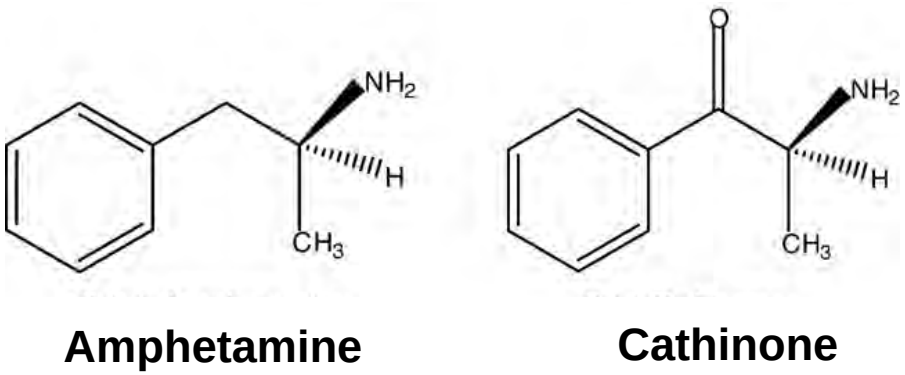

Fig. 4: The chemical structure of amphetamine and cathinone. Modified from [71].

The chemical structure of cathinone resembles that of amphetamine (Figure 4) and exerts sympathomimetic effects by inducing presynaptic release of monoamine neurotransmitters such as dopamine, noradrenaline, and serotonin, and reducing the re-uptake from the synaptic gap [77]; cathinone has also been shown to activate presynaptic alpha-2-adrenergic and 5-hydroxytryptamine-7 receptors inhibiting release of acetylcholine in parasympathetic nerves [78]. Hence, cathinone induces various indirect sympathetic effects on the central and peripheral nervous system including euphoria, restlessness, increased alertness and energy, mydriasis, dry mouth, anorexia, hyperthermia, tachycardia and hypertension [44, 66, 69].

1.2.5 Adverse effects

Cathinone and cathine are controlled substances under the International Convention on Psychotropic Substances (1971), whereas fresh khat leaves are not. The WHO has defined khat as a drug of abuse as it may lead to health and social problems [79].

The growing concerns over the personal and social negative effects of khat, including drug-related violence, financial insecurity, detrimental effects on family life and unemployment are well-recognized but debatable [68, 80].

Chronic khat use is associated with psychological adverse effects, including psychosis [81] and exacerbation of pre-existing psychotic disorder [82, 83]. Khat users may also exhibit dependence; in the majority, however, the degree of associated physical dependence is low while the levels of psychological dependence may be substantial [71].

Khat chewing is also associated with a number of somatic health sequelae (Table 1) and a wide range of consumption-related factors may contribute to khat-related mortality [36].

Table 1: Reported adverse effects of khat on different organ systems

Organ system	Adverse effect
Central nervous system	Insomnia [84], impaired inhibitory control and cognitive functioning [85, 86], disturbances in emotion regulation processes [87], trembling and impaired psychomotor coordination [88], reversible cerebral vasoconstriction [89], stroke [90]
Endocrine system	Poor glycaemic control [91, 92], hyperthermia [93], perspiration [94, 95]
Cardiovascular system	Systemic hypertension [96-98], coronary vasoconstriction [99], acute coronary syndrome [100], myocardial infarction [101-103]
Gastrointestinal system	Periodontal disease [104-106], oral malignancies [107-110], gastritis [106, 111], constipation [106, 111], haemorrhoids [112]
Reproductive system	Spermatozoa abnormalities [113-115], impaired foetal growth [116, 117]
Hepatobiliary system	Acute hepatitis [118-126], chronic liver disease [127-131]

Of special interest within the scope of this thesis was the increasing number of case reports of acute hepatitis [118-126] and cryptogenic cirrhosis [127-131] worldwide, implicating khat as a possible risk factor for the development of both acute and chronic liver disease.

1.2.6 Khat-related hepatotoxicity in humans

Besides some ancient sporadic anecdotal accounts of suspected khat-related liver injury in Yemen and Somalia [53, 94, 111, 132], the first case series suggestive of an association was published in 2005 [133]. D'Souza *et al.* [133] presented a cohort of six young Somalian men in the UK with autoimmune hepatitis (AIH) that did not respond to immunosuppressant therapy. Information about khat usage had not been sought initially but it was confirmed later on that the patients most likely were regular khat users [121].

In 2006, Brostoff *et al*. [118] reported a case of acute hepatitis in a man of East African origin who recently had started chewing khat on a daily basis and developed jaundice and right hypochondrial tenderness. He had evidence of acute mixed hepatocellular/cholestatic hepatitis, which resolved after supportive care and khat cessation.

In 2010, Chapman *et al*. [119] described six UK patients of Somali origin in whom khat abuse was associated with the development of fulminant hepatic failure and provided compelling evidence for a causal effect.

In 2010, Peevers *et al*. [127] published the first case series reporting khat-related cirrhosis and CLD in seven Somalian men in the UK, of whom all were regular khat chewers. All presented with jaundice and abnormal liver function tests, which resolved following khat withdrawal. No other aetiological factors were identified.

The first case-series of khat-related hepatitis outside of the UK were published in 2011 [120, 129]. Stuyt *et al*. [129] reported unexplained CLD in six male immigrants from Somalia and Ethiopia living in the Netherlands and noticed their history of chronic khat use. Further case reports and case series have been published worldwide, including USA [130], Oceania [121], Africa [131], Middle East [125] and Scandinavia [126].

1.2.7 Khat-related hepatotoxicity in experimental models

Animal models have demonstrated evidence of khat-related hepatotoxicity [134]. Short-term toxicological effects of khat have been shown in New Zealander white rabbits fed over a three months period with diets containing different proportions of washed khat leaves; a significant increase in serum liver enzyme activities and evident histological liver injury with both acute hepatocellular degenerative and regenerative activities were observed [135].

A subsequent study, by the same group, examined the long-term toxicological effects of feeding diets containing different levels of khat for six months, and again the serum activities of liver enzymes were significantly elevated; the greatest effects were seen in diets containing higher levels of khat [136]. Histopathological evidence of chronic inflammation of the portal tracts in all animals fed with khat was observed. Moreover, amongst animals fed with a higher dose of khat, porto-portal fibrosis was seen. Although the liver parenchyma architecture remained intact at this stage, spreading fibrosis could consequently lead to cirrhosis.

Further evidence of khat-related hepatotoxicity has been demonstrated in rats given a single daily dose of khat-extract calculated according to body weight and fed by oral gavage for four weeks [137]. Elevated liver enzymes activities and histopathological evidence of ballooning degeneration of hepatocytes with chronic inflammation in the liver was found in both male and female rats.

Al-Qirim *et al*. [138] showed that khat alkaloids induce changes in free radical metabolizing enzyme activity and subsequently induce oxidative stress leading to cell injury. These findings have been confirmed and further explored as khat has been shown generating reactive oxygen species that induce hepatocyte apoptosis through intracellular signal pathways in human liver cells [139].

1.3 Study setting

Our study was carried out in Harar, the capital of the Harari Regional State, which is surrounded by the Oromia Regional State, in the eastern part of Ethiopia (Figure 5).

Fig. 5: Political map of Ethiopia. Modified from [140].

The population in the Harari Regional State was projected to reach 246,000 in 2017, of whom approximately 56% reside in urban areas [141]. The hospitals in Harar are also the main referral health care providers to the people living in surrounding districts (Amharic: ***ወረዳ***, *wereda*) in the East Hararge-Zone and estimated to comprise more than 3.5 million individuals, of whom the rural population constitute more than 90% [141].

1.3.1 Jugal[†] Hospital

Jugal Hospital is a 200-bed governmental hospital in the Harari region, eastern Ethiopia. The hospital is situated within the historic old walled city of Harar Jugal, which was listed as World Heritage Site by UNESCO in 2006 [142]. The hospital is considered as the first governmental hospital in Ethiopia, founded in 1902 by the governor of Harar – Ras Makonnen Woldemikael Gudessa – the father of the eminent Last Emperor of Ethiopia – Haile Selassie I (born Ras Tafari Makonnen Woldemikael).

The hospital was named *Ras Makonnen Hospital* until the 1970's and during the Derg-regime when it was called *Misrak Arbegnoch* – literally meaning '*The eastern Patriots*'. Although it was commonly known as *Jugal Hospital* for a long time, the hospital officially changed its name again around 2010.

The hospital functions as a local hospital serving around 500,000 people. Besides a 30-bed medical department, it has standard surgical and obstetric services, ophthalmology and paediatric outpatient departments, tuberculosis and human immunodeficiency virus (HIV) treatment centres. Available diagnostic services include laboratory services with standard biochemistry and haematology, microscopy and a radiology department with ultrasound and conventional radiography (personal communication: Dr. Tekabe A. Ahmed).

1.3.2 Hiwot Fana Specialized University Hospital

Hiwot Fana Specialized University Hospital is another 200-bed governmental hospital in Harar. The hospital was founded during the Italian occupation (1936-1941) providing health services mainly to Italian soldiers. However, after the liberation, the hospital continued serving the community and is currently functioning as a referral hospital for about 4 million people in eastern Ethiopia.

The hospital has commonly been called *Chefe* and *Murate Hospital*, but from the mid-70's it was named *Hiwot Fana* – literally meaning '*The Path of Life*'. The Harari Regional Health Bureau directed the hospital until 2004 when it became a teaching hospital administrated by the Haramaya University College of Health and Medical Sciences.

[†] There are numerous variations in transcribing the Amharic ጁገል: (Jugel – Jugol – Jugaal – Jogol – Jugla), and although 'Jugel' probably is the most correct transcription, 'Jugal' is more commonly in use.

The hospital has a 40-bed medical department, a surgical department with general surgery and orthopaedic sections, tuberculosis and dermatology clinics, ophthalmology and psychiatry outpatient departments, paediatric and obstetric departments, tuberculosis and HIV treatment centres. Available diagnostic services include a fairly well equipped laboratory performing standard biochemistry and haematology tests, microscopy and a radiology department with ultrasound and conventional radiography (personal communication: Dr. Nejib Y. Ismael).

Medical sub-speciality services are not available in either hospital; thus, all patients with CLD attend a general medical outpatient clinic and, if admitted, are housed on a general medical ward.

2 Aims and objectives

2.1 Main aim

Study the aetiology of CLD in eastern Ethiopia and assess potential risk factors for the development of CLD, including the habitual use of khat (*Catha edulis*).

2.2 Specific objectives

- Explore the aetiological spectrum in study subjects with newly diagnosed CLD in two public hospitals in eastern Ethiopia and the relative contribution of viral hepatitis (**Paper I**).

- Identify potential risk factors for unexplained liver disease, including environmental and/or lifestyle factors (**Paper I**).

- Study the association between khat and CLD (**Paper II**).

- Assess the presence of circulating autoantibodies associated with autoimmune liver disease in healthy Ethiopians without manifest liver disease (**Paper III**).

- Explore the hypothesis that chewing khat triggers an autoimmune process (**Paper III**).

3 Synopsis of results

3.1 Paper I

Unexplained chronic liver disease in Ethiopia: a cross-sectional study

In **Paper I**, 150 incident cases of CLD were included, of whom 108 (72.0%) were men and the median age was 30 (interquartile range 25-40). The CLD was attributed to chronic HBV infection in 55 (36.7%); hepatic schistosomiasis in four (2.7%); alcohol misuse in three (2.0%); chronic HCV infection in two (1.3%); AIH in two (1.3%) and visceral leishmaniasis (VL) in one (0.7%). No patients were identified with hepatitis D virus (HDV) infection, primary biliary cholangitis (PBC), NAFLD, haemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency or malaria. Thus, in the remaining 83 (55.3%) patients the liver disease was unexplained.

The majority (92.7%) of the study population did not drink alcohol; three (2.0%) individuals drank to excess. The overall prevalence of daily khat use was 78.0%.

All 83 patients with unexplained CLD were offered a liver biopsy. However, only five patients ultimately underwent the procedure a median (range) of 33 (20-64) weeks after their initial hospitalization. Foci of pale stained swollen hepatocytes were identified in four of the five patients, with no marked zonal distribution and suggestive of toxic injury. The fifth patient showed no evidence of adaptive parenchymal changes but rather mild mixed steatosis and focal single-cell necrosis. Although the numbers were modest and the differences small, the proliferation index, apoptotic scores and the collagen proportionate area tended to be higher among the three patients who chewed khat.

In conclusion, chronic HBV infection was found in around one-third of patients hospitalized with CLD in eastern Ethiopia. However, in over half of the hospitalized patients the aetiology of the liver disease was unexplained. The prevalence of khat chewing was much higher in the CLD population than expected, suggesting that chewing khat may be an important effect modifier and/or independent risk factor for the development of CLD in this part of the world.

3.2 Paper II

Khat chewing increases the risk for developing chronic liver disease: a hospital-based case-control study

In **Paper II**, we aimed to determine the association between chewing of khat and the risk for developing CLD by comparing the 150 cases with CLD from the preceding cross-sectional study (**Paper I**) to 300 control subjects without CLD. In univariable analysis there was a significant association between khat chewing and the risk of developing CLD (crude odds ratio [OR] 2.64; 95% confidence interval [CI] 1.56-4.58); however, the magnitude of the risk estimate was different in men and women (crude OR 7.37 vs. crude OR 1.04; p=0.001). HBV infection had a considerable confounding effect on the risk estimate (18%), whereas the effects of alcohol consumption (3%), age (2%) and HCV-infection (1%) were minimal.

The final multivariate analysis using a logistic regression model adjusting for age, alcohol use and chronic hepatitis B, demonstrated that the effect of khat on the risk of developing CLD was dependent on its interaction with sex. The effect was strong in men (adjusted OR 5.67; 95% CI 1.85-17.37; p=0.002), but not evident in women (adjusted OR 1.04; 95% CI 0.49-2.19; p=0.922).

Although confined to men, a dose-response relationship between increasing khat exposure and increasing gradient in the risk for developing CLD was observed after adjusting for age, alcohol use and chronic HBV infection. Individuals with low level khat exposure (0.1-5.0 khat years) had an adjusted OR of 3.58 (95% CI 1.05-12.21), individuals with moderate level khat exposure (5.1-40.0 khat years) had an adjusted OR of 5.90 (95% CI 1.79-19.44) and individuals with high-level khat exposure (more than 40.0 khat years) had an adjusted OR of 13.03 (95% CI 3.61-47.02) compared to those who never used khat (test for trend: p=0.0007). These findings were confirmed when khat exposure was employed as a continuous variable in the logistic model (per unit 1.007 (95% CI 1.001-1.013 p=0.019).

In a *post hoc* sensitivity analysis of 80 (53.3%) cases and 269 (89.7%) controls with no identifiable risk factors for CLD except that 66 (82.5%) and 178 (66.2%) respectively were regular khat users, the significant association between khat use and the risk for developing CLD was robust (crude OR 2.41; 95% CI 1.25-4.90; p=0.005). Likewise, the upward trend in the risk for developing CLD with increasing khat exposure observed in the primary analysis

was confirmed, with findings indicative of a dose-response relationship in men but no such relationship in women.

In conclusion, this study found a significant, sex-specific association between khat chewing and the development of CLD. In men, the association was strong and dose-dependent suggesting a causal relationship; no relationship was observed in women. Moreover, this study indicated khat-associated CLD as a new entity likely responsible for a significant proportion of the liver disease observed in countries where khat use is widespread.

3.3 Paper III

High seroprevalence of autoantibodies typical of autoimmune liver disease in eastern Ethiopia – is chewing khat (*Catha edulis*) a triggering factor?

In **Paper III**, the study subjects were the control subjects from the preceding case-control study (**Paper II**). A total of 273 study participants were included and the seroprevalence of anti-nuclear (ANA), smooth muscle (SMA) and anti-mitochondrial antibodies (AMA) was determined, and 169 individuals who reported current khat usage were compared to 104 individuals who never used khat.

Overall, 2.6% of the study subjects were positive for ANA, 15.4% for SMA and 25.6% for AMA. When comparing khat users to non-users, ANA was detected in 4.1% vs. 0% ($p=0.047$), SMA in 16.0% vs. 14.4% ($p=0.730$), and AMA in 24.9% vs. 26.9% ($p=0.704$). ANA was excluded from multivariable analysis since there were no seropositive in the reference group. In a logistic regression model adjusting for age and sex, there was no significant association between khat use and the seropresence of SMA or AMA.

We concluded that this study indicated that khat-related liver injury is mediated through other mechanisms than an autoimmune process. Of note, however, the seroprevalences of SMA and AMA were strikingly high in this Ethiopian population compared to global estimates, questioning their significance in the development of autoimmune liver disease and suggesting that diagnostic algorithms for autoimmune liver diseases developed in North America and Europe might lead to misdiagnosis of patients on the African continent.

4 Methodological considerations

Table 2: Overview of the study participants and study details

Paper I	150 patients with CLD	A cross-sectional study exploring the aetiological spectrum of CLD in two governmental hospitals in eastern Ethiopia.
Paper II	150 patients with CLD and 300 controls without CLD	A case-control study assessing the association between khat and CLD.
Paper III	273 healthy individuals without known autoimmune disease or liver disease	A cross-sectional study nested in the preceding case-control study exploring the seroprevalence of autoantibodies typical for autoimmune liver disease and its association to khat usage.

Paper I-III were all epidemiological studies. Specific inclusion and exclusion criteria are listed in each paper.

4.1 Study design and selection of the study participants

4.1.1 Paper I

Paper I was a cross-sectional study assessing indigenous adult (≥18 years of age) medical in-/ and outpatients presenting for the first time with features of CLD at two governmental hospitals in Harar, eastern Ethiopia between April 2015 and April 2016. The cross-sectional study design is suitable to provide information about the frequency and characteristics of various conditions at a specified time [143].

A total of 244 patients were consecutively recruited and evaluated for eligibility, of whom 150 patients were included, 89 were excluded and five withdrawn (Figure 6).

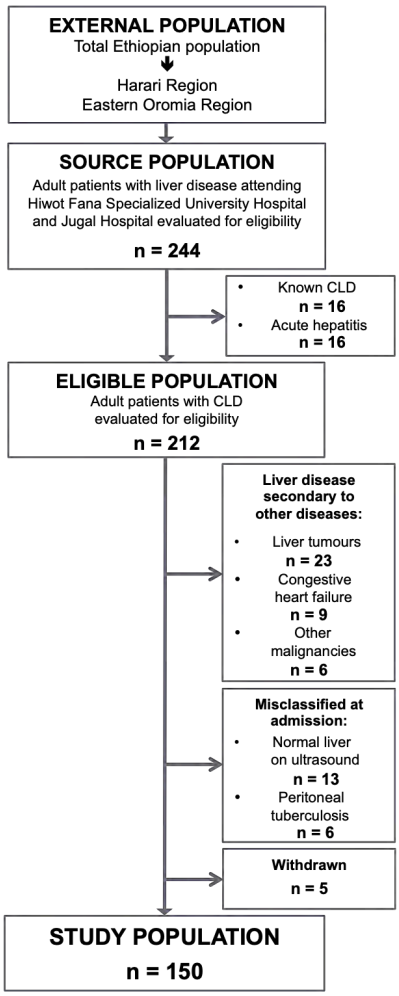

Fig. 6: Flow chart of the study subjects in the cross-sectional study (**Paper I**). Abbreviations: CLD, chronic liver disease.

There are several private hospitals in Harar with more advanced diagnostic facilities available, such as computer tomography, Doppler ultrasound and various coagulation tests. However, since the increased cost of the private health services compared to the subsidized governmental rates may exclude patients in the lower socio-economic strata, we chose to only recruit patients from public hospitals.

In addition to Hiwot Fana Specialized University Hospital and Jugal Hospital, there are another two governmental hospitals in Harar, namely the Harar Police Hospital and the Harar Army Hospital. These hospitals provide health care services exclusively to the employees of the local police force and their respective families and to military personnel stationed in Harar. Thus, we considered it unwise to recruit patients from these hospitals, as the patient groups would have been selected only from distinct occupational groups and also by the fact that both police and military officers serving in the Harari region are seconded staff deriving from other parts of the country, and thus may not be representative of the source population.

We therefore aimed to include broadly from health services largely available for most of the source population by recruiting study subjects from both one local and one referral governmental hospital in the region. However, we cannot preclude that an unknown proportion of patients with CLD may not have been seen by the recruiting medical services for a variety of practical, cultural and socioeconomic reasons.

Moreover, by only including hospitalized patients, selection bias might have occurred by that we either (i) missed to include patients who died at home or never reached the hospital, and thus favouring patients with mild/moderate disease, or (ii) we missed to include patients who had recovered from early disease, and thus leading to an overestimation of severe disease [144]. However, the effect of this potential bias is difficult to estimate.

4.1.2 Paper II

Paper II assessed the association between khat chewing and CLD using a case-control study design. A case-control study is suitable to evaluate the association between an exposure and a disease, and able to calculate a ratio of odds of exposure in cases and controls as a measure of association [143].

The cases derived from the previous cross-sectional study (**Paper I**) and thus comprised of 150 adult (≥18 years of age) incident cases of CLD. The representativeness of the cases was discussed in the previous section (*vide supra*).

Control subjects were consecutively recruited when each case was available, among adults (≥18 years of age) attending (i) the ophthalmology unit; (ii) the dermatology outpatient department; or, (iii) the surgical inpatient or outpatient department at the two hospitals.

The sample size estimation was performed *a priori* based on the inclusion of two controls per case. Based on an estimated prevalence of daily khat use of 20% [37] and the assumption that khat use would be at least twice as common in cases as in controls (OR= 2.00), a minimum of 137 cases and 274 controls were needed. Out of the 370 control subjects recruited, 300 controls were included, 61 were excluded and nine withdrawn (Figure 7).

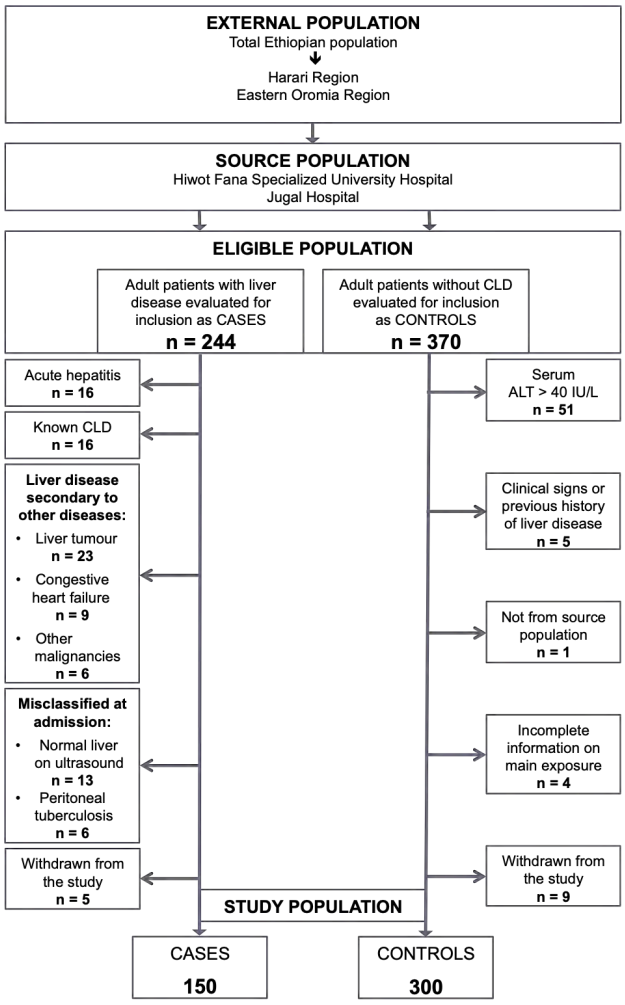

Fig. 7: Flow chart of the study subjects in the case-control study (**Paper II**). Abbreviations: ALT, alanine aminotransferase; CLD, chronic liver disease.

The control subjects should ideally be a random sample of adults (≥18 years of age) free of manifest or previous history of liver disease and recruited from the same source population and over the same time period as the cases derive. However, in this setting, there was no population roster available for a random sample and a random-digit telephone selection would favour households that could afford such a device or service. Hence, out of economic and practical reasons, we decided to use hospital-based controls, both inpatients and outpatients.

As discussed in **Paper II**, the decision to use hospital-based controls might have introduced Berkson's bias [145], as their attendance could have been affected by both exposure and disease. In order to minimize this bias, we included control subjects from a broad span of the hospital departments and outpatient clinics covering a wide range of diseases, as it is unlikely that khat was associated to many of them [146].

Moreover, we did not recruit control subjects from services dealing with illnesses known to be associated with khat usage, such as dental clinics (periodontal disease, dental caries) [147], medical (cardiovascular disease, stroke, gastritis, poorly controlled diabetes) [148] or psychiatric wards [36]. However, this may inadvertently have introduced a possible residual bias as the majority of the controls were outpatients and the majority of the cases inpatients, and we cannot be sure of the direction of this potential bias.

There are, however, also some advantages of using hospital controls. Besides the cost effectiveness and practical convenience, we also acknowledge the advantage that hospital-derived study participants are more likely to be subject to the same intangible selection factors that influenced the cases to attend this particular hospital [143].

We did consider recruiting spouses/relatives/attendants following the cases to the hospitals as controls. These groups may offer a degree of control of confounding factors such as ethnicity, socioeconomic status, and living environment. However, we regarded this group to be inadequate since we largely were assessing factors in which individuals who are closely associated also might have the same degree of exposure.

4.1.3 Paper III

Paper III assessed the seroprevalence of autoantibodies typical of autoimmune liver disease in a healthy population, using a cross-sectional study design. A cross-sectional survey is useful in providing information about demographic and personal characteristics and to assess the prevalence of various serological measurements in certain groups over a given time [143].

However, as discussed in the paper, this study was only designed to determine a point estimate in this hospitalized subgroup, and thus not able to obtain representative estimates on the regional seroprevalence of autoimmune markers, which will need further adequately powered population-based studies.

To explore the hypothesis that khat-related liver injury is mediated through autoimmune mechanisms, we determined the frequency of seropositivity of selected autoantibodies and compared khat users to non-users. However, another limitation to the cross-sectional study design is the classic “chicken or egg” dilemma; since cross-sectional surveys assess both exposure and outcome at a single point in time, they cannot determine whether the exposure preceded or resulted from the outcome.

Although cross-sectional studies are not designed for testing a hypothesis, it may be useful for raising the question of the presence of an association and to pinpoint formulated hypotheses [143]. Hence, we applied this design to this study as a possible first step in postulating khat as a triggering factor of an autoimmune process, not only in patients with overt liver disease but also in healthy individuals.

The study subjects in **Paper III** were the controls in the preceding case-control study (**Paper II**). Out of the 370 control subjects recruited, 310 controls were found eligible, of whom 169 were current khat users, defined for the purpose of this study as reported khat usage within the last 12 months, and 104 had never used khat, and thus classified as 'non-users'. The remaining 37 individuals, who had stopped using khat more than 12 months prior to the study, were excluded from the study (Figure 8).

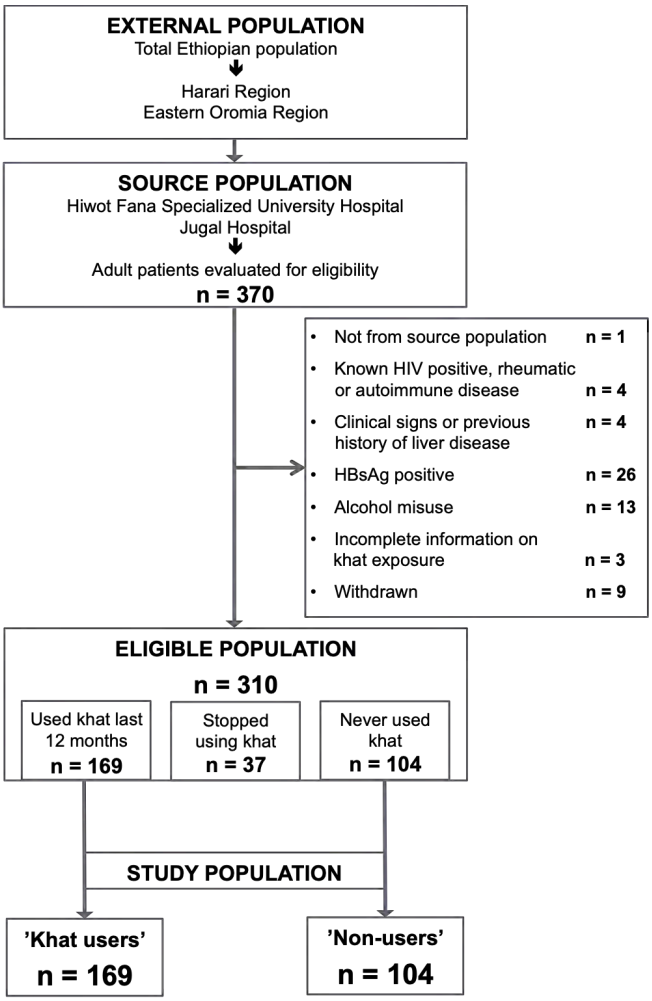

Fig. 8: Flow chart of the study subjects in the cross-sectional survey (**Paper III**). Abbreviations: HBsAg, hepatitis B surface antigen; HIV, human immunodeficiency virus.

In order to investigate the seroprevalence of selected autoantibodies typical for autoimmune liver disease and to reduce the risk of seropositivity due to unspecific cross-reactions, we chose to study apparently healthy individuals without conditions known to be associated with autoimmune markers. However, since the clinical examination followed a standardized proforma focusing on liver stigmata, the presence of other clinical features associated with autoimmune diseases *viz* synovitis, iritis and thyromegaly were not recorded systematically.

In addition, there was a lack of diagnostic tools available identifying autoimmune diseases routinely at the governmental hospitals in this resource-limited setting, and thus there might have been unrecognized cases of autoimmune disease among the study subjects, although the prevalence of autoimmune disease in Ethiopia is expected to be low [149].

Likewise, although all study subjects included in the study did not present with clinical signs of liver disease, the study participants were not screened for asymptomatic liver disease by abdominal ultrasound. Hence, we cannot exclude occult liver injury, in which could hypothetically either represent an ongoing autoimmune liver disease in an early stage or represent a triggering factor for an autoimmune process in the liver, and thus overestimating the seroprevalence of the selected autoantibodies.

4.2 Data collection

All study subjects were consecutively included in order to provide consistent data sampling throughout the study period and reduce the risk of sampling bias. And the study participants were recruited over a one-year period to avoid missing seasonal varieties of disease or environmental exposures.

All study subjects underwent a semi-structured interview with local nurses fluent in their mother tongue using a standardized form (Appendix 1). Demographic data including age, sex, ethnicity, religion, and occupation were recorded. Risk factors for CLD, including a family history of liver disease, previous blood transfusions, tattooing/piercing/scarring, use of dietary grain stored underground, alcohol drinking habits and use of herbal remedies and khat (*Catha edulis*) were explored.

In lack of validated criteria for the quantification of khat exposure, we established a screening tool to assess khat consumption using a visual analogue scale to quantify the khat usage in grams (Figure 9).

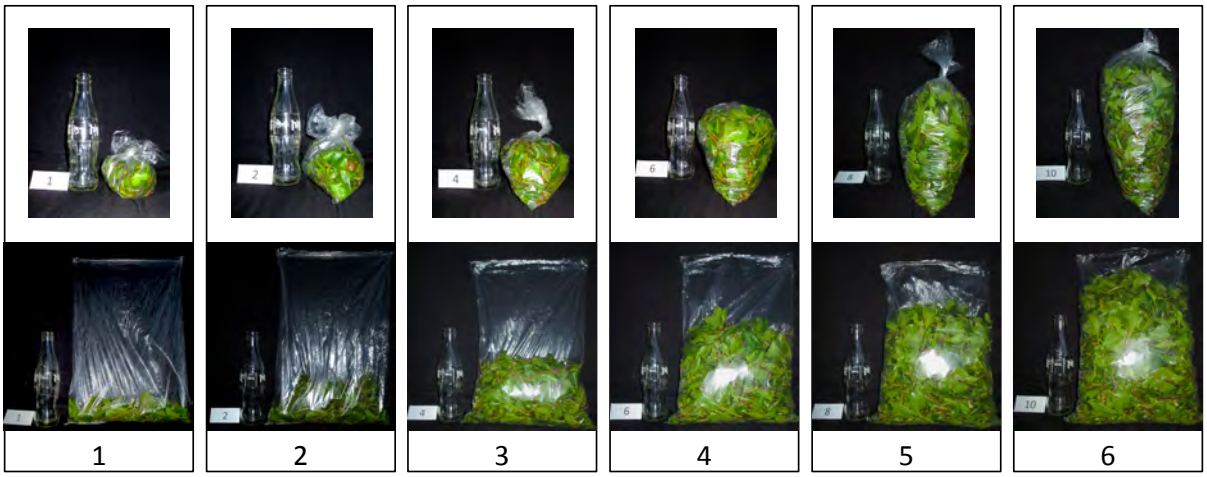

Fig. 9: Visual analogue scale used to quantify the khat usage. (1) 100 grams; (2) 200 grams; (3) 400 grams; (4) 600 grams; (5) 800 grams; (6) 1000 grams.

The frequency of khat chewing was categorized using the Drug Use Disorders Identification Test [150]. By combining the information on khat usage quantified in grams with the frequency and duration of khat use in years, we classified lifetime khat exposure as *khat-years*; one *khat-year* was defined as daily use of 200 grams of fresh khat for one year.

The advantages of this method are that it is inexpensive, simple and intuitive to use, reproducible and suitable for a clinical daily routine even in resource-limited settings. However, since this assessment tool was established for the purpose of this study, the method has not been validated. Other disadvantages are that it might be difficult for some responders to visualize the amount of khat and translate to the corresponding visual analogue scale, and the risk of reporting being interpreted or influenced by the interviewer.

Also, the *khat-year* parameter does not reflect the duration of each khat-session, which may have an impact on the cumulative khat-exposure. To measure the plasma concentration of khat alkaloids would have given a more objective value of khat exposure. However, the elimination half-life is very short and would only reflect the khat consumption within the last 24 hours, and thus does not provide a reliable estimate of the exposure over time [50].

Information on previous and current alcohol use was obtained using a frequency/quantity questionnaire (Appendix 1, p. 2). Average daily intake was quantified in grams by the formula: $\frac{alcohol\ by\ volume \times 0.78 \times volume\ consumed\ (mL)}{100}$, where alcohol by volume (ABV) is the percentage alcohol content of the alcoholic beverage and 0.78 is the specific gravity of alcohol.

The traditional home-brewed Ethiopian beer '*tella'* (Amharic: ጠላ) or '*farsoo'* (Oromo) was considered equivalent to beer with 2-6% ABV, the indigenous Ethiopian honey wine '*tej'* (Amharic: ጠጅ) or *'daadhi'* (Oromo) was considered equivalent to wine with 7-11% ABV, while the traditional home-distilled alcoholic beverage '*areki'* (Amharic: አረቂ) was considered equivalent to spirit alcohol content around 45% ABV [151, 152]. Daily alcohol consumption of >20 g in women and >30 g in men, for a minimum period of six months, was classified as alcohol misuse [14, 153].

As in all observational studies, information bias cannot be excluded. The most important information biases that can lead to misclassification of exposure are recall bias, interviewer bias, and non-response bias.

Recall bias can arise when information on exposure relies on memory, and in a case-control study (**Paper II**) cases may report exposures to risk factors differently than controls. Underreporting or denial of alcohol consumption or other recreational drugs is common and may underestimate its association with CLD; however, the use of khat in eastern Ethiopia is legal and socially accepted, and its usage less likely to be underreported in this context. An advantage in using hospital controls is that they are considered more likely than randomly selected healthy individuals to be aware of antecedent exposures or events, and thus reduce the potential for recall bias [143].

Interviewer bias might lead to differential misclassification of exposure if the interviewer unconsciously does not obtain information about past exposure in the same way in all patients, or favours a certain response from the interviewee differentially. In our study, we used the same standardized interview form for both cases (**Paper I**) and controls (**Paper II & III**); when interviewing control subjects and the interviewer started asking about symptoms of current CLD (Appendix 1, p. 3), the interview was stopped by the principal investigator.

In this study, we also sought to blind the interviewer to the disease status of the interviewee by appointing a dedicated nurse exclusively for interviewing the patients and another nurse for recruiting the patients and other logistical matters. However, although this was achieved in the majority of instances it could not be assured in cases with CLD manifesting obvious features of hepatic decompensation. In order to not highlight clinical features of liver disease prior to the interview, all patients were clinically examined after the interview, and a pre-specified proforma focusing on recording liver stigmata was used (Appendix 2).

Neither the interviewers nor the study subjects were informed about the hypothesis of an association between khat and CLD. Hence, we consider any misclassification of exposure is likely to be non-differential, and the observed effect of khat on CLD would, if anything, be underestimated.

Non-response bias occurs if there are differential response rates to exposure information between cases and controls. However, this seemed not to be a problem in this study, as there were no missing data on exposure information. This may also reflect another advantage of using hospital controls, as they are considered more likely to be willing to participate and cooperate than population-based healthy controls, and thus reduce the risk of bias due to nonresponse [143].

4.3 Diagnostic criteria

The diagnosis of CLD was defined as:

i) the presence of clinical features suggestive of decompensated liver disease *viz* ascites, jaundice, and hepatic encephalopathy;

and

ii) the presence of hepatic surface irregularity and/or parenchyma heterogeneity on abdominal ultrasound.

Historical, clinical, laboratory and imaging data were used to identify the aetiology of the underlying CLD using established criteria with some modifications due to resource-limitations in this setting (Table 3) [154-159].

The strengths and limitations related to these diagnostic criteria are discussed in detail later in the thesis (see section 5.1.1, 5.1.2).

Table 3: Criteria used to assign the aetiology of the liver disease

	Aetiology	Criteria used to assign the diagnosis
1	*Chronic hepatitis B infection*	Evidence of CLD on liver ultrasound **and** positive serum HBsAg.
2	*Chronic hepatitis C infection*	Evidence of CLD on liver ultrasound **and** positive serum anti-HCV **and** positive HCV RNA.
3	*Chronic hepatitis D infection*	Chronic hepatitis B infection **and** positive serum anti HDV IgG confirmed by detection of HDV RNA.
4	*Primary biliary cholangitis*	i. Strongly positive anti-mitochondrial antibodies **and** ii. Cholestatic liver function tests: a. ALP >1.5 x URR **and** b. AST <5 x URR
5	*Autoimmune hepatitis*[a]	i. Strongly positive anti-nuclear antibodies or anti-F-actin **and** ii. Elevated IgG >1.1 x URR
6	*Alcoholic liver disease*	i. Clinical and radiological signs of CLD **and** ii. Daily alcohol consumption >20 g/day in women and >30 g/day in men for 6 months or more.
7	*Non-alcoholic fatty liver disease*	i. Liver ultrasound findings of steatosis **and** ii. Absence of significant alcohol consumption[b] **or** other recognised secondary causes of steatosis **and** iii. [c]BMI >25 kg/m^2
8	*Haemochromatosis*	i. Transferrin saturation >50% **and** ii. Genotyping showing C282Y homozygosity or C282Y/H63D heterozygosity or C282Y/S65C heterozygosity on the HFE gene.
9	*Wilson's disease*	i. Serum caeruloplasmin < 0.140 g/L **and** ii. Age < 40 years
10	*Alpha-1-antitrypsin deficiency*	Serum alpha-1-antitrypsin level < 0.85 g/L.
11	*Malaria*	Positive malaria rapid diagnostic test **and** positive microscopy.
12	*Hepatic schistosomiasis*	Presence of ova from *Schistosoma mansoni* in Kato-Katz thick stool smears **and** typical liver ultrasound findings *viz* periportal thickening/ 'pipestem' fibrosis confirmed by an independent expert.
13	*Visceral leishmaniasis*	Ultrasound findings of hepatosplenomegaly **and** positive rK39 antigen strip test confirmed by positive splenic smear.
14	*Unexplained chronic liver disease*	None of the above

Laboratory reference range: ALP (60-306 U/L); AST (14-40 U/L); IgG (0.8-27.8 g/L) [156].

a. Based on the American Association for the Study of Liver Diseases (AASLD) simplified criteria [157] in the absence of histology.
b. Alcohol consumption <20 g/day in women and <30 g/day in men.
c. Not a part of the AASLD criteria [158] but adopted to exclude cases of starvation-induced steatosis.

Abbreviations: **ALP**, alkaline phosphatase; **anti-HCV**, hepatitis C virus antibody; **anti-HDV**, hepatitis D virus antibody; **AST**, aspartate aminotransferase; **BMI**, body mass index; **CLD**, chronic liver disease; **HBsAg**, hepatitis B surface antigen; **HCV**, hepatitis C virus; **HDV**, hepatitis D virus; **HFE**, high iron Fe; **IgG**, immunoglobulin G; **RNA**, ribonucleic acid; **URR**, upper reference range.

4.4 Laboratory methods

4.4.1 Routine tests performed locally in Harar

Standard haematology and biochemistry tests were performed routinely at the local hospitals by trained local lab technicians dedicated to the project. Complete blood count was performed locally within 24 hours using a Sysmex KX-21N™ haematology analyser (Sysmex, Kobe, Japan). Serum creatinine concentration and serum liver enzyme activities (alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase) were analysed locally within 24 hours, using a semi-automatic biochemistry analyser Dirui DR-7000D (DIRUI Industrial Company, Changchun, China) and HumaLyzer 3000 (HUMAN, Wiesbaden, Germany).

The serum aspartate aminotransferase (AST) to platelet ratio index (APRI) was calculated as $\frac{\frac{AST\ (\text{U/L})}{upper\ reference\ range\ of\ AST\ (\text{U/L})}}{platelet\ count\ (10^9/\text{L})} \times 100$ [160], using a threshold of 0.7 as indicator of significant fibrosis [161]. The Fibrosis-4 (FIB-4) score was calculated as $\frac{age\ (\text{years}) \times AST\ (\text{U/L})}{platelet\ count\ (10^9/\text{L}) \times \sqrt{ALT\ (\text{U/L})}}$, using a threshold of 3.25 to indicate advanced fibrosis/cirrhosis [162].

Baseline screening for HIV, HBsAg, and anti-HCV was undertaken locally by trained local lab technicians dedicated to the project using WHO-approved rapid diagnostic tests (RDTs).

Urine was collected from all women < 45 years of age for determination of human chorionic gonadotropin (hCG) using a pregnancy strip test (Nantong Egens Biotechnology, Jiangsu, China).

Serum and plasma were separated for storage in aliquots at -20º C until transported on ice/dry ice for further analysis either in Addis Ababa, Ethiopia or in Norway. Studies have shown that the biochemical and serological markers of interest are relatively stable for several months under these conditions, provided that they are not exposed to repeated freeze-thaw cycles [163].

4.4.2 Biochemistry performed in Addis Ababa

A broader panel of liver tests (including gamma-glutamyl transferase, total bilirubin, and albumin) was performed by bioengineers at a research laboratory in Addis Ababa at the Aklilu Lemma Institute of Pathobiology using a semi-automatic biochemistry analyser HumaLyzer 3000 (HUMAN, Wiesbaden, Germany).

4.4.3 Biochemistry and serology performed in Norway

Serum specimens were transported on dry ice to Drammen Hospital in Norway and stored at -80 ºC until analysed. Additional biochemical analyses were undertaken by bioengineers at the Department of Medical Biochemistry, Drammen Hospital.

Serum was analysed for immunoglobulin G (IgG), alpha-1-antitrypsin and caeruloplasmin using the IMMAGE® 800 Immunochemistry System (Beckman Coulter, Brea, CA, USA); iron and transferrin concentrations were quantified using ARCHITECT ci16200 (Abbott Diagnostics, Abbott Park, IL, USA).

Total iron binding capacity (TIBC) was calculated as $serum\ transferrin\ (g/L) \times 25.1$ and transferrin saturation as $\frac{serum\ iron\ (\mu mol/L)}{TIBC\ (\mu mol/L)} \times 100\%$. The diurnal variation of serum iron levels is well known and the general opinion is that the serum iron concentration is at its peak in the morning, and thus fasting morning blood samples have been recommended for diagnosing hereditary haemochromatosis [164]. However, studies on diurnal iron fluctuation have shown unequivocal results, suggesting that to restrict the blood sampling to a certain time of the day does not improve the reliability of the results [165].

Likewise, it is shown that there are no diagnostic advantages in using fasting samples [166], and thus no longer considered necessary according to updated established guidelines for the diagnosis of hereditary haemochromatosis [159]. Based on these recommendations, and out of practical convenience, we did not restrict the timing of the sampling or require fasting specimens.

4.4.3.1 Autoantibodies

ANA, SMA, and AMA were determined by enzyme-linked immunosorbent assays (ELISA), using the Phadia™250 Laboratory system (Thermo Fisher Scientific, Waltham, MA, USA) at the Department of Medical Biochemistry, Drammen Hospital in Norway.

4.4.3.1.1 ANA and SMA

ANA was detected by the EliA™ Symphony assay (Phadia, Freiburg, Germany) with a calculated ratio of test sample response to calibrator >1.0 defined as positive, 0.7-1.0 was equivocal, and <0.7 was negative [167, 168]. SMA was determined by QUANTA Lite® Actin IgG (Inova Diagnostics, San Diego, CA, USA) and a cut-off level >30 assay units was classified as positive, as proposed by the manufacturer.

ANA and SMA are serological hallmarks of AIH type 1 and around 75% of the patients are positive for ANA and/or SMA [169]. ANA is a heterogeneous group of autoantibodies targeting an incompletely defined group of antigens related to DNA and nuclear membranes, and is neither organ- nor disease-specific [3].

The two most common methods used in ANA screening are indirect immunofluorescence (IIF) and ELISA. IIF has been used as the gold standard and IIF-based results (in titres) are used in the established criteria for AIH [157, 169]. However, IIF is time-consuming and labour-intensive and the result is reliant on subjective interpretation. Hence, we chose to use ELISA-based screening since it is automated, objective, reproducible, widely used in clinical laboratories and shown to have a similar diagnostic efficiency to IIF, except for some less common autoantibodies associated with systemic sclerosis [167, 168, 170].

SMA is a heterogeneous group of autoantibodies directed against different smooth muscle autoantigens, including actin and non-actin components [171]. Circulating SMA is non-specific and also found in patients with different viral infections, including viral hepatitis. The two most common strategies to determine antibodies to filamentous actin (anti-F-actin) are IIF and ELISA [172].

When distinguishing between different subtypes of SMA in patients with AIH type 1, nearly 90% of SMA-positive patients are shown to have anti-F-actin, and thus anti-F-actin seem to have increased sensitivity for diagnosing AIH type 1 compared to SMA detected by IIF [173, 174]; however, at the cost of a lower specificity and positive predictive value, since anti-F-actin also occur in various other immune-mediated diseases [175].

In our study, we used an ELISA-assay that is based on purified F-actin. In general, the ELISA method is often preferred over IIF since it is automated and much less labour-intensive, standardized and objective, and thus not subject to inter-/ or intra-observer variations.

However, although ELISA anti-F-actin screening is still considered as an appropriate diagnostic workup for AIH [169], studies evaluating the specific ELISA-kit used in our study have demonstrated a high sensitivity but low specificity [174, 176, 177]. Hence, IIF is still considered superior to ELISA, and the possibility of a not AIH-specific SMA-positivity should be taken into account when interpreting the results [169].

4.4.3.1.2 AMA

AMA was determined using the QUANTA Lite® M2 EP (MIT3) assay (Inova Diagnostics) and a cut-off level >25 assay units was classified as positive, as proposed by the manufacturer. AMA is considered as the serological hallmark of PBC since AMA positivity is observed in >90% of patients with PBC [178]. Early studies have described nine subtypes of mitochondrial antigens (M1-M9) [179]. The anti-M2 antibody is the most specific for PBC, targeting the family of the 2-oxo-acid dehydrogenase complexes in the inner mitochondrial membrane, which includes four autoreactive mitochondrial antigens [179, 180].

There are five common strategies to detect AMA: (i) immunoblotting; (ii) enzyme inhibition assay; (iii) luminex beads assay; (iv) IIF; and, (v) ELISA. In our study, we used an ELISA-assay that is based on a hybrid clone called MIT3, which was designed to express three M2-related autoepitopes [181].

IIF and ELISA are the two most common methods in use, in which ELISA tests are preferred in clinical laboratories over IIF because they are rapid, automated, standardized and objective, and thus considered more reliable for detection of AMA [182]. In addition, the MIT3-based ELISA-assays have been shown to be more sensitive and specific than both IIF and the conventional anti-M2 ELISA targeting only one of the M2-related autoepitopes [183-186].

4.4.4 Virology

All patients underwent confirmatory ELISA tests of HBsAg and anti-HCV, which were undertaken in Addis Ababa by trained lab technicians at the Aklilu Lemma Institute of Pathobiology using a fully automated Elisys Uno ELISA Analyzer (HUMAN Diagnostics, Wiesbaden, Germany). Discrepancies between the results of the on-site RDTs and the ELISA results were resolved by a tiebreaker, using a different ELISA assay (ARCHITECT or Bio-Rad, Hercules, CA, USA).

In patients with CLD (**Paper I**) who were HBsAg or anti-HCV positive, frozen plasma specimens were shipped to the Norwegian Institute of Public Health in Oslo for virological analyses. HBV DNA and HCV RNA were measured by polymerase chain reaction (PCR). Plasma was further analysed for HDV antigen and HDV IgG antibody (anti-HDV IgG). HDV RNA was quantified by PCR in samples positive or borderline for anti-HDV IgG. Studies have shown that the viral DNA and RNA in frozen plasma specimens stored at -20 ºC are stable for years, even after several freeze-thaw cycles [187-189].

4.4.5 Parasitology

4.4.5.1 Malaria

In **Paper I**, malaria screening was performed locally using a WHO-approved RDT testing for *Plasmodium falciparum* and *P. vivax* and confirmed by microscopy of blood smears if found positive. Although the malaria prevalence in the Harari region is low [190] and severe liver injury in malaria is rare [191], we tested for malaria mainly to discern a Falciparum-malaria causing acute-on-chronic liver failure requiring urgent adequate anti-malaria treatment [192, 193]. Malaria infection *per se* is not considered as an underlying aetiology for CLD [194].

4.4.5.2 Visceral leishmaniasis

Screening for visceral leishmaniasis (VL) was undertaken locally in patients with unexplained CLD (**Paper I**) using a recombinant K39 antigen (rK39) RDT and confirmed by Giemsa stained splenic smear if found positive.

VL is endemic in Ethiopia with an estimated annual incidence ranging from 2000 up to 5000 cases, which are largely seen in the north-/ and south-western parts of the country [195, 196]; however, to the best of our knowledge, no data are available for the Harari region although the rates are assumed to be low.

According to the Ethiopian national guidelines for diagnosis of visceral leishmaniasis, the rK39 RDT is the method of choice as a screening tool, although it is recommended that a direct agglutination test should be available in hospitals if the rK39 RDT turns out negative in a patient with clinical findings of VL; the definite diagnosis is made by demonstration of amastigotes of *Leishmania donovani* (or *L. infantum* in some cases) by microscopy of tissue aspirates, where a spleen smear has the highest sensitivity [197].

Direct agglutination tests were not routinely available in the hospitals in Harar, but a local pathologist attended the hospitals every other week and undertook a splenic aspiration on special request.

An early study evaluating the rK39 RDT found a high sensitivity (>95%) and specificity (>90%) in the Indian subcontinent but non-acceptable low sensitivity (75-85%) and specificity (70-92%) in East African countries (Sudan, Kenya, and Ethiopia) [198]. However, a recent study from Sudan has demonstrated excellent performance of the rK39 RDT with high sensitivity (97.6-100%) and specificity (92.5-94.5%) [199].

A Cochrane review, which did not include the last study from Sudan, concluded that the rK39 RDT has overall high sensitivity (92%) and specificity (92%) but with regional differences [200]. The specificity of rK39 RDT in East Africa was acceptable (91.1%) and thus rK39 RDTs can replace the direct agglutination and other tests as the basis of therapeutic decision in patients with suspected VL. However, the sensitivity in East Africa (85.3%) is not sufficient for rK39 RDT as a stand-alone test; hence, the Cochrane review recommended that a negative test in a patient with suspected VL should be followed by a second or a different test, which corresponds to the diagnostic algorithm in the Ethiopian national guidelines [197].

4.4.5.3 Schistosomiasis

For the diagnosis of hepatosplenic schistosomiasis among cases with CLD (**Paper I**), we collected one single stool sample on the day of inclusion and local trained lab technicians processed it to five thick smears according to a modified Kato-Katz technique using 41.7-mg templates for detection of the ova of *Schistosoma mansoni* [201].

Detection of ova from *S. mansoni* using a single thick Kato-Katz smear from a single stool specimen has a diagnostic specificity of 100% but a low sensitivity, especially in low prevalence areas with low-intensity infections. Immunoassays detecting circulating cathodic antigen (CCA) have shown better sensitivity than Kato-Katz smears, and are available as urine-based point-of-care (POC) dipstick tests [202].

However, in high prevalence areas, Kato-Katz smears have shown excellent performance as a screening test with up to 100% sensitivity and specificity [203]; and in high-endemic regions both Kato-Katzs and POC-CCA assays have been shown to provide reasonable and

comparable levels of prevalence and therefore both methods are regarded as adequate for morbidity control [204].

In the 1980s the prevalence rates among schoolchildren in the Harar region ranged from 32.4% to 71.1%, but no recent data are available [205]. Based on the assumption that the patients in this study resided in a high prevalence area [30], POC-CCA assays were not included in our set up, and five Kato-Katz slides from a single stool sample were prepared to overcome the lack of sensitivity. Quintet Kato-Katz smears from one stool sample have been shown having a similar diagnostic performance to three Kato-Katz smears from samples collected in two consecutive days [206].

4.4.6 Supplementary analyses

In cases of CLD (**Paper I**) with increased serum transferrin saturation above 50% without any obvious explanation, high iron Fe (HFE) genotyping was undertaken by our collaborators at the Department of Medical Biochemistry at Oslo University Hospital Rikshospitalet in Norway to rule out hereditary haemochromatosis.

The cut-off value of serum transferrin saturation we used (50%) was based on what frequently is referred to as the upper reference range [207]. However, updated guidelines recommend lowering the cut-off value to 45% to increase the sensitivity, although it also lowers the specificity and the positive predictive value [159]. Hence, we might have missed screening some few patients with C282Y-mutation according to established guidelines. However, a transferrin saturation cut-off value of 50% has still a sensitivity >95% for identification of true C282Y-mutation homozygotes [207].

Although the data on HFE genotyping in sub-Saharan Africa is scant, the C282Y mutation is yet to be found in Ethiopians but the H63D mutation has been found in some ethnic groups in central Ethiopia [208]. However, its contribution to iron overload is unclear and mainly linked to C282/H63D compound heterozygotes or H63D homozygotes and considered having a low risk of developing iron-overload-related morbidity [209, 210].

4.5 Abdominal imaging

Since the diagnosis of CLD (**Paper I**) was based on both clinical criteria and the presence of an irregular liver surface and/or liver parenchyma heterogeneity, all patients presenting with features of CLD were assessed with abdominal ultrasound undertaken to a pre-determined standard (Appendix 3) by a local radiologist using a 3.5 MHz convex transducer.

The presence of schistosomal periportal fibrosis was diagnosed using WHO criteria [211]. Still-shot images from the ultrasound scan with the requisite measurements were printed out and re-evaluated by an independent expert.

Abdominal ultrasound is well established as a screening tool and in the diagnostic workup for patients with CLD [212]. As in all operator-dependent techniques, there is a risk for subjective interpretive errors, and the sensitivity and specificity for abdominal ultrasound are reduced compared to computer tomography and magnetic resonance imaging. But it also has many advantages as it is cost-effective, readily available, non-invasive and well tolerated and has not been associated with long-term risk due to ionizing radiation as seen in computer tomography and nuclear medicine methods.

Liver echogenicity has been shown to have poor diagnostic accuracy, whereas liver surface is a useful screen with consistent moderate diagnostic accuracy (sensitivity: 51-73%; specificity: 78-95%) [213]. Due to only moderate sensitivity, we might have missed to include some cases with CLD (**Paper I**); on the other hand, since the specificity of liver ultrasound is relatively high, we can with a high degree of certainty assume that the patients who fulfilled the inclusion criteria represent true cases of CLD.

4.6 Histopathology

Despite the evolution of sensitive and reliable serological markers and increasingly accurate imaging techniques are available, liver biopsy is still regarded as a cornerstone in diagnosing liver disease [214].

In **Paper I**, it was intended that all patients in whom the aetiology of the CLD remained unexplained following investigation would be offered a liver biopsy. However, histopathological investigations were not routinely available in the hospitals due to limited resources both in equipment and trained staff. Hence, we appointed a gastroenterologist from the St. Paul's Hospital Millennium Medical College in Addis Ababa who came to Harar to perform the procedure under ultrasound guidance, using a sterile Menghini technique with local anaesthesia and a 17 G needle Hepafix® (Braun, Melsungen, Germany) [214].

Liver biopsy material was collected in 10% formaldehyde and left in room temperature for a minimum of 4 hours before embedding in paraffin wax. Serial four μm sections were cut and stained with haematoxylin and eosin (H&E) by collaborators at International Clinical Laboratories in Addis Ababa. Biopsies were considered adequate for reading if at least five portal tracts were available for assessment.

The H&E sections and paraffin blocks were shipped to specialist services at the Department of Pathology at Ålesund Hospital in Norway for further staining with Gomori (reticulin), van Gieson (collagen), Masson Trichrome (metachromatic), periodic acid-Schiff (PAS) with and without diastase (glycogen), Perls (iron), and immunohistochemistry was undertaken at the Centre for Pathology at Imperial College London in the UK using Ki-67 as a proliferation marker and activated caspase-3, as an apoptotic marker.

Image analysis to quantify the degree of fibrosis and to calculate the collagen proportionate area (CPA) was carried out on scanned, Sirius Red stained sections as described previously [215]. Histopathologists in Norway and London independently assessed the histological findings blinded to the clinical information; inflammation and fibrosis were graded and staged using the semi-quantitative modified Histological Activity Index [216].

The strengths and limitations related to the liver biopsies will be discussed in detail later in the thesis (see section 5.1.3).

4.7 Statistical analysis

All data were entered into a database built in EpiData Version 3.1 (EpiData Association, Odense, Denmark). In all papers we used simple descriptive statistics to discern differences between the groups of interest. In **Paper I & III**, data were analysed using SPSS version 23.0 and 25.0 (SPSS Inc., Chicago, IL, USA), whereas the statistical analyses in **Paper II** were performed in STATA 14.0 (StataCorp, College Station, TX, USA). All tests were two-sided and the level of significance was set at $p<0.05$.

Pearson Chi-Square test was used for categorical variables and a non-parametric Mann-Whitney U test for continuous variables since the data were not normal-distributed. Non-parametric methods consider ranks instead of absolute values and thus are less influenced by outliers; however, these tests lose statistical power in detecting statistical differences since information is getting lost in the analyses.

In **Paper II**, an explanatory strategy investigating the association between khat chewing and CLD was undertaken [145]. An initial stratified analysis used a Breslow and Day test to pinpoint effect modification (interaction), while confounding was controlled for univariately using the Mantel-Haenszel method. The magnitude of the confounding effect was evaluated by comparing the crude OR and adjusted Mantel-Haenszel ORs.

A logistic regression model was used to study associations between khat chewing and the development of CLD, controlling for multiple confounders and the presence of effect modification. A chi-square test for trend was applied to evaluate a possible dose-response relationship between different exposure levels of khat consumption and the outcome CLD.

The attributable proportion (AP) of CLD cases attributable to khat usage in this study population was estimated as $AP = \frac{Pe\,(\mathrm{OR}-1)}{Pe\,(\mathrm{OR}-1)+1}$ where P_e represents the prevalence of khat exposure in the target population [146].

Moreover, we performed a *post hoc* sensitivity analysis excluding all cases of CLD with an identifiable aetiology, and all control subjects with viral hepatitis and/or history of alcohol misuse were excluded.

In **Paper III**, we used descriptive statistics to compare the presence of autoantibodies in khat users and non-users. Khat users were categorised as 'heavy users' or 'light users' according to the observed median lifetime khat exposure measured in *khat-years*.

Logistic regression models adjusting for age and sex were used to study the association between khat usage and the presence of autoantibodies typical of autoimmune liver disease.

4.8 Ethics and concession

The study was approved by the National Research Ethics Review Committee (NRERC, Ref. No.: 3.10/829/07 and 3.10/129/2016) in Ethiopia and by the Regional Committees for Medical and Health Research Ethics (REK Sør-Øst, Ref. No.: 2014/1146) in Norway. Permission to carry out the research was granted by the hospital management at Hiwot Fana Specialized University Hospital and Jugal Hospital. Permission to transport blood samples and biopsy material to Norway was in accordance with a Material Transfer Agreement obtained from the NRERC in Ethiopia.

The study was conducted in accordance with the Declaration of Helsinki [217]. All study subjects gave their written informed consent to participate in the study and had the right to withdraw from the study at any time without being excluded from further care and treatment from the local hospital.

We performed extensive laboratory investigations to diagnose the underlying aetiologies of the patients CLD, and many efforts have been made to communicate the lab results back to the hospital and the executive physician for further treatment and/or referral to the adequate health institution (Appendix 4).

All patients were offered a follow-up consultation after three months. This appointment had three purposes: (i) to inform the patients about relevant lab test results; (ii) to study the outcome of the disease; and, (iii) to refer the patient for treatment or further follow up if needed.

The appointment was scheduled before the patient was discharged from the hospital and the patient was contacted a few days in advance to remind him/her about the appointment.

The follow-up visit and lab tests were free of charge for the patient, and travel expenses were reimbursed. At the follow-up visit, the patient underwent a basic clinical examination and a blood sample was drawn to re-evaluate a standard liver panel and other relevant tests.

Certain lab results required immediate action by the study team: (i) positive malaria screening prompted adequate treatment according to national guidelines; (ii) patients with a positive HIV RDT confirmed by a second test were referred to the HIV clinic at the hospital for further diagnostics, treatment, and follow-up; (iii) patients with detectable ova from *S. mansoni* in the stool sample were provided treatment with praziquantel according to national guidelines, free of charge for the patient; (iv) patients with positive rK39 RDT were referred for further diagnostics and treatment at Hiwot Fana Specialized University Hospital.

In addition, as an extension of this study, a treatment program for hepatitis B was established at Hiwot Fana Specialized University Hospital. Local staff was trained in the management and follow-up of hepatitis B patients as an integrated part of the hospital. All HBsAg positive study subjects were referred and considered for antiviral treatment following a simplified treatment protocol for chronic hepatitis B in a resource-limited setting. The project is committed to supply the treatment program the antiviral drugs needed for the hepatitis B positive study subjects, free of charge, for 3 years, starting from June 2016.

5 Discussion

There is a paucity of research exploring the underlying aetiologies of CLD in Ethiopia, especially in rural settings, as only a handful studies are available and largely have studied patients deriving from urban settings [23, 26, 218-221].

Simultaneously, in recent years, community-based longitudinal studies, measuring adult mortality in several rural areas of Ethiopia using a verbal autopsy method to assign causes of death, have identified CLD as the leading cause of death in adults less than fifty years of age in rural eastern and south-central Ethiopia [33, 35].

Interestingly, khat chewing has been associated with the development of CLD and its use is widespread in the same rural regions, suggesting khat as an independent risk factor inducing liver inflammation and fibrosis but this has never been formally investigated.

To the best of our knowledge, this present study is the first epidemiological study of CLD in eastern Ethiopia (**Paper I**). Furthermore, this is the first properly designed study (**Paper II**) to assess the association between khat and CLD in humans.

In **Paper III**, we have taken it one step further in the search for a better understanding of the underlying pathogenetic mechanism of khat-related liver injury; by conducting the first explorative laboratory serosurvey in eastern Ethiopia assessing the seroprevalence of autoantibodies typical for autoimmune liver disease in healthy individuals, we investigated the proposed hypothesis that khat chewing might trigger an autoimmune process in predisposed individuals by comparing khat users to non-users.

In the following, we will elaborate on our main findings, their strengths and weaknesses and their consequences for clinical practice.

5.1 Paper I

Generating a hypothesis – what are the risk factors for chronic liver disease in eastern Ethiopia?

In the first part of this study, we explored the aetiological spectrum and potential risk factors for CLD in eastern Ethiopia (**Paper I**). Although chronic hepatitis B was common, we found a remarkably high proportion of unexplained CLD. At the same time, daily khat use was highly prevalent and histological findings indicated toxic liver injury.

Our results suggest a more extensive role for khat in the development CLD than previously reported. The fact that khat use was similar amongst patients with and without other risk factors indicated that khat chewing might act as a sole or an adjuvant cause of liver injury.

5.1.1 Can we trust the CLD diagnosis?

We set out to provide a robust definition of CLD, with the diagnosis out of clinical criteria verified by radiological evidence of hepatic surface irregularity and/or parenchyma heterogeneity. CLD has been defined as hepatic injury lasting for at least six months and may be recognised by elevated liver enzyme activities measured in two blood samples at least six months apart [4]. However, in the present study, none of the patients were included based on laboratory criteria. This was largely due to incomplete medical records and scarce information on previous biochemical history.

Previous laboratory results with evidence of elevated ALT activities for more than six months duration were available in some cases. However, these cases were excluded since they had a previous diagnosis of CLD or attended follow-up consultations at the hospital, and thus represented prevalent cases rather than incident cases.

Although ALT is the most sensitive indicator of hepatocellular injury [3, 222], normal serum ALT activities may be found despite severe liver damage, as seen in patients with a significant loss of liver parenchyma and in end-stage liver disease with reduced enzyme synthesis [3]. Hence, although the cases of CLD in our study only had mild liver enzyme abnormalities, it is not incompatible with the diagnosis of CLD [223]. Interestingly, more than two-thirds of the patients had aspartate-to-platelet ratio index (APRI) and/or FIB4 scores compatible with a diagnosis of significant fibrosis/ cirrhosis [161, 162].

However, it is debatable whether these fibrosis marker panels based on platelets will perform well in sub-Saharan Africa, where thrombocytopenia is a frequent manifestation of endemic tropical diseases such as malaria, dengue fever, leishmaniasis and schistosomiasis [224].

In a study of patients with chronic HBV infection in Gambia and Senegal evaluating non-invasive fibrosis tests by using liver biopsy as the gold standard, APRI had a sensitivity of 9% and a specificity of 98% to detect significant fibrosis in the Gambian cohort, while the sensitivity was 0% and a specificity of 96% to detect significant fibrosis in Senegal [225].

A recent study evaluating non-invasive fibrosis tests among patients with chronic HBV in Ethiopia used transient elastography as a reference and found a similar trend; APRI and FIB-4 scores had low sensitivity but high specificity in this population [226]. Hence, the poor accuracy of APRI/FIB-4 as a clinical tool for diagnosis of liver fibrosis in this setting is largely due to the lack of sensitivity.

Due to resource limitations, the diagnosis of CLD could not be established or confirmed by liver biopsy or advanced imaging *viz* computer tomography, magnetic resonance imaging or endoscopy. Although the wide range of liver diseases defined as CLD usually includes hepatocellular carcinoma, the diagnosis of primary liver cancer was not obtainable since liver biopsies were not routinely performed and histopathology services were not available at the hospitals. Hence, any patient with a detectable liver mass on abdominal ultrasound was excluded from the study.

In the present study, only a small number of patients with unexplained liver disease eventually underwent liver biopsy. As discussed in **Paper I**, the diagnosis of CLD would seem at odds with the lack of histological evidence more than mild fibrosis and inflammation. However, the selection procedure clearly favoured those with the mildest disease, and since the biopsies were undertaken at the earliest five months after the index event, the time interval between presentation and the procedure was sufficiently long for there to have been some resolution of the liver disease.

Nevertheless, the biopsies showed evidence of ongoing disease and combined with the fulfilment of the inclusion criteria, we concluded that the fact that the patients included in this study had CLD could be accepted with a high degree of certainty.

5.1.2 Which diagnoses have we underestimated or missed to identify?

The high proportion of CLD with no identifiable aetiology was intriguingly high compared to global estimates, and raised an important question: Have we underestimated or missed to identify certain underlying causes of CLD in this study population?

5.1.2.1 Chronic HBV infection?

Chronic HBV infection is defined as the seropresence of HBsAg for more than six months [227]. As noted previously, none of the included patients with CLD had available laboratory records dating six months back, and thus the previous HBsAg status of the patients was unknown. In fact, if the patient was known to be HBsAg positive for the last six months, he/she was excluded from the study *per protocol.*

So how did we distinguish acute hepatitis B from chronic hepatitis B without any evidence of persisting HBsAg for at least six months?

The serological hallmark of acute HBV infection is hepatitis B core antibody of IgM class (anti-HBc IgM), which typically last for 4-6 months before it is replaced by anti-HBc IgG [228]. However, anti-HBc IgM may also be seen during flares of hepatitis B in patients with chronic HBV infection, and thus it is difficult to distinguish acute hepatitis B from an acute exacerbation or flare of chronic HBV infection.

Since we did not have any information on the HBsAg status six months prior to inclusion, we modified the diagnostic criteria by adding a radiological criterion requiring evidence of CLD, corresponding to the inclusion criteria of the study in general.

It should be noted that by using these strict diagnostic criteria, we most likely have included patients predominantly with progressed chronic hepatitis B since most individuals in early phases of a chronic HBV infection are asymptomatic or having only mild, nonspecific symptoms with minimal liver injury [228]. And thus, in addition to the limitation of the institution-based cross-sectional study design, only capable of measuring the proportion of disease within a specific hospitalized subgroup, this study cannot estimate the true burden of chronic hepatitis B in this region.

5.1.2.2 Occult HBV infection?

Occult HBV infection (OBI) is defined as the presence of HBV DNA in the liver parenchyma and/or in serum without detectable HBsAg [229]. OBI is regarded as an important risk factor for the development of liver cirrhosis and hepatocellular carcinoma [230].

In our study, all patients were screened for HBV infection by measuring HBsAg using an RDT and a confirmatory ELISA test, as described previously. HBV DNA levels were only measured in the 55 (36.7%) patients with CLD who were HBsAg positive, thus OBI could not be ruled out among the 95 (63.3%) HBsAg-negative patients. However, the role of OBI in cryptogenic cirrhosis is still debated since the pathomechanism of OBI is still unclear and it is not determined whether occult HBV can cause clinically relevant liver injury [230, 231].

By the fact that around 95% of the patients with unexplained CLD in our study presented with decompensated liver disease but only mild abnormalities in the liver transaminase activities, we consider it unlikely that a theoretically suppressed HBsAg with high viral load represents the underlying aetiology of the unexplained CLD in this population.

5.1.2.3 Autoimmune hepatitis

We might have underestimated the frequency of AIH. Liver biopsy is considered essential for diagnosing AIH but was not obtainable in our setting, and thus we made the diagnosis based on the exclusion of other causes of CLD, the presence of circulating ANA and/or SMA and elevated IgG.

We did not screen for other conventional antibodies, such as anti-liver-kidney microsomal antibodies (anti-LKM) and antibody to liver cytosol type 1 (anti-LC1), which are seen in AIH type 2 and constitutes up to 10% of AIH cases [169]. And we did not test for non-standard autoantibodies *viz* antibody to soluble liver antigen (anti-SLA) and atypical perinuclear anti-neutrophil cytoplasmic antibody (atypical pANCA), which could be useful in diagnosing patients negative for conventional autoantibodies [157, 169].

The established simplified diagnostic criteria [157, 169] are based on autoantibody titres determined by IIF, which do not correspond seamlessly to the reactivity shown by the ELISA test. In this study, we based our diagnostic workup on ELISA tests and the serological immune profile of SMA depended on anti-F-actin ELISA. Although a positive anti-F-actin assay may be considered equate to a positive SMA assay in scoring systems [232], actin is not

the only target antigen of AIH-specific SMA reactivity and ELISA can miss the diagnosis in about 20% of the cases [169, 173].

5.1.2.4 Alcoholic liver disease

Alcoholic liver disease is one of the most common underlying aetiologies of CLD [3, 14, 233]. Although there is a general agreement that excessive alcohol consumption is a risk factor for cirrhosis, many other factors influence the risk of alcoholic liver disease; thus, in studies estimating the absolute risk of alcoholic liver disease, various thresholds of alcohol intake have been suggested [234-237].

Becker *et al*. [234] suggested the most conservative thresholds for an increased risk of alcohol-related liver disease at 12 grams/day in women and 24 grams/day in men. However, these thresholds are debatable and considered as controversial by some [235], and we have chosen to define alcohol misuse according to other conservative thresholds considered to increase the risk of alcohol-related cirrhosis, namely 20 grams/day in women [238] and 30 grams/day in men [239] which are in line with the most widely used guidelines [239, 240] and authoritative textbooks [3, 14, 153, 233, 241].

In the present study, the number of patients with alcoholic liver disease was remarkably low. The high proportion of reported alcohol abstinence among patients with CLD might be due to underreporting of alcohol use out of guilt or shame knowing they are being seen for CLD. However, to the potential bias of underreporting, we believe it is relevant that the study was undertaken in a Muslim setting; Harar is said to be the fourth holiest city of Islam and is one of oldest Islamic learning centres in Africa [242, 243].

Since alcohol drinking is socially unacceptable among Muslims and by the fact that most of the patients with CLD were Muslims (92.7%), this might also explain the high proportion of patients who were alcohol abstinent. According to the WHO Global Status Report on Alcohol and Health 2014 the prevalence of lifetime alcohol abstainers in the general population of Ethiopia is 71.2% [15]; and the 2016 Ethiopian Demographic and Health Survey reports an alcohol abstinence prevalence of 86.6% in the Harari region [244], and thus it is not unlikely that the observed high abstinence rates in the present study are representative.

Although the number of patients with alcoholic liver disease in the present study was remarkably low, the observed proportion is in line with previous studies [23, 26, 218-221].

Institutional-based surveys in Addis Ababa from the late 1970's and mid-1980's considered alcoholic related CLD to be rare [23, 219, 220], and in a recent retrospective study of 117 patients with CLD admitted to a governmental hospital in Addis Ababa from 2009 to 2014, only 1.7% were attributed to alcohol [26].

5.1.2.5 Non-alcoholic fatty liver disease

NAFLD is one of the most common liver disorders worldwide and is defined as histological or radiological evidence of hepatitis steatosis and lack of secondary causes of hepatic fat accumulation [18, 153, 158, 241].

In the present study, one of the patients with unexplained CLD who underwent liver biopsy had histological features of mild mixed steatosis (approximately 20%). According to our diagnostic criteria, however, she did not fulfil the criteria for NAFLD since her body mass index (BMI) was 24.7 (including gross ascites). She did not have any other risk factors for NAFLD other than reported months of wasting and fatigue prior to admission with evident hypoalbuminemia (27 g/L). Since the liver biopsy was undertaken one week after the end of the Holy month of Ramadan and the patient was a young devoted Muslim, we considered this case compatible with steatosis due to fasting or malnutrition [18, 245]. However, in retrospect, we cannot exclude NASH in this patient since we did not obtain complete lipid profile or fasting glucose levels in our panel of laboratory tests.

This case also illustrates another weakness of our diagnostic workup, as we have mentioned repeatedly, namely the lack of liver biopsy. Hence, we might have underestimated the frequency of NAFLD since our modified diagnostic criteria did not include liver biopsy but depended on hepatic steatosis detectable on ultrasound.

Although ultrasound is useful in evaluating fatty liver and attractive because it is non-invasive, fast and inexpensive, it has limited sensitivity and an exact quantification of fat is not possible [212]; studies have shown that the threshold for detecting hepatic steatosis on ultrasound is around 20-30% fat on liver biopsy, which means that about one out of five with steatosis appears normal on ultrasound [246, 247].

The estimated overall global prevalence of NAFLD diagnosed by imaging is around 25%, with the highest prevalence reported from the Middle East (32%) and the lowest in Africa (13%) [21]. There is no representative data on the prevalence of NAFLD in Ethiopia,

although it is known that Ethiopia has one of the lowest prevalence rates of obesity worldwide [27]. The data from other populations in sub-Saharan Africa are scarce but suggest that in comparison with Caucasian, Indian and Asian populations, diabetes may be a more important risk factor for NAFLD in Africa than obesity [248].

None of the patients in the present study were obese; other than one case with alcoholic liver disease, none had significant steatosis on hepatic ultrasound and only one had diabetes. Thus, although NAFLD is suggested as playing an important role and possibly under-recognized in patients with unexplained CLD, the prevalence of NAFLD in this study population is likely to be very low.

5.1.2.6 Hepatic schistosomiasis

Although a stool sample was missing in 11 patients, none of these had ultrasound findings suggestive of schistosomiasis and thus the number of cases diagnosed with hepatic schistosomiasis was not affected.

However, we have used strict diagnostic criteria for the diagnosis of schistosomal liver disease, requiring both a positive Kato Katz smear and radiological evidence of periportal thickening/'pipestem fibrosis' confirmed by expert opinion. Hence, by the fact that studies have demonstrated that around 30% of patients with periportal fibrosis due to *S. mansoni* did not have detectable ova in the stool [249], we might have underestimated hepatic schistosomiasis among patients with CLD in this study population.

5.1.2.7 Visceral leishmaniasis

In our study, four (2.7%) patients had a positive rK39 RDT and all were referred for further investigations, treatment, and follow-up at the Hiwot Fana Specialized University Hospital. As far as we know, only one of the patients underwent further diagnostic workup during the study period; the patient had splenomegaly and underwent a splenic aspiration, which turned out negative and thus was not classified as VL in the present study.

Although splenic aspiration was not performed on the second patient with a positive rK39 RDT, the patient had hepatosplenomegaly, findings of elevated serum IgG >2.0 x upper reference range and a history of travelling in a leishmaniasis endemic area, and thus the patient was diagnosed VL.

The two last patients with a positive rK39 RDT had neither hepatosplenomegaly nor history of travelling to endemic areas nor elevated IgG, and although further diagnostics were not available, we found the diagnosis of VL to be unlikely.

For study purposes, all patients with unexplained CLD were screened with the rK39 RDT to rule out VL, although the diagnosis was not suspected.

A weakness using serological methods in cases without adequate medical history or clinical suspicion is that the test cannot distinguish present from past asymptomatic infection or cured disease, and thus we cannot be certain if a positive test represents current disease and thus there is an increased risk of false positive results [200].

The estimated positive predictive value of rK39 RDT in East Africa given a 40% prior probability of VL is 86%, whereas the negative predictive value is 90% [200]. Since VL, most probably, is rarely found in the Harari region, the positive predictive value will decrease dramatically, and thus might explain why we have seen this high proportion of assumed false positive tests. However, as discussed previously, a weakness of this study was that we were solely depending on rK39 RDT in the diagnostic workup, and thus we might have missed diagnosing VL in some cases.

5.1.2.8 Human immunodeficiency virus

In the era of highly active antiretroviral therapy (HAART) and long-term survivors of HIV infection, increasing data suggest that HIV might have a direct effect on the liver [250]. Of note, the polymorphic findings of hepatic steatosis [251], nodular regenerative hyperplasia and non-cirrhotic portal hypertension in HIV-positive patients with CLD [252, 253], indicate a new disease entity possibly related to HAART, although the exact pathogenetic mechanism is still unknown [254].

In our study, one HCV/HIV-coinfected patient had signs of focal nodular hyperplasia on abdominal ultrasound but was simply classified as chronic HCV-infection. Three HIV-positive patients were classified as unexplained CLD, as none had radiological findings of steatosis, nodular hyperplasia or splenomegaly; all three patients had evidence of cirrhosis with irregular/nodular liver surface and moderate/gross ascites. However, since liver biopsy was not available in the diagnostic workup, primary HIV-related liver injury cannot be excluded.

5.1.2.9 Drug-induced liver injury / Herb induced liver injury

The diagnosis of drug-/ or herb-induced liver injury (DILI or HILI) was not obtainable out of several reasons: (i) the definition of 'traditional herbal medicine' in the recorded data was not clearly stated and thus gave inconsistent information on specific drugs or herbs in use (e.g. honey and milk, excessive water intake, camel meat, and ginger-tea were registered as 'traditional herbal medicine'); (ii) questions regarding the timing and dosage (duration/amount) were not defined in the standardized questionnaire and thus not routinely asked for; (iii) traditional herbal remedies are usually based on recipes known only by the local healer and thus precise information was not obtainable; and, (iv) information on previous/baseline liver enzyme activities and/or re-challenge was not available. The Council for International Organisations of Medical Sciences (CIOMS) scale or Roussel Uclaf Causality Assessment Method (RUCAM) were considered but found not applicable due to this information gap [255].

More than two-thirds of the study subjects had scarring and burning marks from traditional medicine, and more than one out of four reported intake of traditional herbal medicine for various diseases including the manifest CLD. Hence, we cannot exclude that these herbal mixtures might even be hepatotoxic and worsen the progress of the disease.

5.1.2.10 Other diseases

As mentioned previously, only limited diagnostic tools were available in this resource-limited setting. Hence, it was not possible to exclude other causes of CLD, including primary sclerosing cholangitis (PSC) and veno-occlusive disease/Budd-Chiari syndrome.

Population-based epidemiological data on PSC are not available in Ethiopia. However, the prevalence and incidence rates of inflammatory bowel disease are low in Africa, and hence, given their inter-association, the prevalence of PSC is also likely to be low [256].

Budd-Chiari syndrome and veno-occlusive disease could not be ruled out without Doppler ultrasound, advanced imaging, and liver biopsy available but was thought to be an unlikely diagnosis based on the clinical and ultrasound findings.

Previous outbreaks of liver disease in the Hirmi Valley in northern Ethiopia were at first diagnosed as cases of veno-occlusive disease [257], but later on ascribed to exposure to a combination of the pyrrolizidine alkaloid acetyllycopsamine and the pesticide

dichlorodiphenyltrichloroethane (DDT) in stored grain [258]. Robinson *et al*. reported a clinical picture of acute illness and the cases were highly clustered with a parallel disease in domestic animals, and thus considered as an unlikely cause of CLD in this study population.

5.1.2.11 Other risk factors

Finally, a number of other risk factors of CLD were not assessed or accounted for. These include (i) toxic liver injury due to exposure to DDT and other pesticides through agricultural use and consumption of unwashed vegetables, grain or khat leaves [259]; (ii) liver injury caused by dietary exposure to aflatoxins from grain stored underground or groundnuts attacked by *Aspergillus* fungi [260, 261]; (iii) nonstandard viral hepatitis, such as GB virus C (GBV-C) or Transfusion Transmitted (TT) virus infection, although their aetiological role is debated [262-264].

5.1.3 Liver biopsies

As discussed in **Paper I**, it was intended, as *per protocol*, that all patients with unexplained CLD would be offered a liver biopsy. However, during the study period, no suitably trained personnel were available to undertake this procedure. This situation was eventually resolved and attempts were then made to contact the study subjects who, after investigation, had unexplained CLD to ask them to return for liver biopsy (Figure 10).

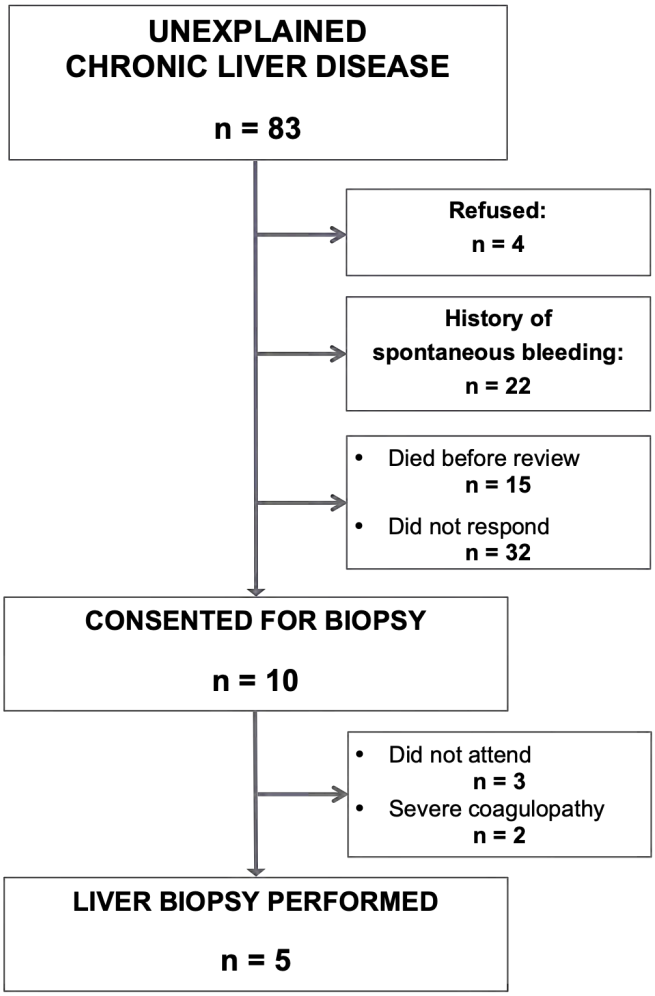

Fig. 10: Selection of patients with unexplained chronic liver disease for liver biopsy.

A large proportion was not contactable or else, if contacted, refused the procedure. In addition, several of the more decompensated patients had died and as the biopsies were to be performed percutaneously, only those with a normal or marginally elevated prothrombin times were considered suitable for biopsy, according to established guidelines [214].

In consequence, liver biopsy was only undertaken in 6% of the patients with unexplained CLD (3% of all study subjects), and we acknowledge that the small number of liver biopsies performed to confirm the established diagnosis is a limitation to this study. If liver biopsies had been available it would have been of value to confirm the presence of CLD and in determining its aetiology.

However, a liver biopsy does not always provide a final or complete diagnosis since a needle biopsy specimen represents only around 1/50 000 of the whole liver, and thus clearly may suffer from inadequate sampling [265]. Although the risk of sampling error can be reduced by undertaking multi-focal biopsies, it was not obtainable in our clinical setting.

The most practical way to minimize sampling error is to obtain a biopsy specimen of sufficient size, which has been suggested as a 16-18 gauge needle biopsy of 25-30 mm in length after formalin fixation, although an accurate diagnosis can be established with only very small biopsy specimens in some cases and 15 mm has been considered sufficient in most studies [266].

In our study, the biopsy specimen lengths ranged from 10-16 mm with 6-10 liver portal tracts. Thus, although we considered more than five portal tracts as representative material for assessment, one could argue that the sampling was not sufficient in size and quality according to established guidelines, and thus should be added as a limitation to the liver biopsy results [214].

Another potential limitation is inter-/ and intra-observer variations in liver biopsy interpretation. In order to minimize the risk of observer variations, semi-quantitative scoring systems were used and two highly qualified pathologists assessed the biopsy specimens independently; one of the pathologists is a general pathologist (prof. em. Roald), whereas the other pathologist has specialization in liver pathology (prof. Goldin); both have long practice in academic context and experience in diagnosing liver disease in African context.

The liver biopsy findings were focal, diffuse and anonymous, with only mild fibrosis and inflammation as described in **Paper I.** Although suggestive of toxic liver injury, the observed histopathological changes were unspecific and not pathognomonic for any disease entity or known causative agent, and might reflect the fact that the selection process favoured only cases with the mildest disease.

5.2 Paper II

Testing a hypothesis – is khat chewing associated with chronic liver disease in eastern Ethiopia?

Besides exploring the underlying aetiologies of CLD in eastern Ethiopia, our cross-sectional study (**Paper I**) was hypothesis generating in that several possible risk factors predisposing for the development of CLD became evident.

In the second part of this study, we chose to focus on the widespread use of khat and the hypothesis: Is khat chewing associated with CLD in this study population?

We tested this hypothesis by a rigidly designed case-control study (**Paper II**) and found a strong and significant association between khat usage and the risk for developing CLD. Furthermore, although confined to men, we found a dose-response relationship between khat exposure and the associated risk of CLD. Evidence from this study, together with previous case reports and animal studies, supports a strong association and suggests a causal relationship between khat chewing and the development of CLD.

5.2.1 Confounding

Confounding is a critical issue in epidemiological studies but can be controlled for by using different methods. The most common methods to prevent confounding in an analytic study design are by randomization, restriction, and matching. In observational studies, restriction and matching can be considered, whereas randomization can only be used in interventional studies.

In the design stage of this case-control study, we considered controlling for confounding through restriction. However, although restriction might have increased the internal validity of the study, it would have reduced the generalizability. Moreover, as indicated in **Paper I,** we suspected that khat not only could be an independent risk factor for CLD but also contribute as an adjuvant cause of liver injury. Hence, we decided not to control for confounding through restriction and included all cases with different aetiologies of CLD in the main analysis.

In the analysis stage, confounding was dealt with by an initial stratification, where the Breslow and Day test pinpointed effect modification and the Mantel-Haenzel method was used to control for potential confounding.

In the *post hoc* sensitivity analysis, we restricted the study population by excluding all cases with an identifiable aetiology or known risk factor for CLD; although the controls did not undergo the same comprehensive investigation as the cases, all controls were screened for the risk factors assumed *a priori* to be the most relevant *viz* viral hepatitis and alcohol abuse, and were excluded from the sensitivity analysis if found positive. The positive association found in the main analysis remained, attesting to the robustness of the findings, and no other significant confounders were identified.

We also considered dealing with confounding by matching cases and controls. However, the advantages of matching to control for confounding effects in the case-control design are limited [145]. Matching may even hamper the efficiency of the study since the matching factor itself cannot be assessed in the analysis in terms of its relationship to the outcome.

Furthermore, if the matching factor is associated with the exposure of interest, the matching variable itself may introduce confounding bias [267]. Hence, matching has been regarded as inappropriate for an exploratory study aiming to answer a general question about what are the underlying causes of the outcome of interest [146]. And as shown in the initial stratified analysis, both age and sex are indeed associated with khat exposure; however, sex came out not as a confounder but rather as an effect modifier, and thus if we had matched on sex we would have been at risk of 'overmatching' and decrease the efficiency.

Hence, in our case-control study, we controlled for sex by stratification (*vide supra*) and adjusted for the confounding effects of age, alcohol and viral hepatitis in a multivariable logistic regression model. However, as mentioned in the discussion section in **Paper II,** there was a number of other potential confounders or effect modifiers that were not assessed or accounted for, including (i) exposure to DDT and other pesticides used in the cultivation of khat which may be present on unwashed leaves [259]; (ii) schistosomiasis which is prevalent in this area; four cases of hepatic schistosomiasis were identified among the cases but controls were not screened for infection; (iii) the use of traditional herbal remedies; (iv) cigarette smoking [5]; and, (v) coffee intake [268].

5.2.2 Causality

Although we found a strong statistical association between khat chewing and CLD, it does not necessarily imply causality. We can use the Bradford Hill's criteria as a guideline to assess a cause-and-effect hypothesis, including (i) information concerning the temporal sequence; (ii) the strength of the association; (iii) consistency of the findings; (iv) the presence of a dose-response relationship; and, (v) the biological plausibility of the hypothesis [269].

5.2.2.1 Temporality

In the cross-sectional study (**Paper I**) we stressed to include only patients with a new diagnose of CLD (incident cases), and patients attending the hospitals for follow-up or exacerbation of previously recognized liver disease were excluded. By excluding prevalent cases, the exposure-disease relationship was less likely to be influenced by altered risk habits, lifestyle change or other interventions from medical follow-up [145].

Information on khat exposure applied to a relevant time period before the clinical diagnosis of CLD. However, despite our good intentions, we may not be able to suggest a temporal relationship by using incident cases since CLD is an indolent disease with no clear onset time.

Previous case reports have observed both acute/sub-acute hepatitis and CLD in Europe and Australia that resolve after cessation of khat chewing but relapse following re-exposure, and thus strongly suggest a causal relationship [119, 121, 122, 127]. However, data on cessation of khat chewing and re-challenge were not available in our case-control study.

5.2.2.2 The strength of the association

The association between khat exposure and the risk of developing CLD observed in the present study was strong, although confined to men. Since khat use is widespread, legal and socially acceptable, the high risk of developing CLD makes khat to be a major contributor to the burden of CLD in the region. Assuming a causal relationship, four out of five men with CLD in our study population can be attributed to khat use.

5.2.2.3 Consistency

Animal models have demonstrated khat-related hepatotoxicity causing acute hepatitis [135], liver fibrosis [136] and histopathological evidence of ballooning degeneration in hepatocytes with chronic inflammation in the liver [137].

Previous case reports have observed acute and sub-acute hepatitis that resolves after cessation of khat chewing [119, 121, 122]. Recent case series from the neighbouring country Somaliland have observed khat-related CLD with histopathological changes of chronic hepatitis, lobular cholestasis and advanced fibrosis [131].

To the best of our knowledge, this is the first case-control study indicating khat chewing as an independent risk factor for developing CLD, and further validation studies in different settings are needed to confirm our findings.

5.2.2.4 Biological gradient

Our case-control study found a gradient relationship in the risk of CLD with increasing levels of khat exposure, although confined to men. Animal studies have also indicated a dose-response relationship between the level of khat exposure and the degree of histological injury [136, 137]. However, this present study was not designed to establish any biologically relevant threshold level at which liver injury occurs.

5.2.2.5 Biological plausibility

Several potential biological mechanisms for khat-related hepatotoxicity have been proposed:

(i) Case reports describe khat-related hepatitis with presence of autoantibodies typical of AIH and liver histopathology corresponding to drug-induced AIH, implicating that khat trigger autoimmune mechanisms leading to liver injury [121, 123, 133].

(ii) Animal studies postulate that khat alkaloids induce changes in free radical metabolizing enzyme activity and subsequently induce oxidative stress causing cell injury [138].

(iii) A study on human liver cells has demonstrated that khat triggers the generation of reactive oxygen species that induce hepatocyte apoptosis through intracellular signal pathways [139].

(iv) Studies on human leukemic cell lines have shown that khat induced apoptosis involving mitochondrial damage and enhanced autophagy [270].

(v) There may be a genetic predisposition to liver injury relating to polymorphisms in the gene controlling CYP2D6, the main enzyme responsible for the hepatic metabolism of the khat alkaloids [75]. A recent study on 40 Ethiopian volunteers found that exposure to khat caused inhibition of CYP2D6 in certain genotypes, and also a marginal inhibition of CYP3A4 activity [271].

5.2.3 Sex-differences in the susceptibility to khat-related liver injury

Interestingly, we found that the effect of khat on the risk for developing CLD was dependent on its interaction with sex. We have undertaken four additional sets of analyses to explore this sex difference further, none of which identified an association between khat use and CLD in women.

Firstly, we categorized khat exposure by quartiles of *khat-years* and stratified by sex. In men, the risk for developing CLD after adjusting for age, alcohol and HBV exposure increased with increasing khat exposure but no such relationship was observed in the women.

Secondly, since the khat usage was significantly lower in women than men, we repeated the analysis using sex-specific exposure levels but still, no association between khat exposure and the development of CLD in women was found (Table 4).

Table 4: Gradient effect of khat consumption, using sex-specific exposure levels

	Women (n=170)					Men (n=280)			
	Cases (n=42)	Controls (n=128)	Adjusted OR[a] (95% CI)	*p*		Cases (n=108)	Controls (n=172)	Adjusted OR[a] (95% CI)	*p*
Khat-years:[b]					***Khat-years:***[c]				
0[d]	19	61	1		**0**[d]	4	40	1	
0.1-5.0	11	37	1.20 (0.48-3.01)	0.703	**0.1-60.0**	70	101	5.27 (1.70-16.33)	0.004
5.1-160.0	12	30	1.20 (0.45-3.24)	0.718	**60.1-250.0**	34	31	10.14 (2.79-36.83)	<0.001

a. Adjusted for the confounding effects of age, alcohol consumption and hepatitis B virus infection.
b. Cumulative frequency of *khat-years* in women: 0.1 is the 25% cumulative frequency (25 percentile), 5.0 is the 75% cumulative frequency (75 percentile), and 160.0 is the maximum.
c. Cumulative frequency of *khat-years* in men: 0.1 is the 25% cumulative frequency (25 percentile), 60.0 is the 75% cumulative frequency (75 percentile), and 250.0 is the maximum.
d. Reference group comprised of unexposed study subjects.

Abbreviations: CI, confidence interval; OR, odds ratio.

Thirdly, we further investigated the khat exposure using per unit increase in *khat-years* as a continuous variable in a logistic model. This, likewise, confirmed a gradient effect in men and the absence of an effect in women.

Finally, we undertook a *post hoc* sensitivity analysis, in which the findings remained robust, and in distinction to the situation in the men, a gradient effect was not found in the women.

So why did we not find a significant association between khat and CLD in women?

This is a key question that we cannot answer based on the data presented in the present study. The health-seeking behaviour is likely to differ between men and women, and thus power deficiency might be one explanation since relatively few women reported high-level khat exposure but the effects would not be easily quantified.

However, there are also other possible explanations, which could be explored further:

(i) Since women chew significantly less khat than men resulting in significant sex-differences in exposure, can the explanation simply be that the levels of exposure in women did not reach toxic thresholds?

(ii) Can it be that there are significant sex-differences in chewing habits, culturally and/or socially, which influence the duration of exposure? It is not unlikely that men spend concentrated blocks of recreational time chewing khat and hence the duration of exposure is prolonged. Women, on the other hand, tend to only chew khat intermittently, and thus even if they chewed the same amount the duration of overall exposure would be very much shorter. As discussed previously, a limitation in the *khat-year* parameter is that it does not comprehend the duration of each khat-session, which undoubtedly will influence the cumulative khat-exposure.

(iii) A third hypothesis is that women are not chewing the young shoots and sprouts but khat leaves taken from the lower parts on the stem. The young top leaves contain a higher concentration of cathinone and other khat-derived substances, and hence the women are less exposed to the potential hepatotoxic agents in khat.

(iv) Can it be related to sex-specific differences in dietary constituents and khat metabolism? Khat is metabolised via CYP2D6, and dietary constituents may result in either induction or inhibition of various isoenzymes, which in term might result in differences in the rate of khat metabolism [272]. It is also possible that there may be sex-specific differences in the number of copy variants of CYP2D6 or differences in frequency of variant single nucleotide polymorphisms [273].

5.3 Paper III

Exploring a hypothesis – does khat chewing increase the seroprevalence of autoantibodies typical of autoimmune liver disease?

In the case-control study **(Paper II)**, we demonstrated a strong and significant association between khat chewing and CLD; however, the pathogenetic mechanism was not addressed. A few previous case reports have described khat-related liver injury mimicking AIH, and the authors speculate that khat might trigger an autoimmune reaction in susceptible individuals [121, 123, 133].

In **Paper I**, however, we found that only 1.3% of the cases with CLD were attributed to AIH. On the other hand, we were also fully aware that we might have missed diagnosing AIH in several cases due to a lack of histopathological investigations, only a limited panel of autoantibodies, and the limitations in using anti-F-actin ELISA, as discussed previously. Hence, in **Paper III** we aimed to investigate this hypothesis further, but from a different angle, by assessing a healthy population in eastern Ethiopia and determine the seroprevalence of autoantibodies typical of autoimmune liver disease and compare khat users to non-users in the search for possible associations between khat and selected autoantibodies.

Of note, the aim of this study was not to prove an association between khat chewing and autoimmune liver disease but rather to gain insight into the pathogenesis of khat-related liver injury. And to minimise the influence of underlying disease on the autoantibody profile, study subjects with manifest liver disease, known autoimmune disease or recognized trigger factors of autoimmunity were excluded and we considered this as a strength of the study; the limitations with this approach have been discussed previously.

In this study (**Paper III**), we found that ANA was more common among khat users compared to non-users; however, the numbers were small and only borderline significant. There were no significant differences between khat users and non-users in the frequency of circulating SMA and AMA. In the further search for an association, we stratified the study subjects according to the level of khat-exposure but still, no associations were found.

Likewise, in the multivariable analysis adjusting for age and sex, no association between khat chewing and the seropresence of SMA or AMA was found.

As discussed in the paper, since none of the ANA seropositive study subjects had concurrent seropresence of SMA or elevated serum IgG, and there was no association between elevated liver transaminase activities and the selected circulating autoantibodies, we concluded that our findings weaken the hypothesis that the pathogenetic mechanism of khat-related liver injury is mediated by autoimmune mechanisms.

This conclusion was for us unexpected and disappointing but still it corresponded well with the observations in **Paper I**. However, given the limitations of the study design, no strong conclusions can be drawn and the findings from this study should be interpreted cautiously.

To further test the hypothesis that khat-related liver injury might be mediated through autoimmune mechanisms, future studies are suggested to follow a study cohort of healthy khat users and non-users, and monitor if the khat users develop liver disease more frequent than the non-users, and register if any changes in the autoantibody profile occur, also after discontinuation of khat use.

Paper III was the first serosurvey assessing selected autoantibodies in a healthy population in eastern Ethiopia. To the best of our knowledge, the only study on autoimmune markers in Ethiopia available is an ancient survey from the Black Lion Hospital in Addis Ababa, which was undertaken in the 1970's studying 107 Ethiopian patients with dyspepsia and 80 healthy controls [149]. Tsega *et al* found that ANA was positive in one patient (0.5%), SMA in 20 (10.7%) and AMA in one (0.5%); all autoantibodies were determined by IIF. Only scant data on the seroprevalence of autoantibodies among healthy individuals in sub-Saharan Africa are available for comparison.

We found that 2.6% of the study subjects were positive for ANA, which was significantly increased compared to the previous study in Ethiopia [149] but at the lower end compared to other sub-Saharan and global estimates ranging from around 3 to 30% in healthy individuals [274-280].

More interestingly, we found that 15.4% were positive for SMA, which was more prevalent than the global estimates around 10-12% and higher than anticipated [149, 281-283]. However, the observations in the present study might not be directly comparable to other studies since there are wide differences in screening assays, quantification methods, and analytic thresholds.

The ELISA technique for detecting SMA is considered inferior to IIF in diagnosing AIH due to its low specificity [169]. Thus the observed high seroprevalence might be a result of the high reactivity frequency of the anti-F-actin ELISA, even in healthy control subjects [176].

However, we have also felt it necessary to ask: Could it be that the seroprevalence of autoantibodies, in general, is high in sub-Saharan Africa due to an increased exposure to various infectious diseases and other environmental triggers of autoimmunity?

It is known that the upper reference range for eosinophil counts and serum IgG in apparently healthy individuals is higher in sub-Saharan African countries [156], and thus may reflect a higher degree of immune activation compared to the Western world. Since the serological tests and assays are largely established in developed countries, and there are scarce epidemiological data on autoimmune markers among healthy individuals in sub-Saharan Africa – is it possible that the conventional cut-off values are inappropriately low for this African population?

Or else, if the observed seroprevalence of SMA (and ANA) in fact is representative for the study population, another interesting question arises: Why have we not observed more cases of AIH in this study population? Hence, further longitudinal studies are needed to study whether the presence of autoantibodies actually lead to the development of clinical liver disease or not. And if not, which genetic and/or environmental factors are in play, protective for autoimmune diseases?

This question becomes even more pertinent in the interpretation of the, in our opinion, most intriguing finding of this study, namely the strikingly high proportion positive for AMA (25.6%), which was more than a twenty-fold increase compared to global estimates [284-288].

Although IIF is still considered as the gold standard, the anti-M2 (MIT3) ELISA-assay we have used is considered having both increased sensitivity and specificity compared to other methods [183-186]. Clearly, this assay was highly effective in detecting AMA and it was most surprising to observe such high seroprevalence.

According to our collaborator responsible for the serological analyses at the Department of Medical Biochemistry, Vestre Viken Hospital Trust, Drammen (personal communication, Dr. Trine Lauritzen), the frequency of positive AMA (and SMA) among the Ethiopian study

subjects in the present study was much higher than the results obtained from routine clinical samples in Norwegian patients using the same assays.

Another interesting feature about this finding was that among the 70 AMA positive healthy individuals, 38 (54.3%) were men. Hence, since testing positive for AMA, especially the subtype AMA-M2, is virtually diagnostic for PBC or at least suggests a significantly increased risk for developing PBC over the next 5-10 years, our findings differ substantially from the sex-ratio usually found among patients with PBC, which predominantly affects women with a female-to-male ratio of 9-10:1 [180].

As discussed repeatedly, the absence of histopathological investigations is a limitation to the diagnostic workup in our study. However, established guidelines do not recommend a liver biopsy for diagnosing PBC, unless AMA is absent or when co-existent AIH or NASH is suspected [178].

By definition, none of the study subjects in **Paper III** had known history or overt symptoms/clinical signs of liver disease but since routine cholestasis markers (alkaline phosphatase, gamma-glutamyl transferase, and bilirubin) were not measured, we cannot exclude asymptomatic PBC.

To the best of our knowledge, there are no epidemiological studies on PBC in Ethiopia or sub-Saharan Africa available [256], but the prevalence is assumed to be one of the lowest in the world [289], which correspond to our findings in **Paper I**, where none of the patients had CLD ascribed to PBC. Hence, we find it less likely that the observed high seroprevalence of AMA represents an epidemic of latent PBC in this apparently healthy study population.

Nevertheless, the natural first step for further investigation of this large cohort of AMA positive individuals will be to rule out PBC. Subsequently, in a most likely still large cohort of AMA positive patients with non-established PBC, extrahepatic disorders should be ruled out since AMA may also be found in patients with various non-hepatic autoimmune diseases and haematological malignancies [290]. As pinpointed previously, the diagnostic facilities for autoimmune and malignant disorders in this resource-limited setting are severely limited. Hence, it is not unlikely that patients with undiagnosed autoimmune disorders or malignancies have been overlooked.

Finally, we could study the significance of this high seroprevalence of AMA using a prospective, longitudinal study design. In a recent study of newly detected AMA positive patients in clinical practice, nearly half did not lead to the diagnosis of PBC [291].

Moreover, among the AMA positive patients with normal alkaline phosphatase, only one in six patients developed PBC after five years; interestingly, this group of patients had increased risk of mortality without any obvious link with circulating AMA [291].

Perhaps our findings in eastern Ethiopia could be a humble beginning that could join other research raising the question of the true significance of AMA in human pathology?

The paradoxical feature of increased immune response but low incidence of autoimmune diseases among African individuals compared to European individuals has been observed for more than 50 years [292]; however, our understanding of the environmental and genetic factors that might contribute to this resistance against autoimmune diseases in Africa is still in its early days [293, 294].

So eventually, we might be obliged to ask: Are the diagnostic algorithms for autoimmune liver disease, which were developed in the Western world, applicable in sub-Saharan Africa?

6 Conclusions

This work set out to explore the aetiological spectrum and potential risk factors for CLD in eastern Ethiopia. From the three studies undertaken and presented in this thesis, certain conclusions can be drawn:

Paper I - A substantial fraction of CLD in eastern Ethiopia is attributed to chronic HBV infection. Khat use is prevalent among CLD patients and may represent an independent or adjuvant risk factor of CLD. After ruling out the most common causes, the underlying aetiology of CLD in eastern Ethiopia is yet to be found in a considerable proportion of the patients. However, certain entities of CLD could not be ruled out in this study and also several other potential environmental factors may contribute and needs to be investigated in more detail.

Paper II – There is a strong association between khat chewing and CLD, suggesting a causal relationship. Given the assumption of causality, more than 80% of CLD among men in this region is attributable to khat chewing. Whether exposure to khat has an effect on the development of CLD in women is still unclear. There is no obvious explanation for the sex difference in the susceptibility to khat-related liver injury in the present study.

Paper III – The hypothesis that khat triggers an autoimmune response leading to liver injury mimicking AIH is weakened. This study also found a strikingly high seroprevalence of AMA in healthy Ethiopians. Whether this finding represents undiagnosed PBC or other autoimmune disease or exposure to environmental factors is not clear. These issues have to be resolved preparatory to a prospective, longitudinal study that could assess the significance of this high seroprevalence of AMA.

7 Future perspectives

CLD is a major contributor to morbidity and mortality worldwide, and the leading cause of death in adults in eastern Ethiopia. And thus, there is an urgent need for further investigations to facilitate public health efforts in alleviating the burden of CLD in Ethiopia and other countries in sub-Saharan Africa.

Our study has pinpointed chronic hepatitis B and khat usage as major contributors to the development of CLD, of which both are preventable and avoidable. However, since there are certain limitations of using an institution-based study design, additional population-based studies are requested to investigate the burden of CLD in the region and to obtain a regional estimate of the burden of viral hepatitis. Furthermore, community-based studies are needed to explore the habitual use of khat in more detail.

The health consequences of khat chewing, both on an individual and a public health level, are profound. Since khat is increasingly available and habitual khat chewing is expanding worldwide, the results of this study can be widely extrapolated to any country where khat is chewed including diasporic communities worldwide.

Since our study is the first controlled study demonstrating the aetiological importance of khat chewing in humans, there is a pressing need for validation studies from other settings and study populations, evaluating the value of our findings.

The hypothesis that khat-related liver injury is mediated through autoimmune mechanisms in susceptible individuals was weakened by our findings and the pathogenetic mechanism of khat-related liver injury is still unclear and warrants further studies.

Another unresolved issue is why some individuals seem to develop severe consequences of their khat abuse, whereas others are apparently unharmed. One recent hypothesis speculates that genetic variants associated with low or non-functional drug metabolism through CYP2D6 in the liver can explain these differences. Poor metabolizers would accumulate khat alkaloids and be at increased risk of hepatotoxic effects. By studying CYP2D6 activities in cases with CLD and history of khat use compared to a control group of khat users without CLD, we could test this hypothesis.

Finally, we do not have any obvious explanations for the sex-differences in the risk for developing khat-related liver injury observed in our study and further efforts are warranted to elucidate this differential susceptibility.

More than 50 years ago, Blahoš *et al* stated that chronic liver diseases in Harar «*represent not only a medical but a social problem.*» [32, p. 192]; yet, to the best of our knowledge, no studies on liver diseases in this part of Ethiopia had been undertaken before this study. The intention of this thesis was to start addressing this major public health issue and hopefully contribute by identifying plausible underlying risk factors.

Mounting evidence have demonstrated various adverse health effects related to khat use, and this study has sought to contribute by adding new knowledge to the aspects of liver disease related to khat use.

We acknowledge that the khat controversy is multifaceted and complex, and requires a multidisciplinary approach to decide on policy, regulations, and legislation. At the same time, we plea that it will not take another 50 years before any action is taken to start addressing the emerging public health issue implied by this thesis:

Catha edulis and chronic liver disease in eastern Ethiopia.

Although one single study cannot be decisive, we sincerely hope that this work could pave the road for further studies and a better understanding of khat-related liver disease and inspire to concerted actions in preventing its occurrence and thus alleviate the burden it imposes.

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9 Appendices

9.1 Appendix 1

Study ID no._____

I N T E R V I E W

..... HOSPITAL

Name_____Hospital ID no._____

NB!! ALL DATES FOLLOWING EUROPEAN CALENDAR

Admission date: …..../…..../20.....… Date of filling form:…..../…..../20.....…

Interviewer_____ Language_____

Sex *M□ F□* Age_____ and/or Date of Birth:…../…../19……

Occupation_____ Ethnic group_____

Address_____Region_____

Subcity_____Kebele_____

Phonenumber_____

CHEMICALS / KHAT / DRUGS

Exposure to chemicals/pesticides *Y□ N□ Specify (type/brand? for what purpose? how often?)*

_____

_____

Do you eat grain that was stored in the ground? *Y□ N□ Specify (type grain? storage time? insecticides sprayed directly on grain Y□ N□)*_____

_____

_____

Do you chew khat? *Never □* *<1 day/MONTH □* *2-4 days/MONTH □*

2-3 days/WEEK □ *>4 days/week □* *Stopped□ when:*_____

On a typical chewing day, how much do you chew (hours, bundles)?_____

_____

For how many years have you chewed khat?_____

Do you use any additional substances during chewing khat (e.g beer, alcohol, groundnuts) ?

*Y□ N□ Specify:*_____

Other drugs/herbs/traditional medicine *Y□ N□ Specify:*_____

_____

Study ID no._____

Do you drink alcohol? *Never* □ *<1 day/MONTH* □ *2-4 days/MONTH* □
2-3 days/WEEK □ *>4 days/week* □ *Stopped*□ *when:*_____
IF YES*:* On a typical drinking day, what and how much do you drink (be exact)?_____

_____

IF YES*:* For how many years have you been drinking?_____

MEDICATION

Presently on any prescribed medication? *Y*□ *N*□ ***IF YES:*** *Drug name, dosage and started when:*

_____

_____

Complementary or alternative medicines over the past 6 months (e. g. paracetamol, ibuprofen, diclofenac, short courses of antibiotics)? *Y*□ *N*□
IF YES: *Specify dosage + start/stop dates):*_____

_____

_____

IF STRONG SUSPICION ON DILI – fill out RUCAM score (additional form)

MEDICAL HISTORY

Previous liver disease/jaundice? *Don't know* □ *Y*□ *Year*_____*N*□
IF YES*: Diagnosis*_____

_____

Previous hepatitis test? *Don't know* □ *Y*□ *N*□ *Date…../…../…....Result*_____
Previous blood transfusion *Y*□ *N*□ *Year*_____
Previous tattoo/scarring/piercing *Y*□ *N*□ *Year*_____
Other previous diseases/surgery_____

_____

_____

Liver disease in the family? *Don't know* □ *Y*□ *N*□ *Specify*_____

_____

Contact with icteric patients past 6 months? *Y*□ *N*□ *Specify*_____

_____

Domestic animals sick or died past 6 months? *Y*□ *N*□ *Specify (when, type animal, death cause, swollen belly?)* _____

_____

FOR WOMEN *Pregnant? Y* □ _____*weeks N* □ *Breastfeeding Y* □ _____*months N* □

CURRENT DIAGNOSIS OF ADMISSION/OPD CHECK UP:

Diagnosis_____

Study ID no._____

CURRENT LIVER RELATED SYMPTOMS

Duration of current symptoms _____ weeks / months / years

YES	**NO**	
□	□	Jaundice
□	□	Abdominal pain
□	□	Abdominal swelling
□	□	Oedema
□	□	Fever
□	□	Rash
□	□	Nausea/vomiting
□	□	Diarrhoea
□	□	Arthralgia/myalgia
□	□	Haematemesis
□	□	Confusion
□	□	Weight loss/wasting
□	□	Other_____

9.2 Appendix 2

141105/version6/AJSO Study ID no._____

CLINICAL EXAMINATION

..... HOSPITAL

Name_____Hospital ID no._____

Admission date: ……/……/20.....… Date of filling form:……/……/20.....…

Examiner_____

CLINICAL EXAMINATION

Weight_____Height_____Temp_____BP_____HR_____

General state *Well* □ *Ill-looking* □ *Bedridden* □

Oedema *N*□ +□ ++□ +++□

Eyes *N* □ *Jaundice* □ *Kayser-Fleischer rings* □ *Pale conjunctivae* □

Skin *N* □ *Rash* □ *Jaundice* □ *Spider nevi* □ *Palmar erytema* □ *Traditional tattoo/scar* □

Chest (for men) *N* □ *Gynecomastia* □

Heart *N* □ *Abnormal* □ *Specify* _____

Lungs *N* □ *Abnormal* □ *Specify* _____

Abdomen *N* □ *Ascites* □ *Caput medusa* □ *Firm liver* □ *Hepatomegaly* □ *Splenomegaly* □

CNS *N* □ *Confused* □ *Coma* □

Other:_____

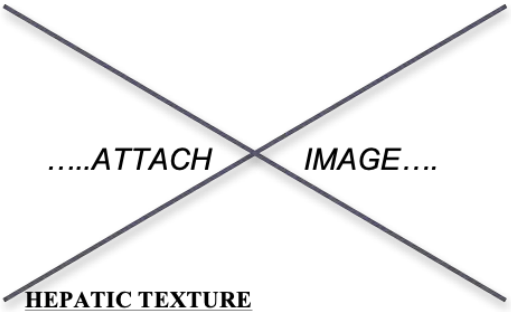
_____

_____

9.3 Appendix 3

141020/version4/SO

Study ID no._____



LIVER DISEASE STUDY
HIWOT FANA SPECIALIZED UNIVERSITY HOSPITAL ☐
JUGAL HOSPITAL ☐
- ULTRASOUND LIVER

Examiner_____
Patient_____
Hospital ID no._____
Date of filling form: ……/……/20.....

HEPATIC TEXTURE

Normal ☐ Heterogenous ☐ Course / irregular ☐ Steatosis ☐

*Comments:*_____

LIVER SURFACE

Normal ☐ Mild uneven ☐ Nodular / irregular ☐

*Comments:*_____

LIVER SIZE

Normal ☐ Small ☐ Enlarged ☐ Liver span _____ cm

*Comments:*_____

HEPATIC VESSELS

Smooth ☐ Obscured ☐ Irregular / narrowed ☐

*Comments:*_____

ASCITES – amount of fluid

No ☐ Small ☐ Moderate ☐ Gross ☐

*Comments:*_____

GALLDUCTS

Normal ☐ Dilated ☐ Strictures ☐ Common duct stones ☐ Gallbladder stones ☐

*Comments:*_____

SCHISTOSOMIASIS LIVER FIBROSIS *No signs ☐ Yes, suspected schistosomal fibrosis ☐*

If yes above: Image pattern score_____

Wall thickness 2nd branching portal branch (measure 2-3 branches)

1) inner-to-inner _____ mm outer-to-outer_____mm

2) inner-to-inner _____ mm outer-to-outer_____mm

3) inner-to-inner _____ mm outer-to-outer_____mm

Portal vein diameter _____ mm

*Comments:*_____

LIVER TUMOURS

No ☐ Single ☐ Multiple ☐ Size: ……..cm

*Comments:*_____

SPLENOMEGALY

No ☐ Moderate ☐ Large ☐ Spleen span _____ cm

*Comments:*_____

OTHER COMMENTS: (use the back of the sheet)_____

9.4 Appendix 4

Hospital ID no._____
Study ID no._____

LABORATORY RESULTS - LIVER DISEASE STUDY

…… HOSPITAL

Patients name:…….. Age:…. Male ☐ Female ☐

Dr:……...……... Date:…….

		1 (hospital lab Harar)	2 (research lab Addis/Norway)
Date		...../...../20.....	...../...../20.....
Lab test	**Normal range**	**Result**	**Result**
Hemoglobin	M: 12.2-17.7g/dl F: 9.5-15.8 g/dl		
WBC	3.1-9.1 10^9/L		
-PMN	1.0-5.3 10^9/L		
-lympho	1.2-3.7 10^9/L		
-eosino	0.0-1.5 10^9/L		
Platelets	126-438 10^9/L		
SGPT (ALT)	8-40 IU/L		
SGOT (AST)	14-40 IU/L		
ALP	48-164 IU/L		
γGT	7-61 IU/L		
Bilirubin total	0.2-2.2 mg/dL		
Albumin	35-52 g/L		
Creatinine	0.53-1.23 mg/dL		
**Iron sat%	15-50 %		
IgG	0.8-27.8 g/L		
Ceruloplasmin (<40y)	0.20-0.60 g/L		
**Alpha-1-antitrypsin	1.0-1.7 g/L		

* = only in AH patients; ** = only in CLD patients

Hospital ID no._____
Study ID no._____

	1 (hospital lab Harar)			2 (research lab Addis/Norway)
Date	...../...../20.....			...../...../20.....
Lab test	**Result**			**Result**
Pregnancy test (women <45years)				
HIV RDT				
Malaria RDT				
*HAV IgG/IgM RDT	IgG	IgM		
*HEV IgM RDT				
HBsAg RDT	InTec	Healgen	Determine	
Anti-HCV RDT	OneStep	SD Bioline		
Schisto stool test				
HBsAg				
anti-HBs				
anti-HBc				
anti-HCV				
*anti-CMV IgM				
*anti-EBV IgM				
ANA				
AMA-M2				
SMA				
LKM-1				
**Leishmania DAT				
HBeAg				
Anti-HBe				
HDV Ag				
Anti-HDV				
Anti-HBc IgM				
HBV DNA				
HCV DNA				

* = only in AH patients; ** = only in CLD patients

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Unexplained chronic liver disease in Ethiopia: a cross-sectional study

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Abstract

Background: Hepatitis B virus (HBV) infection is assumed to be the major cause of chronic liver disease (CLD) in sub-Saharan Africa. The contribution of other aetiological causes of CLD is less well documented and hence opportunities to modulate other potential risk factors are being lost. The aims of this study were to explore the aetiological spectrum of CLD in eastern Ethiopia and to identify plausible underlying risk factors for its development.

Methods: A cross-sectional study was undertaken between April 2015 and April 2016 in two public hospitals in Harar, eastern Ethiopia. The study population comprised of consenting adults with clinical and radiological evidence of chronic liver disease. The baseline evaluation included: (i) a semi-structured interview designed to obtain information about the ingestion of alcohol, herbal medicines and local recreational drugs such as khat (*Catha edulis*); (ii) clinical examination; (iii) extensive laboratory testing; and, (iv) abdominal ultrasonography.

Results: One-hundred-and-fifty patients with CLD (men 72.0%; median age 30 [interquartile range 25–40] years) were included. CLD was attributed to chronic HBV infection in 55 (36.7%) individuals; other aetiological agents were identified in a further 12 (8.0%). No aetiological factors were identified in the remaining 83 (55.3%) patients. The overall prevalence of daily khat use was 78.0%, while alcohol abuse, defined as > 20 g/day in women and > 30 g/day in men, was rare (2.0%). Histological features of toxic liver injury were observed in a subset of patients with unexplained liver injury who underwent liver biopsy.

Conclusion: The aetiology of CLD in eastern Ethiopia is largely unexplained. The widespread use of khat in the region, together with histopathological findings indicating toxic liver injury, suggests an association which warrants further investigation.

Keywords: Hepatotoxicity, Epidemiology, *Catha edulis*, Viral hepatitis, Sub-Saharan Africa

Background

'Chronic liver disease' (CLD) is the term used to describe disordered liver function lasting for six or more months. It results from a process of progressive destruction and regeneration of the liver parenchyma and encompasses a wide range of liver pathologies including: chronic hepatitis, cirrhosis and hepatocellular carcinoma. CLD is a major cause of morbidity and mortality, and was responsible for an estimated 1.3 million deaths worldwide in 2015 [1]. The commonest causes of CLD are chronic infection with hepatitis B (HBV) or C (HCV), alcohol misuse and non-alcoholic fatty liver disease (NAFLD) [2].

Ethiopia is a low-income country in East Africa with a population of nearly 100 million [3]. The prevalence of CLD in Ethiopia is largely unknown but is assumed to be high [4]. The estimated seroprevalence of hepatitis B surface antigen (HBsAg) in Ethiopia is 6.0% [5] and of HCV-antibody (anti-HCV) 3.1% [6]. Although these data are extracted predominantly from institution-based studies and may not be representative of the situation nationwide, chronic HBV infection is thought to be a major cause of CLD in this region [4].

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Community-based, longitudinal studies have been undertaken in several rural areas of Ethiopia, in recent years, using a verbal autopsy method to assign causes of death [7–9]. CLD was the leading cause of death in the age group 15–49 years in Kersa in eastern Ethiopia (13.7%) [9] and in Butajira in central Ethiopia (11.3%) [7]. In contrast, CLD was the cause of death in only 3.5% of adults of the same age in Kilte Awlalo in northern Ethiopia [8]. One suggested explanation for this difference is the relative availability of khat (*Catha edulis*), an indigenous plant which is chewed for its psychotropic effects. Khat chewing has been associated with the development of CLD [10]; its use is widespread in eastern [11] and south-central Ethiopia [12] but much less so in northern parts of the country [13].

One of the most important aspects of CLD prevention is the identification and management of potential risk factors. Public health efforts to reduce the toll of CLD in Ethiopia and other countries in sub-Saharan Africa will be considerably hampered if information on avoidable or treatable risk factors is unavailable. Thus, the aims of this study were to explore the aetiological spectrum of CLD in eastern Ethiopia and to identify plausible underlying risk factors for CLD using a hospital-based cross-sectional design.

Methods

Study setting and participants

A cross-sectional study of indigenous adults, aged ≥18 years, presenting for the first time with features of CLD was undertaken in two governmental hospitals in Harar, eastern Ethiopia between April 2015 and April 2016. CLD was defined as: (i) the presence of clinical features suggestive of decompensated liver disease viz. ascites, jaundice and/or hepatic encephalopathy; and (ii) the presence, on ultrasound, of hepatic parenchyma heterogeneity and/or surface irregularity. Patients presenting with severe acute hepatitis defined as liver injury of < 6 weeks duration, serum alanine aminotransferase (ALT) activity of > 100 U/L and the absence of coarsened echotexture and surface irregularity on ultrasonography, were excluded. Also excluded were patients with liver dysfunction secondary to comorbidities viz. congestive cardiac failure, biliary obstruction and septicaemia. Patients who had previously diagnosed CLD were excluded since they might represent a subgroup with more severe liver disease, or might have altered their risk habits in response to previous medical advice.

Patient assessment

Suitable patients presenting to the regional Hiwot Fana Specialized University Hospital, and the local Jugal Hospital underwent a semi-structured interview by local nurses fluent in their mother tongue. Demographic data were recorded and potential risk factors for CLD were explored. Information on past and current use of alcohol was obtained and quantified in grams. Daily alcohol consumption of > 20 g in women and > 30 g in men, for a minimum period of 6 months, was classified as alcohol misuse. Information on khat usage was obtained using a visual analogue scale and quantified in grams. The frequency and duration of khat use in years was used to classify lifetime khat exposure as *khat-years*. Approximately 100–300 g of fresh khat leaves are chewed in a typical session [14]; thus, one khat-year was defined as daily use of 200 g of fresh khat for 1 year.

Clinical examination was undertaken using a prespecified proforma.

Laboratory tests

Blood was collected by venous puncture for immediate processing; serum and plasma were separated and stored in aliquots at − 20 °C until transported on ice/dry ice for analysis either in Ethiopia or Norway. Full blood counts were performed using a KX-21 N™ haematology analyser (Sysmex, Kobe, Japan). Standard biochemical tests were analysed using a semi-automatic biochemistry analyser DR-7000D (DIRUI, Changchun, China) and HumaLyzer 3000 (HUMAN, Wiesbaden, Germany). The serum aspartate aminotransferase (AST) to platelet ratio index (APRI) was calculated as $\frac{\frac{\text{AST (U/L)}}{\text{upper reference range of AST (U/L)}}}{\text{platelet count } (10^9/\text{L})} \times 100$ [15], using a threshold of 0.7 as indicator of significant fibrosis [16]. The Fibrosis-4 (FIB-4) score was calculated as $\frac{\text{age (years) x AST (U/L)}}{\text{platelet count } (10^9/\text{L}) \text{ x } \sqrt{\text{ALT (U/L)}}}$, using a threshold of 3.25 to indicate advanced fibrosis/cirrhosis [17].

HBsAg was measured using the rapid diagnostic test (RDT) Determine™ (Alere, Waltham, MA, USA); anti-HCV was measured using the SD BIOLINE HCV RDT (Standard Diagnostics, Yongin-si, Republic of Korea). Confirmatory testing of HBsAg and anti-HCV was undertaken using enzyme-linked immunosorbent assays (Elisys Uno, HUMAN, Wiesbaden, Germany; or Architect, Abbott Diagnostics, IL, USA). HBV DNA and HCV RNA were measured in patients who tested positive for HBsAg or anti-HCV by polymerase chain reaction using RealTime HBV, m2000 system (Abbott Molecular, Abbott Park, IL, USA). Plasma was analysed for hepatitis D virus (HDV) antigen and HDV-antibody using the ETI-DELTAK-2 and ETI-AB-DELTAK-2 assay (DiaSorin, Turin, Italy), respectively.

Human immunodeficiency virus (HIV) screening was performed using the KHB HIV (1 + 2) Antibody RDT (Shanghai Kehua Bio-Engineering, Shanghai, China) and confirmed using the HIV 1/2 STAT-PAK® RDT (Chembio Diagnostics, Medford, NY, USA). Malaria screening was performed using the SD BIOLINE Malaria Ag P.f/

P.v RDT (Standard Diagnostics) and confirmed by microscopy of blood smears.

Serum was analysed for immunoglobulin G using the IMMAGE® 800 Immunochemistry System (Beckman Coulter, Brea, CA, USA). Serum iron and transferrin concentrations were quantified using ARCHITECT ci16200 (Abbott Diagnostics). Total iron binding capacity (TIBC) was calculated as $25.1 \times$ serum transferrin (g/L) and transferrin saturation as $\frac{\text{serum iron } (\mu\text{mol/L})}{\text{TIBC } (\mu\text{mol/L})}\ 100\%$.

Anti-nuclear, anti-mitochondrial and anti-actin antibodies were analysed by the Phadia™250 Laboratory system (Thermo Fisher Scientific, Waltham, MA, USA) using the EliA™ Symphony assay (Phadia, Freiburg, Germany), QUANTA Lite® M2 EP (MIT3) and QUANTA Lite® Actin IgG (Inova Diagnostics, San Diego, CA, USA).

A stool sample was collected and five thick smears processed according to a modified Kato-Katz technique using 41.7-mg templates for detection of the ova of *Schistosoma mansoni* [18].

Patients who, after initial screening, appeared to have unexplained CLD underwent more extensive testing including: measurement of serum alpha-1-antitrypsin and caeruloplasmin concentrations using the IMMAGE® 800 Immunochemistry System (Beckman Coulter); high iron Fe (*HFE*) genotyping, if the serum transferrin saturation was increased above 50%, without obvious explanation; and, screening for visceral leishmaniasis using a recombinant K39-antigen strip test IT-LEISH® (Bio-Rad) and confirmed by Giemsa stained splenic smear.

Urine from all women < 45 years of age was tested for human chorionic gonadotropin (hCG) using a HCG Pregnancy Strip Test (Nantong Egens Biotechnology, Jiangsu, China).

Abdominal imaging

Abdominal ultrasonography was undertaken to a predetermined standard by a local radiologist using a 3.5 MHz convex transducer Flexus SSD-1100 (Aloka, Tokyo, Japan). The diagnosis of CLD was based on the presence of an irregular liver surface and/or liver parenchyma heterogeneity [19]. The presence of schistosomal periportal fibrosis was diagnosed using WHO criteria [20] and re-evaluated by an independent expert.

Determination of the aetiology of the CLD

Historical, clinical, laboratory and imaging data were used to identify the aetiology of the underlying CLD using published criteria (Table 1) [21–25].

Liver biopsy and histopathology

It was intended that all patients in whom the aetiology of the CLD remained unexplained following investigation would be offered a liver biopsy. However, during the period April 2015 to April 2016, no suitably trained personnel were available to undertake this procedure. This situation was eventually resolved and the patients were subsequently contacted and asked to return for liver biopsy. In the interim several of the more decompensated patients had died and as the biopsies were to be performed percutaneously, only those with a normal or marginally elevated prothrombin time were considered suitable [26].

The procedure was performed, under ultrasound guidance, using a sterile Menghini technique with local anaesthetic and a 17G needle Hepafix® (Braun, Melsungen, Germany). Serial four μm sections were cut and stained with haematoxylin and eosin; Gomori (reticulin); van Gieson (collagen); Masson Trichrome (metachromatic); periodic acid-Schiff (PAS), with and without diastase (glycogen); and Perls (iron). Histopathologists in Norway and London independently assessed the histological findings blinded to the clinical information; inflammation and fibrosis were graded and staged using the semi-quantitative, modified Histological Activity Index [27]. Subsequent immunohistochemistry was undertaken using Ki-67 as a proliferation marker (Dako, catalogue number M724, concentration 1/100 with pre-treatment) and activated caspase-3, (Cell Signalling Technology, catalogue number 9664, concentration 1/100 with pre-treatment) as an apoptotic marker. Image analysis to quantify the degree of fibrosis and to calculate the collagen proportionate area (CPA) was carried out on scanned, Sirius Red stained sections [28].

Statistical methods

Statistical analyses were performed in SPSS 23.0 (SPSS Inc., Chicago, IL, USA). Categorical variables were summarized as frequencies, while continuous variables were presented as median and interquartile range (IQR). Comparisons between groups were performed using the Pearson χ^2-test for categorical variables and Mann-Whitney U-test for continuous variables. A p-value < 0.05 was considered significant. The *Strengthening the Reporting of Observational studies in Epidemiology* (STROBE) statement guidelines were followed [29].

Ethics

The study was approved by the National Research Ethics Review Committee (Ref. No.: 3.10/829/07 and 3.10/129/2016) in Ethiopia and by the Regional Committees for Medical and Health Research Ethics (Ref. No.: 2014/1146) in Norway. The study was conducted in accordance with the Declaration of Helsinki [30]. Written informed consent was obtained from all participating individuals.

Table 1 Criteria used to assign the aetiology of the liver disease

Aetiology		Criteria used to assign diagnosis
1	Chronic hepatitis B infection	Evidence of CLD on liver ultrasound and positive serum HBsAg.
2	Chronic hepatitis C infection	Evidence of CLD on liver ultrasound and positive serum anti-HCV and positive HCV RNA.
3	Chronic hepatitis D infection	Chronic hepatitis B infection and positive serum anti HDV IgG confirmed by detection of HDV RNA.
4	Primary biliary cholangitis	i. Strongly positive anti-mitochondrial antibodies and ii. Cholestatic liver function tests: a. ALP > 1.5 x URR and b. AST < 5 x URR
5	Autoimmune hepatitis[a]	i. Strongly positive anti-nuclear antibodies or anti-actin and ii. Elevated IgG > 1.1 x URR
6	Alcoholic liver disease	i. Clinical and radiological signs of CLD and ii. Daily alcohol consumption > 20 g/day in women and > 30 g/day in men for 6 months or more.
7	Non-alcoholic fatty liver disease	i. Liver ultrasound findings of steatosis and ii. Absence of significant alcohol consumption[b] or other recognised secondary causes of steatosis and iii. BMI > 25 kg/m^2 [c]
8	Haemochromatosis	i. Transferrin saturation > 50% and ii. Genotyping showing C282Y homozygosity or C282Y/H63D heterozygosity or C282Y/S65C heterozygosity on the HFE gene.
9	Wilson's disease	i. Serum caeruloplasmin < 0.140 g/L and ii. Age < 40 years
10	Alpha-1-antitrypsin deficiency	Serum alpha-1-antitrypsin level < 0.85 g/L.
11	Malaria	Positive malaria rapid diagnostic test and positive microscopy.
12	Hepatic schistosomiasis	Presence of ova from *Schistosoma mansoni* in Kato-Katz thick stool smears and typical liver ultrasound findings viz. periportal thickening/'pipestem' fibrosis confirmed by an independent expert.
13	Visceral leishmaniasis	Ultrasound findings of hepatosplenomegaly and positive K39 antigen strip test confirmed by positive splenic smear.
14	Unexplained chronic liver disease	None of the above

Abbreviations: *ALP* alkaline phosphatase, *anti-HCV* hepatitis C virus antibody, *anti-HDV* hepatitis D virus antibody, *AST* aspartate aminotransferase, *BMI* body mass index, *CLD* chronic liver disease, *HBsAg* hepatitis B surface antigen, *HCV* hepatitis C virus, *HDV* hepatitis D virus, *HFE* high iron Fe, *IgG* immunoglobulin G, *URR* upper reference range

Laboratory reference ranges: ALP (60–306 U/L); AST (14–40 U/L); IgG (0.8–27.8 g/L) [21]

[a]Based on the American Association for the Study of Liver Disease (AASLD) simplified criteria [22] in the absence of histology

[b]Alcohol consumption < 20 g/day in women and < 30 g/day in men

[c]Not a part of the AASLD criteria [23] but adopted to exclude cases of starvation-induced steatosis

Results

Study population

A total of 244 patients with liver disease were admitted to hospital during the study period. Of these, 212 patients presented with a new diagnosis of probable CLD and were evaluated for inclusion. The final study population comprised of 150 cases with newly diagnosed CLD (Fig. 1).

Aetiological spectrum

The aetiology of the liver disease was identified in 67 (44.7%) of the 150 patients and ascribed to chronic HBV infection in 55 (36.7%); hepatic schistosomiasis in four (2.7%); alcohol misuse in three (2.0%); chronic HCV infection in two (1.3%); autoimmune hepatitis in two (1.3%) and visceral leishmaniasis in one (0.7%). No cause was identified in the remaining 83 (55.3%) patients, in whom the liver disease was, therefore, unexplained.

Demography

Overall, there were more men (72.0%) than women; the median age was 30 (IQR 25–40) years (Table 2). The majority of the study subjects were Muslim (92.7%). The overall reported prevalence of daily khat use was 78.0%. Khat use was more common among men than women (92.6% vs. 40.5%; $p < 0.001$); overall khat exposure was also higher in men than women (36 vs. 0.6 khat-years; $p < 0.001$).

Women were more likely to have unexplained CLD than men (71.4% vs. 49.1%; $p = 0.013$). Otherwise there were no significant differences in demographic features between the aetiology known/unknown groups.

Clinical presentation

The majority of the patients presented with clinical features suggestive of hepatic decompensation (Table 3). Patients with unexplained CLD were more likely to

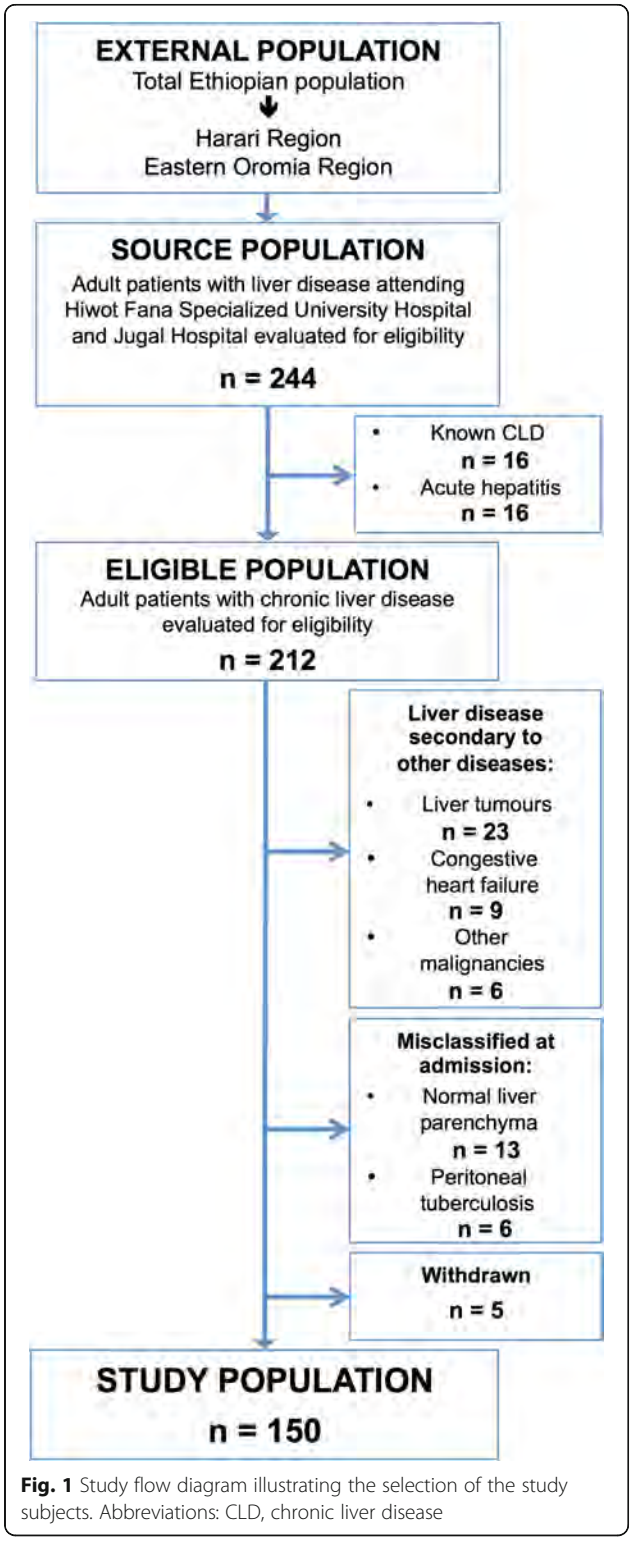

Fig. 1 Study flow diagram illustrating the selection of the study subjects. Abbreviations: CLD, chronic liver disease

present with abdominal swelling than those in whom the aetiology was known (92.8% vs. 76.1%; $p = 0.004$). Otherwise there were no distinguishing clinical features between the aetiology known/unknown groups.

Table 2 Demographic features of the study subjects with chronic liver disease, by aetiology

Variable	All patients ($n = 150$)	Aetiology known ($n = 67$)	Aetiology unknown ($n = 83$)
Sex (n, % men)	108 (72.0)	55 (82.1)	53 (63.9)*
Age (years)	30 (25–40)	30 (20–40)	30 (25–40)
Ethnic group			
Oromo	134 (89.3)	59 (88.1)	75 (90.4)
Amhara	9 (6.0)	5 (7.5)	4 (4.8)
Somali	5 (3.3)	2 (3.0)	3 (3.6)
Gurage	2 (1.3)	1 (1.5)	1 (1.2)
Religion			
Islam	139 (92.7)	60 (89.6)	79 (95.2)
Christianity	11 (7.3)	7 (10.4)	4 (4.8)
Occupation			
Farmer	100 (66.7)	46 (68.7)	54 (65.1)
Unemployed	14 (9.3)	5 (7.5)	9 (10.8)
Housewife	11 (7.3)	2 (3.0)	9 (10.8)
Student	8 (5.3)	5 (7.5)	3 (3.6)
Day worker	5 (3.3)	3 (4.5)	2 (2.4)
Public servant	4 (2.7)	1 (1.5)	3 (3.6)
Health professional	2 (1.3)	2 (3.0)	0
Other	6 (4.0)	3 (4.5)	3 (3.6)
Pregnant	3 (2.0)	1 (8.3)	2 (6.7)
Previous blood transfusion	23 (15.3)	9 (13.4)	14 (16.9)
Family history of liver disease	8 (5.3)	4 (6.0)	4 (4.8)
Dietary grain stored underground	53 (35.3)	25 (37.3)	28 (33.7)
Weeks of storage	24 (12–52)	24 (12–52)	24 (12–52)
Traditional herbal medicine	40 (26.7)	16 (23.9)	24 (28.9)
History of alcohol consumption:			
Never	139 (92.7)	61 (91.0)	78 (94.0)
Current	6 (4.0)	5 (7.5)	1 (1.2)
Stopped	5 (3.3)	1 (1.5)	4 (4.8)
Alcohol abuse[a]	3 (2.0)	3 (4.5)	0
History of daily use of khat	117 (78.0)	56 (83.6)	61 (73.5)
Khat-years[b]	20 (3–70)	20 (3–75)	18 (1–60)

Data are presented as number (%) or as median (interquartile range) unless otherwise noted

*$p < 0.05$; significance of the difference between the aetiology known/unknown group

[a]Daily consumption of > 20 g/day in women and > 30 g/day in men for 6 months or more

[b]One khat-year is defined as daily use of 200 g fresh khat for 1 year

Laboratory findings

Overall, the alterations in laboratory variables were mild (Table 4). A total of 92 (61.7%) patients had an APRI score of > 0.7 while 43 (28.9%) had a FIB-4 score of > 3.25. Patients with unexplained CLD had a lower median serum ALT activity (30 U/L [IQR 21–51] vs. 41 [IQR

Table 3 Clinical characteristics and ultrasound findings in the study subjects with chronic liver disease, by aetiology

Variable	All patients ($n = 150$)	Aetiology known ($n = 67$)	Aetiology unknown ($n = 83$)
Symptoms			
Abdominal swelling	128 (85.3)	51 (76.1)	77 (92.8)*
Epigastric pain	12 (80.0)	56 (83.6)	64 (77.1)
Weight loss	119 (79.3)	54 (80.6)	65 (78.3)
Fever	77 (51.3)	35 (52.2)	42 (50.6)
Arthralgia/myalgia	75 (50.3)[a]	33 (49.3)	42 (51.2)[a]
Nausea	69 (46.3)[a]	30 (45.5)[a]	39 (47.0)
Diarrhoea	64 (42.7)	27 (40.3)	37 (44.6)
Haematemesis	53 (35.3)	27 (40.3)	26 (31.3)
History of jaundice	47 (31.3)	24 (35.8)	23 (27.7)
Clinical findings			
Ascites	138 (92.0)	60 (89.6)	78 (94.0)
Splenomegaly	99 (66.0)	48 (71.6)	51 (61.4)
Jaundice	28 (18.7)	16 (23.9)	12 (14.5)
Caput medusae	25 (16.7)	8 (11.9)	17 (20.5)
Hepatic encephalopathy	16 (10.7)	7 (10.4)	9 (10.8)
Traditional scarring/burning	101 (67.3)	46 (68.7)	55 (66.3)
Ultrasound findings			
Ascites	138 (92.0)	60 (89.6)	78 (94.0)
Smooth liver surface	4 (2.7)	3 (4.5)	1 (1.2)
Mild uneven liver surface	44 (29.3)	15 (22.4)	29 (34.9)
Nodular liver surface	102 (68.0)	49 (73.1)	53 (63.9)
Heterogeneous echotexture	62 (41.3)	22 (32.8)	40 (48.2)
Coarse echotexture	87 (58.0)	44 (65.7)	43 (51.8)
Hepatic steatosis	1 (0.7)	1 (1.5)	0
Periportal fibrosis	21 (14.0)	9 (13.4)	12 (14.5)
In-hospital death	9 (6.0)[a]	4 (6.0)	5 (6.1)[a]

Data are presented as number (%) or as median (interquartile range) unless otherwise noted
[a]One observation missing
*$p < 0.05$; significance of the difference between the aetiology known/unknown group

24–58]; $p = 0.032$) and values above the upper reference range (URR) were observed in proportionately fewer patients than amongst those in whom the aetiology was known (31.3% vs. 50.7%; $p = 0.016$). Otherwise there were no distinguishing laboratory features between the aetiology known/unknown groups.

Abdominal ultrasound findings

The commonest findings on liver ultrasound were an irregular/nodular liver surface (68.0%), coarse liver texture (58.0%) and ascites (92.0%) (Table 3). There were no significant differences in abdominal ultrasound findings between the aetiology known/unknown groups.

Table 4 Laboratory findings in the study subjects with chronic liver disease, by aetiology

Laboratory variable	All patients ($n = 150$)	Aetiology known ($n = 67$)	Aetiology unknown ($n = 83$)
ALT (U/L)	34 (22–55)	41 (24–58)	30 (21–51)*
> URR	60 (40.0)	34 (50.7)	26 (31.3)*
AST (U/L)	44 (28–81)	52 (31–83)	41 (28–78)
> URR	84 (56.0)	41 (61.2)	43 (51.8)
ALP (U/L)	317 (207–416)	315 (250–423)	320 (200–385)
> URR	80 (53.3)	37 (55.2)	43 (51.8)
GGT (U/L)	29 (19–48)	29 (21–47)	29 (18–52)
> URR	30 (20.0)	14 (20.9)	16 (19.3)
Total bilirubin (μmol/L)	19 (10–38)	21 (12–51)	17 (10–31)
> URR	36 (24.0)	20 (29.9)	16 (19.3)
Albumin (g/L)	37 (28–50)	36 (30–50)	37 (27–50)
< LRR	63 (42.0)	27 (40.3)	36 (43.4)
Creatinine (μmol/L)	80 (62–97)	80 (62–97)	71 (62–88)
> URR	23 (15.3)	11 (16.4)	12 (14.5)
< LRR	19 (12.7)	8 (11.9)	11 (13.3)
Platelet count (10^9/L)	125 (76–206)	123 (71–186)	147 (76–223)
< LRR	75 (50.0)	36 (53.7)	39 (47.0)
IgG (g/L)	23.9 (17.1–32.5)	27.0 (16.7–34.2)	21.6 (17.2–30.6)
> URR	55 (36.7)	31 (46.3)	24 (28.9)
HIV infection[a]	4 (2.7)	1 (1.5)	3 (3.6)
Kato-Katz smear positive	23 (16.5)[b]	13 (21.3)[c]	10 (12.8)[d]
APRI score > 0.7[e]	92 (61.7)[f]	46 (69.7)[f]	46 (55.4)
FIB-4 score > 3.25[g]	43 (28.9)[f]	20 (30.3)[f]	23 (27.7)
APRI score > 0.7 OR FIB-4 score > 3.25	94 (63.1)[f]	46 (69.7)[f]	48 (57.8)

Data are presented as number (%) or as median (interquartile range)
Laboratory reference ranges: ALT (8–40 U/L); AST (14–40 U/L); ALP (60–306 U/L); GGT (7–61 U/L); Bilirubin (3–38 μmol/L); Albumin (35–52 g/L); Creatinine (47–109 μmol/L); Platelet count (126–438 × 10^9/L); IgG (0.8–27.8 g/L) [21]
[a]One patient with chronic HCV was co-infected with HIV.
[b]Stool sample missing in 11 patients.
[c]Stool sample missing in six patients.
[d]Stool sample missing in five patients.
[e]APRI: (AST (U/L)/URR of AST (U/L))/platelet count (10^9/L) × 100
[f]One observation missing.
[g]FIB-4: age (years) x AST (U/L)/(platelet count (10^9/L) x √ALT (U/L))
*$p < 0.05$; significance of the difference between the aetiology known/unknown group
Abbreviations: *ALP* alkaline phosphatase, *ALT* alanine aminotransferase, *APRI* aspartate aminotransferase to platelets ratio index, *AST* aspartate aminotransferase, *GGT* gamma-glutamyltransferase, *HCV* hepatitis C virus, *HIV* human immunodeficiency virus, *IgG* immunoglobulin G, *LRR* lower reference range, *URR* upper reference range

Histopathology

Of the 83 patients with unexplained CLD, 15 (18.1%) died during or shortly after admission; 35 (42.2%) could not be contacted; four (4.8%) refused to undergo the procedure, while 24 (28.9%) were unsuitable because of a severe coagulopathy. Thus, only five (6.0%) patients

underwent liver biopsy a median (range) of 33 (20–64) weeks after their initial hospitalization (Table 5); three (Cases 1/2/4) had a history of khat chewing.

Microscopically none of the specimens showed more than mild fibrosis and inflammation (Table 6). Foci of pale stained swollen hepatocytes were identified in Cases 1–4, with no marked zonal distribution; these stained negative for PAS and were thus suggestive of toxic injury (Case 1; Fig. 2a-f). The fifth patient showed no evidence of adaptive parenchymal changes but mild mixed steatosis and focal single cell necrosis (Fig. 2g-h). Although the numbers were modest and the differences small, the proliferation index, apoptotic scores and the CPA tended to be higher among the three patients (Cases 1/2/4) who chewed khat compared to the two who did not (Cases 3/5).

Discussion

This study aimed to explore the aetiological spectrum and underlying risk factors for the development of CLD in eastern Ethiopia. Chronic HBV infection was the major identified risk factor, explaining the development of CLD in roughly one-third of the patients. However, an aetiological factor was identified in less than 10% of the remainder. Thus, in over half of the included cases the aetiology of the liver disease was unexplained.

Of prime importance in this study was the surety of the diagnosis of CLD. The criteria used were stringent and required not only that patients had clinical evidence of decompensated liver disease but also evidence of hepatic parenchyma heterogeneity and/or surface irregularity on ultrasound. The liver function test abnormalities were mild but this is not incompatible with the diagnosis of CLD. Over two-thirds of the patients had APRI and/or FIB-4 scores compatible with a diagnosis of significant fibrosis/cirrhosis. The histological findings in the five patients who underwent liver biopsy would seem at odds with a diagnosis of CLD; however, these patients fulfilled the inclusion criteria at presentation and biopsies undertaken after a considerable delay still showed evidence of ongoing disease. Thus, the fact that the patients included in this study had CLD can be accepted with a high degree of certainty.

The proportion of patients, in the present study, in whom the CLD was aetiologically unexplained is substantially higher than might be expected. In the 1980's more than 50% of cases of CLD worldwide did not have an ascribed cause compared with the current global estimate of approximately 5% [31–33]. Thus, the prevalence of unexplained CLD in this area of eastern Ethiopia is ten-fold higher than would be expected. No observational studies exploring the aetiological spectrum of CLD in eastern Ethiopia or in sub-Saharan Africa are available for comparison.

The seroprevalence of HBsAg in the present population was high while the seroprevalence of anti-HCV was low. There are no representative population-based prevalence studies on viral hepatitis in this part of Ethiopia. However, a recent study of blood donors in eastern Ethiopia found similar seroprevalence rates to those reported here [34].

No data are available on the prevalence of NAFLD in Ethiopia although it is known that Ethiopia has one of the lowest prevalence rates of obesity worldwide [35]. The data that are available from other populations suggest that the overall prevalence of NAFLD in sub-Saharan Africa is low [36]. In a case-control study undertaken in Nigeria, 16.7% of patients with type II diabetes mellitus were found to have NAFLD compared with only 1.2% of non-diabetic control subjects, suggesting that, in comparison with Caucasian, Indian and Asian populations, diabetes may be a more important risk factor for NAFLD in Africa than obesity [37]. None of the patients in the present study was obese; other than one case with alcoholic liver disease, none had significant steatosis on hepatic ultrasound and only one had diabetes. Thus, the prevalence of NAFLD in this study population is likely to be very low.

The prevalence of daily khat use identified in the present study was much higher than previously reported [11, 13]. A regional study in Harar city found that 20.9% of 1890 secondary school students chewed khat daily; the lifetime prevalence of khat chewing was 24.2% [11]. The 2011 Ethiopian Demographic and Health Survey identified an overall prevalence of khat chewing of 15.3%. However, there are significant regional variations in the prevalence from 53.2% in the Harari region in eastern Ethiopia to 1.1% in the Tigray region in northern Ethiopia [13]. Khat use is more widespread amongst Muslims than Christians and amongst men than in women [11, 13], which accords with the findings in the present study.

There are a number of case reports which implicate khat as a factor in the development of both acute [38] and chronic liver disease [39–41]. In addition, khat-related hepatotoxicity has been convincingly demonstrated in animal models [42]. The fact that khat use was similar amongst patients with and without other risk factors indicated that it may act as a sole or an adjuvant cause of liver injury. Although only a limited number of liver biopsies was undertaken, the histological findings of focal parenchymal changes mirror those observed in animal models [42] and are supportive of toxic liver injury. However, the design of this study does not allow a definitive conclusion to be made, and further studies to assess causality are needed.

This study had a number of strengths despite the resource limitations at the Ethiopian sites. First: the

Table 5 Characteristics of the five patients with unexplained chronic liver disease who underwent liver biopsy

No.	Sex	Age (yr)	Alcohol use	Khat use	Main symptoms and signs	Main ultrasound findings	ALT (U/L)	AST (U/L)	ALP (U/L)	Albumin (g/L)	Bilirubin (μmol/L)	APRI score[a]	FIB-4 score[b]	Biopsy delay (wk)
1	Female	26	Never	100 g/day, 10 yr	Abdominal swelling; abdominal pain; nausea; diarrhoea; fatigue Ascites	Heterogenous liver texture Mild uneven liver surface Gross ascites Moderate splenomegaly	15	16	88	50	9	0.20	0.53	20
2	Male	25	Never	200 g/day, 5 yr	Abdominal pain; Diarrhea; arthralgia / myalgia	Heterogenous liver texture Mild uneven liver surface Periportal fibrosis Moderate splenomegaly	15	19	107	46	19	0.74	1.92	33
3	Female	30	Never	Never	Abdominal swelling; abdominal pain Ascites; umbilical hernia; non-specific rash	Heterogenous liver texture Mild uneven liver surface Gross ascites Reduced liver span	43	44	258	20	5	0.68	1.25	64
4	Male	25	36 g/day × 1/ wk., 3 yr	400 g/day × 3/ wk., 5 yr	Abdominal pain; nausea; diarrhea; arthralgia/myalgia Splenomegaly	Heterogenous liver texture Mild uneven liver surface Splenomegaly	100	98	300	58	31	1.34	1.34	22
5	Female	25	Never	Never	Abdominal swelling; fatigue; peripheral oedema. Ascites	Heterogenous liver texture Mild uneven liver surface Gross ascites Reduced liver span	39	62	385	27	17	0.76	1.22	58

Abbreviations: *ALP* alkaline phosphatase, *ALT* alanine aminotransferase, *APRI* aspartate aminotransferase to platelets ratio index, *AST* aspartate aminotransferase, *URR* upper reference range
Laboratory reference ranges: ALT (8–40 U/L); AST (14–40 U/L); ALP (60–306 U/L); Albumin (35–52 g/L); Bilirubin (3–38 μmol/L) [21]
[a]APRI: (AST (U/L)/URR of AST (U/L))/platelet count (10^9/L) × 100
[b]FIB-4: age (years) x AST (U/L)/(platelet count (10^9/L) x √ALT (U/L))

Table 6 Histopathological findings of the five patients with unexplained chronic liver disease who underwent liver biopsy

No	Sex	Age (yr)	General microscopy	Parenchymal changes	Ishak-score
1	Female	26	Mild portal and lobular hepatitis with sinusoidal lymphocytosis. Variation of hepatic cord thickness. Normal bile ducts and intact liver plate.	Focal adaptive parenchymal changes with diffuse hepatocyte swelling/ clarification. No steatosis or haemosiderosis. Collagen proportionate area: 5% Proliferation index: 8% Apoptosis index: 3%	Fibrosis = 2 Necroinflammation = 2 • piecemeal 0 • lobular 1 • confluent 0 • portal 1
2	Male	25	Normal architecture with normal portal areas, no inflammation. Normal bile ducts and intact liver plate.	Adaptive parenchymal changes with diffuse hepatocyte swelling/ clarification. No steatosis or haemosiderosis. Collagen proportionate area: 6% Proliferation index: 12% Apoptosis index: 5%	Fibrosis = 1 Necroinflammation = 0
3	Female	30	Normal architecture with normal portal areas, no inflammation. Normal bile ducts and intact liver plate.	Mild adaptive parenchymal changes with diffuse hepatocyte swelling/clarification. No steatosis or haemosiderosis. Collagen proportionate area: 3% Proliferation index: 6% Apoptosis index: 1%	Fibrosis = 1 Necroinflammation = 1 • piecemeal 0 • lobular 1 • confluent 0 • portal 0
4	Male	25	Normal architecture with normal portal areas, no inflammation. Normal bile ducts and intact liver plate.	Adaptive parenchymal changes with diffuse hepatocyte swelling/clarification. No steatosis or haemosiderosis. Collagen proportionate area: 8% Proliferation index: 15% Apoptosis index: 6%	Fibrosis = 0 Necroinflammation = 0
5	Female	25	Normal architecture with normal portal areas. Normal bile ducts and intact liver plate	Mild mixed steatosis ≈ 20% Focal single cell necrosis, a few apoptotic hepatocytes, a few parenchymal granulocytes. No adaptive changes. Collagen proportionate area: 3% Proliferation index: 3% Apoptosis index: 2%	Fibrosis = 0 Necroinflammation = 1 • piecemeal 0 • lobular 1 • confluent 0 • portal 0

sample size was large and the prospective inclusion of study subjects provided consistent data sampling throughout the study period. Second: robust clinical, laboratory and ultrasound criteria were used to define CLD. Third: the aetiology of the liver injury was determined following a comprehensive, standardized clinical evaluation, multicentre laboratory testing using high-performance diagnostics, abdominal ultrasound with expert review, and, in a small number, histological examination of liver biopsy material.

The study also has its limitations. First: selection bias cannot be excluded, as an unknown proportion of patients with CLD may not have been seen by the recruiting medical services for a variety of practical, cultural and socioeconomic reasons. Second: liver biopsies were undertaken in only a small number of patients with unexplained CLD; the selection procedure for liver biopsy undoubtedly favoured those with the mildest disease and the time interval between presentation and the procedure was sufficiently long for there to have been some resolution of the liver disease. Nevertheless, the histological findings provided useful confirmatory evidence of toxic liver injury in some. Third: issue could be taken with the criteria used to diagnose schistosomal liver disease. Positive assignment required a positive stool smear and radiological evidence of periportal thickening/'pipe stem' fibrosis confirmed by expert opinion; thus, the diagnosis may have been underestimated. Fourth: HBV DNA levels were not measured in 95 HBsAg-negative patients and thus the presence of occult HBV could not be ruled out in this subgroup [43]. However, the pathogenetic mechanism of occult HBV infection is still not clear [44] and the role of occult HBV in unexplained CLD is still debated [33]. Approximately 95% of the patients with unexplained CLD in the present study had decompensated disease on presentation but only low-grade abnormalities in the liver transaminase activities. Thus, it is unlikely that occult HBV infection was the underlying cause of the unexplained CLD in this population. Finally: the diagnosis of CLD was not confirmed by advanced imaging, endoscopy or, in the majority, by histological examination of liver biopsy material. Furthermore, certain causes of CLD could not be ruled out due to resource limitations, including: primary sclerosing cholangitis, veno-occlusive disease/Budd-Chiari syndrome and injury from other hepatotoxins.

CLD has recently been reported as the leading cause of death in adults less than 50 years of age in eastern

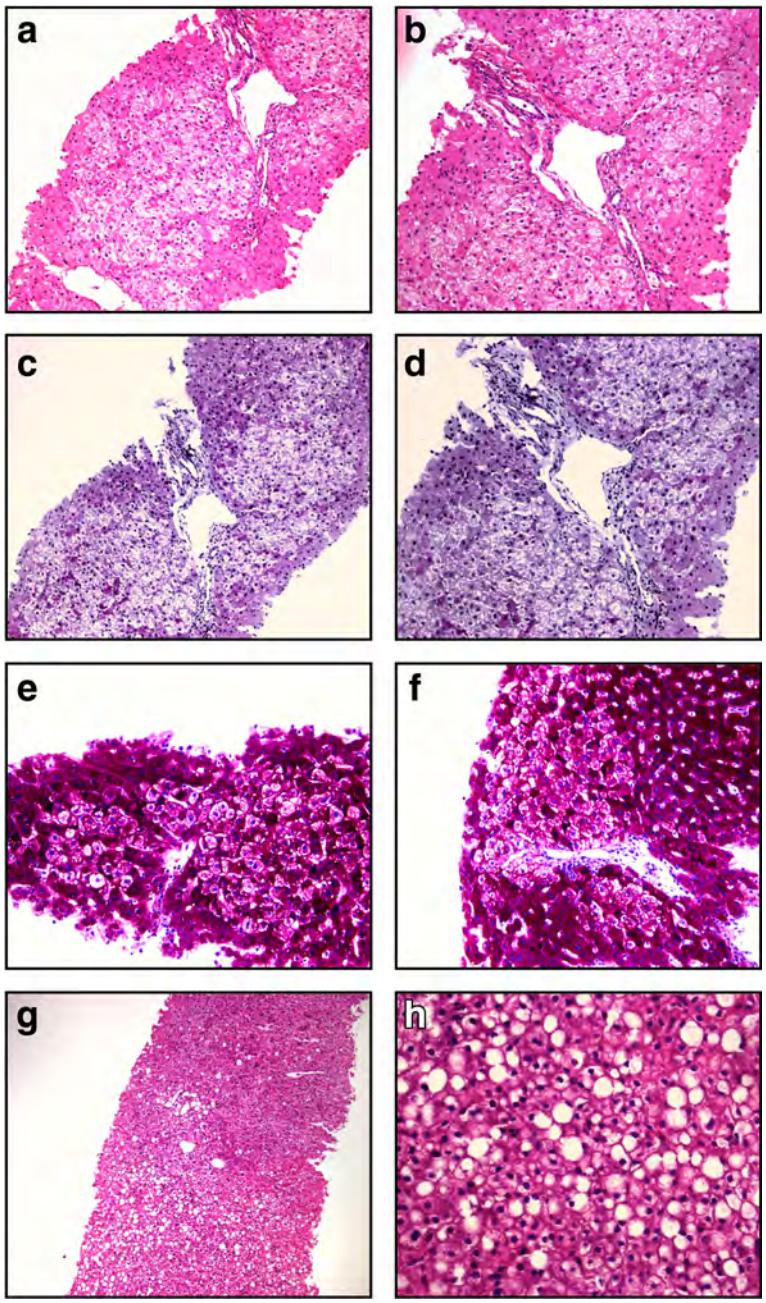

Fig. 2 Liver histology in Ethiopian patients with unexplained chronic liver disease. **a** Case 1: Adaptive parenchymal changes with focal diffuse swollen pale stained hepatocytes stretching through all zones. H&E, 100×. **b** Case 1: Intact liver plate, normal bile ducts and patent central vein. Sinusoidal lymphocytosis. H&E, 200×. **c** + **d** Case 1: Masson Trichrome stain negative indicated non-cirrhotic liver disease. 100×, 200×. **e** + **f** Case 1: Swollen hepatocyte clarification negative for periodic acid-Schiff stain in approximately 30%. 200×. **g** Case 5: Mild mixed steatosis. H&E, 100×. **h** Case 5: Mixed macro- and microvesicular steatosis with focal single cell necrosis and sporadic parenchymal granulocytes. H&E, 400×. Abbreviations: H&E, haematoxylin and eosin

Ethiopia [9]. If, as identified in the present study, a high proportion of the CLD is 'unexplained' then it may be difficult, if not impossible, to prevent its occurrence and hence to reduce the burden it imposes. If, however, as suggested in the present study, exposure to the recreational substance khat is of major aetiological importance, then there is an urgent need to further investigate this possibility with analytic studies designed to assess causality. There are campaigns in place to radically reduce the burden of viral liver disease worldwide [45], and this is undoubtedly vital. However, if khat was found to be a major contributor to the development of CLD, then given its widespread use,

legal status and social acceptability it would be a much more difficult problem to deal with requiring concerted governmental action in the countries and communities involved.

Conclusions

Chronic HBV infection was found in around one third of patients hospitalized with CLD in eastern Ethiopia. However, in over half of the patients the aetiology of the liver disease was unexplained. The prevalence of khat chewing was much higher in the CLD population than expected, suggesting khat as an effect modifier and/or independent risk factor for development of CLD in this part of the world. Further epidemiological studies, which include appropriate comparison groups, should be undertaken to assess whether khat plays a causal role in the development of CLD.

Abbreviations

ALT: Alanine aminotransferase; anti-HCV: Hepatitis C virus antibody; anti-HDV: Hepatitis D virus antibody; APRI: Aspartate aminotransferase to platelet ratio index; AST: Aspartate aminotransferase; CLD: Chronic liver disease; CPA: Collagen proportionate area; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; NAFLD: Non-alcoholic fatty liver disease

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AJ, NB and SGG conceived and designed the study with substantial contributions from NYI, TAA, TL and KSJ. SMSO, NYI and TAA were responsible for the inclusion of patients and data collection. TL and KSJ were responsible for the laboratory work, and BR and RDG for the pathological examinations. SMSO, AJ, MYM performed the statistical analysis. SMSO, AJ, MYM and AMDR drafted the first version of the manuscript, and all authors critically revised the manuscript and approved it.

Ethics approval and consent to participate

The study was approved by the Regional Committee for Medical and Health Research Ethics in Norway and the National Research Ethics Review Committee in Ethiopia, as well as the pertinent institutional ethical review boards. Written informed consent was obtained from all study participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Khat Chewing Increases the Risk for Developing Chronic Liver Disease: A Hospital-Based Case–Control Study

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The chewing of the leaves of *Catha edulis* (khat) has been implicated in the development of liver disease, but no controlled observations have been undertaken. The objective of the present study was to determine whether khat chewing is associated with development of chronic liver disease (CLD). A case–control study was conducted at two public hospitals in Harar, Ethiopia, between April 2015 and April 2016. A consecutive sample of 150 adult hospital attendees with CLD were included as cases, and 300 adult hospital attendees without clinical or laboratory evidence of CLD were included as controls. Khat consumption was quantified in "khat years"; 1 khat year was defined as daily use of 200 g of fresh khat for 1 year. A logistic regression model was used to control for confounders. There was a significant association between chewing khat and the risk for developing CLD (crude odds ratio, 2.64; 95% confidence interval [CI], 1.56-4.58). In men, this risk, following adjustment for age, alcohol use, and chronic hepatitis B/C infection, increased with increasing khat exposure; thus, compared to never users the adjusted odds ratios were for low khat exposure 3.58 (95% CI 1.05-12.21), moderate khat exposure 5.90 (95% CI 1.79-19.44), and high khat exposure 13.03 (95% CI 3.61-47.02). The findings were robust in a *post hoc* sensitivity analysis in which individuals with identifiable risk factors for CLD were excluded. *Conclusion:* A significant association was observed between chewing khat and the risk for developing CLD, and in men the association was strong and dose-dependent, suggesting a causal relationship; as the prevalence of khat chewing is increasing worldwide, these findings have major public health implications. (HEPATOLOGY 2018;68:248-257).

The leaves and shoots of the evergreen shrub *Catha edulis* (khat) are chewed to reduce fatigue, to increase performance, and for the pleasurable effects, which include euphoria, loquacity, and excitement. Khat chewing is common in the Horn of Africa, the Arabian Peninsula, and the East Coast of Africa, where it has been part of the social and cultural heritage for centuries.[1] Over the past three decades khat has become increasingly available worldwide and its use perpetuated and even adopted by the wider diaspora.[2] The global prevalence of khat chewing is unknown; the proposed figure of 20 million daily users is probably an underestimate.[3]

Khat leaves, typically 100-300 g, are chewed in sessions lasting several hours; and the juice of the masticated leaves is swallowed. Alkaloids, of which cathinone (aminopropiophenone) is the most important, are extracted during chewing; the buccal mucosa plays a major role in the absorption.[4] The state of alertness and euphoria associated with khat usage is most likely induced by cathinone, which exerts effects on the central nervous system similar to those of amphetamine.[4,5]

Abbreviations: ALT, alanine aminotransferase; AOR, adjusted odds ratio; AP, attributable proportion; CI, confidence interval; CLD, chronic liver disease; CYP, cytochrome P450; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; OR, odds ratio.

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The World Health Organization has defined khat as a drug of abuse as it may lead to health and social problems. Chronic khat use is associated with psychological adverse effects, including psychosis and exacerbation of preexisting psychotic disorders.[6] Khat users may also exhibit dependence; however in the majority of users the degree of associated physical dependence is low, although the levels of psychological dependence may be substantial.[5]

Khat chewing is also associated with a number of somatic health sequelae, including myocardial infarction, systemic hypertension, upper gastrointestinal cancers, cognitive impairment, and impaired fetal growth.[7] Khat has also been implicated in the development of both acute hepatitis[8-12] and chronic liver disease (CLD)[13-16] in several case series. Chapman et al. reported on 6 patients of Somali origin, living in the United Kingdom, in whom khat abuse was implicated in the development of fulminant hepatic failure.[12] Stuyt et al. reported unexplained CLD in 6 male immigrants from Somalia and Ethiopia living in The Netherlands and noted their history of chronic khat use.[14] However, because khat is illegal in Europe, North America, and Australia and the users are mainly from closed immigrant communities, no controlled observations are available.

Decompensated CLD is one of the most frequent reasons for admission to medical wards in eastern Ethiopia; in >50% of the cases the liver disease is "unexplained."[16] Khat chewing is widespread throughout the country but more especially in the eastern regions where khat cultivation predominantly takes place.[17,18] The number of people chewing khat has increased rapidly in recent years with the habit gaining popularity in all segments of the community; hence, the overall prevalence of khat chewing in Ethiopia is estimated at 15.3% but with wide regional variations.[18] Although khat chewing is traditionally a male habit, a recent community-based study in pregnant women in eastern Ethiopia reported a prevalence of khat usage of 34.6%.[19]

The aim of the present study was to determine the association between use of khat and the risk for developing significant CLD in eastern Ethiopia, using a case–control design exercising controls for potential confounding variables.

Participants and Methods

STUDY SETTING AND PARTICIPANTS

This prospective, hospital-based, case–control study was undertaken between April 2015 and April 2016 in Harar, eastern Ethiopia. Inpatients and outpatients attending two large public hospitals, the Jugal Hospital and the Hiwot Fana Specialized University Hospital, were eligible for inclusion. Medical subspecialty services are not available in either hospital; thus, all patients with CLD attend a general medical outpatient clinic and, if admitted, are housed on a general medical ward.

Cases comprised adult (≥18 years of age) outpatients and inpatients with a new diagnosis of CLD defined, for the purposes of this study, as (1) the presence of clinical features suggestive of decompensated liver disease (*viz.* ascites, jaundice, and/or hepatic encephalopathy) and (2) the presence, on ultrasound, of hepatic parenchyma heterogeneity and/or surface irregularity. Patients presenting with severe acute hepatitis, defined as liver injury of <6 weeks duration, serum alanine aminotransferase (ALT) activity of >100 U/L, and the absence of coarsened echotexture

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and surface irregularity on ultrasonography, were excluded. Also excluded were patients with a previously established diagnosis of CLD or with liver dysfunction secondary to alternative conditions (*viz.* congestive cardiac failure, hepatic malignancies, biliary obstruction, and septicemia).

As khat use is associated with a number of adverse medical and psychiatric sequelae, controls could not be selected from among the general medical or psychiatric populations. Controls therefore comprised adult hospital outpatients or inpatients under the care of the ophthalmology, dermatology, or surgical service, none of whom had a history or clinical evidence of CLD or a serum ALT above the upper limit of the laboratory reference range of 40 U/L.

PATIENT ASSESSMENT

All patients were assessed using standardized checklists and semistructured interviews to gather information on predetermined demographic and clinical variables. Interviews were conducted by local nurses fluent in the native language of study participants, and interviewers were blinded to the disease status of interviewees. Neither study subjects nor interviewers were told that the study hypothesis was of a likely association between khat chewing and the development of CLD.

Information on the use of khat was obtained and quantified in grams using a visual analogue scale. The frequency of khat chewing was categorized using the Drug Use Disorders Identification Test.[20] The frequency and duration of khat usage were used to classify lifetime khat exposure in "khat years," with 1 khat year defined by daily use of 200 g of fresh khat for 1 year. Exposed subjects were defined as all past or current khat users, regardless of the amount chewed. Those who had never used khat were defined as "never users."

Information on the frequency and quantity of previous and current alcohol use was obtained using a frequency/quantity questionnaire; average daily intake was quantified in grams using the following equation:

Alcohol concentration by volume $\times$ 0.78 $\times$ volume consumed (mL)/100

where alcohol concentration by volume is the percentage alcohol content of the local alcoholic beverages[21] and 0.78 is the specific gravity of alcohol. Alcohol abuse was defined as consumption of >20 g/day for women and >30 g/day for men.[22] Exposed subjects were defined as past or current users of alcohol, regardless of the amount consumed.

Clinical examination was conducted after the patient interview using a prespecified standard. Particular note was made of any features suggestive of CLD including cutaneous and peripheral liver stigmata and features of hepatic decompensation.

LABORATORY TESTS

Blood was collected by venipuncture, and the serum and plasma were separated within 2 hours and aliquots stored at −20°C until analyzed. Routine blood tests including serum ALT and aspartate aminotransferase activities were analyzed using a semiautomatic biochemistry analyzer, Dirui DR-7000D (DIRUI, Changchun, China) and HumaLyzer 3000 (HUMAN, Wiesbaden, Germany). Hepatitis B surface antigen (HBsAg) screening was undertaken locally using the World Health Organization–approved rapid diagnostic test Determine (Alere, Waltham, MA); hepatitis C virus (HCV) antibody screening was undertaken locally using the World Health Organization–approved rapid diagnostic test SD BIOLINE HCV (Standard Diagnostics, Yongin-si, Republic of Korea). All sera were subsequently transported on ice to Aklilu Lemma Institute of Pathobiology in Addis Ababa for confirmatory testing of HBsAg and HCV antibody using an enzyme-linked immunosorbent assay method (Elisys Uno, HUMAN; or Architect, Abbott Diagnostics, IL). Discrepancies between rapid tests and enzyme-linked immunosorbent assay results were resolved using a second enzyme-linked immunosorbent assay (Architect; or Bio-Rad, Hercules, CA).

ABDOMINAL IMAGING

Abdominal ultrasonography was undertaken, to a predetermined standard, by a local radiologist using a 3.5-MHz convex transducer (Aloka Flexus SSD-1100; Aloka, Tokyo, Japan). The diagnosis of CLD was based on the presence of an irregular liver surface and/or liver parenchyma heterogeneity.[23] Other features suggestive of the presence of CLD, such as ascites, splenomegaly, and a collateral circulation, were also noted and recorded.

SAMPLE SIZE CALCULATION

A sample size estimation was performed *a priori* based on the inclusion of 2 controls per case and the conventional type I error of 5% and power of 80%. Based on an estimated prevalence of daily khat use of

20%[24] and the assumption that khat use would be at least twice as common in cases as in controls (odds ratio [OR], 2.00), a minimum of 137 cases and 274 controls would be needed for an adequately powered study. Individuals with other risk factors for CLD were retained in the main analysis because it was assumed that khat chewing could act as either a sole or an adjuvant cause of liver injury.

STATISTICAL METHODS

Categorical variables were summarized as frequencies and continuous variables by the median and interquartile range. Comparisons between cases and controls were performed using the Mann-Whitney U test for continuous variables and the Pearson chi-squared test for categorical variables.

An explanatory strategy investigating the association between khat chewing and CLD was undertaken and quantified as OR with its 95% confidence interval (CI).[25] An initial stratified analysis evaluated effect modification (interaction) and confounding by other variables considered in the protocol. A Breslow and Day test of homogeneity between strata was performed to pinpoint effect modification (interaction). Confounding was controlled univariately using the Mantel-Haenszel method. The magnitude of the confounding effect was evaluated by comparing the crude OR and the adjusted Mantel-Haenszel OR. When cells contained zero counts, a value of 0.5 was added to each cell frequency before calculating the stratum-specific OR and the Mantel-Haenszel summary OR. A logistic regression model was used to control for multiple confounders and the presence of effect modification.[25] The relationship between levels of khat exposure and the risk for developing CLD was explored by (1) using a chi-squared test for trend with khat exposure categorized by khat year quartiles and (2) using the per unit increase in khat years as a continuous variable in the logistic regression model.

The attributable proportion (AP) was estimated as:

$$AP = \frac{Pe(\mathrm{OR}-1)}{Pe(\mathrm{OR}-1)+1}$$

where *P*e is the prevalence of khat exposure in the target population.[26]

A *post hoc* sensitivity analysis was performed excluding all cases of CLD with an identifiable etiology after a comprehensive panel of tests, which included parasitology and serological testing for viral, autoimmune, and genetic liver diseases.[16] Controls did not undergo the same comprehensive panel of tests or abdominal ultrasound, but all had serum ALT activities <40 U/L and were screened for the risk factors assumed *a priori* to be the most relevant (*viz.* alcohol abuse and viral hepatitis); those with a history of alcohol abuse or evidence of chronic hepatitis B and C infection were excluded from the sensitivity analysis.

Statistical analyses were performed in STATA 14.0 (StataCorp, College Station, TX). The study was compliant with the Strengthening the Reporting of Observational Studies in Epidemiology statement guidelines.[27]

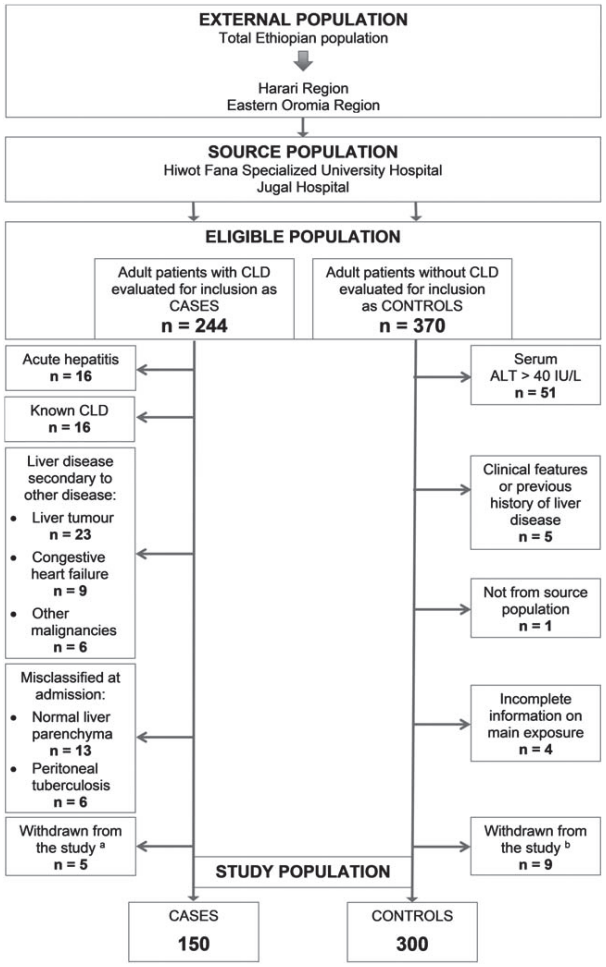

FIG. 1. Selection of study subjects. [a]Two patients refused blood sampling and withdrew their consent; 3 patients deteriorated rapidly and were withdrawn from the study for compassionate reasons. [b]Nine patients refused blood sampling and withdrew their consent.

TABLE 1. Characteristics of Cases and Controls Included in the Study

Characteristic	Cases (n = 150)	Controls (n = 300)	*P*
Men	108 (72.0)	172 (57.3)	0.002
Age (years)	30 (25-40)	30 (24-52)	0.125
Religion			<0.001
Islam	139 (92.7)	198 (66.0)	
Christianity	11 (7.3)	102 (34.0)	
History of alcohol consumption			<0.001
Never	139 (92.7)	233 (77.7)	
Current	6 (4.0)	52 (17.3)	
Stopped	5 (3.3)	15 (5.0)	
Alcohol abuse*	3 (2.0)	9 (3.0)	0.758
History of khat use	127 (84.7)	203 (67.7)	<0.001
Khat years†	20 (3-70)	2 (0-20)	<0.001
Men	36 (6-75)	10 (0-40)	
Women	0.6 (0-7)	0.1 (0-5)	
HBsAg-positive	55 (36.7)	22 (7.3)	<0.001
HCV antibody–positive	2 (1.3)	0 (0.0)	0.111

Data are presented as number (%) or as median (interquartile range).
*Defined as consumption of >20 g alcohol/day in women and >30 g/day in men.
†One khat year is defined by daily use of 200 g of fresh khat for 1 year.

ETHICS

The study was conducted in accordance with the Declaration of Helsinki[28] and approved by the National Research Ethics Review Committee (ref. no. 3.10/829/07) in Ethiopia and by the Regional Committees for Medical and Health Research Ethics (ref. no. 2014/1146) in Norway. Written informed consent was obtained from all study subjects.

Results

CHARACTERISTICS OF THE STUDY POPULATION

In total 244 potential cases and 370 controls were screened for inclusion. The final study population comprised 150 cases with CLD and 300 controls without liver disease (Fig. 1). Of these, 31 (20.7%) cases and 265 (88.3%) controls were outpatients, while 119 (79.3%) cases and 35 (11.7%) controls were inpatients. Cases were significantly more likely than controls to be male, Muslim, nondrinkers of alcohol, and HBsAg-positive and to use khat (Table 1).

Men were more likely than women to be HBsAg-positive, among both cases (41.7% versus 23.8%; $P = 0.042$) and controls (9.9% versus 3.9%; $P = 0.049$). Moreover, the proportion reporting current or previous khat use was significantly higher among men than women (cases, 96.3% versus 54.8%; $P < 0.001$; controls, 77.9% versus 53.9%; $P < 0.001$). The median level of khat exposure was also significantly higher in men compared to women (Table 1).

ASSOCIATION BETWEEN KHAT EXPOSURE AND CLD

In univariable analysis, there was a significant association between chewing khat and the risk for developing CLD (crude OR, 2.64; 95% CI, 1.56-4.58; $P < 0.001$). The magnitude of the risk estimate was different in men and women (Table 2). Chronic hepatitis B virus infection had a considerable confounding effect on the risk estimate (18%), whereas the effects of age (2%) and alcohol consumption (3%) were minimal. The variable HCV infection contained too few positive results to be included in both stratified and multivariable analyses.

The final multivariable analysis showed that the effect of khat on the risk for developing CLD was dependent on its interaction with sex when adjusting for age, alcohol use, and chronic hepatitis B virus infection (interaction khat × sex; $P = 0.013$). The effect was strong in men (adjusted odds ratio [AOR], 5.67; 95% CI, 1.85-17.37; $P = 0.002$) but not evident in women (AOR, 1.04; 95% CI, 0.49-2.19; $P = 0.922$).

An upward trend in the risk for developing CLD with increasing khat exposure was found after adjusting for hepatitis B virus infection, alcohol use, and age; this was indicative of a dose–response relationship in men but not in women (Table 3). When khat exposure was employed as a continuous variable in the logistic regression model, the findings pointed in the same direction; per 1 khat year increment, the odds of CLD was significantly increased in men (AOR, 1.007; 95% CI, 1.001-1.013; $P = 0.019$) but not in women (AOR, 1.012; 95% CI, 0.998-1.027; $P = 0.102$).

PROPORTION OF CLD ATTRIBUTABLE TO KHAT EXPOSURE

Assuming that the relationship between khat exposure and the risk for developing CLD is causal, more than half of the CLD cases in this study population were attributable to khat usage (AP, 52.6%; 95% CI, 33.1-72.0). This effect showed a marked difference in relation to sex; in men, the AP was 83.2% (95% CI,

TABLE 2. Association Between Khat and the Risk of Developing CLD Controlling for Potential Confounders

Characteristic	Cases (n = 150) Khat exposure Yes	Cases (n = 150) Khat exposure No	Controls (n = 300) Khat exposure Yes	Controls (n = 300) Khat exposure No	OR (95% CI)	OR_{MH} (95% CI)	Breslow and Day test of homogeneity (*P*)
Crude (n)	127	23	203	97	2.64 (1.56-4.58)		
Sex							
Men	104	4	134	38	7.37 (2.52-29.18)	*	0.001
Women	23	19	69	59	1.04 (0.49-2.22)		
Age							
18-29 years	47	10	71	57	3.77 (1.68-9.07)	2.57 (1.55-4.28)	0.177
≥30 years	80	13	132	40	1.86 (0.91-4.03)		
Alcohol consumption							
Yes	10	1	42	25	5.95 (0.75-268.43)	2.57 (1.54-4.30)	0.396
No	117	22	161	72	2.38 (1.36-4.26)		
HBsAg							
Positive	50	5	18	4	2.22 (0.39-11.51)	2.16 (1.27-3.67)	0.966
Negative	77	18	185	93	2.15 (1.19-4.04)		
HCV antibody							
Positive	1	1	0	0	†		
Negative	126	22	203	97			

*Cannot be calculated because sex is an effect modifier of khat use in chronic liver disease.
†Cannot be calculated since none of the controls are anti-HCV positive.
Abbreviation: OR_{MH}, Mantel-Haenszel summary OR estimate.

66.4-100), while in women it was only 1.9% (95% CI –35.6 to 39.3).

SENSITIVITY ANALYSIS

Seventy (46.7%) of the 150 cases and 31 (10.3%) of the 300 controls had documented risk factors for CLD other than khat use (Supporting Fig. S1). The remaining 80 (53.3%) cases and 269 (89.7%) controls had no identifiable risk factors for CLD except that 66 (82.5%) and 178 (66.2%), respectively, were regular khat users (Supporting Table S1).

The significant association between khat use and the risk for developing CLD was robust in this subpopulation of 80 cases and 269 controls (crude OR, 2.41; 95% CI, 1.25-4.90; $P = 0.005$). The sex differences were slightly more pronounced than in the total cohort (Supporting Table S2). No other significant confounders were identified.

The upward trend in the risk for developing CLD with increasing khat exposure observed in the primary analysis persisted in the sensitivity analysis, with findings indicative of a dose–response relationship in men but no such relationship in women (Supporting Table S3). Similarly, the findings from the primary analysis when khat exposure was analyzed as a continuous variable were reproduced in the sensitivity analysis; per 1 khat year increment, the odds of CLD was

TABLE 3. Gradient Effect of Khat Use on the Frequency of CLD, Stratified by Sex

	Men (n = 280) Cases (n = 108)	Men (n = 280) Controls (n = 172)	Men (n = 280) AOR (95% CI)*	Women (n = 170) Cases (n = 42)	Women (n = 170) Controls (n = 128)	Women (n = 170) AOR (95% CI)*
Khat years, by quartiles†						
Q1: 0	4	40	1.0 (reference)	19	61	1.0 (reference)
Q2: 0.1-5.0	21	40	3.58 (1.05-12.21)	11	37	1.21 (0.48-3.06)
Q3: 5.1-40.0	34	51	5.90 (1.79-19.44)	7	22	0.98 (0.32-3.00)
Q4: 40.1-250	49	41	13.03 (3.61-47.02)	5	8	1.97 (0.46-8.45)
P for trend			<0.001			0.801

*Adjusted for the confounding effect of age, alcohol consumption, and chronic hepatitis B infection.
†One khat year is defined by daily use of 200 g of fresh khat for 1 year.

significantly increased in men (AOR, 1.008; 95% CI, 1.001-1.015; $P = 0.034$) but not in women (AOR, 1.014; 95% CI, 0.997-1.031; $P = 0.108$).

Discussion

A strong and significant association was observed in the present study between chewing khat and the risk for developing CLD. No risk factors for liver injury were identified in 53% of the cases with CLD despite extensive investigations, except that >80% of them used khat. Additionally, although confined to men, a clear dose–response relationship was observed between khat exposure and the associated risk for CLD. In previous case reports, resolution of the liver injury has been recorded following cessation of khat, while relapse following reexposure has also been documented.[9,12,13] Moreover, khat has been shown to be hepatotoxic in animals, manifesting as a spectrum of liver injury.[29-31] Evidence from the present study, together with previous case reports and animal studies, supports a strong association and suggests a causal relationship between khat chewing and the development of CLD.

The mechanism of the khat-related hepatotoxicity is unknown, but several plausible biological explanations have been proposed. First, although there were no compelling features of an autoimmune process in the cases in the present study, previous case reports have documented low titers of autoantibodies and histological features reminiscent of an autoimmune hepatitis in patients suspected of having khat-related liver injury.[9,10,32] Second, the khat-related alkaloids are metabolized extensively in the liver by the enzyme cytochrome P450 2D6 (CYP2D6) and have short half-lives.[4,33] The finding in one case series of very high concentrations of khat alkaloids in a sample of explanted liver many weeks after the last exposure to khat suggests that accumulation of khat and/or its metabolites may be important.[12] Thus, polymorphisms in the gene controlling CYP2D6 may play a role in determining individual susceptibility to khat-related liver injury, as may possession of variants in other genes implicated in the risk for developing CLD *per se*.[34] Third, the possibility that the liver injury might relate not to khat itself but to contaminants such as herbicides and pesticides or to contamination with heavy metals or toxogenic fungi has to be considered but is thought to be unlikely.[35,36] Rodents given diets containing uncontaminated khat leaves showed elevated liver enzymes and necroinflammatory change on histology, supporting the contention that the natural substances contained in khat leaves are responsible for the hepatic injury.[29,30] Finally, khat has been shown to trigger generation of reactive oxygen species in human cells *in vitro*, resulting in hepatocyte apoptosis.[37] Similar histological features have been described in humans following ingestion of ecstasy, another amphetamine-related drug.[38]

There is no obvious explanation for the observed sex differences in the susceptibility to khat-related liver injury observed in the present study. Khat usage was generally lower in women than men, but even women with moderate-level or high-level khat exposure did not seem to be at significantly increased risk of CLD. This apparent differential susceptibility to khat-related hepatotoxicity could be explained by one or more of the following: (1) the levels of exposure in women may not reach the threshold for toxicity; (2) there are sex differences in chewing habits which might influence the duration of exposure, with men spending concentrated blocks of recreational time chewing khat—and hence the duration of exposure is prolonged—while women tend to chew khat intermittently so that the duration of overall exposure is much shorter; (3) exposure to certain dietary or environmental agents may result in either induction or inhibition of CYP2D6 activity, causing changes in khat metabolism; sex-specific differences in exposure to these agents may, therefore, play a role. It is also possible that there are sex-specific differences in the number of copy variants of CYP2D6 or differences in the frequency of variant single-nucleotide polymorphisms. None of these possibilities, however, can be addressed using the data from the present study.

This study had a number of strengths. First, data were collected over a 1-year period, thereby controlling for possible seasonal variations in khat availability and hence exposure. Second, by using newly diagnosed cases, the exposure–disease relationship was less likely to be influenced by altered risk habits, lifestyle change, or other interventions based on previous medical advice.[25] Finally, there were no missing data either for the main exposure variables or for the potential confounders.

The study also had its limitations. First, selection bias cannot be excluded; an unknown proportion of patients with CLD may not have been seen by the recruiting medical services for a variety of practical, cultural, and socioeconomic reasons. In addition, the decision to use hospital patients as controls might have

introduced Berkson's bias[25] as their attendance could have been affected by both exposure and disease. An attempt was made to minimize this risk by the selection of control subjects from a range of hospital departments and not from specialties dealing with illnesses known to be associated with khat usage.[7,39,40] However, this may inadvertently have resulted in introduction of possible bias as the majority of the controls were outpatients and the majority of the cases inpatients, and we cannot be sure whether this potential bias goes toward or away from the null.

Second, as in any case–control study, information bias cannot be excluded. Underreporting or denial of alcohol consumption or other recreational drugs is common in observational studies and may result in an underestimation of the degree of confounding. The fact that the cases in the present study reported less exposure to alcohol may suggest information bias, but it more likely reflects the fact that the majority of the cases were Muslims compared with only two thirds of the controls. Of note, the 2016 Ethiopian Demographic and Health Survey reported a prevalence of abstinence from alcohol of 86.6% in the Harari region,[41] and thus the high abstinence rate in the present study seems representative. Although data from Ethiopia are scarce, the small proportion of people with alcohol-related CLD in the present study is in line with previous findings.[42,43] The use of khat in eastern Ethiopia, on the contrary, is legal and socially accepted, and its usage is less likely to be underreported in this context. Because neither the study subjects nor the interviewers were informed about the primary aim of this study, any misclassification of khat exposure is likely to be nondifferential, so the observed effect of khat on the development of CLD would, if anything, be underestimated.[25]

Third, abdominal ultrasound was not performed in the controls, and hence, some may have had undiagnosed CLD. However, undiagnosed cirrhosis is rare in the general population, usually <1% of adults in population-based studies,[44,45] so it is unlikely that this would have affected our results significantly.

Fourth, power deficiency might be one explanation why women appeared less susceptible to khat-related liver injury as relatively few women reported high-level khat exposure.

Finally, we cannot exclude residual confounding by factors not accounted for in our analysis, such as cigarette smoking, coffee intake, the use of traditional herbal remedies, and exposure to dichlorodiphenyltrichloroethane and other potentially hepatotoxic pesticides through consumption of unwashed khat leaves.[46,47]

In conclusion, a strong and significant association was observed between khat chewing and CLD, strengthening the hypothesis that khat is implicated in the development of CLD. In men, the association was strong and dose-dependent, suggesting a causal relationship. This study identified khat-associated CLD that may be responsible for a significant proportion of the liver disease observed in countries where khat use is widespread. As the prevalence of khat chewing is expanding within the wider diaspora, these findings have important public health implications.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29809/suppinfo.

Supplementary table 1 – Characteristics of the cases and controls included in the subgroup analysis

Characteristic	Cases (n = 80)	Controls (n = 269)	*p*
Men	53 (66.3)	147 (54.6)	0.065
Age (years)	30 (25-40)	30 (24-50)	0.595
Religion			<0.001
Islam	78 (97.5)	183 (68.0)	
Christianity	2 (2.5)	86 (32.0)	
History of alcohol consumption			<0.001
Never	75 (93.8)	216 (80.3)	
Current	1 (1.3)	44 (16.4)	
Stopped	4 (5.0)	9 (3.3)	
History of khat use	66 (82.5)	178 (66.2)	0.005
Khat years [a]	19 (3-71)	2 (0-20)	<0.001
Men	40 (14-78)	9 (0-40)	
Women	0.5 (0-5)	0.1 (0-4)	

Data are presented as number (%) or as median (interquartile range).

[a] One khat year is defined by daily use of 200 grams of fresh khat for one year.

Supplementary table 2 – The association between khat use and the risk of developing chronic liver disease in the subgroup analysis

Characteristic	Cases (n = 80)		Controls (n = 269)		OR (95% CI)	OR_{MH} (95% CI)	Breslow and Day test of homogeneity (*p*)
	Khat exposure		Khat exposure				
	Yes	No	Yes	No			
Crude (n)	66	14	178	91	2.41 (1.25 – 4.90)		
Sex							
Men	51	2	113	34	7.67 (1.83– 67.92)		0.015
Women	15	12	65	57	1.10 (0.44 – 2.80)	★	
Age							
18-29 years	24	6	63	53	3.37 (1.21 – 10.75)	2.33 (1.24 – 4.38)	0.309
≥ 30 years	42	8	115	38	1.73 (0.72 – 4.65)		
Alcohol consumption							
Yes	5	0	31	22	7.86 (0.41– 149.40)	2.25 (1.20 – 4.22)	0.382
No	61	14	147	69	2.05 (1.07 – 3.91)		

★ Cannot be calculated because sex is an effect modifier of khat use in chronic liver disease.

Abbreviations: CI, confidence interval; OR, odds ratio; OR_{MH}, Mantel-Haenszel summary odds ratio estimate.

Supplementary table 3 – Gradient effect of khat use on the frequency of chronic liver disease, stratified by sex in the subgroup analysis

	Men (n=200)			Women (n=149)		
	Cases (n=53)	Controls (n=147)	AOR (95% CI) [a]	Cases (n=27)	Controls (n=122)	AOR (95% CI) [a]
Khat years, by quartiles [b]						
Q1: 0	2	36	1.0 (reference)	12	59	1.0 (reference)
Q2: 0.1 – 3.0	2	27	1.10 (0.14 – 8.43)	6	32	1.16 (0.38 – 3.53)
Q3: 3.1 – 30.0	20	43	7.73 (1.65 – 36.32)	6	22	1.57 (0.50 – 4.99)
Q4: 30.1 – 250	29	41	15.94 (3.18 – 79.91)	3	9	2.19 (0.45 – 10.62)
p for trend			<0.001			0.752

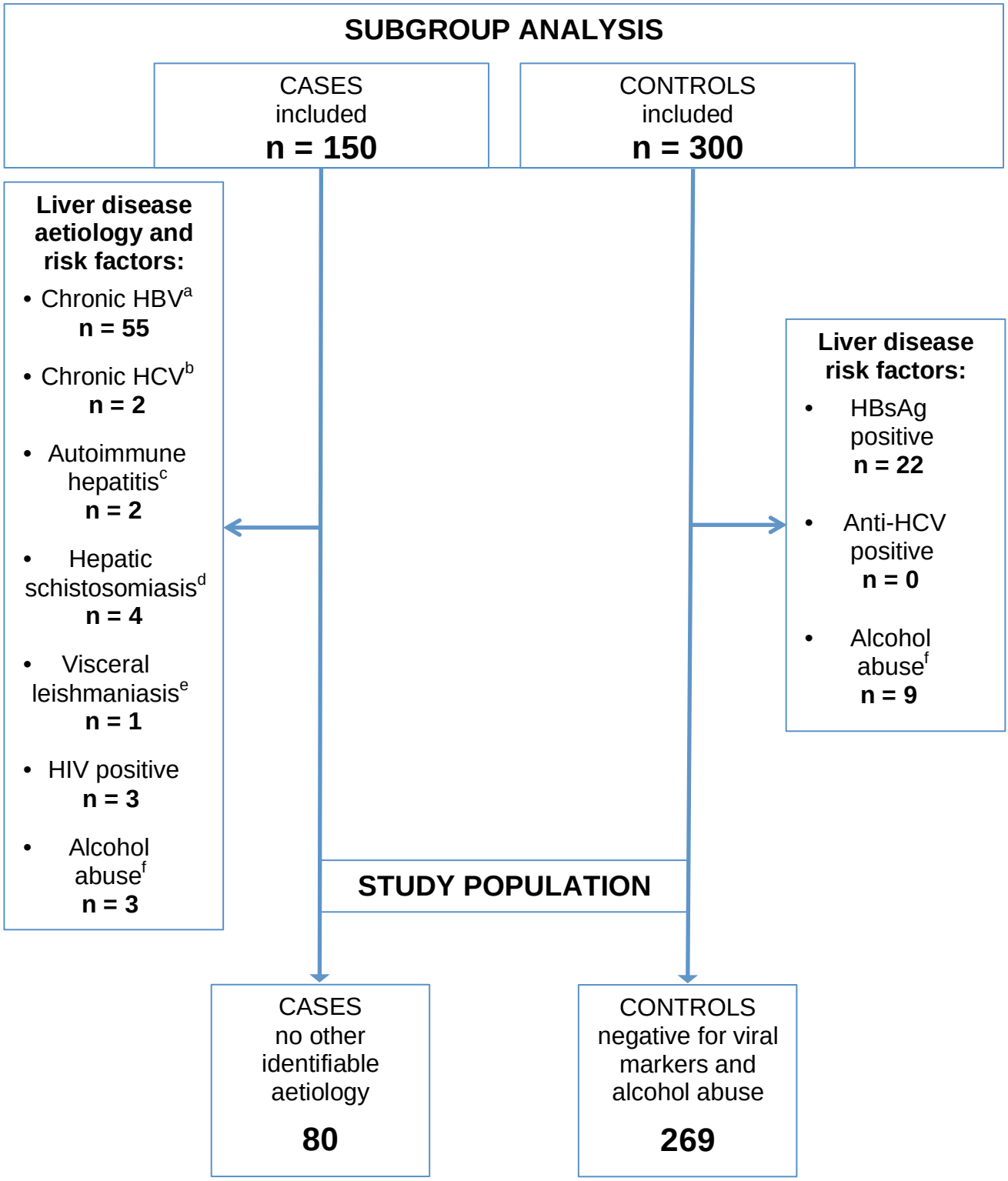
Abbreviations: AOR, adjusted odds ratio; CI, confidence interval.

[a] Adjusted for the confounding effect of age and alcohol consumption.

[b] One khat year is defined by daily use of 200 grams of fresh khat for one year.

Supplementary figure 1 – Subgroup analysis: selection of study subjects

Abbreviations: anti-HCV, hepatitis C antibody; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IgG, immunoglobulin G.



a. Defined as ultrasonographic evidence of chronic liver disease and positive HBsAg.
b. Defined as ultrasonographic evidence of chronic liver disease and positive anti-HCV.
c. Diagnosed by exclusion of alternative diagnoses and strongly positive anti-nuclear antibodies or anti-actin and elevated IgG (>1.1 x upper limit of normal).
d. Diagnosed by finding ova from *Schistosoma mansoni* in stool samples and typical ultrasonographic findings of hepatic schistosomiasis confirmed by an independent expert reviewer and absence of viral markers.
e. Diagnosed by positive recombinant K39 antigen strip test and hepatosplenomegaly confirmed by abdominal ultrasound.

III

Manuscript

High seroprevalence of autoantibodies typical of autoimmune liver disease in eastern Ethiopia - is chewing of khat (Catha edulis) a triggering factor?

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Abstract

Background: Recent studies have identified chewing of khat (*Catha edulis*) as an independent risk factor for liver injury; however, the pathogenetic mechanism remains poorly understood. Case series have found markers of autoimmune hepatitis in patients with khat-related liver disease, suggesting that khat chewing might trigger an autoimmune response. The aims of the present study were i) to assess the prevalence of autoantibodies typical for autoimmune liver diseases in a healthy population in Ethiopia, and ii) to explore the hypothesis that khat usage triggers autoimmunity. *Methods:* Consenting adults (≥18 years) without known autoimmune disease or manifest liver disease were included. One-hundred-and-sixty-nine individuals with current khat use were compared to 104 individuals who never used khat. Seroprevalence of anti-nuclear (ANA), anti-smooth muscle (SMA) and anti-mitochondrial antibodies (AMA) were determined and compared between the groups using logistic regression models to adjust for age and sex. *Results:* Overall, 2.6% of the study subjects were positive for ANA, 15.4% for SMA and 25.6% for AMA. When comparing khat users to non-users, ANA was detected in 4.1% vs. 0% (p=0.047), SMA in 16.0% vs. 14.4% (p=0.730), and AMA in 24.9% vs. 26.9% (p=0.704). ANA was excluded from multivariable analysis since there were no seropositive in the reference group. After adjusting for sex and age, no significant association between khat use and SMA or AMA was found. *Conclusions:* No association between khat usage and the seropresence of SMA or AMA was found, weakening the hypothesis that khat-related liver injury is mediated through autoimmune mechanisms. However, the seroprevalences of AMA and SMA were strikingly high in this Ethiopian population compared to global estimates, suggesting that diagnostic algorithms for autoimmune liver diseases developed in Europe and North America might lead to misdiagnosis of patients on the African continent.

Introduction

Catha edulis (khat) is an evergreen shrub indigenous to East Africa and the Arabian Peninsula that is cultivated for its use as a natural stimulant [1]. Khat leaves contain more than 40 different compounds, including three alkaloids - cathinone (aminopropriophenone), cathine (norpseudoephedrine) and norephedrine, which are structurally related to amphetamine and exert a psychostimulatory effect on the central nervous system [2]. Fresh young leaves and twigs are chewed to increase performance, evoke alertness and to attain a state of euphoria enhancing social interaction [3]. The habit of khat chewing is common in East Africa and the Arabian Peninsula where it is considered as a part of the social and cultural heritage which has prevailed for ages, probably as far back as the 13th century [4]. Over the last three decades, the Horn of Africa and Middle East have been engulfed by war and instability, leading to mass migration and hence a wider spread of khat consumption within immigrant communities wordwide [5]. In addition, there is a growing interest in plant-derived and uncontrolled psychoactive substances among youth in Europe and the USA [6, 7]. The global prevalence is unknown but estimates range up to 20 million daily users, which most likely is an underestimation [1].

Cathinone and cathine are controlled substances under the international Convention of Psychotropic Substances (1971), whereas fresh khat leaves are not. The World Health Organization has defined khat as a drug of abuse since it is associated with negative social and health consequences [8]. In addition to psychological adverse effects such as psychosis [9] and exacerbation of pre-existing psychotic disorder [10], khat use is associated with a wide range of somatic health problems, including acute and chronic liver disease [11].

In an earlier case-control study, we demonstrated a strong and significant association between khat chewing and chronic liver disease in Ethiopia [12]; however, the mechanism of liver injury was not addressed. A few previous case reports have described khat-related liver injury mimicking autoimmune hepatitis, and the authors speculate that khat might trigger an autoimmune reaction in susceptible individuals [13-15]. The hallmark of autoimmune hepatitis is the presence of certain autoantibodies, of which antinuclear antibodies (ANA) and anti-smooth muscle antibodies (SMA) are the most important. Other diagnostic criteria include elevated levels of immunoglobulin G (IgG), typical histopathological changes in the liver and the absence of active viral hepatitis [16].

The prevalence of autoimmune disease and autoantibodies in Ethiopia is largely unknown. In an ancient study, Tsega *et al.* studied records from 7966 medical in-patients in four different

hospitals in Addis Ababa between 1971 and 1978, of whom 0.2% had rheumatoid arthritis and 0.05% had systemic lupus erythematosus. In a substudy of 107 Ethiopians with dyspepsia and 80 healthy controls recruited from 1975 to 1978, SMA were found in 20 (10.7%); ANA in one (0.5%) and anti-mitochondrial antibodies (AMA) in one (0.5%) [17].

In the present study, which was nested in a previously published case-control study [12], we aimed to assess i) the seroprevalence of autoantibodies typical of autoimmune liver diseases in a well-defined study population in Ethiopia, and ii) to explore the hypothesis that khat usage triggers autoimmunity. Results from this study might pave the road for a better understanding – and ultimately better treatment – of khat-related liver injury.

Material and methods

Study setting and participants

A case-control study was undertaken at Hiwot Fana Specialized University Hospital and Jugal Hospital in Harar, Ethiopia, between April 2015 and April 2016, as previously described [12]. Study subjects for the present analysis were the controls from the previous study, and comprised of adults aged 18 years and above attending the ophthalmology, dermatology or surgical services during the study period. Individuals with conditions associated with autoimmune markers were excluded from the analysis, such as: i) known human immunodeficiency virus infection, rheumatic or autoimmune disease; ii) history of alcohol misuse, defined as >20 g/day in women and >30 g/day in men [18]; iii) clinical signs or previous history of liver disease; or, iv) positive serum hepatitis B surface antigen (HBsAg) or hepatitis C virus antibody (anti-HCV).

Patient assessment

All study participants underwent a semi-structured interview by local nurses fluent in their mother tongue. Demographic data including age, sex, ethnicity, religion and occupation were recorded. Current diagnosis, previous medical history, alcohol drinking habits and use of herbal remedies and khat (*Catha edulis*) were explored.

In lack of validated criteria for the quantification of khat usage, we established a screening tool to assess khat consumption as described in previous publications [12, 19]. By combining information on khat usage quantified in grams using a visual analogue scale (Figure 1) with the frequency and duration of khat usage categorized using the Drug Use Disorders Identification Test [20], we classified lifetime khat exposure as *khat-years*. Approximately 100-300 g of fresh khat leaves are chewed in a typical session [21]; thus one *khat-year* was defined as daily use of 200 g of fresh khat for one year.

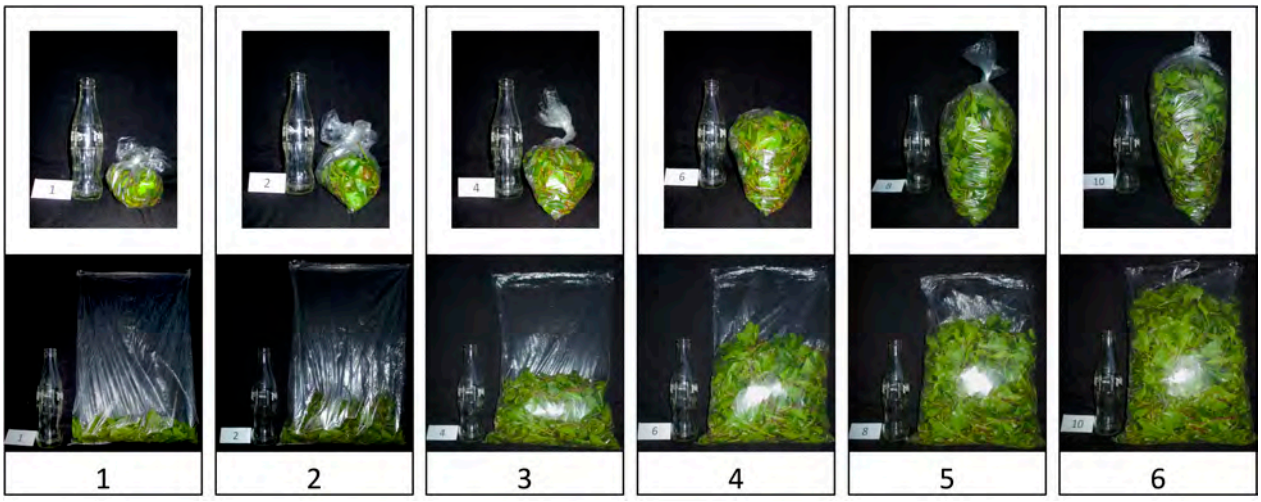

Fig. 1: The visual analogue scale used to quantify the use of khat in grams.
1) 100 grams; **2)** 200 grams; **3)** 400 grams; **4)** 600 grams; **5)** 800 grams; **6)** 1000 grams.

Clinical examination was undertaken using a pre-specified protocol. Study subjects with features suggestive of manifest liver disease, such as jaundice, ascites, hepatosplenomegaly, caput medusae or spider angioma, were excluded from the analysis.

Laboratory tests

Blood was collected by venous puncture for immediate processing; serum was separated for storage in aliquots at -20 ºC. Standard biochemical tests were analysed locally using a semi-automatic biochemistry analyser DR-7000D (DIRUI, Changchun, China) and HumaLyzer 3000 (HUMAN, Wiesbaden, Germany).

Validated rapid diagnostic tests were used to screen for HBsAg and anti-HCV; results were confirmed using enzyme-linked immunosorbent assays as previously described [12].

Serum specimens were transported on dry ice to Drammen Hospital in Norway and stored at -80 ºC until analysed. Autoimmune markers were determined by the Phadia™250 Laboratory system (Thermo Fisher Scientific, Waltham, MA, USA). ANA was detected using the EliA™ Symphony assay (Phadia, Freiburg, Germany) with a calculated ratio of test sample response to calibrator >1.0 defined as positive, 0.7-1.0 was equivocal, and <0.7 was negative [22, 23]; SMA was determined by QUANTA Lite® Actin IgG (Inova Diagnostics, San Diego, CA, USA) and a cut-off level >30 assay units was classified as positive, as proposed by the manufacturer [24]; AMA was determined using QUANTA Lite® M2 EP (MIT3) (Inova Diagnostics) and a cut-off level >25 assay units was classified as positive, as proposed by the manufacturer [25]. Serum was

analysed for IgG using the IMMAGE® 800 Immunochemistry System (Beckman Coulter, Brea, CA, USA).

Patient selection and sample size calculation

All control subjects from the previous case-control study [12] were evaluated for inclusion. Eligible study participants were categorised into three groups according to reported khat usage: i) individuals who never used khat were classified as ‘non-users’; ii) individuals who had stopped chewing khat for more than one year were termed ‘stopped chewing khat’; and, iii) individuals with current khat use, defined as reported khat usage within the last 12 months, were classified as ‘khat users’.

Statistical methods

Categorical variables were summarized as frequencies, whereas continuous variables were presented as median and interquartile range (IQR) since the data was not normally distributed. Comparisons between groups were performed using the Pearson’s chi-square test for categorical variables, and Mann-Whitney U test for continuous variables. Khat users were further categorised as ’heavy users’ or ’light users’ according to the median lifetime khat exposure measured in *khat-years*. In the multivariable analysis a logistic regression model was used to control for confounders.

The statistical analyses were performed in SPSS 25.0 (SPSS Inc., Chicago, IL, USA). All tests were two-sided and a *p*-value <0.05 was considered significant throughout the study. The *Strengthening the Reporting of Observational studies in Epidemiology* (STROBE) statement guidelines were followed [26].

Ethics

The study was approved by the National Research Ethics Review Committee (NRERC, Ref. No.: 3.10/829/07) in Ethiopia and by the Regional Committees for Medical and Health Research Ethics (REK Sør-Øst Ref. No.: 2014/1146) in Norway. The study was conducted in accordance with the Declaration of Helsinki [27]. Written informed consent was obtained from all study subjects.

Results

Study population

A total of 370 individuals were recruited and evaluated for eligibility, of whom 310 fulfilled the inclusion criteria. Of these, 169 study subjects had a history of using khat within the last 12 months, and 104 had never chewed khat. Thus, the final study population comprised of 169 'khat users' and 104 'non-users' (Figure 2).

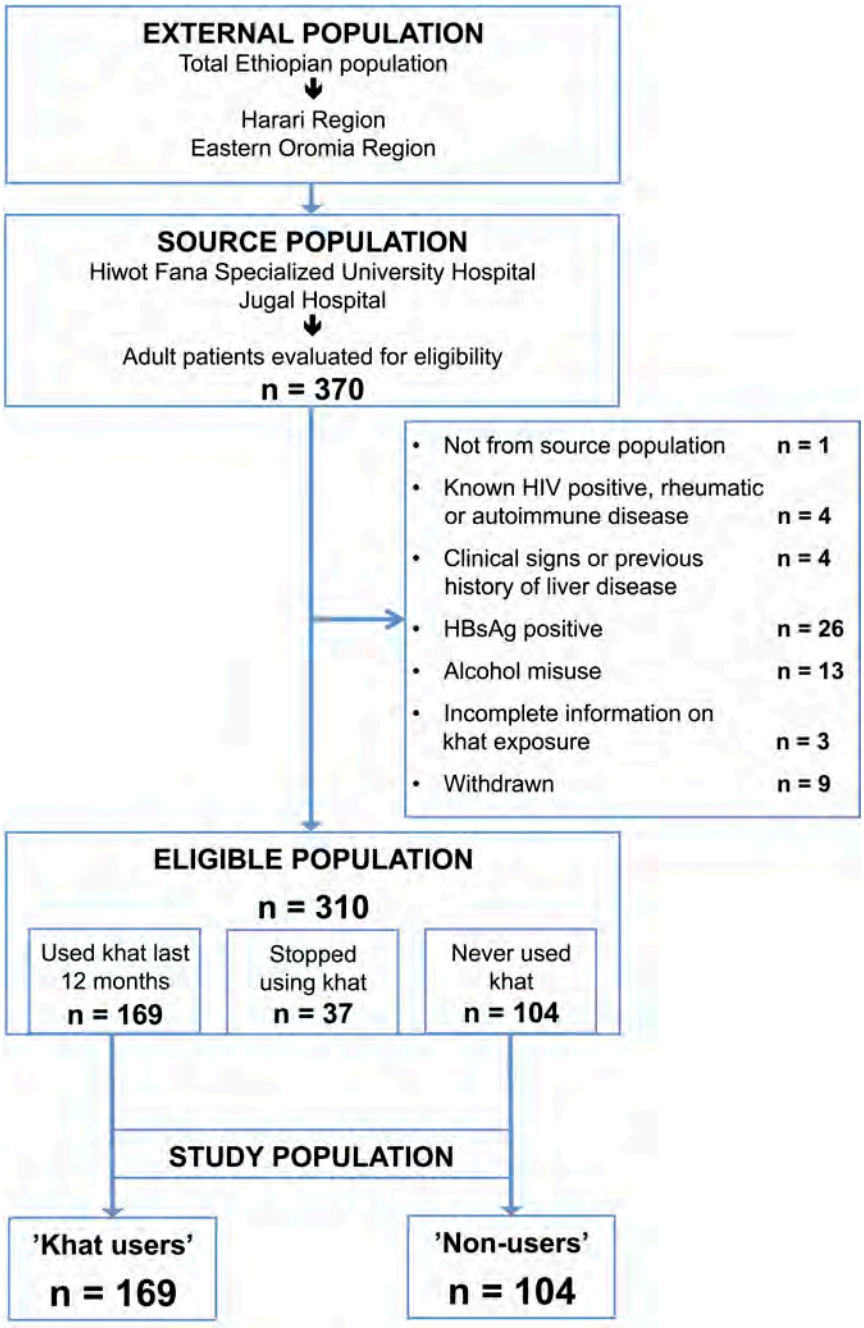

Fig. 2: Study flow diagram illustrating the selection of the study subjects.

Abbreviations: HBsAg, hepatitis B surface antigen; **HIV**, human immunodeficiency virus

Demography

Overall, there were more men (57.5%) than women among the study participants, and the median age was 30 (IQR 24-50) years (Table 1). Study subjects in the non-user group were younger than the 'heavy khat users' (>15 *khat-years*) (median 27 *vs*. 40 years of age; $p<0.001$). The khat users were more likely than the non-users to be male, ethnic Oromo, Muslim and farmers. Overall, men were more likely to have a history of khat use within the last 12 months than women (74.5% *vs.* 44.8%; $p<0.001$). Moreover, among the khat users, men reported higher khat exposure than women (median 23 *vs.* 4 *khat-years*; $p<0.001$).

Table 1 – Demographic features of the study participants, by khat consumption level.

Variable	Non-users (n=104)	Light khat users[1] (n=86)	Heavy khat users[2] (n=83)	Significance (*p*) Non-users *vs.* Light khat users	Significance (*p*) Non-users *vs.* Heavy khat users
Men	40 (38.5)	47 (54.7)	70 (84.3)	***0.026***	***<0.001***
Age (years)	27 (22-52)	27 /24-40)	40 (30-55)	0.497	***0.001***
Ethnic group:					
Oromo	48 (46.2)	67 (77.9)	78 (94.0)		
Amhara	42 (40.4)	15 (17.4)	4 (4.8)	***<0.001***	***<0.001***
Other	14 (13.5)	4 (4.7)	1 (1.2)		
Religion:					
Islam	44 (42.3)	69 (80.2)	79 (95.2)	***<0.001***	***<0.001***
Christianity	60 (57.7)	17 (19.8)	4 (4.8)		
Occupation:					
Farmer	9 (8.7)	28 (32.6)	65 (78.3)		
Housewife	26 (25.0)	10 (11.6)	1 (1.2)		
Student	14 (13.5)	10 (11.6)	0	***<0.001***	***<0.001***
Public servant	17 (16.3)	3 (3.5)	3 (3.6)		
Health professional	5 (4.8)	3 (3.5)	2 (2.4)		
Other	33 (31.7)	32 (37.2)	12 (14.5)		
Alcohol use[3]	24 (23.1)	17 (19.8)	6 (7.2)	0.581	***0.003***
Khat-years[4]	0	2 (0.5-10)	60 (30-100)	***<0.001***	***<0.001***

Data are presented as number (%) or as median (interquartile range).

1. ≤15 khat-years
2. >15 khat-years
3. ≤20 grams/day in women and ≤30 grams/day in men.
4. One khat-year was defined as daily use of 200 grams fresh khat for one year.

Laboratory findings

The overall median serum alanine aminotransferase (ALT) activity was 23 U/L (IQR 17-32) and the overall median serum aspartate aminotransferase (AST) activity was 28 U/L (IQR 21-38) (Table 2).

Table 2 – Laboratory findings in the study participants, by khat consumption level

Variable	Non-users (n=104)	Light khat users[1] (n=86)	Heavy khat users[2] (n=83)	Significance (*p*) Non-users *vs.* Light khat users	Significance (*p*) Non-users *vs.* Heavy khat users
ALT (U/L)	22 (16-31)	23 (18-31)	25 (18-34)	0.382	***0.015***
AST (U/L)	26 (19-35)	26 (21-33)	32 (24-47)	0.893	***0.003***
IgG (g/L)	14.9 (12.8-17.0)	15.2 (12.8-17.1)	15.3 (12.6-18.2)	0.924	0.347
ANA positive	0	4 (4.7)	3 (3.6)	***0.040***	0.086
SMA positive	15 (14.4)	10 (11.6)	17 (20.5)	0.570	0.274
AMA positive	28 (26.9)	20 (23.3)	22 (26.5)	0.563	0.949

Data are presented as number (%) or as median (interquartile range).
Laboratory reference range: ALT (8-40 U/L); AST (14-40 U/L); IgG (0.8-27.8 g/L) [28].

1. ≤15 khat-years[3]
2. >15 khat-years[3]
3. One khat-year was defined as daily use of 200 grams fresh khat for one year.

Abbreviations: **ALT**, alanine aminotransferase; **AMA**, anti-mitochondrial antibodies; **ANA**, anti-nuclear antibodies; **AST**, aspartate aminotransferase; **IgG**, immunoglobulin G; **SMA**, anti-smooth muscle antibodies.

Serum liver transaminase activities were elevated amongst heavy khat users compared to non-users (ALT: median 25 *vs.* 22 U/L, p=0.015; AST: median 32 *vs.* 26 U/L, p=0.003); however, by definition, none of the study participants presented with clinical signs of liver injury or recognized liver disease. When comparing study subjects with circulating autoimmune markers to seronegative study subjects, there was no difference in the proportions with elevated liver transaminase activity (20.2% vs. 22.5%; p=0.655). Overall median serum level of IgG was 15.1 g/L (IQR 12.8-17.4) and only 4 (1.5%) had IgG above the upper reference range [28], with no significant differences between the groups (non-users: 1.9% *vs.* khat users: 1.2%; p=0.984).

The overall proportion of study subjects with circulating autoantibodies was 2.6% for ANA, 15.4% for SMA and 25.6% for AMA. None of the study subjects with a positive ANA had presence of SMA or elevated serum IgG. Khat-users were more likely than non-users to be ANA positive (4.1% *vs.* 0%; p=0.047); however, there was no significant difference in the seropresence of ANA between heavy users and non-users (3.6%% vs. 0%; p=0.086). No significant differences in SMA or AMA seroprevalence between khat users and non-users were found (SMA: 16.0% *vs.* 14.4%, p=0.730; AMA: 24.9% vs. 26.9% (p=0.704).

Since there were no observations of ANA seropositive among the non-users, the outcome variable 'ANA positive' could not be included in the multivariable analysis. In multivariable analysis adjusting for age and sex, no significant association between khat use and SMA or AMA was found (Table 3).

Table 3 – Association between khat and the frequency of circulating SMA or AMA, by khat level

Variable	Crude OR (95% CI)	Significance (*p*)	Adjusted OR[1] (95% CI)	Significance (*p*)
SMA				
Non-users[2]	1		1	
Light users[3]	0.78 (0.33-1.84)	0.571	0.89 (0.37-2.12)	0.785
Heavy users[4]	1.53 (0.71-3.28)	0.276	1.80 (0.76-4.26)	0.180
AMA				
Non-users[2]	1		1	
Light users[3]	0.82 (0.42-1.59)	0.563	0.81 (0.42-1.60)	0.551
Heavy users[4]	0.98 (0.51-1.88)	0.949	1.21 (0.58-2.52)	0.608

1. Adjusted for the confounding effects of age and sex.
2. Reference group.
3. ≤15 khat-years[5]
4. >15 khat-years[5]
5. One khat-year was defined as daily use of 200 grams fresh khat for one year.

Abbreviations: AMA, anti-mitochondrial antibodies; **CI**, confidence interval; **OR**, odds ratio; **SMA**, anti-smooth muscle antibodies.

Discussion

In the present study, the overall seroprevalence of ANA was low, whereas a substantial proportion were SMA and/or AMA positive. No significant association between khat use and circulating SMA or AMA was found. In univariable analysis the association between khat and ANA was borderline significant; however, there was no significant difference in the seropresence of ANA between heavy users and non-users. Since there were no ANA-positive observations in the reference group, it was not possible to further explore the association between khat and ANA in multivariable analysis.

Previous case reports have proposed khat-induced autoimmune response causing acute and chronic liver injury in patients of Somali and Yemeni origin with seropresence of ANA and/or SMA [13-15]. In the present study, khat users were more likely to be ANA positive than non-users, however, numbers were small and the increased proportion of ANA-positive khat users compared to non-users was of borderline significance, and hence the observed association is at best dubious. Although there may be an association between khat use and circulating ANA, ANA is not specific for autoimmune hepatitis but is also found in patients with other autoimmune diseases, viral infections, a wide range of other liver diseases and even in subgroups of healthy subjects [29, 30]. Similar to ANA, SMA also lacks organ-/ and disease specificity, but is still considered the most specific marker of autoimmune hepatitis [29]; the combined seropositivity for ANA and SMA together with elevated serum IgG increase the specificity and the diagnostic accuracy [16, 31, 32].

Our findings weaken the hypothesis that the pathogenetic mechanism of khat-related liver injury is mediated by autoimmune mechanisms, since i) there was no association between khat use and circulating SMA; ii) none of the ANA seropositive study subjects in the present study had concurrent seropresence of SMA or elevated serum IgG; and, iii) there was no association between elevated liver transaminase activities and the selected circulating autoantibodies. Moreover, these findings correspond to our earlier study of 150 patients with chronic liver disease attending the same hospital from where the study subjects in the present study were recruited, of whom only two (1.3%) patients were attributed to autoimmune liver disease [19]. Although only a limited number of liver biopsies was undertaken in the previous study, the histological findings were supportive of toxic liver injury [19] and mirror those observed in animal models [33] and previous case reports of khat-induced liver injury in patients with a mixed clinical picture of autoimmune hepatitis together with histological evidence compatible with toxic origin [14, 34-36]. However, to distinguish drug-induced liver injury with presence of autoantibodies from autoimmune liver disease is difficult, and was beyond the scope of this study. Future studies should explore this further by following up patients with khat-related liver injury and circulating autoantibodies, and study the seropresence of autoantibodies and manifest liver disease after discontinuation of khat use.

The high proportion of study participants with positive AMA and/or SMA in the present study was intriguing. To the best of our knowledge, the survey undertaken by Tsega *et al* [17] in the 1970's is the only study on autoimmune markers in Ethiopia, and only scant data on the seroprevalence of autoantibodies among healthy individuals in sub-Saharan Africa are available. In a study of autoantibodies among 152 elderly individuals (median age 66 years) in rural south-west Cameroon, AMA was found in 0.7%, while 9% were SMA positive [37]. The strikingly high overall prevalence of AMA (25.6%) in the present study was more than twenty-fold the global estimates [38, 39]. The global prevalence of SMA in the general population worldwide is estimated to be around 10-12% [40-42]; hence, the observed proportion of SMA positive (15.4%) in the present study was higher than anticipated. However, there are wide differences in quantification methods, analytic thresholds and screening assays available and the observed results in the present study might not be directly comparable to other studies. Nevertheless, the proportion with a positive AMA and/or SMA among the Ethiopian study subjects was much higher than the results obtained from routine clinical samples in Norwegian patients using the same assays (personal communication, Dr. Trine Lauritzen).

In Cameroon, Njemini *et al* [37] found that 9% of healthy elderly were ANA positive. In Sierra Leone, a study of 70 women treated for vesicovaginal fistulas were screened for ANA and as many as 28.5% were found positive [43]. Oyeyinka *et al* [44] studied the presence of ANA in 111 plasma samples from healthy Nigerians aged 6 to 95 years of age, of whom 4 (3.6%) were positive. Hence, the observed seropositivity of ANA (2.6%) in the present study, was low compared to Sub-Saharan and worldwide estimates ranging up to around 30% in healthy controls, although different cut-off titres determining ANA as positive have been used [45-48].

In general, autoimmune diseases more frequently affect women than men [49]. Although men tend to develop autoimmune hepatitis at a younger age than women and have a higher relapse rate, men appear to have reduced susceptibility to the development of autoimmune hepatitis and a better prognosis than women [50]. Studies worldwide have found women significantly more frequently SMA positive than men [37, 40, 41]. However, in the present study, there were no sex differences in the presence of autoimmune markers, which correspond with the previous study undertaken in Ethiopia [17].

The remarkably high seroprevalence of AMA and SMA observed in this present study of healthy Ethiopians compared to global estimates raises an important question: Are the diagnostic algorithms for autoimmune diseases, which were developed in Europe and North America, applicable in sub-Saharan Africa? The presence of autoimmune markers might be explained from ethnic variations in autoimmune response or other environmental factors than khat, as our findings indicate. However, this study was not designed to determine the regional seroprevalence of autoimmune markers and further adequately powered population-based studies are needed to obtain representative estimates.

This study had a number of strengths, most importantly that the sample size was large and the study subjects underwent a rigorous quantification of khat usage and state-of-the art testing for autoimmune markers. Study subjects with manifest liver disease, known autoimmune disease or recognized trigger factors of autoimmunity were excluded to minimise the influence of underlying disease on the autoantibody profile.

The study also has its limitations. Firstly, the ideal study group would be healthy individuals randomly selected from the source-population. However, in lack of a population roster, the study subjects in the present study were selected among inpatients and outpatients from several hospital departments. Although participants with conditions known to influence on autoimmunity were excluded, there might still have been undiagnosed cases of autoimmune disease among the study

subjects. However, the prevalence of autoimmune disease in Ethiopia is expected to be low [17], so the confounding effect is likely to be small. Secondly, underreporting or denial of alcohol consumption or other recreational drugs is common. Alcohol consumption has been identified as a protective factor against autoimmune diseases [51, 52], and thus underreporting would, if anything, underestimate the effect of khat exposure. The use of khat in eastern Ethiopia, however, is legal and socially accepted, and its usage less likely to be underreported in this context. Thirdly, a number of other predictors of autoimmunity were not explored. Of note, cigarette smoking may trigger an autoimmune response [53] and smoking habits were not assessed in the present study, and hence we cannot exclude that cigarette smoking might exert confounding effects not accounted for in our analysis. Finally, cross-sectional studies are not designed for testing a hypothesis but may be useful for raising the question of the presence of an association [54].

Conclusions

In the present study, there was no association between khat chewing and the seropresence of SMA or AMA. ANA was more common among khat users compared to non-users, but numbers were small and only borderline significant. Our findings weaken the hypothesis that khat-related liver injury is mediated through autoimmune mechanisms. Of note, the seroprevalence of AMA and SMA were strikingly high in this Ethiopian population compared to global estimates, suggesting that diagnostic algorithms for autoimmune liver diseases developed in Europe and North America might lead to misdiagnosis of patients on the African continent.

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