

## Title Page

# Strategies to Diagnose Non-Alcoholic Steatohepatitis: A Novel Approach to Take Advantage of Pharmacokinetic Alterations

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Drug Disposition to Diagnose NASH

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### List of nonstandard abbreviations:

ABC: ATP-binding cassette

ADR: adverse drug reaction

ALT: alanine transaminase

APAP: acetaminophen

AST: aspartate transaminase

BMI: body mass index

CT: computed tomography

CYP: cytochrome P450

EASL: European Association for the Study of the Liver

FLI: fatty liver index

GGT: gamma-glutamyl transferase

HSI: hepatic steatosis index

HU: Hounsfield unit

LAP: lipid accumulation product

miRNA: microRNA

MR: magnetic resonance

MRE: magnetic resonance elastography

MRP: multidrug resistance-associated protein

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

OATP: organic anion transporting polypeptide

PDFF: proton density fat fraction

US: ultrasound

VCTE: vibration-controlled transient elastography

WC: waist circumference

## **Abstract:**

Non-alcoholic steatohepatitis (NASH) is the progressive form of non-alcoholic fatty liver disease (NAFLD) and is diagnosed by a liver biopsy. Due to the invasiveness of a biopsy, the majority of patients with NASH are undiagnosed. Additionally, the prevalence of NAFLD and NASH creates the need for a simple screening method to differentiate patients with NAFLD versus NASH. Non-invasive strategies for diagnosing NAFLD versus NASH have been developed, typically relying on imaging techniques and endogenous biomarker panels. However, each technique has limitations, and none can accurately predict the associated functional impairment of drug metabolism and disposition. The function of several drug metabolizing enzymes and drug transporters have been described in NASH that impacts drug pharmacokinetics. The aim of this review is to give an overview of the existing non-invasive strategies to diagnose NASH, and to propose a novel strategy based on altered pharmacokinetics using an exogenous biomarker whose disposition and elimination pathways are directly impacted by disease progression. Altered disposition of safe and relatively inert exogenous compounds may provide the sensitivity and specificity needed to differentiate patients with NAFLD and NASH to facilitate a direct indication of hepatic impairment on drug metabolism and prevent subsequent adverse drug reactions.

**Significance Statement:** This review provides an overview of the main non-invasive techniques (imaging and panels of biomarkers) used to diagnose non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) along with a biopsy. Pharmacokinetic changes have been identified in NASH and this review proposes a new approach to predict NASH and the related risk of adverse drug reactions, based on the assessment of drug elimination disruption using exogenous biomarkers.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is a broad range of chronic liver diseases characterized by the accumulation of fat in the liver, called steatosis, not related to the consumption of alcohol (Chalasani *et al.*, 2018). NAFLD may develop into its progressive form, non-alcoholic steatohepatitis (NASH), which is accompanied by inflammation and hepatocyte ballooning, with risk for fibrosis (Ahmed *et al.*, 2015). Notably, NASH is considered the second most common indication for liver transplant in patients with chronic liver diseases (Goldberg *et al.*, 2017; Younossi *et al.*, 2021). The progression to NASH from simple NAFLD increases the risk of comorbidities such as cardiovascular disorders, metabolic diseases, and complications including cirrhosis and hepatocellular carcinoma (Singh *et al.*, 2015; Adams *et al.*, 2017). Therefore, the ability to screen for NAFLD accurately and safely, and more specifically NASH, is crucial to public health.

An epidemiologic meta-analysis estimated the global prevalence of NAFLD at 25.24% (Younossi *et al.*, 2016). The analysis suggested significant geographic disparities with highest prevalence of NAFLD in the Middle East and South America (31.79% and 30.45%, respectively) and lowest in Africa (13.48%) (Younossi *et al.*, 2016). This study also illustrated disparities related to the diagnostic methods: only the studies using imaging to diagnose NAFLD were included in the calculation of the prevalence because of their better accuracy (Younossi *et al.*, 2016). However, the pooled regional NAFLD prevalence were drastically lower when calculated based on blood test diagnosis than on imaging (for example 12.89% with blood test versus 24.13% with imaging in North America) (Younossi *et al.*, 2016). These results suggest an underestimation using blood tests in diagnosing NAFLD, probably due to fluctuating liver enzymes in the disease (Younossi *et al.*, 2016). Among biopsied NAFLD patients, the analysis showed the prevalence of NASH was 59.10% (Younossi *et al.*, 2016). However, the overall

prevalence of NASH in the general population is actually estimated at only 1.5-6.45%, probably because diagnosis requires histological confirmation by biopsy (Younossi *et al.*, 2018a).

Liver biopsy is considered the “gold standard” to diagnose NAFLD and to assess its progression to NASH (Younossi *et al.*, 2018b). The NAFLD activity score (NAS) is a commonly proposed method to measure disease progression, consisting of a compilation of unweighted factors calculated from biopsy (steatosis, lobular inflammation, and hepatocyte ballooning), with their sum indicating the degree of NAFLD (Kleiner *et al.*, 2005). The sum ranges from 0 to 8, and a result  $\geq 5$  indicates NASH, whereas a score  $\leq 2$  is described as “no-NASH” (Brunt *et al.*, 2011). Although liver biopsy is the “gold standard” for diagnosing NAFLD and NASH, this technique is not always consistent because of inter- and intra-operator errors that can occur in both biopsy sampling and NAFLD activity scoring (Bedossa, 2014). Due to the invasiveness of the procedure, there is a risk of pain, bleeding, and infection (Rockey *et al.*, 2009; Arab *et al.*, 2018; Chalasani *et al.*, 2018). Performing a biopsy without other strong indication of disease progression or future complications can be excessive in most patients, especially considering the lack of approved treatments for NAFLD and NASH (Younossi *et al.*, 2018c).

In the United States, systematic screening of NAFLD is not recommended because the gray-area between NASH and “no-NASH” of early-onset disease is difficult to assess, unless indicators suggest advanced fibrosis (Chalasani *et al.*, 2018). However, some studies indicate that patients with NAFLD may have an increased risk for adverse drug reactions (ADRs), including drugs largely used in the general population such as acetaminophen, corticosteroids, antibiotic, or antidiabetic drugs (Chalasani *et al.*, 2015; Massart *et al.*, 2017; Allard *et al.*, 2019; Lammert *et al.*, 2019). Identifying these NAFLD and NASH patients at risk of ADRs may be valuable to propose personalized treatments and limit ADR onset and their consequences on health. Thus, other modes of diagnosing NAFLD are often explored prior to biopsy, avoiding this invasive procedure when possible, and relying mostly on blood tests, risk factors and imaging

modalities (Younossi *et al.*, 2018b). Although these techniques are accurate to detect steatosis and fibrosis, no individual test has an accuracy comparable to biopsy to draw a distinction between NAFLD and the non-fibrotic early stage of NASH, nor do they offer any information regarding the increased risk for ADRs (Chalasani *et al.*, 2018). Consequently, there is a need for more accurate, non-invasive tests to diagnose NASH at its earliest onset, and for a direct functional assessment of liver impairment that may indicate an increased risk of ADR due to altered drug metabolism and transport capacity. The ability to predict NASH and the related risk of ADRs would be a powerful tool in the hands of clinicians tracking disease progression in patients. Herein is an overview of the current non-invasive methods used for diagnosing NASH, as well as a proposed novel strategy to predict disease stage based on drug disposition.



## Imaging Diagnosis

Non-invasive imaging techniques have been developed to make NASH diagnosis simpler and safer to apply than biopsy and offer an advantage for patients and clinicians (Madrazo, 2017). Imaging techniques enable a complete surveillance of the liver, which is valuable when the distribution of fat or other pathological features in a liver is not uniform. In these cases, a biopsy-based diagnostic can fail if the sample is not collected in the damaged part of the liver, while imaging techniques offer a more global overview of the liver (Sumida *et al.*, 2014).

### *Imaging NAFLD*

The first and most common method developed for the diagnosis of NAFLD is ultrasound (US), where the appearance of hepatomegaly with hyperechogenic hepatic tissues (showing more echogenicity than the kidneys), vascular blurring, and deep attenuation on ultrasonography are consistent with liver steatosis (Table 1) (Obika and Noguchi, 2012; Madrazo, 2017). Steatosis is reported to be detectable by US when more than 20% of hepatocytes contain histologically visible fat (Shannon *et al.*, 2011). However, the accuracy of US in detecting hepatic steatosis is affected by the presence of severe fibrosis, morbid obesity, or in individuals with mild steatosis with a fatty change of less than 23% - 30% (Sumida *et al.*, 2014; Zhou *et al.*, 2019). Recently, researchers have tried to improve this technique by developing US adaptative sound speed as a score of steatosis (Imbault *et al.*, 2017, 2018; Dioguardi Burgio *et al.*, 2019). Although this procedure is associated with significant inter- and intra-observer variability and is operator-dependent, it remains the first-line imaging test used in clinical practice especially for screening of suspected NAFLD because of its lack of invasiveness, wide availability, and relatively low cost (Singh *et al.*, 2013; European Association for the Study of the Liver (EASL) *et al.*, 2016).

Liver imaging by computed tomography (CT) is available to assess steatosis by measuring the attenuation which is correlated with the degree of intrahepatic fat accumulation (Table 1) (Li *et al.*, 2018b). Hepatic attenuation is inversely associated with the fat content, which means that a

fatty liver has a lower attenuation on CT images than a normal liver (Lee and Park, 2014). The most common CT method of diagnosing NAFLD involves the determination of liver attenuation of scans compared to that of the spleen (the liver-to-spleen attenuation difference). Normal liver has an attenuation value of 50-65 Hounsfield units (HU), which is generally 8-10 HU higher than that of the spleen (Li *et al.*, 2018b). In contrast, the attenuation value of the liver may decrease to less than 40 HU when fatty infiltration occurs, and a lower hepatic attenuation of 10 HU relative to the spleen is consistent with hepatic steatosis (Madrazo, 2017). Nonenhanced CT is more commonly used than enhanced CT and outperforms US in evaluating the severity of steatosis, achieving a specificity of 100% and sensitivity of 82% for diagnosing higher degrees of hepatic steatosis (>30%) (Park *et al.*, 2006). Nevertheless, the inconvenience of CT is that this method is associated with the use of radiation involving larger doses than the more common conventional X-ray imaging procedures and is considered a carcinogenic risk (Brenner and Hall, 2007). For these reasons, CT is not routinely used to screen for NAFLD in clinical practice.

The more recent introduction of magnetic resonance (MR) expanded the ability to diagnose NAFLD via imaging. MR determines steatosis by signal intensity differences on opposed-phase or fat saturation (Table 1) (Springer *et al.*, 2010). The sensitivity and specificity of MR for detecting histologically confirmed steatosis ( $\geq 5\%$ ) are both approximately 90% (Li *et al.*, 2018b). Several MR sequences have been developed to biochemically assess the liver such as MR spectroscopy, opposed-phase imaging, or complex-based chemical shift imaging-based MR (Madrazo, 2017; Zhou *et al.*, 2019). An advanced and robust technique for assessing hepatic steatosis is MR-derived proton density fat fraction (PDFF, Table 1) (Dulai *et al.*, 2016). It enables the determination of the liver fat fraction using MRI-visible protons bound to fat in the liver to quantify steatosis by dividing all protons in the liver. Thus, the liver signal on MRI is split into water and fat signal components by measuring gradient echoes at appropriately spaced

echo times, to quantify the percentage of liver fat (Yokoo *et al.*, 2011). A robust correlation was reported between the results with PDFF and histological assessment of steatosis with higher performances for PDFF, with no impact of obesity on the results (Tang *et al.*, 2013; Zhou *et al.*, 2019). PDFF has been shown the most accurate imaging method for measuring hepatic fat content and is even more sensitive than the histology-determined steatosis grade in quantifying longitudinal changes (Noureddin *et al.*, 2013). It allows fat mapping of the entire liver and can be determined with any clinical MR platform so it can easily be used as an imaging biomarker to quantify changes in liver fat. Compared to CT, PDFF offers the advantage to be a non-radiative modality allowing for a follow-up of the disease over the time with promising sensitivity to assess longitudinal changes.

However, the applications of these imaging methods are still relatively limited in the detection of inflammation, which is more important than steatosis in terms of its high risk for fibrosis, cirrhosis, and hepatocellular carcinoma (Li *et al.*, 2018b). It is also difficult to distinguish NAFLD from NASH with these routing imaging techniques because NASH consists of various parameters, including liver fibrosis which can cause progressive liver damage, and none of these techniques can predict the degree of liver fibrosis (Zhou *et al.*, 2019).

### *Imaging NASH*

Elastography is a method developed more recently which can help to distinguish NASH from simple steatosis. It consists of measuring the degree of tissue stiffness by quantifying the speed of propagation of a shear wave through tissue (Madrado, 2017). Scales for grading tissue stiffness have been compared to the degree of fibrosis seen on biopsies of the liver (Deffieux *et al.*, 2015). This technique allows the presence of fibrosis in tissues to be predicted by the degree of stiffness, which is often a sign of steatohepatitis present in NASH and not yet in NAFLD. Elastography can be performed both by US and MR (Zhou *et al.*, 2019).

The first Food and Drug Administration (FDA) approved and widely used elastography method is the Vibration-Controlled Transient Elastography (VCTE, Fibroscan<sup>®</sup>, Echosens<sup>®</sup>) employing US (Table 1) (Sandrin *et al.*, 2002; Wong *et al.*, 2010). This technology assesses hepatic stiffness by quantifying the speed of shear wave emitted by a vibrator in the intercostal space and followed by US (Li *et al.*, 2018b). When hepatic tissue has fibrous elements, the US transmission wave is faster than in normal tissues and the velocity is positively related to liver stiffness (Zhou *et al.*, 2019). VCTE is largely used because of its exceptional quantification capacity but the detection is limited to a local scale of the tissue. A more recent approach based on VCTE, controlled attenuation parameter (CAP), simultaneously measures stiffness and steatosis (Table 1) (Myers *et al.*, 2012). Even though, the CAP method has been considered a promising non-invasive test for diagnosing and staging of hepatic steatosis, a recent systematic review reported the limits of this technique, such as the high rates of missed or wrong diagnosis in patients with a high stage of steatosis and obesity (Pu *et al.*, 2019). This limitation restricts its application, especially when patients present with metabolic syndrome, which is often the case for this diagnostic. MR elastography (MRE) is also based on shear wave propagation but with MR modality, using a modified phase-contrast sequence and an external mechanical actuator to induce and non-invasively visualize propagating tissue shear waves (Table 1) (Cui *et al.*, 2015; Doycheva *et al.*, 2016; Li *et al.*, 2018b). MRE offers the advantage to perform 3-dimensional imaging and to assess the entire liver with a high success rate. It is not affected by steatosis and may be applied in patients with obesity, ascites, or intestinal interposition between the liver and anterior abdominal wall (Yin *et al.*, 2007; Venkatesh *et al.*, 2013). However, MRE is expensive and requires heavy equipment, which makes it difficult for wide applications. More competitive, feasible, and easy to implement methods are needed for diagnosing NASH, especially for initial NASH without fibrosis.

## Biomarkers and Panels

### *Assessing NAFLD*

While NAFLD is occasionally identified incidentally by imaging, elevated aminotransferase levels are the first indication of hepatic impairment for most patients. However, liver enzymes alone are neither sufficient, nor reliable to diagnose NAFLD. A study suggested that up to 80% of NAFLD patients may present normal levels of alanine transaminases (ALT) (Browning *et al.*, 2004). Thus, several panels of biomarkers have been developed, striving to detect hepatic steatosis and to guide further investigations, by way of liver biopsy or imaging (Piazzolla and Mangia, 2020).

Among the scoring systems and panels of biomarkers that have been well validated, the Fatty Liver Index (FLI) has been demonstrated as a simple and accurate predictor of NAFLD (Table 2) (Bedogni *et al.*, 2010). The FLI uses easy-to-obtain parameters such as body mass index (BMI), waist circumference (WC), serum levels of triglycerides and gamma-glutamyl-transferase (GGT). FLI has shown good performance in detecting fatty liver but a poor ability to distinguish moderate-to-severe steatosis from mild steatosis (Fedchuk *et al.*, 2014). A simplified index, adjacent to the FLI, is the Lipid Accumulation Product (LAP) which relies only on the measurement of WC and the serum level of triglycerides, weighted by gender (Table 2). This index was first developed to identify cardiometabolic disorders but has been shown to be a simple and reasonably accurate predictor of steatosis (Bedogni *et al.*, 2010). Both FLI and LAP have been developed in Caucasian populations but demonstrated difficulties in other populations because of differences in BMI and WC. For this reason, Lee *et al.* developed the Hepatic Steatosis Index (HSI) which was assessed in a large cohort of Korean patients (Table 2). This index is calculated based on aspartate transaminase (AST) and ALT ratio, BMI, gender information, and Type 2 Diabetes with a good prediction for NAFLD (Lee *et al.*, 2010).

Nevertheless, the HSI accuracy decreases in obese children and similarly to the FLI, the HSI poorly distinguishes moderate-to-severe steatosis from mild steatosis (Fedchuk *et al.*, 2014).

Interestingly, while the above-mentioned scores were validated against US imaging for steatosis, the NAFLD Liver Fat Score (NLFS) was standardized against MR spectroscopy. This score includes as variables the fasting serum insulin and AST levels, the AST/ALT ratio, and the presence of metabolic syndrome and/or Type 2 Diabetes (Table 2). However, the inclusion in the formula of serum insulin level, which is not a routine test, is a limitation to its wide clinical use (Piazzolla and Mangia, 2020). Another test, validated with liver biopsy as reference, is the commercial biomarker panel SteatoTest<sup>®</sup> (Biopredictive, Paris, France). It uses the combination of several biochemical parameters (serum levels of total cholesterol, triglycerides, glucose, bilirubin, GGT,  $\alpha$ 2-macroglobulin, haptoglobin, apolipoprotein A1, and ALT) associated to age, gender, and BMI (Table 2) (Poynard *et al.*, 2005). The accuracy of this test is limited by the potential variability of BMI and total bilirubin, which is why a second version of this test has recently been developed, excluding these two parameters (Poynard *et al.*, 2019).

Given the lack of evidence for cost-effectiveness of systematic screening for NAFLD, the American Association for the Study of Liver Disease (AASLD) does not currently recommend it unless there is a suspicion of advanced fibrosis (Chalasani *et al.*, 2018). EASL, however, has advised its screening in patients with obesity, Type 2 Diabetes, or metabolic syndrome (European Association for the Study of the Liver (EASL) *et al.*, 2016). Although these scores have potential in predicting NAFLD, they will most likely serve as indicators in the early screening steps to detect hepatic steatosis risk in patients. Nevertheless, these tools are valuable in assessing NAFLD to predict NASH at an early stage, alongside other biomarkers used for inflammation and fibrosis diagnosis.

### *Assessing NASH*

While screening for NAFLD is a major element to identify NASH patients, there is a gap between non-invasive prediction of NAFLD and NASH. No test currently distinguishes NAFLD from NASH patients in clinical settings beside liver biopsy. However, several steps have been made toward creating predictors of NASH that have utility in helping to identify which patients need a biopsy. NASH is a progressive form of NAFLD in which the steatosis is often associated with inflammation, fibrosis, and hepatocyte ballooning or other hepatocellular injury. Several biomarkers and biomarker panels associated with these features have been studied as predictors of the disease. A few of the major biomarkers are discussed here.

One of the most studied biomarkers for NASH is Cytokeratin-18 (CK-18) fragment levels, a marker of hepatocyte apoptosis cleaved during the period of cell death (Wieckowska *et al.*, 2006). This biomarker has been considered promising in NASH diagnosis, but some issues were identified later, such as limited sensitivity at the individual level and considerable variability in the suggested cutoffs and their respective diagnostic accuracy (Castera *et al.*, 2019). Other individual biomarkers explored are inflammatory markers such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin 8 (IL-8), but also showed moderate performances with variability across studies (Qi *et al.*, 2017). Other biomarkers to assess fibrosis and collagen levels, particularly pro collagen III (Pro-C3), which reflects the expression of extracellular matrix turnover, have been explored (Tanwar *et al.*, 2013).

However, each biomarker considered individually for the diagnosis of NASH has been inadequate given the complexity of the disease. To improve performance, a variety of multiple predictive panels have been developed over the two last decades, combining several biomarkers assessing different features of the disease to offer a general overview of disease stage. One of the first panels is the HAIR score, named according to the parameters included in the panel: hypertension, ALT, and insulin resistance (Table 2) (Dixon *et al.*, 2001). Later,

Palekar *et al.* generated a panel of six markers combining age, sex, AST, BMI, AST/ALT ratio, and serum hyaluronic acid (Table 2) (Palekar *et al.*, 2006). The NASHTest<sup>®</sup> (Biopredictive, Paris, France) combines 13 parameters (age, sex, height, weight, serum levels of triglycerides, cholesterol,  $\alpha$ 2-macroglobulin, apolipoprotein A1, haptoglobin, GGT, ALT, AST, and total bilirubin, Table 2) and enables a classification of the disease in three categories called NASH, borderline NASH, and no-NASH (Poynard *et al.*, 2006). More recently, the new NASH ClinLipMet score was developed, derived from a previous panel, the NASH Clin score, but improved by adding metabolic syndrome-based factors (Zhou *et al.*, 2016). The originality of this panel is to combine genetic, clinical, lipodomic, and metabolomic markers (glutamate, isoleucine, glycine, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6, AST, fasting insulin, and PNPLA3 genotype) (Table 2). However, this panel is not widely used because of the measurements of fasting insulin and PNPLA3 which are complex and costly (Zhou *et al.*, 2019). These scores are predictive of the disease with a sensitivity generally around 80% in the tested cohorts, but none are able to precisely differentiate NASH from simple NAFLD (Obika and Noguchi, 2012). Combination of these panels with others assessing fibrosis (the most clinically relevant factor for disease outcomes) can improve diagnostic accuracy. Several panels have initially been developed for the clinical diagnosis of hepatitis but can be adapted to NASH. Among them, a simple test is the Fib-4 test which includes age, platelet count, AST, and ALT (Table 2) (Vallet-Pichard *et al.*, 2007). Shah *et al.* validated its use in patients with NAFLD in a study demonstrating its superiority to seven other panels of fibrosis biomarkers (Shah *et al.*, 2009). The study involved some easy panels widely used in clinical practice such as the BARD score containing the parameters of BMI, AST/ALT ratio, and the presence of Type 2 Diabetes, or the NAFLD fibrosis score (NFS), consisting in age, BMI, hyperglycemia, AST/ALT ratio, platelets, and albumin (Table 2) (Angulo *et al.*, 2007; Ruffillo *et al.*, 2011). Both American and European guidelines recommend the use of Fib-4 and NFS to identify patients with NAFLD at risk for fibrosis (European Association for the Study of the Liver (EASL) *et al.*, 2016).



Beyond these well-known and validated panels of biomarkers, a variety of novel blood-based biomarkers are actually emerging, detected in circulating extracellular vesicles (Piazzolla and Mangia, 2020). Recent studies showed that the release of some ectosomes and exosomes is increased in NAFLD and NASH patients and that the molecules transported in these vesicles could be used to detect the disease (Kornek et al., 2012; Chen et al., 2018; Malhi, 2019). Different proteins may be of interest such as the CD10 protein whose increase in urinary exosomes was associated with steatosis and fibrosis in transgenic mice, with a change detected at the early stage of the disease and consistent over the time of the progression (Conde-Vancells et al., 2010). Other promising biomarkers are small non-coding microRNA (miRNA) which transcriptionally regulate gene expression (Newman et al., 2020). Diverse vitro and vivo studies reported upregulation of miRNA observed in NAFLD and NASH (Lee et al., 2005; Pirola et al., 2015). The most relevant of them is the miRNA-122 which is involved in lipid metabolism and whose serum concentrations have been shown upregulated in NASH while the hepatic concentrations were downregulated, probably due to an increase of the release (Szabo and Csak, 2016).

### **Altered Drug Elimination as a Predictor of NASH**

A major concern for patients with chronic liver diseases is the impairment of liver function and consequently the related risk of drug elimination disruption. The imaging techniques and biomarker panels discussed thus far assess different features of NAFLD and NASH diseases but do not provide information about the impairment of the pharmacokinetic process. Non-invasively predicting the risk of drug toxicity associated with these liver impairments has been elusive so far and would be an imperative predictive tool for patients. Because the liver plays a key role in the metabolism and disposition of numerous drugs, a novel strategy to distinguish patients with NASH from NAFLD could be developed to exploit alterations in the function of drug metabolizing enzymes and transporters that are specific to NASH. Here, we offer our

perspective regarding the opportunities and challenges of this new and non-invasive method which could be used to easily assess disease progression, as well as a direct indication of hepatic impairment to reduce the risk of ADRs.

The liver plays a central role in the pharmacokinetics of drugs. Drug metabolizing enzymes and transporters provide the underlying absorption, distribution, metabolism, and elimination (ADME) mechanisms, and NASH has been shown to cause disease-specific alterations to the function of numerous enzymes and transporters (Li *et al.*, 2018a). Several of these metabolic pathways have been largely characterized and changes in elimination could be used as a predictive tool so long as the mechanistic alterations in enzyme and transporter functions are specific to the progression to NASH.

Many phase I biotransformation enzymes were analyzed for mRNA, protein, and enzymatic activity in patients with varying progression of NAFLD to NASH (Fisher *et al.*, 2009). The main changes identified were a decrease in cytochromes CYP1A2 and CYP2C19 mRNA, protein levels, and enzymatic activities, while they were increased for CYP2C9 and CYP2A6 (Figure 1). Another study conducted in pediatric patients confirmed a significant reduction in CYP2C19 activity, which appears to be the cytochrome most highly impacted by NASH (Li *et al.*, 2017). A decrease in CYP3A4 mRNA expression and CYP3A activity has also been reported in NAFLD patients (Woolsey *et al.*, 2015) (Figure 1). Enzymes responsible for phase II biotransformation showed that sulfotransferase SULT1C4 increased in mRNA and protein expression in NASH (Hardwick *et al.*, 2013) (Figure 1). Additionally, other enzymes involved in the antioxidant response may also be impacted by NASH such as the NAD(P)H:quinone oxidoreductase 1 and glutathion transferases whose mRNA, protein expressions and activity are increased in NASH patients (Hardwick *et al.*, 2010) (Figure 1).

Regarding transporters, most of the hepatic ATP-binding cassette family (ABC) have been reported to have increased protein expression levels in NASH patients (Hardwick *et al.*, 2011;

Tanaka *et al.*, 2012; Okushin *et al.*, 2016). These proteins are involved in the efflux of substrates from the liver into bile (e.g. P-glycoprotein (P-gp) and the Breast Cancer Resistance Protein (BCRP)) mediating biliary efflux at the canalicular membrane, or from the liver into blood (Multidrug Resistance-Associated Protein (MRP) 1, MRP3, MRP4, MRP5, MRP6) responsible for sinusoidal efflux (Figure 1). Interestingly, the canalicular transporter MRP2 was described to have an altered localization, resulting in a loss of its efflux activity (Canet *et al.*, 2015) (Figure 1). Contrary to the ABC transporters, the majority of uptake transporters of the Solute Carrier family (SLC) expressed in the liver have been shown to have decreased protein expression levels in NASH patients (Vildhede *et al.*, 2020). These transporters are localized on the sinusoidal membrane where they mediate the hepatic uptake of substrates (Figure 1). The study included the three main Organic Anion Transporting Polypeptides (OATP) 1B1, OATP1B3, OAT2B1, but also the Organic Anion Transporters (OAT) 2 and OAT7, and the Sodium Taurocholate Protein (NTCP), all of which had decreased expression in NASH (Vildhede *et al.*, 2020).

Several studies have demonstrated altered pharmacokinetics in NASH patients using drugs whose elimination is dependent on the pathways described previously. A study performed with morphine reported that both hepatically-derived morphine glucuronides (morphine-3- and morphine-6-glucuronides) had increased maximal concentrations ( $C_{max}$ ) and area under the curves (AUC) in the serum of NASH patients, and that the degree of this increase was correlated to the severity of the disease (Ferslew *et al.*, 2015). A similar study was conducted with acetaminophen (APAP) in a pediatric population and found that NASH patients had increased serum and urinary levels of APAP-glucuronide associated with decreased serum levels of APAP-sulfate (Canet *et al.*, 2015). An impact on midazolam pharmacokinetics has also been detected in a cohort of NASH patients presenting 2.4-fold higher plasma levels compared with controls (Woolsey *et al.*, 2015).

One of the advantages of a pharmacokinetic biomarker is the potential for a single drug to be dependent upon multiple NASH-altered ADME processes that create a potentiated change in disposition. Much like how adding characters to the length of a password increases the security of identification, drugs using enzymes and transporters specifically impacted by NASH may increasingly differ in disposition and elimination. Additionally, pharmacokinetic disposition includes a component of directionality, where disposition of metabolites can be eliminated into bile, or retained in plasma, with consequent changes that can appear in liver impairment. Drugs that rely on metabolizing enzymes, and uptake and efflux transporters that are each individually altered at the stage of NASH could have dramatic differences in NASH patients when compared to patients with healthy livers and the potential difference could allow a selective diagnosis. The specificity of this approach needs to be tested since many other factors may alter drug disposition, especially the absorption phase if the drug is given orally. However, the selection of a drug undergoing several overlapping mechanisms altered by NASH increases the specificity of this approach. The validation also requires repeated tests to assess the feasibility and practicality, and other parameters such as the sensitivity and robustness of the method.

An advantage of this approach against the previous methods already existing is that the altered drug disposition might be used as a direct measure of hepatic function to indicate risks for ADRs from drugs that are dependent on enzymes and transporters altered in NASH. Hence, an exogenous probe drug strategy could have tremendous benefit in the drug development process as a companion diagnostic test for drugs with the potential for ADRs due to hepatic impairment. For such a test, the drug selected as the companion diagnostic should be validated by establishing a correlation between the pharmacokinetic changes observed for both the companion diagnostic and the drug being developed.

For exogenous diagnostics, additional safety standards must be considered. It is intuitive that the potential for any toxicity caused by the diagnostic probe drug must be significantly less than

the disease to be diagnosed. As such, the ideal drug would need to be previously approved by the FDA for use in humans, have a long track record with minimal safety concerns, and have little to no pharmacologic effect at the dose used for diagnosis. Since the object of the exogenous probe drug is to identify pharmacokinetic changes rather than pharmacodynamic changes, it is possible to use a subtherapeutic dose of the probe drug, as low as possible to be reasonably quantified in plasma and/or urine. This drug validated as a predictor of NASH would be administered in a single dose and the test could be performed with a simple blood and/or urine sample in order to assess the concentration of the drug and/or its metabolites. The results measured would be compared to thresholds for healthy and disease individuals validated beforehand.

## Conclusion

The development of an accurate, non-invasive diagnostic tool would streamline the diagnosis of NASH patients and provide a direct indication of hepatic impairment that could identify individuals at risk for future ADRs. Currently, no approved pharmacotherapy is available for NASH patients, but several drugs are in the pipeline. It is critical that the population in need of these therapies is readily identifiable. Using the novel strategy of pharmacokinetic changes of exogenous probe drugs has the capacity to provide a precise diagnostic tool. This strategy represents an unexplored, but mechanistically promising approach to directly assess hepatic impairment via exogenous predictors and opens new clinical opportunities.

## Authorship contributions

*Wrote or contributed to the writing of the manuscript:*

Marie, Tripp, and Cherrington

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## Figure legend

### **Figure 1 – Main pharmacokinetic changes reported in NASH compared to a healthy liver.**

The activity of the influx transporters (OATP1B1, OATP1B3, OATP2B1, OAT2, OAT7, NTCP) is decreased while the activity of the efflux transporters (BCRP, P-gp, MRP3, MRP4, MRP5, MRP6) is increased, with a mislocalization of MRP2. The activity of the phase I metabolism enzymes is both decreased (CYP1A2, CYP219, CYP3A4) and increased (CYP2C9, CYP2A6) while some of the phase II metabolism enzymes (SULT1C4, NAD(P)H:quinone oxidoreductase 1, glutathion transferase) present an increase of the activity.

## Tables

Table 1 – Overview of the main imaging techniques used for diagnosing NAFLD and NASH.

Approaches	Applications	Limitations
<b>CAP</b>	Stiffness and steatosis simultaneously measured based on VCTE.	High rates of missed or wrong diagnosis in the patients with a high stage of steatosis and obesity.
<b>CT</b>	Steatosis by measurement of attenuation which is correlated with the degree of intrahepatic fat accumulation.	Radiations.
<b>MR</b>	Steatosis by signal intensity differences on opposed-phase or fat saturation.	Price and equipment.
<b>MR-PDFF</b>	Steatosis by dividing all protons in the liver using MRI-visible protons bound to fat.	Price and equipment.
<b>MRE</b>	Stiffness based on shear wave propagation using MR modality.	Price and equipment.
<b>US</b>	Steatosis based on appearance of hepatomegaly with hyperechogenic hepatic tissues, vascular blurring, and deep attenuation.	Accuracy may be affected by the presence of severe fibrosis, morbid obesity, or in individuals with mild steatosis.
<b>VCTE</b>	Stiffness by quantifying the speed of shear wave emitted by a vibrator in the intercostal space and followed by	Detection limited to a local scale of the tissue.

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Panels	Applications	$\alpha$ 2-macroglobulin	Albumin	Apolipoprotein A1	Age	ALT	AST	AST/ALT ratio	Bilirubin	BMI	Cholesterol	Gender	GGT	Glucose	Glutamate	Glycine	Haptoglobin	Height	Hyaluronic acid	Hypertension	Insulin	Isoleucine	Lysophosphatidylcholine 16:0	Downloaded from <a href="https://www.aspetjournals.org">https://www.aspetjournals.org</a> at ASPET Journals on April 8, 2024	Phosphoethanolamine 40:6	Platelet count	PNPLA3 genotype	Type 2 diabetes	Triglycerides	Waist circumference	Weight		
BARD score	Fibrosis							✓		✓																			✓				
Fib-4	Fibrosis				✓	✓	✓																				✓						
FLI	Steatosis									✓			✓																	✓	✓		
HAIR score	NASH					✓														✓	✓												
HIS	Steatosis							✓		✓		✓																	✓				
LAP	Steatosis											✓																		✓	✓		
NFS	Fibrosis		✓		✓			✓		✓				✓													✓		✓				
NASH ClinLipMet score	NASH						✓								✓	✓					✓	✓	✓			✓		✓					
NASHTest®	NASH	✓		✓	✓	✓	✓		✓		✓	✓	✓				✓	✓												✓		✓	
NFLS	Steatosis						✓	✓													✓			✓					✓				
Palekar score	NASH				✓		✓	✓		✓		✓							✓														
SteatoTest®	Steatosis	✓		✓	✓	✓			✓	✓	✓	✓	✓	✓			✓													✓			

Table 2 - Overview of the main panels of biomarkers used for diagnosing NAFLD and NASH and the parameters involved in each panel.

## Footnotes

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Figure 1  
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Healthy liver

NASH liver

