Non-invasive diagnosis of nonalcoholic fatty liver disease and associated liver disease in at-risk populations

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1. Abstract

English

BACKGROUND: Liver transplant (LT) recipients, patients with type 2 diabetes (T2D), and people with HIV represent at-risk populations for nonalcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC). These populations require monitoring to prevent hepatic failure. We aimed to: 1) provide a review on non-invasive tools (NITs) for NAFLD and liver fibrosis in patients with T2D; 2) determine incidences and predictors of NAFLD and NASH in LT recipients using NITs, namely Fibroscan with controlled attenuation parameter (CAP) and cytokeratin 18 (CK-18), and evaluate the diagnostic performance of NITs compared to liver histology; and 3) determine the rate of surveillance of HCC, and the reasons for suboptimal surveillance, in HIV-infected patients.

METHODS: Aim 1) We performed a search and included the definitions, strengths, limitations, and accuracy of NITs in diagnosing NAFLD and liver fibrosis in patients with T2D; 2) We performed a prospective study involving patients who received LT between 2015-18. Post-LT, patients were followed for 5 visits. Fibroscan with CAP and CK-18 measurements were recorded. Incidences of NAFLD and NASH diagnosed non-invasively were described. Multivariable Cox regression models assessed predictors of NAFLD and NASH. The performance of the NITs to diagnose NAFLD, NASH and liver fibrosis was compared to liver histology; 3) We conducted a retrospective study of the LIVEr disease in HIV Cohort. We included patients eligible for HCC surveillance from the Cohort. Inclusion criteria included: HIV infection and HIV/HCV co-infection with liver stiffness measurement (LSM) ≥10 kPa, HIV/HBV co-infection regardless of LSM score; and a follow-up time of at least 12 months. Optimal surveillance was defined as at least yearly examination by ultrasound (US) with or without twice a year alpha fetoprotein (AFP). Individual reasons for suboptimal surveillance were reported.

RESULTS: 1) NITs to diagnose NAFLD and liver fibrosis in patients with T2D include biomarkers (such as Fatty Liver Index, Fib-4, NAFLD fibrosis score, CK-18, PRO-C3, Enhanced liver fibrosis test, FibroTest and SteatoTest) and imaging tools (such as US, Fibroscan, MRI-proton density fat fraction, magnetic resonance spectrometry and magnetic resonance

elastography); 2) 40 LT recipients (mean age 58, and 53% transplanted due to NASH) were included. During a median follow-up of 16.8 months, 63% developed NAFLD and 48.5% developed NASH. On multivariate Cox regression analysis, BMI was an independent predictor of both NAFLD (aHR 1.1, 95% 1.0-1.2) and NASH (aHR 1.1, 95% CI 1.0-1.3). A hazard plot showed that BMI>25 was a significant predictor as compared to BMI<25 for both NAFLD and NASH (log rank of <0.0001 and 0.009, respectively). Compared to liver histology, Fibroscan with CAP and a combination of CAP and CK-18 had a diagnostic accuracy of 76% and 82% to diagnose NAFLD and NASH, respectively; 3) 186 patients met the inclusion criteria for HCC surveillance (25% HIV mono-infected, 46% HIV/HCV coinfected, and 28% HIV/HBV coinfected). The surveillance rate by US was similar among the groups; however, surveillance rate by AFP was lower in the HIV mono-infected group (p<0.0001). Overall, among the reasons for suboptimal surveillance rates, patient-level factors were more frequent in HIV/HCV co-infected patients. Surveillance for HCC was discontinued in 52% of HIV mono-infected and 45% of HIV/HCV co-infected patients because of a reduction in LSM during follow-up. During a mean follow-up of 55 months, incidence of HCC was 2.2%.

CONCLUSION: The progression of liver disease diagnosed by NITs is common in at-risk populations. There is still a gap regarding the role of NITs in the diagnosis of NAFLD and liver fibrosis in at-risk populations. Future studies should aim to implementation of NITs and optimization of surveillance.

French

CONTEXTE : Les transplantés hépatiques, les patients atteints de diabète de type 2 (DT2) et les personnes avec l'infection VIH représentent des populations à risque pour la stéatose hépatique non alcoolique (NAFLD) et le carcinome hépatocellulaire (CHC). Ces populations nécessitent une surveillance pour prévenir les complications. Objectives : 1) fournir une revue des outils non invasifs (NIT) pour la NAFLD chez les patients atteints de DT2 ; 2) déterminer les incidences de la NAFLD et de la NASH chez les receveurs de greffe hepatique (GH) utilisant des NIT; et 3) déterminer le taux de surveillance du CHC, chez les patients avec le VIH.

MÉTHODES : Objective 1) Nous avons etudié les définitions, les forces, les limites et la performance des NIT dans le diagnostic de la NAFLD et de la fibrose hépatique chez les patients atteints de DT2 ; 2) Nous avons réalisé une étude prospective des patients ayant reçu une GH entre 2015-18. Des mesures non-invasives de fibroscan avec paramètre d'atténuation contrôlée (CAP) et la cytokératine 18 (CK-18) ont été enregistrées pendant 5 visites après la GH. Les incidences de NAFLD et de NASH diagnostiquées avec le NIT ont été décrites. Des modèles de régression multivariables de Cox ont évalué les prédicteurs de la NAFLD et de la NASH. La performance des NIT pour diagnostiquer la NAFLD et la NASH a été comparée à l'histologie du foie ; 3) Nous avons mené une étude rétrospective de la cohorte “LIVEr disease in HIV” (LIVEHIV). Nous avons inclus les patients avec VIH éligibles pour la surveillance du CHC. Les critères d'inclusion comprenaient: L'infection par le VIH et la co-infection VIH/VHC avec une mesure du Fibroscan ≥10 kPa, la co-infection VIH/VHB indépendamment du score Fibroscan. La surveillance optimale a été définie comme un examen au moins annuel par échographie (US) avec ou sans dosage semestriel de l'alpha-fœtoprotéine (AFP). Les raisons individuelles d'une surveillance sous-optimale ont été rapportées.

RÉSULTATS : 1) Les NIT pour diagnostiquer la NAFLD et la fibrose hépatique chez les patients atteints de DT2 comprennent des biomarqueurs (Fib-4, le score de fibrose de la NAFLD, CK-18, PRO-C3, le test de fibrose hépatique amélioré, FibroTest et SteatoTest) et des outils d'imagerie (l’US, le Fibroscan, la fraction graisseuse de densité de protons de l'IRM, la spectrométrie de résonance magnétique et l'élastographie de résonance magnétique) ; 2) 40 receveurs de GH ont été inclus. Au cours d'un suivi médian de 16,8 mois, 63% ont développé une NAFLD et 48,5% une NASH. Dans l'analyse de régression multivariée de Cox, l'IMC était un prédicteur indépendant de la NAFLD (aHR 1,1, 95% 1,0-1,2) et de la NASH (aHR 1,1, 95% CI 1,0-1,3). Comparé à l'histologie du foie, le Fibroscan avec CAP et une combinaison de CAP et de CK-18 avaient une précision diagnostique de 76% et 82% pour diagnostiquer la NAFLD et la NASH, respectivement ; 3) 186 patients répondaient aux critères d'inclusion pour la surveillance du CHC. Le taux de surveillance par ultrasonographie était similaire entre les groupes; cependant, le taux de surveillance par AFP était plus faible dans le groupe des mono-infectés par le VIH comparé aux patients co-infectés ($p<0,0001$). Parmi les raisons expliquant les taux de surveillance sous-optimaux, les facteurs liés au patient étaient plus fréquents chez les patients co-infectés par le VIH/VHC. La surveillance du CHC a été interrompue chez 52 % des

patients mono-infectés par le VIH et 45 % des patients co-infectés par le VIH/VHC en raison d'une réduction des au cours du suivi.

CONCLUSION : La progression de la maladie hépatique diagnostiquée par les NIT est fréquente dans les populations à risque. Il existe encore des lacunes concernant le rôle des NIT dans le diagnostic de la NAFLD et de la fibrose hépatique dans les populations à risque. Les études futures devraient viser la mise en œuvre des NIT et l'optimisation de la surveillance.

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3. Contribution of Authors

Diagnosis of NAFLD and liver fibrosis in patients with T2D

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AA was involved in all steps from study concept and design, acquisition of literature data, interpretation of data, and drafting of manuscripts. KP and VAF were involved in study conception, and critical revision. GS was involved in study conception and design, critical revision and overall study supervision.

NAFLD in LT recipients diagnosed by CK-18 and TE.

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AA was involved in acquisition of data, analysis and interpretation of data, and drafting of manuscripts. PS, PM, MD, PW, PG and TC were involved in study conception and design, critical revision of manuscripts. GS was involved in studies conception and design, interpretation of data, critical revision of manuscript and overall study supervision.

HCC surveillance in HIV-infected patients

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study concept and design, critical revision of manuscripts. GS was involved in studies concept and design, interpretation of data, critical revision of manuscript and overall study supervision.

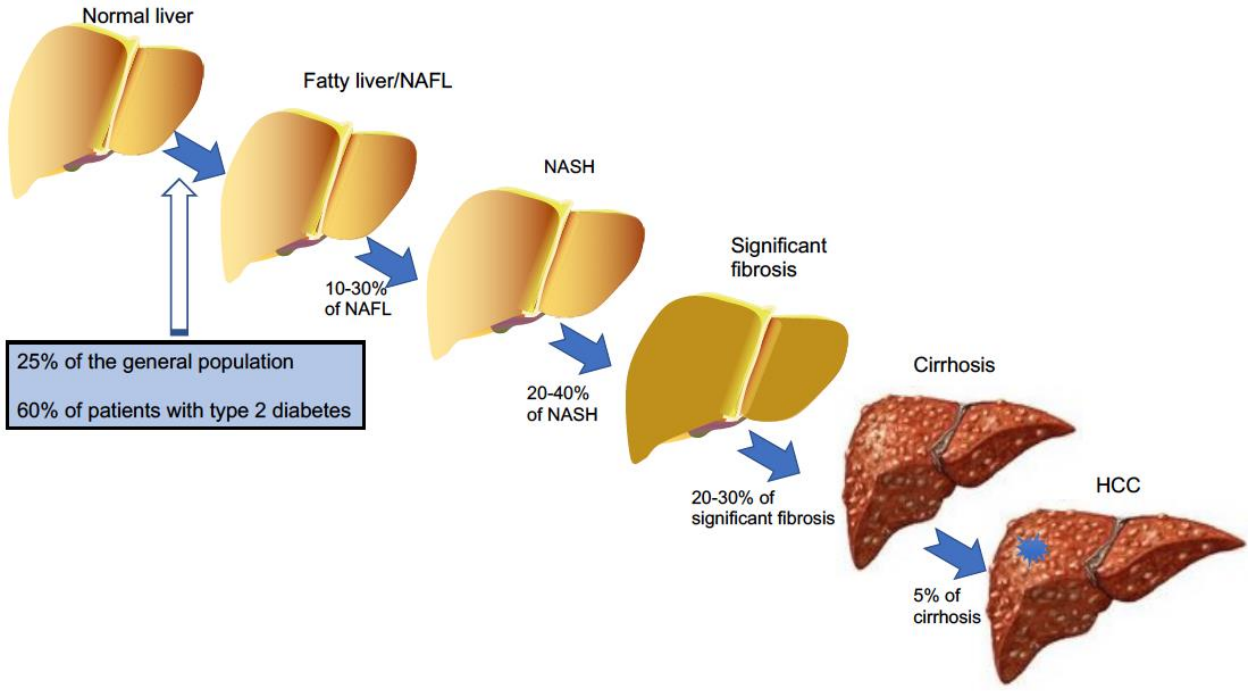
AA wrote, formatted, and revised the whole thesis. GS critically revised all thesis sections.

4. Introduction

Liver diseases are a major health concern and one of the leading causes of death in Canada(1). Nonalcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC) significantly contribute to these figures: NAFLD affects 25% of the Canadian general population, while HCC is the second leading cause of cancer-related death in the world(2, 3).

NAFLD is characterized by a fat overload involving over 5% of the liver weight in the absence of other causes of liver disease. It is a disease spectrum ranging from bland steatosis or nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) (Figure 1).

Figure 1. Progression of liver disease in patients with NAFLD.



If left untreated, NAFL may progresses to NASH, a severe liver disease characterized by a chronic state of necroinflammation that over time leads to liver scarring (fibrosis), cirrhosis, HCC and death(4, 5). Patients with T2D (type 2 diabetes), liver transplant (LT) recipients and people living with HIV represent at-risk populations for NAFLD and HCC.

T2D is closely associated with NAFLD. This close association is due to common underlying pathogenic mechanisms, involving insulin resistance, inflammation, and oxidative stress(6, 7).

T2D represent one of the main risk factors for the development of NASH and liver fibrosis before and after LT(8). It is also reported that T2D is closely associated with HIV(9). Moreover, patients with T2D have higher risk of developing HCC from all etiologies of liver disease, including NAFLD(10).

In recent years, there has been a shift in the etiologies of liver diseases leading to LT: chronic hepatitis C virus (HCV) is declining, while NAFLD is on the rise. NASH is now the second leading indication for LT in North America and is projected to become the main indication over the next 10 years(11, 12). In contrast to alcoholic liver disease, the mitigation of NASH risk factors is not a requirement for transplant eligibility. Hence, risk factors for NASH may persist or worsen after LT, placing these recipients at risk for recurrence. *De novo* NASH in patients transplanted for other etiologies of liver disease can also occur due to excess of metabolic risk factors following LT, including diabetes, rapid weight gain, hypertension, and hyperlipidemia. Immunosuppressive medications may also play a role, as both corticosteroids and calcineurin inhibitors promote diabetes, hypertension and hypercholesterolemia(13, 14).

People with HIV, alone or co-infected with HCV or hepatitis B virus (HBV), have a higher risk of developing HCC than other populations with liver disease(15). In fact, HCC may be more aggressive in those with HIV infection(16). This is due to underlying mechanisms, including oxidative stress, direct activation of stellate cells, and HIV interaction with hepatocytes. HCC incidence continues to grow in patients with HIV despite the use of highly active antiretroviral therapy medications. This is because the incidence of HCC is more influenced by the age and HCV status of the patient(16).

The global prevalence of NAFLD, T2D and HIV continues to rise causing the prevalence of associated complications such as NASH, liver fibrosis and HCC, to increase as well(3, 17, 18). Early identification of liver diseases in these high-risk populations is pivotal to prompt initiation of targeted surveillance as well as provide options for current and investigational therapeutic interventions to reduce disease progression to hepatic failure and mortality.

With time, there has been a shift away from liver biopsy, because it is invasive, costly, and prone to sampling error, and a shift towards using NITs for the diagnosis of liver diseases as they are cost-effective and easy to perform. Most of the literature has focused on the use of NITs to

diagnose NAFLD and liver fibrosis however there are no reviews that combine the role of all NITs to diagnose NAFLD and liver fibrosis in the specific context of T2D.

Several retrospective studies have shown that NAFLD is common after LT, however only a few prospective studies were done on this topic(19, 20).. This is mainly because liver biopsy is an is an impractical tool for serial measurements. Recently, NITs, specifically serum biomarker cytokeratin 18 (CK-18) and the ultrasound-based transient elastography (TE) with controlled attenuation parameter (CAP), have been implemented to diagnose NAFLD and NASH. No study has employed CK-18 to diagnose NASH in LT recipients thus far.

Guidelines advocate for non-invasive surveillance of HCC in high-risk populations using ultrasound (US) with or without alpha-fetoprotein (AFP)(21, 22). These populations include patients with HIV, alone or co-infected with HCV or HBV. Regular surveillance is important because prognosis is better in the early stages of HCC. Early treatment prevents progression to advanced HCC, hence reducing the burden on the healthcare system and reducing mortality. Studies have shown that HCC surveillance is underutilized in patients with cirrhosis due to different etiologies(23, 24). There is limited data on the use of surveillance of HCC in HIV-infected patients.

We aimed to: Specific aim 1) provide an updated review on non-invasive diagnostic tools for NAFLD and liver fibrosis in the specific context of T2D; Specific aim 2) determine the incidence and predictors of NAFLD and NASH in LT recipients using TE with CAP and CK-18, and evaluate the diagnostic performance of the NITs compared to liver histology; Specific aim 3) determine the rate of surveillance of HCC, as well as the reasons for suboptimal surveillance, in HIV-infected patients, with and without HBV and HCV co-infection. Given results from previous studies, we hypothesized that LT recipients will have a high incidence of NAFLD and NASH and that NITs will have a good accuracy as compared to liver biopsies performed as part of clinical care. We also hypothesized that HCC surveillance will be underutilized: numerous factors at the patient, clinician and healthcare system levels could in fact interfere with this monitoring.

5. A comprehensive review of the relevant literature

Specific aim 1: Diagnosis of NAFLD and liver fibrosis in patients with T2D

According to the World Health Organization (WHO), the prevalence of T2D is estimated to be 8.5% of the global population. Importantly, prevalence of NAFLD is increased in patients with T2D compared to the general population, with figures as high as 60–80%(25, 26). A meta-analysis of 24 studies involving 35,599 patients with T2D reported a pooled prevalence of NAFLD at 59.67% (95% confidence interval [CI] 54.31–64.92%)(27). NAFLD and T2D not only coexist due to shared metabolic risk factors but may also act synergistically to drive associated adverse outcomes, such as cardiovascular disease. T2D is associated with the progression of NAFL to NASH and liver fibrosis. In a cross-sectional study using 2011–2014 data from the National Health and Nutrition Examination Survey (NHANES), T2D was the strongest predictor of advanced liver fibrosis, with an odds ratio of 18.20 (95% CI 4.7–70.1), adjusted for age, sex, race/ethnicity, body mass index (BMI), presence of hypertension and presence of diabetes mellitus, twice higher than the odds ratio reported for obesity(28). Moreover, higher rates of HCC and liver cirrhosis have been reported in patients with T2D. On the other hand, NASH is an independent risk factor for development of T2D and is associated with a higher prevalence of both micro- and macrovascular complications in patients with T2D(29). Additionally, patients with coexisting NAFLD and T2D may experience poorer glycemic control, worsening hyperinsulinemia, greater atherogenic dyslipidemia and hypertension(30, 31). Modelling studies in the United States showed that, among NASH cases in 2015, an estimated 20% have advanced liver fibrosis. By 2030, this number is expected to increase over 160% to 7.94 million cases and will account for 29% of NASH cases. Compensated cirrhosis cases among the NASH population may increase by 163% from 1.16 million cases to 3.05 million during 2015–2030, along with an estimated 800,000 excess liver deaths over this period(32). Similar alarming rates have been reported in Canada, where the prevalence of advanced liver fibrosis may nearly double by 2030(33). However, it is important to recognize that these are estimates based on modelling and there is a dearth of prospective epidemiological data, which is urgently needed. The alarming increase of NAFLD, particularly in patients with T2D, resulted in changes in the 2019 guidelines of the American Diabetes

Association (ADA), which for the first-time recommend screening patients with T2D or prediabetes for NASH-related liver fibrosis(34). The question on whether screening patients with diabetes for liver fibrosis related to NAFLD and determining the most efficient diagnostic tool to do so has become a major research topic of interest. The American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of Liver (EASL), and the ADA collectively agree that T2D is an important risk factor for developing NASH and related liver fibrosis. Table 1 reports the guidance on whether patients with T2D should be screened for NAFLD-related liver fibrosis according to these different guidelines.

The AASLD does not recommend population screening for NAFLD-related liver fibrosis, citing a reference concluding that screening is not cost-effective(36). However, Noureddin et al. compared strategies of screening and treatment versus no screening and treatment in hypothetical patients with NAFLD and T2D by a Markov model, showing that screening approaches using US with transaminases and followed by TE may be cost-effective compared with a no screening strategy(37). Another study using the Markov model by Zhang et al. also suggested that, compared to no screening, screening for NASH annually with NAFLD fibrosis score (NFS)/TE/CK-18 algorithm confirmed with magnetic resonance elastography (MRE) may be cost-effective in patients with T2D(38). Table 2 describes the detailed approaches of the two studies. However, a true cost-effective analysis can only be done when an effective and approved intervention is available. This review will focus on the role of diagnostic NITs and strategies specifically studied in T2D to diagnose NASH and to stage NAFLD-related liver fibrosis.

Table 1. Comparison between the AASLD, EASL and ADA guidelines for screening for NAFLD in T2D.

	ADA[34]	**AASLD**[7]	**EASL**[35]	**Further elaboration**
Screening for NAFLD/fibrosis in T2D	Routinely screen in prediabetics and T2D	Do not routinely screen but have a high index of suspicion for NAFLD and NASH in patients with T2D.	Routinely screen irrespective of liver enzyme levels	Focus on screening for NASH
Initial screening tools	Ultrasound and ALT Followed by serum fibrosis biomarkers or MRE or TE	No information provided Biomarkers or TE can be used to identify those at low or high risk for advanced fibrosis.	Ultrasound, transaminases, steatosis biomarkers Followed by fibrosis biomarkers or TE for intermediate risk fibrosis, confirmation with biopsy for high-risk fibrosis.	Clear cut-offs for steatosis (Lower threshold for steatosis as high prevalence of obesity). Simpler tools such as AST or more specific tools instead of tools affected by glucose levels or treatments.
Follow up	Routine follow up	No information provided	Steatosis absent with normal transaminases – follow up 3-5 years Low risk fibrosis – Repeat and follow up in 2 years. Fibrosis or abnormal liver enzymes – reassess every year Cirrhosis – surveillance every 6 months	Clear follow up times required.
When to refer to hepatology	Significant/advanced liver fibrosis by NITs (≥F2)	No information provided	Abnormal liver enzymes and/or medium to high-risk fibrosis and cirrhosis	Specify cut-offs for low, intermediate, and high-risk fibrosis using serum biomarkers and imaging tools

** AASLD American Association for the Study of Liver Diseases, EASL European Association for the Study of the Liver, ADA American Diabetes Association, NAFLD nonalcoholic fatty liver disease, T2D type 2 diabetes, NASH nonalcoholic steatohepatitis, ALT alanine aminotransferase, TE transient elastography, MRE magnetic resonance elastography, AST aspartate aminotransferase.*

Table 2. Cost-effectiveness of screening strategies for NAFLD in diabetes.

Study details	Intervention	Comparator	Cost-effective outcomes
Noureddin et al. Hypothetical cohort of 55-year-old patients with NAFLD and T2D followed over 1 year cycles until death.	6 screening approaches 1. Ultrasound + AST then if NAFLD/NASH likely → liver biopsy 2. Ultrasound + AST then if NAFLD/NASH likely → TE. If TE was suggestive of ≥F2 → liver biopsy 3. Ultrasound + AST then if NAFLD/NASH likely → TE. 4. Ultrasound + ALT then if NAFLD/NASH likely → liver biopsy 5. Ultrasound + ALT then if NAFLD/NASH likely → TE. If TE was suggestive of ≥F2 → liver biopsy. 6. Ultrasound + ALT then if NAFLD/NASH likely → TE.	No screening	Median incremental cost effectiveness ratio: 1. Not cost-effective 2. Not cost-effective 3. Cost-effective ($35,274/QALY) 4. Not cost-effective 5. Not cost-effective 6. Cost-effective ($36,740/QALY)
Zhang et al. Screening in a high-risk population with diabetes	NFS/TE/CK-18 sequential strategy with MRE confirmation for advanced fibrosis	Screening in the general population	Incremental cost effectiveness ratio: Cost-effective ($7,991/QALY)

** NAFLD nonalcoholic fatty liver disease, T2D type 2 diabetes, AST aspartate aminotransferase, NASH nonalcoholic steatohepatitis, ALT alanine aminotransferase, TE transient elastography,AST aspartate aminotransferase, QALY quality-adjusted life year, NFS NAFLD fibrosis score, CK-18 cytokeratin 18, MRE magnetic resonance elastography.*

Specific aim 2: NAFLD in LT recipients diagnosed by CK-18 and TE.

About 20% and 10% of LT recipients develop *de novo* NAFLD and NASH, respectively(14). Recurrent NAFLD and NASH can be as frequent as 62% and 33%, respectively. NAFLD is a common occurrence within 6 months, whereas the onset of NASH occurs in a period of 6 months to 1 year in several studies(39). Due to these reasons, LT recipients may require monitoring to detect changes to the liver graft and prevent hepatic failure and mortality. The majority of studies evaluating recurrent NAFLD and NASH in LT recipients have been of retrospective nature, with no serial monitoring. Hence, longitudinal, prospective data on frequency of NAFLD and NASH are lacking in the first months following LT. Protocol biopsies have long been used to identify liver disease recurrence and guide management. However, liver biopsy is invasive, costly and prone to sampling error(40). Recent NITs for the diagnosis of hepatic steatosis and fibrosis include the measurement of liver stiffness by TE and the associated CAP(5, 41-43). The accuracy of TE for the diagnosis of liver graft fibrosis seems similar to the non-transplant population(44). Only one study has investigated the accuracy of CAP in the post-transplant setting(45). Serum CK-18 has been proposed for the non-invasive diagnosis of NASH. CK-18 is the major intermediate filament protein in the liver and one of the most prominent substrates of caspases during hepatocyte apoptosis. Apoptotic cell death of hepatocytes is associated with release of caspase-cleaved CK-18 fragments into the bloodstream. Apoptotic activity occurs in NASH but not in NAFL, as such the presence of CK-18 fragments in the blood may differentiate the two conditions(46-49). In a meta-analysis of over 1,600 patients CK-18 predicted the presence of NASH with a pooled area under the curve (AUROC) of 0.82(50). One report suggests that CK-18 could also have a prognostic value in predicting one-year survival post-LT(51). Thus far, no study has employed CK-18 to diagnose NASH in LT recipients. We aimed to determine the incidence and predictors of NAFLD and NASH in liver LT using TE with CAP and CK-18 and evaluate the diagnostic performance of the NITs compared to liver histology.

Specific aim 3: HCC surveillance in HIV-infected patients

HIV continues to be an important public health concern affecting approximately 37.6 million people worldwide in 2020 (www.unaids.org). In Canada, there were approximately 63,110 people living with HIV in 2016, an increase of 2,945 people (5%) since 2014. According to

statistics Canada, every 4 hours one person was infected with HIV in 2018. Due to continuous transmission, the number of newly infected HIV patients is expected to increase. With the development of highly active antiretroviral therapy, HIV related illnesses are decreasing, and patients tend to live healthier lives. However, infected patients are still prone to diseases not related to HIV. HCC has emerged as a major disease in this population. The incidence of HCC is increasing with a reported incidence of 16 cases per 100,000 population(52). It is the second leading cause of cancer-related death in the world with a 5-year survival rate of only 10-15%(2, 3, 53). HCC is responsible for a significant economic burden on healthcare systems. A study from the USA using National Inpatient Sample database revealed that total inpatient charges related to HCC doubled from $1.0 billion in 2005 to $2.0 billion in 2009(54). HCC is closely associated with HIV(55) and the main reason for this is the co-infection with chronic viral hepatitis (HBV and HCV). Globally, the estimated number of HIV patients co-infected with HBV and HCV is 2.7 million and 2.3 million, respectively(56). Studies have shown an increased incidence of HCC in populations with HIV/HBV or HIV/HCV(57-59). The main mechanisms in which HCC develops in these groups is the ongoing inflammation caused by viral hepatitis, leading to cirrhosis; and HIV-related reduced immune response causing hepatitis viral loads to increase, leading to inflammation and tumorigenesis(56).

Guidelines from the EASL, AASLD and the Asian Pacific Association for the Study of the Liver (APASL) advocate surveillance for HCC in high-risk populations every 6 months using US. Surveillance aims to reduce mortality rates and lessen the burden on the health care systems. In a meta-analysis of 47 studies involving 15,158 patients, HCC surveillance was associated with early HCC detection and improved overall survival (60). Surveillance was also shown to reduce HCC-related mortality in patients with chronic hepatitis B(61). Additionally, early diagnosis of HCC provides patients with an eligibility to a variety of treatment options, including hepatic artery embolization, tumor resection and chemotherapy, as opposed to fewer treatment options with a late diagnosis(60). The recommended tools for the surveillance of HCC are the US and AFP. The US is an easy to use and cost-effective tool with excellent accuracy for detection of liver masses. A meta-analysis of 13 studies determined that the performance of US to detect HCC at any stage had a pooled sensitivity of 94%(62). AFP is a widely available serum marker which is commonly used as an adjunct to US for the detection of liver cancers. There are conflicting reports of the role of AFP in detecting HCC when combined with US. A meta-

analysis of 19 studies showed that using a combination of US and AFP compared to using US alone was less specific at detecting early HCC and was not cost-effective(63). Another study of 1,597 patients with cirrhosis concluded that when using a cutoff level at 20 ng/ml and AFP level increase of ≥2 times from its nadir in the past 12 months, along with US, attained a sensitivity of 99.2% and specificity of 68.3%, whereas using US alone had a sensitivity of 92.0% and a specificity of 71.5%(64). A randomized control trial involving 18,816 people with HBV reported a 37% reduction of HCC mortality with a compliance rate of approximately 60% using a combination of US and AFP for surveillance(65). Despite guidelines have not specifically recommended the use of AFP as part of the screening tools for HCC, these recent data suggest an increase in diagnostic accuracy for HCC detection when US is combined with AFP. For this reason, we defined adequate surveillance as at least yearly examination by US with or without twice a year determination of AFP in our study. We aimed to determine the rate of surveillance of HCC, as well as the reasons for suboptimal surveillance, in HIV-infected patients, with and without HBV and HCV co-infection.

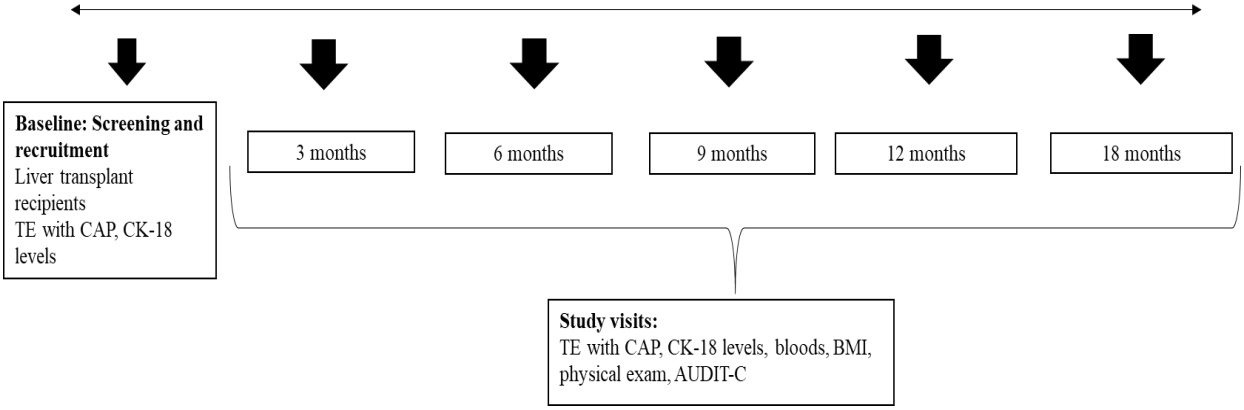
6. a) Methods

Specific aim 1 –Diagnosis of NAFLD and liver fibrosis in patients with T2D: We performed a search of studies on NITs used to diagnose NAFLD and liver fibrosis pertaining to patients with T2D. We included the definitions and roles of specific NITs, their strengths and limitations, and their accuracy in diagnosing NAFLD, and liver fibrosis in patients with T2D. We also included studies of a combination of NITs for specialist referral for NAFLD and related fibrosis in patients with T2D.

Specific aim 2 – NAFLD in LT recipients diagnosed by CK-18 and TE: We performed a prospective, longitudinal study conducted at a single center, the McGill University Health Center (MUHC) Solid Organ Transplant Unit. It involved all eligible and consecutive adult patients who received LT between 2015 to 2018. Inclusion criteria were the following: age >18 years; patient and graft survival >6 months; a minimum follow-up of 1 year. Exclusion criteria were any of the following: LT due to chronic hepatitis C, genotype 3; patients who received liver grafts

involving more than 10% steatosis; failure of TE with CAP examination or unreliable measurement at study entry. Post-transplant, patients were followed for a total of 5 study visits over a period of 18 months (Figure 2).

Figure 2. Study plan showing baseline and study visits.



Since 1990, a computerized database on all LT recipients has been maintained into which demographic data, clinical diagnosis, laboratory results, and prescription information have been prospectively entered. Patients were followed until March 2020 or were censored either when they died, developed the outcome, or at their last study visit. A complete medical history and physical examination was done at each study visit, along with the following parameters: body mass index (BMI), laboratory tests for hematology, blood chemistry. The questionnaire Alcohol Use Disorders Identification Test (AUDIT-C) was administered(66). TE with CAP measurement and plasma to measure CK-18 were also acquired at each study visit. TE examination was performed in patients fasting for at least 3 hours using FibroScan 502 Touch (Echosens, Paris, France). The same two experienced operators performed all elastographic measurements. The standard M probe was used in all patients. The XL probe was used in cases of failure of TE with the M probe or if BMI >30 Kg/m2. The following criteria were applied to define the result of TE as reliable: at least 10 validated measurements and an interquartile range (IQR) <30% of the median liver stiffness measurement (LSM)(67). The immunosuppressive regimen used as a

standard by the LT program is induction with anti-thymocyte globulin and started on tacrolimus and mycophenolate mofetil as maintenance immunosuppression and rapid prednisone taper. Overweight and obesity were defined as BMI >25 and >30 Kg/m2, respectively. Liver biopsy was performed at discretion of the treating transplant hepatologist, as part of standard of care. All biopsies were obtained with 16G Tru-Cut type needle and interpreted by two experienced liver pathologists. The stage of fibrosis was reported according to the Kleiner classification(68). The threshold used to define significant liver fibrosis was histological stage 2 out of 4 by (>F2). The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3) and hepatocellular ballooning (0–2). A diagnosis of NASH was made if NAS ≥5(68). The CAP cut-off used for diagnosis of NAFLD was 270 dB/m, as recently reported in LT recipients(45). Plasma stored at -80C was used for quantitative measurement of CK-18 levels by the Human cytokeratin ELISA kit (MJS Biolynx inc, Brockville Ontario, Canada). A cut-off of CK-18 >130.5 U/L was used to indicate significant hepatocyte apoptosis, diagnostic for NASH when combined with CAP >270 dB/m(69, 70). Liver fibrosis (stage >1 out of 4) was diagnosed as liver stiffness measurement ≥7.4 kPa(45). The following simple serum fibrosis biomarkers were also computed: hepatic steatosis index (HSI), defined as 8 x AST/ALT + BMI (+2, if female; +2, if diabetes)(71), fibrosis-4 (FIB-4), calculated as [age (years) x AST]/[platelet count (10^9/L) x √ALT)(72), and AST to platelet ratio (APRI), calculated as [(AST level/AST (upper limit of normal))/platelet count (10^9/L) X 100](73). Liver fibrosis was defined as FIB-4 >3.64 and APRI >1, as previously described in the LT setting(74). The performance of the NITs to diagnose NAFLD, NASH and significant liver fibrosis was compared to liver histology and measured with the following: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, positive and negative likelihood ratios (LR + and LR−, respectively). Correlation coefficients of LSM and CAP with serum biomarkers were calculated using the Pearson correlation analysis. We estimated incidence rates of NAFLD, NASH and significant liver fibrosis by dividing the number of participants developing the outcome by the number of person-years (PY) of follow-up. Clopper-Pearson exact model was used to calculate confidence intervals (CI) for incidence rates. Multivariable time-dependent Cox regression models were constructed to assess predictors of NAFLD and NASH and included covariates that were determined a priori to be clinically important. The final model was adjusted for sex, BMI and ALT. We generated Kaplan-Meier

curves to illustrate and compare the cumulative incidence of NAFLD and NASH in overweight vs. normal weight patients. The log rank test was used to evaluate differences among distributions. Changes in LSM, FIB-4 and APRI scores during follow-up were illustrated using a spaghetti plot.

Specific aim 3 - HCC surveillance in HIV patients: This was a retrospective analysis of the LIVEr disease in HIV (LIVEHIV) Cohort. The LIVEHIV Cohort is a prospective screening program for liver fibrosis and NAFLD in people living with HIV running at the MUHC since 2013. Patients undergo HCV and HBV serology, US, TE with CAP, and routine liver tests. It includes over 1,000 patients with active HIV. We included patients eligible for HCC surveillance from the Cohort. Inclusion criteria were the following: HIV mono-infection and HIV/HCV co-infection with LSM ≥10 kPa, HIV/HBV co-infection regardless of LSM score; and a follow-up time of at least 12 months. Patients with decompensated liver cirrhosis were excluded. Optimal surveillance was defined as at least yearly examination by US with or without twice a year determination of AFP. We performed the analysis to determine surveillance using a bar chart and Pearson's chi-square test. Individual reasons for suboptimal surveillance were also reported using Pearson's chi-square test and pie charts.

Continuous variables were expressed as mean (standard deviation), and categorical variables were presented as numbers (%). We considered an association with the outcome significant when the 95% confidence interval (CI) excluded one. All tests were two-tailed and with a significance level of $\alpha = 0.05$. Statistical analysis was performed using IBM® SPSS Statistics.

6. b) Results

Specific aim 1: diagnosis of NAFLD and liver fibrosis in patients with T2D:

Liver biopsy

Liver biopsy is the gold standard test to assess for transition NAFL to NASH(75). Histological examination allows for direct visualization of the liver parenchyma, therefore liver biopsy is

considered a reference tool to stage liver fibrosis. It also remains the only available technique, recognized by government agencies, to diagnose NASH(76). The most frequently used histologic classification adopted for the diagnosis and staging of NASH is the NAFLD activity score (NAS). The diagnosis is based on three components (steatosis, score 0–3; lobular inflammation, score 0–3; hepatocyte ballooning, score 0–2), with a cumulative NAS score ≥ 5 indicating severe NASH changes(68). Liver fibrosis is evaluated separately from NAS and is classified into 5 stages, from no (stage 0)/minimal fibrosis (stage 1) to cirrhosis (stage 4). Stage >2 liver fibrosis, which is also defined as significant liver fibrosis, is a threshold that indicates a progressive liver disease which will eventually lead to cirrhosis and end-stage complications (Figure 1). Advanced liver fibrosis is defined as stage >3, while cirrhosis is stage 4(77). Other scoring systems that combine Steatosis, Activity and Fibrosis (SAF score) have recently been adopted for grading and staging NAFLD patients(78). However, liver biopsy remains an invasive and costly procedure, and cannot be used as a large-scale screening tool or for serial measurements(40). Liver biopsy is also liable to sampling error when an insufficient amount of tissue is collected(79). The diagnosis of NASH and liver fibrosis can be overlooked or inconsistently graded due to sampling error and significant heterogenous distribution of histologic changes in the liver(80). Additionally, inter- and intra-observer pathologist variability further reduces reliability of biopsy to diagnose NASH and liver fibrosis(81). In a prospective multicenter study involving 150 patients with NAFLD, liver biopsies were evaluated by pathologists at local institutions and by pathologists specialized in liver pathology. The study concluded that the concordance rates to evaluate NASH and fibrosis among the two groups of pathologists was poor(82). As a result of these liver biopsy limitations, there has been a shift towards using NITs to stage liver fibrosis related to NASH and to diagnose NAFL (hepatic steatosis) as they are cost-effective and reproducible. These include serum biomarkers and imaging assessments which, when used together, provide information on the various degrees of liver steatosis and fibrosis. Initially, these NITs were limited and developed to detect significant fibrosis due to chronic hepatitis C. With time, they are increasingly used in clinical practice and have become acceptable alternatives to liver biopsy to evaluate NAFLD and to stage related liver fibrosis.

Liver transaminases

Liver transaminases are routinely used in the assessment of all liver diseases, including NAFLD. Specifically, alanine aminotransferase (ALT) is the liver enzyme that has been shown to be higher in patients with diabetes and NAFLD as compared to diabetic patients without NAFLD(83). However, liver transaminases alone have limited accuracy for diagnosing NAFLD or liver fibrosis, and a significant proportion of both diabetic and non-diabetic patients with NASH might have normal aminotransferase levels(84). In a prospective study involving 173 patients, ALT had a low accuracy in predicting NASH and liver fibrosis with an area under the receiver operating characteristic curve (AUROC) of 0.61 (95% CI, 0.49-0.74) and 0.57 (95% CI, 0.47-0.68), respectively. At 1-fold upper limit of normal (ULN), the sensitivity and specificity values of ALT to predict NASH were 75% (95% CI 58–92%) and 47% (95% CI 39– 55%), respectively. At 0.5xULN, the sensitivity and specificity values of ALT to predict NASH were 100% (95% CI 100–100%) and 11% (95% CI 6–16%), respectively. Similarly, at 1xULN, the sensitivity and specificity values to predict significant fibrosis were 64% (95% CI 51–78%) and 47% (95% CI 38–56%), respectively. At 0.5xULN, the sensitivity, and specificity values to predict significant fibrosis were 93% (95% CI 86–100%) and 10% (95% CI 5–15%), respectively(85). Thus, the use of transaminases alone to diagnose NAFLD can be challenging as it does not reflect the severity or stage of the disease nor does it confirm the diagnosis.

Biomarkers

Serum biomarkers are generally used as the initial screening tools for NAFLD along with or as a precursor to imaging studies. The EASL recommends that whenever imaging tools are not available, serum biomarkers are an acceptable alternative for the diagnosis of NAFLD(35). They are generally considered cost-effective and widely available tools. Although there are numerous studies on their value in patients with chronic liver diseases, only a few biomarkers have been assessed in patients with T2D, most of them studied in small populations with mixed results. These include either clinical scores such as Fatty Liver Index (FLI), Fibrosis-4 (FIB-4) index and NFS, or patented biomarkers performed in commercial laboratories, such as N-terminal type III collagen propeptide (PRO-C3), Enhanced liver fibrosis test (ELF™) (Siemens Healthineers, Erlangen, Germany), and FibroTest-ActiTest and SteatoTest/NashTest (BioPredictive, Paris,

FR). FLI and SteatoTest are used to evaluate steatosis, while FIB-4, NFS, PRO-C propeptides, and Enhanced Liver Fibrosis Test are used to evaluate liver fibrosis.

Imaging tools

With advances in technology, several imaging tools are emerging as accurate diagnostic tools for detecting and quantifying hepatic steatosis and fibrosis. Since biomarkers adopt two cut-off values, one to rule out and one to rule in NAFLD and liver fibrosis, a significant proportion of patients could fall within the underdetermined risk zones (or grey area) using biomarkers alone. Imaging methods to assess liver stiffness and fat quantification are generally used as a second step in determining the degree and extent of liver pathologies. Where they are readily available, some centers use imaging tools as a first step for screening for NAFLD-related liver fibrosis in T2D patients. Certain imaging tools have shown excellent correlation to liver histology, potentially allowing for avoiding liver biopsy for staging advanced fibrosis or grading steatosis if these imaging tools are available.

Biomarkers

Biomarkers for steatosis

a. Fatty Liver Index (FLI)

FLI is a model for the prediction of fatty liver calculated using the formula: FLI = ey/(1 + ey) × 100, where y = 0.953 × ln(triglycerides, mg/dL) + 0.139 × BMI, kg/m2 + 0.718 × ln (gamma glutamyltransferase [GGT], U/L) + 0.053 x waist circumference, cm - 15.745(86). It was constructed to include two of the strongest risk factors for fatty liver, namely BMI and waist circumference. A score of FLI <30 rules out fatty liver while a score of FLI ≥60 rules in fatty liver(86). In studies with patients without diabetes, FLI was shown to accurately detect fatty liver with an AUROC of 0.834 (95% CI: 0.825–0.842)(87). However, in patients with diabetes the biomarker is not as accurate. In a study of 220 patients with T2D, the AUROC for FLI to identify hepatic steatosis was only 0.647 (95% CI 0.58–0.71) with a low correlation of 0.281 (95% CI 0.158 to 0.403) compared to magnetic resonance spectrometry (MRS), a highly sensitive imaging method considered the gold standard to quantify liver fat(88). Another study where 162 participants with diabetes were diagnosed with steatosis using FLI score >60, FLI erroneously diagnosed fatty liver in 43 participants (24.0%) and failed to diagnose it in 5 participants (2.7%)

in comparison to MRS(89). The reason could be because FLI was not developed for the population with diabetes. Based on these results, FLI alone does not appear to be an accurate tool to diagnose fatty liver; however, it could be used to risk stratify patients with T2D for further investigations. The main drawback of this tool is the possibility of false positivity as its components fluctuate with acute conditions.

b. SteatoTest

The components of the SteatoTest include BMI, serum cholesterol, triglycerides, glucose adjusted for age and gender, GGT, total bilirubin, alpha-2-macroglobulin, apolipoprotein A1, haptoglobin and ALT. The predetermined SteatoTest threshold score for moderate to severe steatosis is 0.57(90).This commercial panel has been studied in small cohorts of patients with diabetes. Recently, Poynard et al. concluded that the performance of SteatoTest was not different in patients with T2D compared to patients without T2D when using the Obuchowski measure(91). Similar to FLI, it may give false positive diagnosis of NAFLD in patients with T2D. Two studies showed that SteatoTest underperforms in detecting NAFLD proven by MRS in patients with diabetes, with an AUROC of 0.73 (95% CI 0.65 to 0.81) and 0.674 (95% CI 0.608–0.736)(88, 92). Another study showed that the SteatoTest erroneously diagnosed NAFLD in 20.1% of participants with diabetes compared to MRS and failed to diagnose it in 8.9% participants(89). The SteatoTest is not the best non-invasive tool to diagnose NAFLD in patients with diabetes but has a prognostic value in overall survival in patients with T2D(93).

<u>Biomarkers for fibrosis</u>

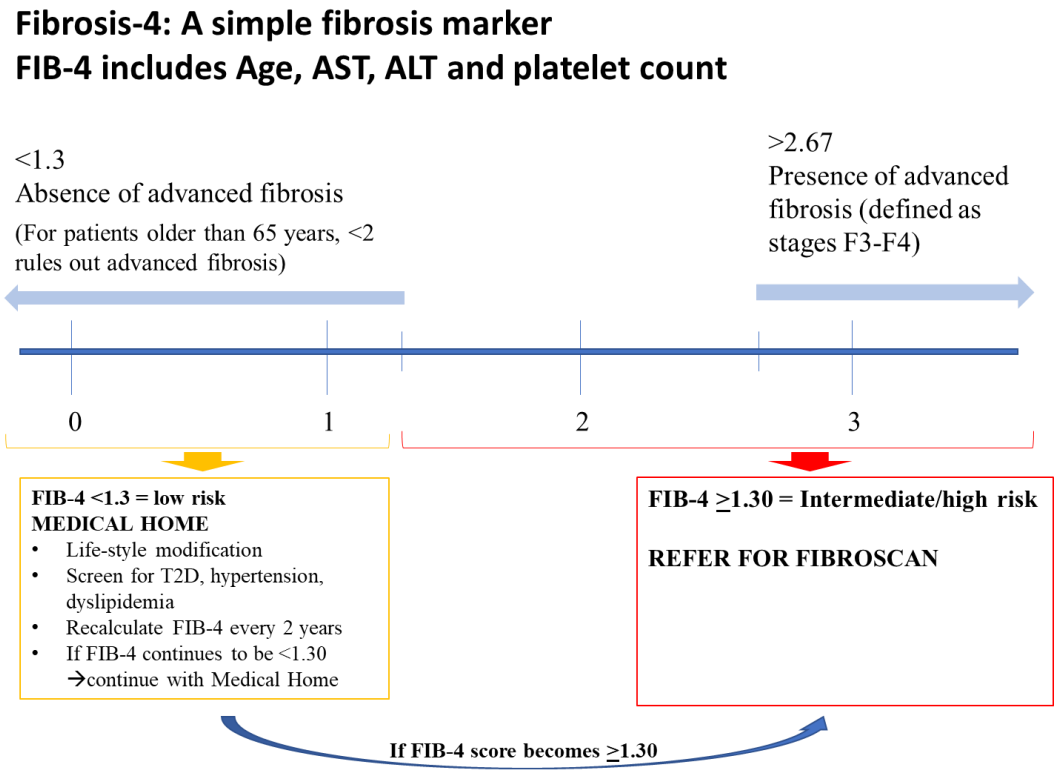
a. Fibrosis-4 (FIB-4) index

The FIB-4 index is a commonly used biomarker to detect advanced fibrosis in the context of NAFLD. As its name indicates, it has four components to determine fibrosis: age, aspartate aminotransferase (AST), ALT, and platelet count, initially developed for detection of advanced fibrosis in patients with HIV and hepatitis C co-infection(94). For NAFLD, a FIB-4 index of >2.67 has a positive predictive value of 50% for advanced fibrosis with 97% specificity while a score <1.3 has a high negative predictive value for advanced fibrosis at 95%(95). A meta-analysis of 6 studies with 1910 NAFLD patients indicated summary sensitivity and specificity of 31.9% and 95.7%, respectively for FIB-4 >2.67 for advanced fibrosis. FIB-4 index score <1.3

has been proposed to increase test sensitivity, and this threshold is now used as a screening test to exclude advanced fibrosis in NAFLD patients(96, 97). However, ~30% of patients may have FIB-4 scores 1.3–2.67 and are categorized as being "indeterminate" for advanced fibrosis due to unreliable test accuracy in this range (Figure 3).

Figure 3. Clinical use of FIB-4 to screen for NAFLD-related liver fibrosis in patients with T2D.

https://www.mdcalc.com/fibrosis-4-fib-4-index-liver-fibrosis



FIB-4 is among the best studied biomarkers in the population with diabetes. A recent study concluded that the performance of FIB-4 was not different in patients with T2D vs. patients without T2D when using the Obuchowski measure(91). In a study of 191 patients with diabetes who underwent MRS followed by liver biopsy if NAFLD was present, FIB-4 had an AUROC of 0.83 and 0.86 to detect moderate-to-advanced fibrosis and advanced fibrosis, respectively(98). Bertot et al. reported that in 124 patients with diabetes and biopsy proven NAFLD, FIB-4 had an

AUROC of 0.79 (95% CI 0.71–0.87) and 0.80 (95% CI 0.71–0.90) for predicting fibrosis stages F3–4 and F4, respectively. However, the same study indicated that FIB-4 had a lower accuracy for predicting F4/cirrhosis among patients with diabetes compared to patients without diabetes(99). Otherwise, Nones et al. concluded that FIB-4 had an AUROC of 0.830 for detecting stage ≥F2 fibrosis in 67 patients with diabetes and NAFLD confirmed by histology, with high negative predictive value (93.48%) for ruling out severe liver fibrosis (stages 3 and 4)(100). The FIB-4 may also be used to monitor disease progression. A large study by Filozof et al. in T2D patients reported that FIB-4 values obtained over several years detect progressive fibrotic changes and help identify patients at risk of liver outcomes.(101) The FIB-4 is a simple, low cost, and easily available test; however, its limitations include reduced specificity in older adults. For this reason, a higher cut-off of 2 to rule-out advanced liver fibrosis has been proposed in patients older than 65 years(102).

b. NAFLD fibrosis score (NFS)

This score was first developed in 2007 to specifically identify patients with advanced liver fibrosis in NAFLD(103). The parameters included in the score are age, BMI, the presence of diabetes or impaired fasting glucose, AST, ALT, platelets, and albumin. A score >0.676 rules in advanced fibrosis and identifies patients who are at higher risk for development of liver-related complications and mortality while a score of < -1.455 rules out advanced fibrosis(103). A meta-analysis of 10 studies with 3057 NAFLD patients indicated summary sensitivity and specificity of 72.9% and 73.8%, respectively to exclude advanced fibrosis for NFS < -1.455(97). As for its potential role in the population with diabetes, Nones et al. suggested that the NFS model had a high negative predictive value (93.6%) in patients with diabetes and severe liver fibrosis (stages F3–4)(100). Bertot et al. reported a similar accuracy of NFS to detect advanced fibrosis in patients with diabetes as compared to patients without diabetes(99). Similar to the FIB-4 index, ~30% of patients may have indeterminate range scores with poor test accuracy for advanced fibrosis, requiring second-line non-invasive serum tests or imaging elastography for further evaluation prior to consideration of liver biopsy.

c. FibroTest

FibroTest is a commercial panel that measures the levels of fibrosis by using the following: alpha2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin, and GGT, adjusted for

age and gender. FibroTest scores range from zero to 1.00 with higher values indicating a greater probability of significant lesions. A FibroTest score >0.58 is suggestive of advanced liver fibrosis(104). It is a validated tool for the measurement of fibrosis in patients with NAFLD, viral hepatitis and other chronic liver diseases(61). FibroTest has been studied to detect advanced fibrosis in patients with T2D, however the results were suboptimal. Jacqueminet et al. used FibroTest to screen 1131 patients with diabetes and no known chronic liver diseases, and found advanced fibrosis in 5.6%, which was confirmed using liver stiffness measurement in only 50% of the patients(105). Bril et al. reported that the performance of FibroTest to identify patients with moderate or advanced fibrosis was 0.67 (95% CI 0.58 to 0.76) and 0.72 (95% CI 0.61 to 0.83), respectively in T2D patients with NAFLD proven by MRS(88). FibroTest has shown to provide prognostic value for predicting overall survival in patients with T2D(93). A limitation of this test is the possibility of producing false positive results affected by acute hepatitis, acute hemolysis, inflammation, or extrahepatic cholestasis, so its interpretation requires careful analysis(61).

d. PRO-C3 propeptides

During the early process of liver fibrosis, there is increased synthesis and release of collagen in the interstitial space, mainly type III collagen(106). PRO-C3 is a biomarker that detects the formation of type III collagen and can be measured in the serum using ELISA methods(107). Initial studies showed that PRO-C3 reflects liver fibrogenesis in chronic liver diseases such as chronic hepatitis C and could also mirror liver dysfunction in HIV positive patients receiving antiretroviral therapy(106, 108, 109). PRO-C3 also has promising value in the diagnosis of NAFLD related fibrosis in patients with T2D. In a cohort of 191 patients with T2D, PRO-C3 performed well for the diagnosis of significant and advanced liver fibrosis (AUROC 0.88 [95% CI 0.80–0.95], and 0.81 [95% CI 0.74–0.88], respectively). After 18 months, PRO-C3 changes were associated with changes in fibrosis stages. The same study reported that, compared to other biomarkers, performance of PRO-C3 was similar to that of FIB-4(98). A recent study in 213 patients with T2D and biopsy, with F3–4 prevalence of 19%, indicated that AST, PRO-C3 and another well validated simple fibrosis biomarker, AST to platelet ratio index (APRI), had higher AUROC (0.85–0.90) compared to NFS, FibroTest, and FIB-4 (0.64–0.78) for advanced fibrosis. A sequential combination of AST followed by PRO-C3 was able to reduce the number of

biopsies required to diagnose advanced fibrosis(110). Importantly, PRO-C3 is not routinely available, and is not established as a standalone diagnostic for NAFLD.

e. Enhanced liver fibrosis test (ELF)

The ELF is a commercial panel that measures the degree of fibrosis with three direct markers: hyaluronic acid, procollagen III amino-terminal peptide, and tissue inhibitor of matrix metalloproteinase 1. An ELF score ≥9.8 accurately indicates the presence of advanced fibrosis in patients with chronic liver diseases, including NAFLD(111, 112). The United Kingdom National Institute for Health and Care Excellence (NICE) guidelines recommend using the ELF test as the first-line test for advanced fibrosis in patients with NAFLD. In a meta-analysis of nine studies involving 1826 patients, the pooled sensitivity and specificity values of ELF test for assessment of significant liver fibrosis were 83% (95% CI 0.80–0.86) and 73% (95% CI 0.69–0.77), respectively; and for evaluation of advanced liver fibrosis were 78% (95% CI 0.74–0.81) and 76% (95% CI 0.73–0.78), respectively; and for estimation of cirrhosis were 80% (95% CI 0.75–0.85) and 71% (95% CI 0.68–0.74), respectively(113). The ELF test has also been shown to outperform liver biopsy for risk stratifying patients with cirrhosis for liver related complications, such as HCC(114). For its role in the population with diabetes, one study of 252 patients from an endocrine clinic or primary care facility concluded that ELF ≥9.8, with concordant liver stiffness measurement (LSM) ≥8.2 kPa, and had a high negative predictive value (91.7%) and positive predictive value (95.8%) for excluding and identifying significant liver fibrosis, respectively(115). The main drawbacks are its availability and cost-effectiveness. Furthermore, its utility in identifying advanced fibrosis in patients with T2D requires validation.

f. AST to platelet ratio index (APRI)

APRI provides an estimation of liver fibrosis and is calculated by dividing the AST level (IU/L), expressed as the number of times above the upper limit of normal (ULN), by platelet count (109/L): AST (/ULN) × 100/platelet count (109/L)(73). APRI is a simple, inexpensive test and has been shown to have an acceptable accuracy in diagnosing patients with hepatitis C(116). There are very few studies on its role in diagnosing NAFLD-related fibrosis in patients with T2D. One study showed that in 1429 patients with T2D assessed for advanced fibrosis using different biomarkers, APRI greatly varied in the diagnosis of advanced fibrosis as compared to NFS, FIB-4 and AST-ALT ratio. The study also showed that a large proportion of patients fell in

the indeterminate zone risk using APRI(117). However, Bril et al. concluded that APRI has an AUROC of 0.86 (95% CI 0.80–0.91) to diagnose advanced fibrosis in T2D(110). Further larger studies are required to determine its accuracy in patients with diabetes.

Biomarkers for NASH

a. Cytokeratin-18 (CK-18)

CK-18 is the major intermediate filament protein in the liver and one of the most prominent substrates of caspases during hepatocyte apoptosis. During hepatocyte apoptosis, caspase-cleaved CK-18 fragments are released into the bloodstream(48). Several studies have shown that CK-18 tends to be higher in patients with NAFLD or NASH as compared to patients without NAFLD(49, 118). Similarly, several studies involving patients with NAFLD and T2D have also shown that CK-18 was higher in patients with NAFLD than without(119, 120). Of note, CK-18 levels are influenced by inflammation due to various liver injuries and diseases(121, 122). They are also influenced by T2D, thresholds for optimal CK-18 levels are not established, and assays are not routinely available. For these reasons, CK-18 appears to have limited diagnostic utility as a standalone marker for NASH. To date, there is no single biomarker that can accurately diagnose NASH in patients with diabetes. In one study, Bril et al. used several biomarkers (CK-18, NashTest 2, and others) to identify NASH in patients with T2D and concluded that none of the biomarkers had optimum performance(110). In another study by the same author, the SteatoTest, Acti-Test and NashTest-2 also underperformed to identify T2D patients with NAFLD [AUROC 0.73 (95% CI 0.65 to 0.81), 0.70 (95% CI 0.63 to 0.77) and 0.69 (95% CI 0.62 to 0.76), respectively].

Imaging tools

Imaging tools to diagnose steatosis

a. Ultrasound

The ADA and EASL recommend ultrasound as one of the first-line imaging method for detecting NAFLD. It is a commonly available, affordable and easy to use tool of imaging for steatosis of the liver. Ultrasound provides a 4-point qualitative scoring system to grade the severity of NAFLD: normal, mild, moderate and severe(123). The accuracy of the ultrasound for the

detection of moderate and severe fatty liver compared to liver biopsy is reported to be >80%(124). B-mode ultrasound has also shown to be an effective screening tool of NAFLD in patients with diabetes. Two different studies by Mantovani et al. showed that the prevalence of NAFLD in a population of Italian patients with T2D detected on ultrasound was 71.2% and 73.7%(125, 126). Targher et al. conducted a cross-sectional study enrolling 2839 patients with diabetes in Italy and found a 71.1% age-adjusted prevalence of NAFLD in men and 68% in women based on ultrasonographic evaluation(127). In keeping with diagnostic tools in NAFLD, ultrasound also comes with limitations. The main one is being an operator-dependent technique which can result in significant inter-observer variability(128). Ultrasound is also unable to quantify the amount of steatosis present in the liver, or provide any staging of NAFLD-related fibrosis, and it cannot detect mild hepatic steatosis (<30% steatosis)(129). Additionally, its sensitivity is reduced by the presence of morbid obesity(130). For these reasons, ultrasound is less accurate in detecting steatosis as compared to other liver imaging tools.

b. Vibration controlled transient elastography (TE)

Over the last decade or so, TE has been preferred to ultrasound for the detection of hepatic steatosis and it also provides simultaneous information on liver fibrosis. This technology utilizes an ultrasound-based device that measures shear wave velocity as a surrogate of liver stiffness. The stiffer the liver, the more fibrotic it is. The patient must be fasting for at least 3 h prior to the test. Several ultrasonound-elastography devices are now available. The most validated of these methods in chronic liver disease is TE (FibroScan, Echosens, Paris, France). This device also includes two probes (M and XL) for use in adult patients depending on body habitus and includes software for rapid and accurate technique to quantitate liver steatosis using controlled attenuation parameter (CAP) measured in decibels per meter (dB/m), and liver stiffness value measured in kilo Pascals (kPa). Compared to liver biopsy, TE has a high accuracy for detecting both steatosis and fibrosis(131, 132). A meta-analysis involving 2735 patients reported optimal cutoffs of CAP are 248 dB/m for steatosis grade 1, 268 dB/m for grade 2 and 280 dB/m for grade 3, with pooled sensitivities and specificities of 69% and 82% for ≥grade 1, 77% and 81% for ≥grade 2, and 88% and 78% for grade 3(131). As for liver fibrosis, based on patient data from 6 independent prospective cohorts involving 6295 participants, the optimal liver stiffness thresholds for patients with metabolic risk factors for NAFLD is 9.1 kPa for fibrosis stages

≥F2(133). Several studies demonstrated that TE has an important screening role in the diagnosis of NAFLD and hepatic fibrosis in patients with T2D. Kwok et al. examined 1918 patients with T2D and reported a prevalence of NAFLD and advanced fibrosis by Fibroscan of 72.8% and 17.7%, respectively(25). Similarly, de L´edinghen et al. examined 277 patients with diabetes and the prevalence of severe fibrosis to be 15.5%(104). In 137 patients with diabetes, Mantovani et al. reported a proportion of significant liver fibrosis of approximately 18% with an LSM cut-off ≥7 kPa and nearly 10% with an LSM cut-off ≥ 8.7 kPa(126). Sporea et al. used the following TE cutoffs to discriminate between fibrosis stages: 8.2 kPa for F ≥ 2, 9.7 kPa for F3–4, and 13.6 kPa for F4, and the following CAP cutoffs to discriminate between steatosis grades: 274 dB/m (grade 1), 290 dB/m (grade 2), 302 dB/m (grade 3). The authors also reported a prevalence of grade 3 steatosis of 60.3%, while 19.4% had advanced fibrosis in 776 patients with diabetes(134). Unlike standard ultrasound, TE is not as widely available. It may also produce unreliable results in acute hepatic inflammation, congestion, cholestasis, ascites, or portal vein thrombosis, and is contraindicated in pregnant patients or patients with intra-cardiac devices. Failure to obtain any measurements or chances of unreliable results can happen especially with operator inexperience or in patients with morbid obesity(135). As obesity is very common in patients with diabetes, the role of TE for routine screening of steatosis and advanced fibrosis could be challenging. However, the use of the XL probe in these patients might yield more reliable results(136).

There are several other ultrasonographic-based imaging tools that have proven to detect and stage NAFLD related liver fibrosis, such as point shear wave elastography and acoustic radiation force impulse; however, there are very few to no studies of their role in patients with T2D.

c. Magnetic resonance imaging-proton density fat fraction (MRI-PDFF)

MRI-PDFF is an imaging-based biomarker which quantifies liver fat content. It uses PDFF, which reflects the concentration of mobile triglycerides within any tissue and expresses it in percentages, accurately quantifying fat in all nine segments of the liver(137). It has been shown to perform better than CAP to predict histologic steatosis grade(138). A cross-sectional study of 104 consecutive adults compared the performance of MRI-PDFF with CAP for diagnosis of steatosis with respect to findings from biopsy. MRI-PDFF had an AUROC of 0.99 (95% CI 0.98–1.00) for diagnosing any steatosis (grades 1–3 versus 0) while CAP had an AUROC of 0.85 (95% CI 0.75–0.96). Using a threshold of 3.71%, MRI-PDFF had a sensitivity of 95.8%, and

specificity of 100% for diagnosing steatosis while using a threshold of 261 dB/m, CAP had a sensitivity of 71.8%, and a specificity of 85.7% for diagnosing steatosis(139). MRI-PDFF has been studied in the population with diabetes, with acceptable results. In a study by Doycheva et al. of 100 consecutively enrolled patients with diabetes, the prevalence of NAFLD on MRI-PDFF was 65%(138).89 Another study of 179 French patients with T2D reported a prevalence of NAFLD on MRI-PDFF of 68.7%. The main advantages of MRI as an imaging tool are its excellent accuracy and the lack of the limitations intrinsic to ultrasound and TE. However, the cost and limited availability of MRI-PDFF are its limitations.

d. Proton magnetic resonance spectrometry (MRS)

MRS, a specialized imaging technique of the MRI, was initially established for brain imaging to characterize tumors, infections and other diseases. With the development of technology, it now offers greater information regarding the biochemical composition of intraabdominal organs. It uses a highly sensitive and reliable technique quantifying intracellular lipid content of the liver and correlates well with liver biopsy results(140). Hepatic triglyceride content of >5.5% indicates steatosis(140-142). As for its role in patients with diabetes, studies reported high prevalence rates of NAFLD using MRS. Based on a hepatic triglyceride content >5.5%, one study found NAFLD prevalence of 60.3% in 101 patients with diabetes(143). In another study, which enrolled 103 mainly obese patients with diabetes, MRS found a prevalence of NAFLD of 50%(84). The main limitations include its high cost, low availability as it requires advanced processing methods that not every MRI is routinely equipped with, as well as its inability to measure fat content of the entire liver, but only in small regions of interest(144).

<u>Imaging tools to diagnose liver fibrosis</u>

a. Magnetic resonance elastography (MRE)

The MRE is considered the most accurate non-invasive tool to detect liver fibrosis(145, 146). It uses additional hardware and software on the conventional MRI to image the propagation of acoustic shear waves and produces cross-sectional images of the liver. Several analysis data have found that MRE to be more accurate than TE in identifying liver fibrosis(139, 147, 148). A study in 100 T2D patients reported a MRE prevalence of advanced fibrosis of 7.1%.(138) MRE is a safe tool that images the entire liver rather than a small sample of the organ. It also provides

accurate results in obese patients(149). MRE may have higher diagnostic accuracy than TE for advanced fibrosis, but there is limited availability and increased cost of MR-based imaging compared to ultrasound-elastography for routine use in clinical practice. As for TE, several factors can interfere with the results such as hepatic inflammation, congestion, or massive ascites.

Care pathways for specialist referrals

The association between T2D and NAFLD is established, and 10–15% of patients may have advanced fibrosis. The consequences of advanced NAFLD and T2D impose a heavy burden on the health care system. For the primary care physician or diabetologist, it is vital to identify T2D patients with advanced liver fibrosis in order to select patients for referral and co-management with a hepatologist. Indeed, patients with advanced liver fibrosis may require specific surveillance including screening for HCC and esophageal varices arising from portal hypertension. Individual biomarkers or imaging tools as single tests are not reliable at identifying advanced fibrosis in at-risk populations; hence combinations of serum and imaging tests into diagnostic algorithms have been implemented to provide more reliable screening results. In tertiary center NAFLD cohorts, simple tests such as FIB-4 or NFS can exclude advanced fibrosis with good negative predictive values but require secondary confirmatory tests such as TE to rule-in advanced fibrosis. Although the optimal combination of NITs for screening in community populations with a low prevalence of advanced NAFLD has not been established, several pragmatic approaches combining FIB-4, NFS, proprietary markers such as ELF (Enhanced Liver Fibrosis), and ultrasound-elastography have been proposed for clinical practice. A few studies have evaluated the current guidelines for the diagnosis of NAFLD in patients with T2D by combining several NITs to risk stratify patients for specialist referral. Sberna et al. evaluated the EASL, European Association for the Study of Diabetes (EASD) and European Association for the Study of Obesity (EASO) guidelines using four non-invasive test combinations. Their study concluded that these practice guidelines would lead to an excessive number of patients being referred for specialist care and are not feasible in patients with T2D(89) (Table 3). This was confirmed by another study that evaluated both EASL-EASD-EASO and German practice guidelines for the diagnosis of NAFLD in patients with T2D (Table 3)(150). Instead, they proposed simpler referral methods for screening; a higher AST resulted in a referral rate of 25%

and the Fibroscan-AST (FAST) method which also incorporates CAP into a predictive score for NASH F2–4, resulted in 12% requiring referral(151). The FAST score is proposed to be useful for the risk stratification of NAFLD and related fibrosis and for treatment decisions(151). Ciardullo et al. evaluated EASL-EASD-EASO practice guidelines using FLI and FIB-4 which resulted in reduced referral of patients (28.3% with standard FIB-4 and 13.4% with age adjusted FIB-4)(117) (Table 3). It is important to note that these studies were performed on small cohorts and do not differentiate between abnormal and severely abnormal test results. Additionally, acceptable referral rates are difficult to standardize across clinical practices and vary by region and healthcare payer systems. Therefore, a conclusion regarding whether these proposed pathways are practical and cost-effective for screening and secondary referral for the population with diabetes cannot be made with currently available data.

Specific aim 2 – NAFLD in LT recipients diagnosed by CK-18 and TE:

Characteristics of patients at study entry

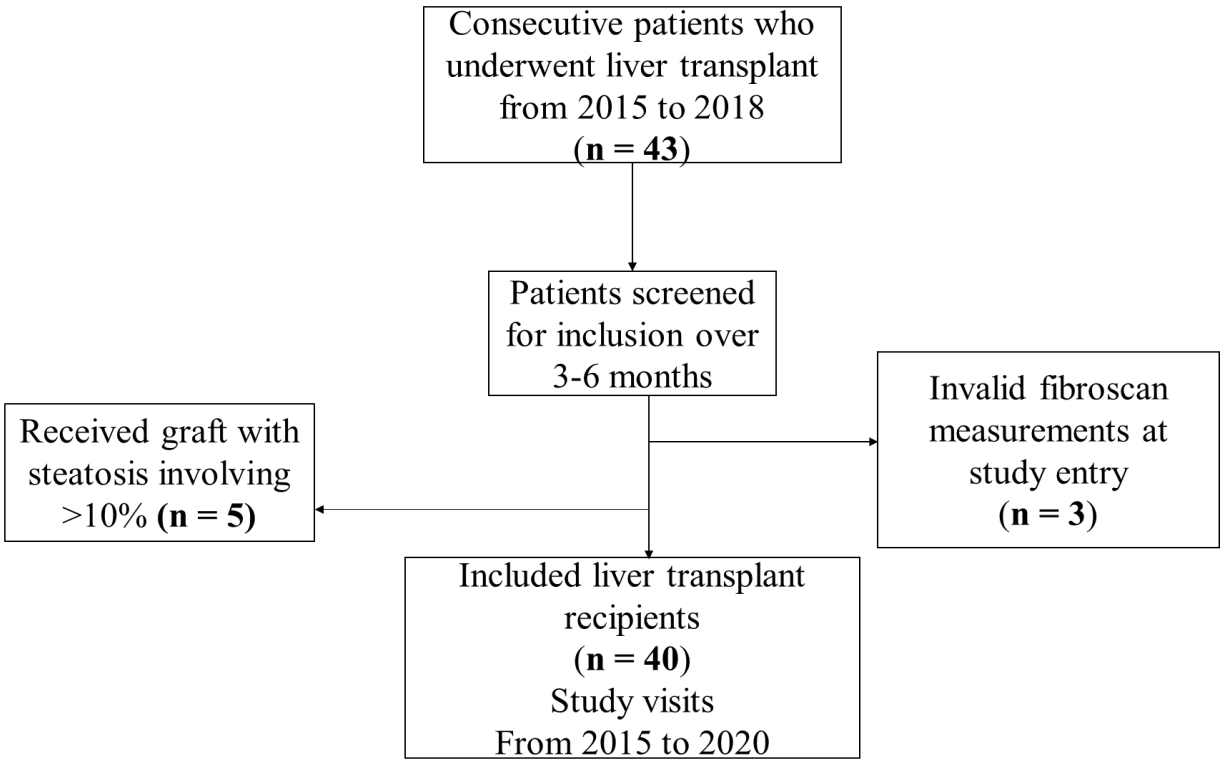
After applying exclusion criteria, 40 LT recipients were included in this prospective study (Figure 4). The main demographic, clinical and biochemical characteristics of the study population at baseline are summarized in Table 4. Univariable analysis by outcome category of NAFLD and NASH is also reported. Overall, mean age was 57.3 years and 70% of patients were male. The most frequent indications for LT were NASH and HCC. Metabolic comorbidities were frequent, with overweight, diabetes and hypertension affecting 40%, 35% and 37.5% of the patients, respectively. Patients who developed NAFLD and NASH during the follow-up period were more frequently transplanted for NASH and on tacrolimus as immunosuppressant.

Table 3. Referral rates of diagnostic algorithms proposed by current guidelines in T2D patients.

Author	*Study type*	*Guidelines/methods used*	*Tools used*	*Referral to hepatologist*
Sberna et al.[89]	Retrospective N= 179	EASL-EASD-EASO guidelines	Increased liver enzymes with: 1. Positive NFS & normal/abnormal FLI 2. Positive FibroTest & normal/abnormal SteatoTest 3. Positive NFS & normal/abnormal MRS 4. Positive FibroTest & normal/abnormal MRS	84.9% 34.6% 68.7% 33.5%
Blank et al.[150]	Prospective, cross-sectional N= 184	EASL-EASD-EASO guidelines	High transaminases, FiB-4, NFS	60-77%
			Ultrasound, NFS	
		German guidelines		76% required LSM, 25% referred
			AST>ULN	25%
		Newsome et al.	FAST (LSM, CAP, AST)	35% for rule out cut off and 12% for rule in cut off
Ciardullo et al.[117]	Retrospective, cross-sectional N= 1023	EASL-EASD-EASO guidelines	FLI & FIB-4	28.3% (13.4% with age-adjusted FIB-4)

***** *EASL-EASD-EASO European Association for the Study of the Liver-European Association for the Study of Diabetes-European Association for the Study of Obesity, NFS NAFLD fibrosis score, MRS magnetic resonance spectrometry, FIB-4 fibrosis-4 index, LSM liver stiffness measurement, ULN upper limit of normal, FAST Fibroscan® aspartate aminotransferase, CAP controlled attenuation parameter, FLI fatty liver index, AST aspartate aminotransferase.*

Figure 4. Flow diagram of the study recruitment.



Diagnostic accuracy of NITs compared to liver histology and correlation between TE with CAP and serum biomarkers

During the study period, 35 liver biopsies (mean length 1.7 cm, standard deviation 0.4) from 24 patients were available. The median time between liver biopsies and non-invasive diagnostic testing was 38.6 ± 30 days. Table 5 shows the performance of NITs compared to liver histology. The diagnostic accuracy of CAP and HSI for NAFLD was 76% and 45.7%, respectively. The diagnostic accuracy of a combination of CAP ≥ 270 dB/m and CK-18 >130.5 to diagnose NASH was 82%. The diagnostic accuracy of LSM, FIB-4 and APRI for liver fibrosis was low at 57.8%, 48.7% and 54.1%, respectively. There was a medium positive correlation between CAP and HSI of 0.4. There was a medium positive correlation between LSM and FIB-4 of 0.4, and a weak positive correlation between LSM and APRI of 0.1.

Table 4. Characteristics of patients at study entry.

	Whole Cohort *N=40*	*Patients who developed NAFL* *n=22*	*Patients who developed NASH* *n=17*
Age	57.3 ± 8.5	55.5 ± 9.2	56.3 ± 7.9
Male	28 (70)	18 (82)	14 (82)
Ethnicity			
Caucasian	32 (80)	19 (86)	15 (88)
Other (Asian, Black, Arab)	8 (20)	3 (14)	2 (11)
Etiology of Liver Disease			
NASH	21 (52.5)	13 (52)	12 (70)
HCC	9 (22.5)	2 (9)	2 (12)
HCV (excluding genotype 3)	8 (20)	6 (27)	3 (18)
Alcoholic liver disease	1 (2.5)	1 (4.5)	0
Other	1 (2.5)	0	0
BMI in kg/m^2	24.8 ± 4.6	26.2 ± 5.1	26.6 ± 4.5
BMI >25	18 (40)	14 (64)	12 (70)
Comorbidities			
Diabetes	14 (35)	9 (41)	8 (47)
Hypertension	15 (37.5)	7 (32)	8 (47)
Dyslipidemia	6 (15)	6 (27)	5 (29)
MELD-Na Score	<9	<9	<9
Laboratory			
AST, U/L	27.6 ± 33	31.8 ± 41.2	34.5 ± 45.1
ALT, U/L	32.8 ± 42.8	37.6 ± 52.6	40.6 ± 57.7
GGT, U/L	177.5 ± 256.6	177.7 ± 271.4	188.1 ± 297.6
Bilirubin, µmol/L	17 ± 15.9	18.2 ± 17.3	18 ± 18.2
INR	1.25 ± 1.39	1.05 ± 0.12	1.04 ± 1.3
Albumin, g/L	39.6 ± 3.69	38.7 ± 4.3	39.4 ± 3.9
Platelets, 10^9/L	172.3 ± 86.9	185 ± 92.5	170.5 ± 93.6

Table 5. Diagnostic accuracy of NITs compared to liver histology

	Steatosis on liver histology		***NASH on liver histology***	***Fibrosis on liver histology***		
	CAP	**HSI**	**CAP+CK-18**	**LSM**	**FIB-4**	**APRI**
Sensitivity	58%	64.3%	75%	62%	7.1%	14.3%
Specificity	86%	33%	83%	54.2%	73.9%	78.3%
PPV	70%	39%	37%	54.2%	14.3%	28.6%
NPV	79%	58%	96%	61.9%	56.7%	60%
LR+	4.28	0.96	4.50	1.35	0.27	0.66
LR-	0.48	1.07	0.3	0.70	1.26	1.10
Accuracy	76%	45.7%	82%	58.7%	48.7%	54.1%

Incidence and predictors of NAFLD and NASH by CAP and CK-18

During a median follow-up of 16.8 months (IQR 15.6-18.0), 22 patients (63.0%) developed NAFLD (incidence rate: 71.0 per 100 PY, 95% CI 45.0-78.0), and 17 patients (48.5%) developed NASH (incidence rate: 48.6 per 100 PY, 95% CI 31.4-66.0). On multivariate Cox regression analysis, BMI was an independent predictor of both NAFLD (adjusted HR 1.1, 95% 1.0-1.2) and (adjusted HR 1.1, 95% CI 1.0-1.3) (Table 6). To further elaborate on the effect of high BMI on the incidence of NAFLD and NASH, a hazard plot was performed and showed that overweight was a significant risk factor for both NAFLD and NASH (log-rank $p<0.01$) (Figure 5).

Changes in LSM, FIB-4 and APRI during follow-up

Given the low accuracy for the non-invasive fibrosis tests, we studied changes in LSM, FIB-4 and APRI during the follow-up. While the majority of patients had an LSM ranging from 2.5 to 15 kPa, there were patients who developed marked increases, and these were observed in the first six months of follow-up (Figure 6a). Similarly, while most of the patients had FIB-4 and APRI

ranging from 1 to 2.5 and from 0.5 to 1.5, respectively, there were patients who developed marked increases during the first six months of follow-up (Figure 6b and 6c).

Table 6. Risk factors for post-LT development of NAFLD and NASH using univariate and multivariate Cox regression analysis.

	NAFLD			
	Univariate analysis		**Multivariate analysis**	
	HR (95% CI)	**P value**	**HR (95% CI)**	**P value**
Sex	0.6 (0.4-1.2)	0.10	0.9 (0.3-1.7)	0.50
Age	1.0 (0.9-1.0)	0.60		
BMI	1.1 (1.0-1.2)	<0.01	1.1 (1.0-1.2)	<0.01
Diabetes	1.7 (1.0-2.7)	0.02		
Dyslipidemia	4.6 (1.7-12.8)	<0.01		
ALT	1.0 (0.9-1.0)	0.09	1 (0.9-1.0)	0.30
	NASH			
	Univariate analysis		**Multivariate analysis**	
	HR (95% CI)	**P value**	**HR (95% CI)**	**P value**
Sex	0.6 (0.3-1.1)	0.1	0.9 (0.4-2.1)	0.80
Age	1.0 (0.9-1.0)	0.9		
BMI	1.1 (1.0-1.2)	0.01	1.1 (1.0-1.3)	<0.01
Diabetes	1.3 (0.7-2.1)	0.3		
Dyslipidemia	4.4 (1.5-13)	0.007		
ALT	1.0 (1.0-1.0)	0.03	1 (0.9-1.0)	0.10

Legend: Results given as mean (standard deviation) or n (%). aHR, adjusted hazard ratio; ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; HR, hazard ratio; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Figure 5. Hazard ratio based on BMI in NAFLD (left) with a P-value of <0.0001 by log rank and in NASH (right) with a p-value of 0.009 by log rank.

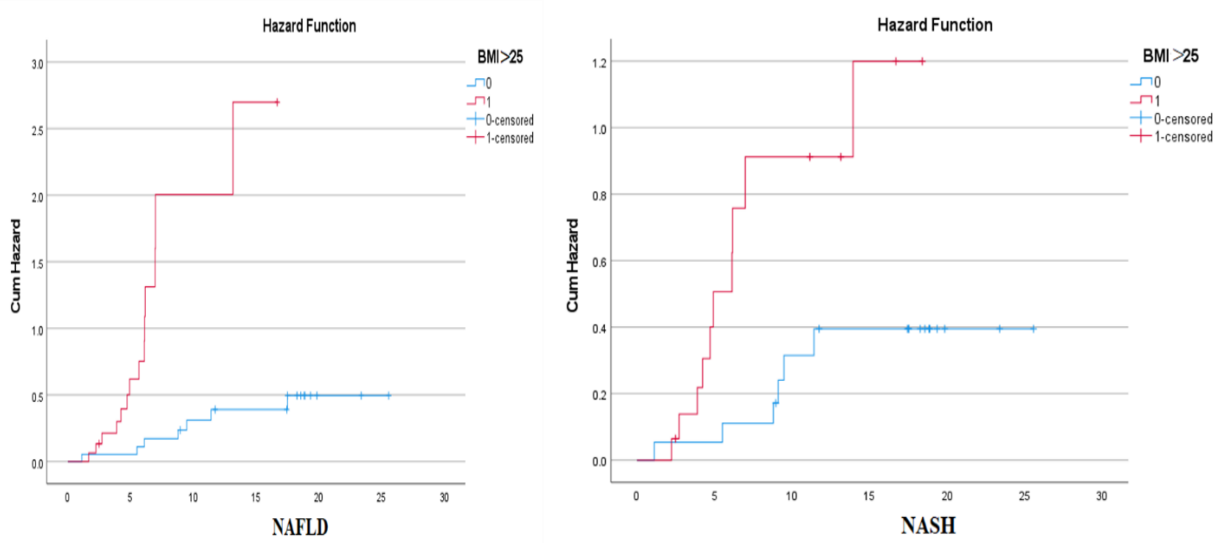


Figure 6a. Changes of LSM of the population during the study period.

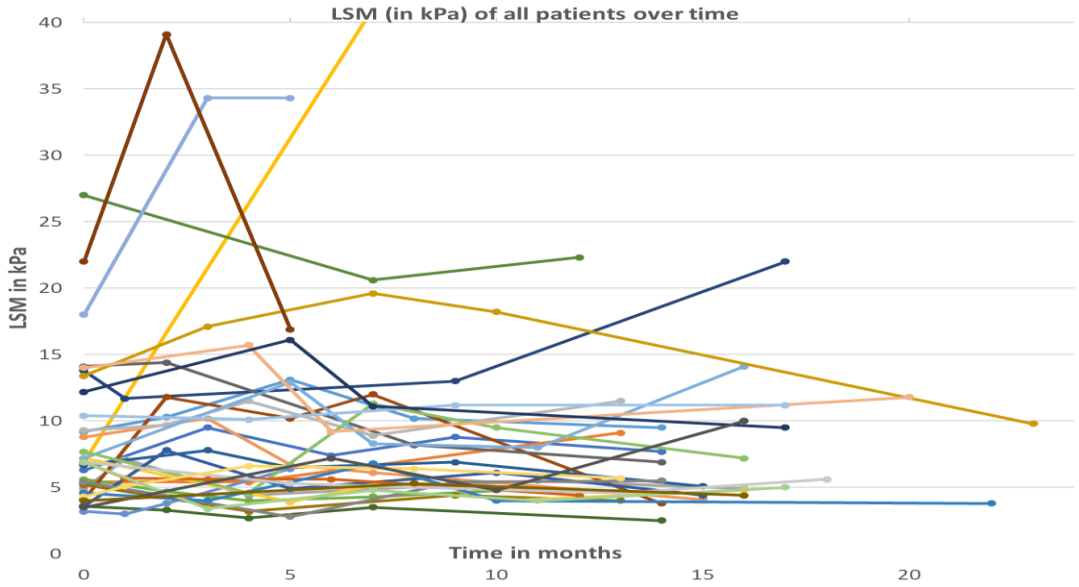


Figure 6b. Changes of FIB-4 of the population during the study period.

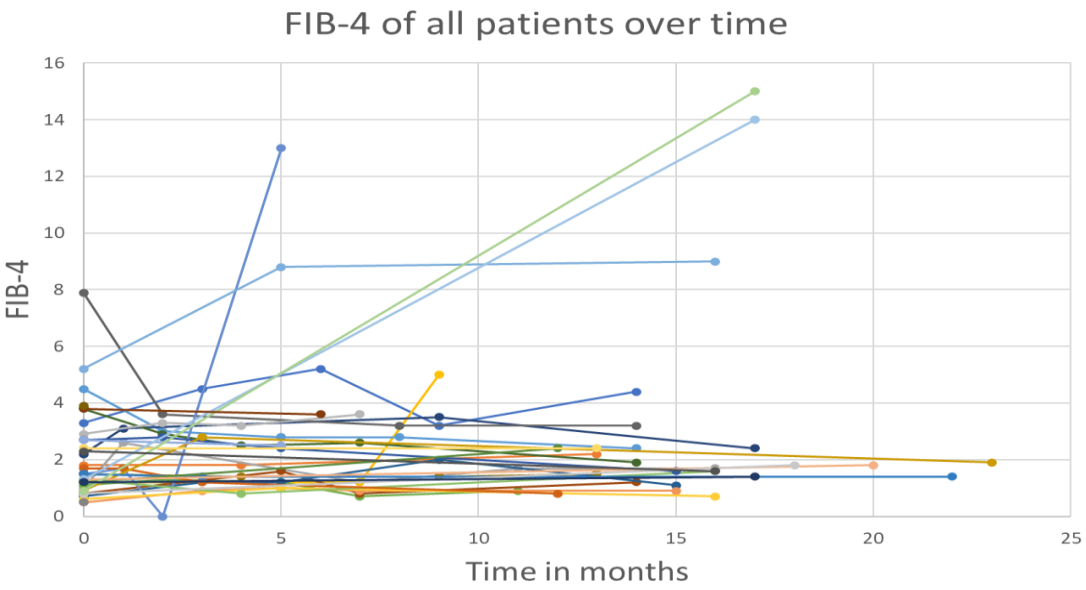
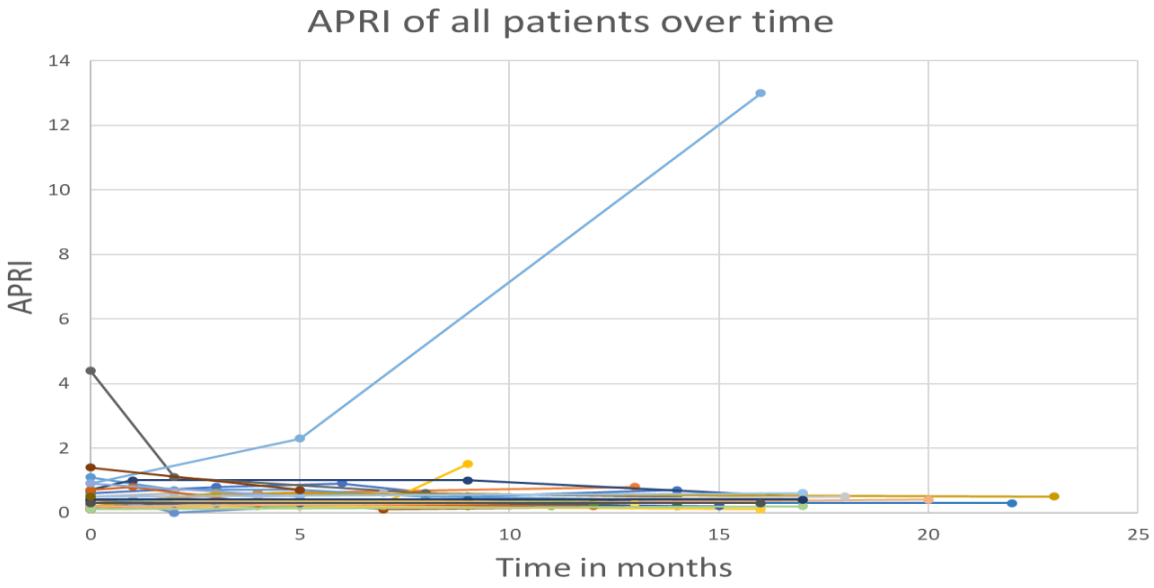


Figure 6c. Changes of APRI of the population during the study period.



Specific aim 3 - HCC surveillance in HIV patients:

Characteristics of patients at study entry

186 patients met the inclusion criteria for HCC surveillance (Figure 7). The main demographic, clinical and biochemical characteristics of the study population at baseline are summarized in Table 7. Overall, mean age was 58, 76% male, 66% Caucasian: 25% with HIV mono-infection, 46% with HIV/HCV, and 28% with HIV/HBV.

Figure 7. Flow diagram of the study recruitment.

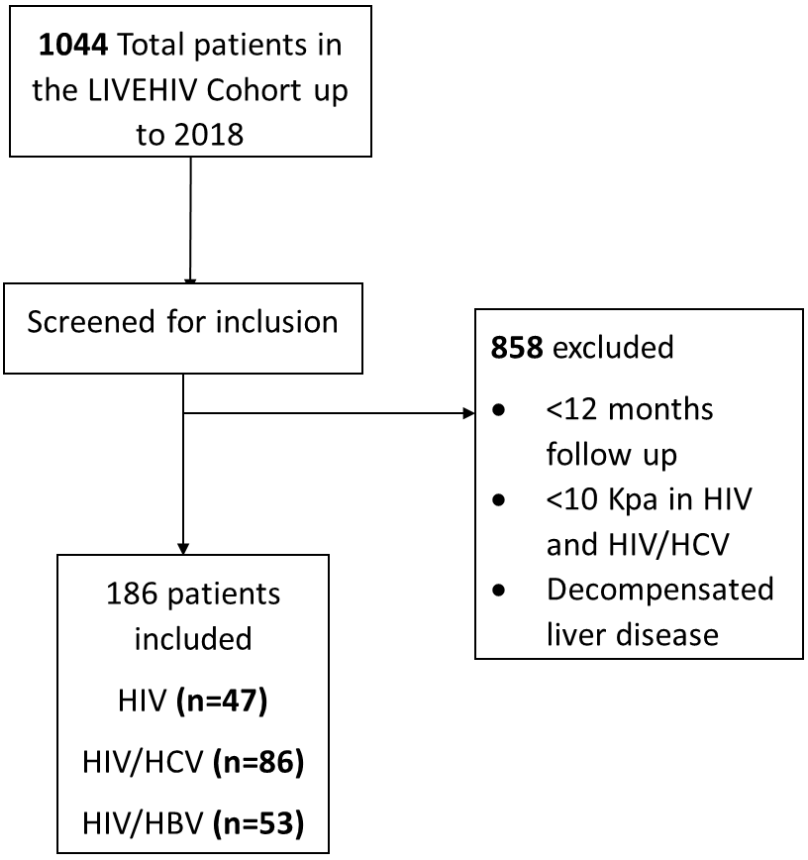


Table 7. Baseline characteristics reported as mean (standard deviation) for continuous variables and percentage for categorical variables.

	HIV mono-infected n=47	HIV/HCV co-infected n=86	HIV/HBV co-infected n=53
Age	61±7.7	56±8	57±10
Sex (Male) in %	83%	71%	77%
Duration of HIV in years	25.5±8.2	25±7	20±9
% of patients with undetectable HIV load (<50 copies)	64%	65%	47%
CD4 Count	679±330	476±243	638±311
Follow up time in months	24.5±17.4	38.7±24	32.6±18
Liver Stiffness Measure/LSM	13.6±11.2 (range 10-63.9)	16±11 (range 10-51.4)	7.2±6.6 (range 2.7-39.7)

Surveillance rate, reasons for suboptimal surveillance, and incidence of HCC

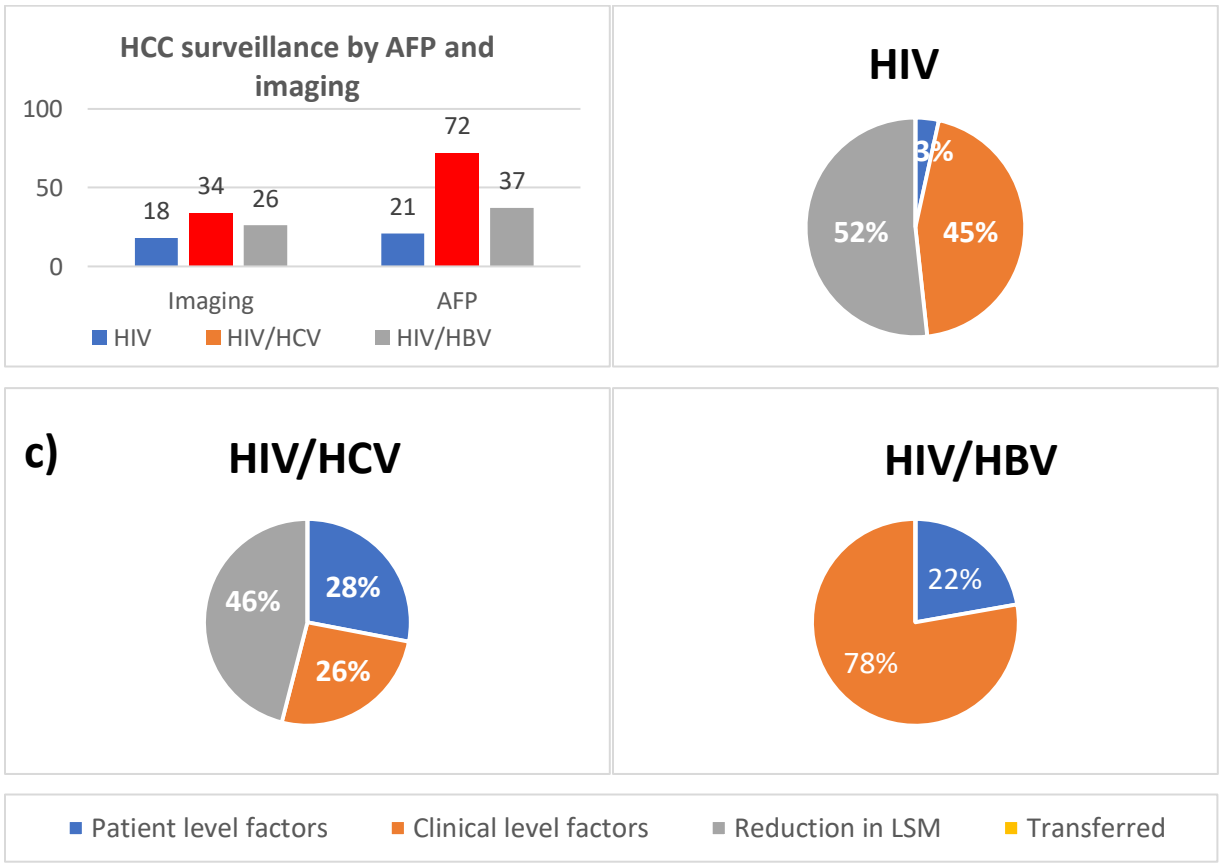
Surveillance rates of ultrasound and AFP were recorded. 38% of HIV mono-infected patients, 40% of HIV/HCV co-infected patients and 49% of HIV/HBC co-infected patients had optimal surveillance of HCC using ultrasound. Using AFP, 45% of HIV mono-infected patients, 84% of HIV/HCV co-infected patients and 70% of HIV/HBC co-infected patients had optimal HCC surveillance. The surveillance rate to ultrasound was similar among the three groups. However, surveillance rate to AFP was lower in the HIV mono-infected group ($p<0.0001$) (Figure 8a). In all three groups, 70% of patients had optimal surveillance with AFP, while 42% had optimal surveillance with ultrasound.

Among the reasons for suboptimal surveillance rates, clinical level factors constituted 45% of HIV mono-infected patients, 26% of HIV/HCV co-infected patients and 78% of HIV/HBV co-infected patients, while patient level factors constituted 3% of HIV mono-infected patients, 28% of HIV/HCV co-infected patients and 22% of HIV/HBV co-infected patients. All in all, patient-level factors were more frequent in HIV/HCV co-infected group($p=0.03$). Surveillance for HCC

was discontinued in 52% of HIV mono-infected and 46% of HIV/HCV co-infected patients because of the reduction in LSM during the follow-up (p<0.001). (Figure 8b, and c).

During a mean follow-up time of 55 months, incidence of HCC was 2.2% (0.5 per 100 person-years, 95% CI 0.07-0.66).

Figure 8. a) Surveillance of HCC by AFP and imaging by group. Reasons for suboptimal surveillance of HCC in **b)** HIV, **c)** HIV/HCV and **d)** HIV/HBV infected patients.



7. Discussion

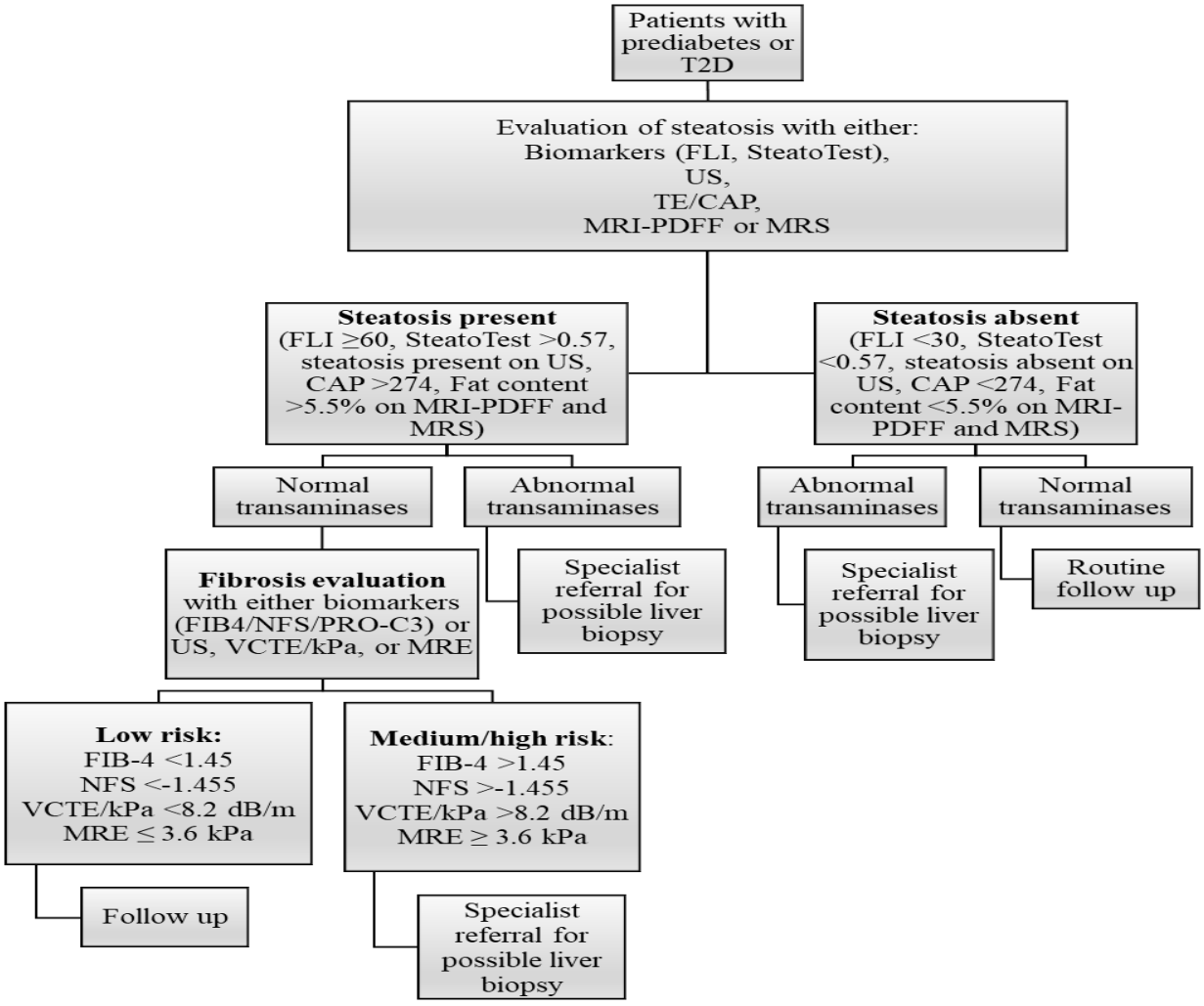
We can deduce the following from our review and studies: 1) Based on available studies, imaging techniques have shown to be reliable in diagnosing NAFLD and liver fibrosis in patients with T2D, however there is still uncertainty on whether the current biomarkers are useful for

assessment and progression of NAFLD and fibrosis(88, 92, 99); 2) Our original analysis reported that NAFLD and NASH diagnosed non-invasively are frequent occurrences in LT recipients. NAFLD and NASH are mainly driven by high BMI. We also reported that the diagnostic accuracy of NITs for NAFLD and NASH is good and, non-invasive fibrosis tests have a low accuracy in the first months following LT; Finally, 3) we observed suboptimal surveillance of HCC among patients in the LIVEHIV Cohort mainly due to clinical-level factors.

As for our current knowledge, there is still an important gap regarding the role of biomarkers in the diagnosis of NAFLD and related liver fibrosis in patients with T2D. Furthermore, there is a shortage of studies regarding the role of NITs in the diagnosis of NASH in this population. Many of currently available biomarkers were not designed to specifically reflect liver changes in patients with diabetes. Some of those tests rely heavily on diabetic status and impaired blood glucose that could overestimate or underestimate the prevalence of NAFLD and advanced fibrosis in the population with diabetes. Optimal diagnostic thresholds have not been established for T2D and vary with prevalence of disease severity between study cohorts. Additionally, repeating these tests in the same individual can produce different results because of fluctuations in fasting plasma glucose levels due to compliance with the diet, and the use of different hypoglycemic agents that interfere with many parameters of these panels(88, 89, 92). This results in significant variability in the detection of NAFLD and advanced fibrosis and discordance between diagnostic test scores(152). Extensive and long-term data on use of these tests is required to determine their accuracy to monitor disease progression, potential treatment response, and prognostic values. For now, they can be used to help identify patients at risk of progressive liver changes and the need for further surveillance. Another major gap in the literature is the role of NITs in patients with type 1 diabetes. Due to the presence of insulin resistance, as well as increasing obesity in this group, patients are at a high risk for NAFLD and related liver diseases(153-155). Type 1 diabetes usually occurs earlier in life, which places patients at a higher risk of developing associated complications such as NAFLD. The prevalence of type 1 diabetes is also expected to rise in the next decades and the risks of developing NAFLD and associated liver diseases is expected to rise as well. Therefore, it is extremely important to implement studies on the role and accuracy of NITs in this population. Another important point worth mentioning is the current knowledge on care pathways for specialist referral. Current international and national guidelines were designed to risk stratify and improve patient care and

diagnosis of NAFLD-related fibrosis; however, these guidelines were not specifically designed for patients with T2D. They do not specify which tools should be used to diagnose fibrosis and steatosis, nor their specific cut-off values. They also lack clear definitions for what is meant by intermediate and high-risk patients, and this in turn results in over- or under-referrals to the Hepatologist. The ideal pathways for secondary care assessment include easy to use, readily available, cost-effective, and accurate tools that provide early diagnosis and acceptable referral rates for specialist care. We constructed a flowchart with a proposed combination of NITs to diagnose NAFLD and related liver fibrosis in patients with T2D (Figure 9).

Figure 9: Flowchart of our proposed combination of NITs to diagnose NAFLD in patients with T2D.



Our choice of these specific tools is based on the performance and cost of these individual tools from the result section. Of note, our proposed combination of tools has not been studied in patients with T2DM and could be far from ideal. In order to identify the optimal referral rate and to better understand which tools are useful in screening T2D patients, larger studies involving the current or different non-invasive test combinations are required in specific populations. These studies should focus on tools that are not affected by the diabetic status of the patients, possibly NITs with lower index thresholds indices and simpler biomarkers to better risk stratify and refer them to specialist care. Studies on PRO-C3 and TE have shown promising results, but without further validation using these tools, their exact role in the diagnostic pathway for T2D has yet to be defined. Studies should focus on diagnosing and screening for NASH and advanced liver fibrosis rather than diagnosing NAFLD or simple steatosis, with clear cut-offs of test results and definitions of risk for further investigations. The differences in screening guidelines are partly due to the limited knowledge on the cost-effectiveness of screening in this high-risk population, therefore future studies should also focus on the cost effectiveness of screening NASH and NAFLD-related liver fibrosis on a large scale in patients with T2D (Table 8).

In our prospective analysis, we have shown that NAFLD and NASH diagnosed noninvasively are frequent occurrences in the first 18 months from LT. Similar to results reported in previous retrospective studies, the majority of incident NAFLD and NASH in our population occurred within the first year of LT (156-158). The main predictor of these events was high BMI, thus underlying the importance of controlling the weight beginning from the first 3 months post-LT. We compared the performance of NITs to liver biopsy. We used a CAP cutoff ≥270 dB/m, as referenced by Siddiqui et al., and compared it to the presence steatosis grade 0 vs 1-3 on liver biopsy (45). Our results showed a lower sensitivity (58% vs 74%), however the specificity (86% vs 87%), PPV (70% vs 78%) and NPV (79% vs 84%) were similar. The variations can be explained by the different population sizes, number of available liver biopsies and the timing of the study conducted within the first 18 months from LT When HSI was compared to histology, it showed less accuracy than CAP as demonstrated before in other studies on non-LT populations (159, 160). Secondly, we used a combination of CK-18 >130.5 with CAP ≥ 270 dB/m and compared it to the presence of NASH (NAS ≥ 5 or proven NASH) on liver histology.

Table 8. A summary of the NITs for screening for NAFLD in T2D.

Diagnostic tools	*Components*	*Cut-offs to rule out/in advanced fibrosis*	*Advantages*	*Disadvantages*
Biomarkers				
FLI	Waist circumference, BMI, triglyceride, GGT	<30/≥60	Affordable Widely available Useful to risk stratify patients for further investigations	Risk of unreliable results hence not accurate when used alone.
SteatoTest	BMI, serum cholesterol, triglycerides, serum glucose, and the FibroTest-ActiTest (GGT, total bilirubin, α 2-macroglobulin, apolipoprotein A1, haptoglobin and ALT	0.57	Affordable Widely available May have prognostic value in T2D patients.	Risk of unreliable results hence not accurate when used alone.
FIB-4	Age, AST, ALT, platelets	<1.3/>2.67	Affordable Widely available Useful in detecting disease progression	Risk of unreliable results hence not accurate when used alone.
NFS	Age, BMI, diabetes, AST-to-ALT ratio, platelets, and albumin	<-1.455/ >0.676	High negative predictive value in severe liver fibrosis.	Risk of unreliable results hence not accurate when used alone.
FibroTest	α2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin, and GGT.	>0.58 is suggestive of advanced or severe fibrosis.	Useful for detecting advanced fibrosis. Provides prognostic value for predicting overall survival.	Possibility of producing false positive results affected by acute conditions.
PRO-C3		13.1-15.6 ng/ml for advanced fibrosis	Measures active fibrosis.	Not routinely available. Not established as a standalone diagnostic tool for NAFLD.

ELF	Hyaluronic acid, procollagen III amino-terminal peptide, tissue inhibitor of matrix metalloproteinase 1	≥9.8 indicates advanced fibrosis	Accurately rules out fibrosis.	Cost. Availability. Requires validation in T2D population.
<u>Imaging tools</u> ***Ultrasound***			Widely available Inexpensive. Good accuracy in detecting fibrosis and steatosis compared to biopsy.	Reduced sensitivity and specificity to detect steatosis <30%. Affected by obesity Operate dependent.
TE	CAP and LSM	S1/mild – 274 dB/m, S2/moderate – 290 dB/m, S3/severe – 302 dB/m F ≥ 2–8.2 kPa, F ≥ 3–9.7 kPa, and F4 - 13.6 kPa.	Widely available. Inexpensive. Easy to use and reproducible. Accurate to detect stages of fibrosis and steatosis.	Requires patient to be fasting ≥3 hours prior. Chances of failure or unreliable results in ascites, morbid obesity, acute liver conditions and pregnancy.
MRI-PDFF		Presence of hepatic steatosis ≥5%	Reproducible. High accuracy to quantify steatosis in all liver segments.	Cost. Not widely available.
MRS		HTGC of >5.5% indicates steatosis	High accuracy to quantify steatosis.	Cost. Not widely available. Its inability to measure fat content of the entire liver.
MRE			Highly accurate. Detects different stages of fibrosis. Safe. Images entire liver. Not affected by obesity.	Cost. Not widely available. Unreliable results in acute liver conditions.

** FLI fatty liver index, BMI body mass index, GGT gamma glutamyl-transferase, ALT alanine aminotransferase, T2D type 2 diabetes, AST aspartate aminotransferase, NFS NAFLD fibrosis score, pro-C3 N-terminal type III collagen propeptide, NAFLD nonalcoholic fatty liver disease, ELF, TE, CAP controlled attenuation parameter, LSM liver stiffness measurement, MRI PDFF Magnetic Resonance Imaging-Proton Density Fat Fraction, MRS magnetic resonance spectroscopy, MRE magnetic resonance elastography.*

To our knowledge, this is the first study to use CK-18 combined with CAP to detect NASH in LT patients. Compared to one meta-analyses of over 1,600 patients that assessed the accuracy of CK-18 (cut-off range: 121.6– 380.2 U/L) in non-transplanted patients with NASH, our results are similar for both sensitivity (75% vs 78%) and specificity (83% vs 87%)(50). Compared to another more recent meta-analysis of over 1,400 patients that evaluated the diagnostic value of CK-18 for the diagnosis of NASH, our results also reported similar sensitivity (75% vs 75%), specificity (83% vs 77%), LR+ (4.5 vs 3.3), and LR- (0.3 vs 0.3)(156).

There are two interesting points worth mentioning. Firstly, our cut-off values of all the non-invasive biomarkers reported a higher NPV then a PPV which could indicate that these tests are more efficient at ruling-out NAFLD, NASH and liver fibrosis rather than ruling-in these diseases, as described in a previous study(45, 97, 132). However, their ability to minimize the need for liver biopsy in this clinical setting still requires further validation. Secondly, while we combined CK-18 with CAP to diagnose NASH, our results are very closely related to the results of the two meta-analyses which used CK-18 alone to diagnose NASH. This makes us question the role of combining CAP with CK-18 to diagnose NASH. Two studies investigated the combined use of CK-18 with TE to detect fibrosis and found either no significant improvement or only some improvement in AUROC by combining CK-18 and TE compared to using a single test(157, 158). Yet, other studies have shown that combining CK-18 with other biomarkers improves the accuracy to diagnose NASH(159, 160). Our analysis must be replicated in a larger sample using different combinations of biomarkers to better understand this. Our results are comparable to a recent cross-sectional study by Mikolasevic et al. which reported a prevalence of liver steatosis of 68.6% and severe liver steatosis of 46.8% in LT recipients using CAP and LSM(161). Our incidence rates are also comparable to previous published meta-analysis and

retrospective studies, while minor variations are most likely due to the difference in populations, the cut-off values to define steatosis/NAFLD and NASH, and the absence of the use of CK-18 as a diagnostic tool in those studies(161-164). On multivariate Cox regression analysis, high BMI was the main risk factor for development of NAFLD and NASH in patients post LT, conceding with results from previous studies(161, 162). Obesity is an independent risk factor for the development of NAFLD and NASH and can occur or continue to be present even during the first month's post LT Indeed, other studies have shown that the maximum weight gain occurs in the first year post LT mainly because of the use of immunosuppressive medications(165, 166). Diabetes and dyslipidemia were significant risk factors on univariate analysis, also in line with previous results(161). The presence of these risk factors poses a risk for development of fatty deposits in the graft and progression to NAFLD and NASH. Therefore, strategies must be implemented both before and after LT to control and prevent the progression of liver disease. These strategies include weight reduction with low carbohydrate diet and performing regular exercise, avoiding alcohol and smoking, control of comorbid metabolic diseases, and controlling immunosuppression medications post-LT. We also reported a low performance of non-invasive fibrosis tests during the first 18 months following LT. Similar findings have been reported previously in post LT patients with HCV recurrence. El-Meteini et al concluded that TE and APRI were insignificantly correlated with the degree of fibrosis in liver biopsy done at 3 months post-transplant in 31 patients (P value of .134, .535)(167). Kabbany et al reported that APRI and FIB-4 had a poor diagnostic accuracy compared to liver biopsy for the presence of advanced fibrosis in 93 patients post LT due to NAFLD and HCV(168). Kamphues et al also concluded that APRI and FIB-4 are not feasible to assess liver fibrosis in 135 post LT patients(169). Indeed, some of our patients experienced an important variation in LSM, FIB-4 and APRI particularly during the first 6 months post LT however, we suspect that this could be due to different reasons. Inflammation due to congestion or cholestasis, which is common post LT, could be one reason for the inaccuracy of fibrosis tests. Another reason could be due to fluctuations in the individual biochemical components, such as liver enzymes and platelets, especially during the first 6 months following LT as patients are have started receiving and adjusting their immunosuppressive medications. We also suspect that since a majority of our liver recipients had a BMI >25, obesity could have interfered with the LSM results(135). Still, our study and the

previous published results studies were performed on small cohorts, therefore a final conclusion regarding the accuracy of non-invasive fibrosis tests cannot be made based on these results.

In our retrospective analysis, we established that surveillance of HCC among patients in the LIVEHIV Cohort was suboptimal. The surveillance rate to AFP was lower in the HIV mono-infected group compared to the HIV/HCV and HIV/HBV groups ($p<0.0001$). The main explanation for this is that AFP is automatically added in blood requests of patients with HIV/HCV and HIV/HBV. The main reason of suboptimal surveillance was due to clinical-level factors, which is due to under-recognition of at-risk populations, suboptimal knowledge about surveillance guidelines, and limited patient time in the clinic. Patient-level factors constituted an important percentage among the reasons for suboptimal surveillance rates particularly in the HIV/HCV group, and was mostly associated to difficult access to care, drug/alcohol misuse, and psychiatric conditions. Surveillance for HCC was discontinued in a large percentage of the HIV mono-infected and HIV/HCV co-infected patient groups because of the reduction in LSM during the follow-up, attributed to antiviral/antiretroviral treatment. Low surveillance rates of HCC have been reported in previous studies. A large systemic review including 50 554 HCC cases from around the world reported a 37% pooled proportion of HCC diagnosed by surveillance(170). In a systemic review by Singal et al involving 9 studies of patients with various etiologies of cirrhosis with different surveillance strategies using AFP and imaging, 5 of the 9 studies showed surveillance rates for HCC below 30% and a pooled surveillance rate of 18.4%. Low surveillance rates were associated with low socio-economic status of the patients(171). Another study by the same author involving 397 cirrhotic patients reported that only 20% received HCC surveillance and the main reasons were under-recognition of liver disease and the absence of surveillance orders(24). A third study reported that only 2% of cirrhotic patients had consistent HCC surveillance, while 33% and 65% had inconsistent and no surveillance for HCC(172). As mentioned initially, HCC is a growing medical problem and surveillance must be implemented to prevent the cascade of consequences that can occur from this disease. Unfortunately, because surveillance is a multi-step process, numerous factors can interfere with its success. To improve HCC surveillance, strategies should focus on tackling concerns at the patient, physician and clinical system levels. Strategies that can be applied to reduce obstacles at the physician and clinical level include improving awareness of at-risk populations by arranging refresher lectures

about updated surveillance guidelines to health care providers; and implementing automated systems to accurately identify high risk patients as well as remind healthcare providers to repeat ultrasound and AFP testing. As for improving concerns at the patient level, several strategies can be done which include improving patient awareness about their disease and the benefits of surveillance by providing counselling sessions and medical brochures; providing supportive services to patients with socio-economic issues; and creating easy pathways to help patients with scheduling surveillance appointments.

There are limitations to our study. Some of the published results of NITs in patients with T2D that we have proposed in Figure 9 have not been compared to reference methods such as liver biopsy or MRI assessment for steatosis/fibrosis. Hence, the role of these NITs is still unclear. Nevertheless, our proposed flowchart was based on the low costs and acceptable accuracy results that were published. As for the NAFLD in liver recipient aim, the sample size was small and involved a single study site with a bias of patient demographics and treatment strategies and outcomes. These limitations could have interfered with the interpretation of the results. Nevertheless, our incidence rates and predictors are similar to previous retrospective studies(162-164). The overall study length was short (16.8 months) however, our aim was to study early graft changes in LT recipients using NITs. Additionally, not all patients had available liver biopsy to compare with NITs. Regardless of this, the results obtained from our study provide rationale for the use of NITs to frequently monitor this patient population, which could not be feasible with liver biopsy. The main limitations of HCC surveillance aim are its retrospective nature, as well as and the presence of only one outcome, the HCC rate, with no details regarding the stage of the HCC at detection, eligibility for treatment, and type of treatment received.

8. Conclusion

Overall, our study has concluded that progression of liver disease is common in at-risk populations. Various NITs and panels have been developed to diagnose NAFLD and related liver fibrosis, among which some have shown to be useful in screening patients with T2D. Nevertheless, their exact role in the population with diabetes is understudied. While imaging

tools show promise, the role of current biomarkers to predict advanced fibrosis stage remains unclear, and there are emerging biomarkers such as genetic polymorphisms that require further study in T2D patients. CAP and CK-18 are promising NITs for diagnosing NAFLD and NASH in LT recipients, however LSM and other fibrosis biomarkers are not reliable tests in detecting liver fibrosis in the first months post-transplant. Finally, HCC surveillance is suboptimal in patients with HIV and efforts should focus on improving surveillance strategies at the patient, physician, and clinical system levels. Future studies should focus on evaluating NITs and optimizing surveillance resulting in improved outcomes.

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