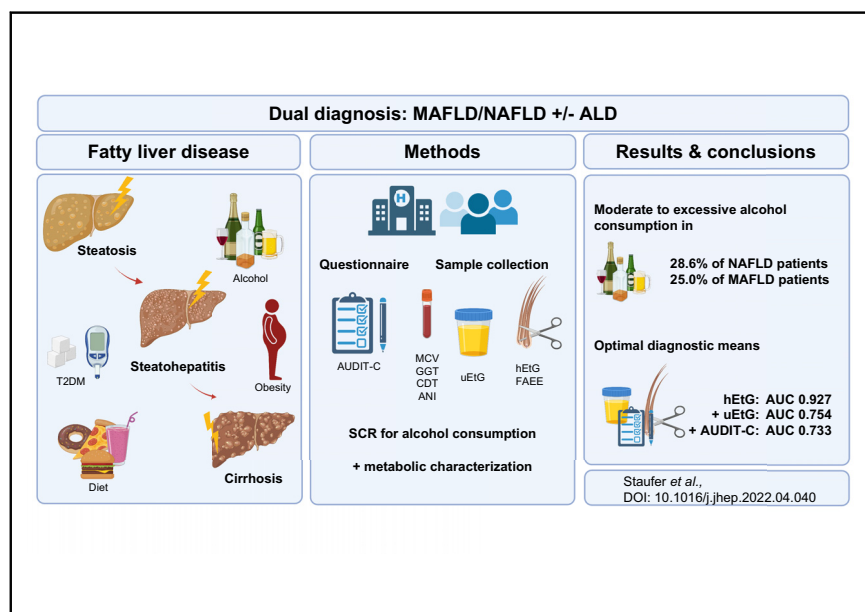


# Ethyl glucuronide in hair detects a high rate of harmful alcohol consumption in presumed non-alcoholic fatty liver disease

## Graphical abstract



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## Lay summary

Fatty liver disease can be caused by metabolic factors and/or alcohol consumption. The diagnosis of non-alcoholic fatty liver disease (NAFLD) is based on the exclusion of harmful alcohol consumption, while metabolic dysfunction-associated fatty liver disease (MAFLD), which has been proposed as a new name for NAFLD, is based on the presence of metabolic comorbidities and allows for alcohol consumption. Herein, we show that up to 29% of patients diagnosed with NAFLD and 25% with MAFLD are at risk of alcohol-related liver damage. We show that ethyl glucuronide (a metabolite of alcohol) in the hair and urine can accurately detect potentially harmful alcohol consumption in these patients – as such, these tests should be integrated into routine diagnostic work-up for patients with fatty liver disease.

## Highlights

- Alcohol intake may be a relevant contributor to the progression of fatty liver disease.
- NAFLD and ALD have not been reliably distinguished by established diagnostic means.
- We found relevant alcohol intake in 29% of patients with NAFLD, and 25% with MAFLD.
- hEtG and uEtG showed good to excellent accuracy to detect alcohol intake.
- They should be used for routine diagnostic work-up in addition to AUDIT-C.

# Ethyl glucuronide in hair detects a high rate of harmful alcohol consumption in presumed non-alcoholic fatty liver disease

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**Background & Aims:** Non-alcoholic fatty liver disease (NAFLD) and alcohol-related liver disease (ALD) cannot reliably be distinguished by routine diagnostics, and the role of alcohol consumption in metabolic dysfunction-associated fatty liver disease (MAFLD) remains unclear. We investigated alcohol consumption in patients with presumed NAFLD and ALD using novel objective alcohol markers.

**Methods:** In total, 184 consecutive patients were included in this prospective observational study. Alcohol intake was assessed by ethylglucuronide in hair (hEtG) and urine (uEtG); the utility of these measures for alcohol detection was compared to Alcohol Use Disorders Identification Test-Consumption (AUDIT-C), carbohydrate deficient transferrin (CDT), mean corpuscular volume (MCV), gamma-glutamyltransferase (GGT), and ALD/NAFLD index (ANI). Clinical characteristics of patients with NAFLD and ALD were re-assessed after reclassification based on repeated moderate ( $\geq 10$  g < 60 g EtOH/day) and excessive ( $\geq 60$  g EtOH/day) alcohol consumption, and patients were retrospectively reclassified based on MAFLD criteria.

**Results:** Repeated moderate to excessive alcohol consumption was detected in 28.6%, 28.5%, and 25.0% of patients with presumed NAFLD, ALD or MAFLD, respectively. ANI score, AUDIT-C, uEtG, and hEtG showed AUCs of 0.628, 0.733, 0.754, and 0.927 for the detection of repeated moderate to excessive alcohol consumption, respectively. The indirect markers CDT, MCV and GGT were not reliable. Patients with repeated moderate or excessive alcohol consumption were significantly more often male, had a significantly lower BMI, and suffered significantly less often from type 2 diabetes or impaired glucose tolerance.

**Conclusions:** In total, 28.6% of patients with presumed NAFLD, and 25.0% with MAFLD are at risk of alcohol-related liver

damage. AUDIT-C, uEtG and hEtG should be used to screen for alcohol consumption in patients with fatty liver disease.

**Lay summary:** Fatty liver disease can be caused by metabolic factors and/or alcohol consumption. The diagnosis of non-alcoholic fatty liver disease (NAFLD) is based on the exclusion of harmful alcohol consumption, while metabolic dysfunction-associated fatty liver disease (MAFLD), which has been proposed as a new name for NAFLD, is based on the presence of metabolic comorbidities and allows for alcohol consumption. Herein, we show that up to 29% of patients diagnosed with NAFLD and 25% with MAFLD are at risk of alcohol-related liver damage. We show that ethyl glucuronide (a metabolite of alcohol) in the hair and urine can accurately detect potentially harmful alcohol consumption in these patients – as such, these tests should be integrated into routine diagnostic work-up for patients with fatty liver disease.

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide.<sup>1</sup> The global prevalence of NAFLD and its progressive subtype non-alcoholic steatohepatitis (NASH) in the general population is estimated at 25%, and 3% to 5%, respectively.<sup>1,2</sup> NAFLD/NASH is closely associated with obesity and insulin resistance as part of the metabolic syndrome.<sup>3</sup> Seven percent of the normal-weight population,<sup>4,5</sup> and up to 93% of individuals with obesity are affected by NAFLD.<sup>6,7</sup> The proportion of NASH markedly increases from up to 8% to 40% depending on the number and severity of metabolic co-morbidities.<sup>7-9</sup>

The pathogenesis of NAFLD/NASH is multifactorial and incompletely understood. It has been mechanistically linked to (1) dietary factors,<sup>10,11</sup> (2) dysbiosis and deregulation of the gut endothelial barrier,<sup>12</sup> (3) changes in short chain fatty acids and bile acids,<sup>13</sup> as well as (4) single nucleotide polymorphisms (SNPs) and genetic variants of the patatin-like phospholipase

Keywords: non-alcoholic fatty liver disease; alcoholic liver disease; metabolic dysfunction - associated fatty liver disease; ethyl glucuronide; harmful alcohol consumption.

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domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*), Membrane-bound O-acyltransferase domain-containing protein 7 (*MBOAT7*), or 17 $\beta$ -hydroxysteroid dehydrogenase type 13.<sup>14,15</sup> Furthermore, increased endogenous ethanol (EtOH) levels due to alcohol-producing microbiota,<sup>16</sup> or insulin-dependent impairment of alcohol dehydrogenase activity in liver tissue in the presence of insulin resistance have been discussed as contributing factors to the development of NAFLD/NASH.<sup>17</sup>

To account for the heterogeneity in pathogenesis of fatty liver, an expert panel recently proposed the term metabolic dysfunction-associated fatty liver disease (MAFLD) defined as the evidence of hepatic steatosis in addition to either overweight/obesity, and type 2 diabetes mellitus (T2DM) or evidence of metabolic dysregulation irrespective of alcohol consumption or its amount.<sup>18</sup>

The impact of alcohol intake on the development and progression of NAFLD/NASH has remained unclear, and appears to be dependent on sex, and the dose and type of alcohol. Very low alcohol consumption of <10 g EtOH/day has even been considered protective, whereas data on moderate consumption are conflicting.<sup>19–22</sup> In contrast, excessive alcohol consumption of >60 g EtOH/day for at least 2 weeks has been found to cause steatosis in 90% of patients, of whom 20% to 40% may develop fibrosis, and 10% to 35% may develop steatohepatitis.<sup>23</sup>

Alongside the increasing burden of obesity, more than 50% of global alcohol is consumed by populations in the American, European and Western Pacific Region.<sup>24</sup> Worldwide, the average amount of pure EtOH ingested by people consuming alcohol is 32.8 g/day.<sup>24</sup> In 2016, it was estimated that 55.5% of the world population, 76.5% of the European population, and 83.1% of the American population consumed alcohol.<sup>24</sup> The harmful use of alcohol accounts for 5.3% of all deaths worldwide, leading to mortality rates exceeding those caused by diabetes.<sup>24</sup> In total, ALD accounts for 48% of cirrhosis-associated deaths in the US.<sup>24</sup> Thus, early diagnosis of patients with potentially harmful alcohol consumption is needed to provide adequate treatment.

The present study investigated alcohol consumption in patients with NAFLD and ALD, who were diagnosed according to currently applied routine diagnostic criteria including retrospective reclassification according to the recently proposed MAFLD criteria. We compared the current gold standard of alcohol detection to the direct long-term alcohol parameter ethyl glucuronide in hair (hEtG), and the direct very sensitive short-term parameter ethyl glucuronide in urine (uEtG), both being EtOH metabolites, as well as ALD/NAFLD Index (ANI).<sup>25</sup> We analysed their diagnostic accuracy for repeated moderate to excessive alcohol consumption in patients with presumed NAFLD and hence their ability to identify those at risk of alcohol-related liver damage. Patients with NAFLD were reclassified based on objective alcohol parameters, and clinical features of patients with and without alcohol-related liver damage were compared.

## Patients and methods

### Study population

We prospectively enrolled consecutive patients with NAFLD and ALD referred to 3 liver outpatient clinics in Austria from March 2013 through November 2018. Diagnosis of NAFLD was established by the presence of liver steatosis based on ultrasound, magnetic resonance imaging, computed tomography and/or liver

biopsy (steatosis in >5% of hepatocytes). As defined by the current EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease 2016,<sup>26</sup> NAFLD was additionally defined by diagnosis of exclusion, *i.e.* by excluding secondary causes by means of laboratory testing and detailed medical history (other chronic liver diseases such as viral hepatitis, autoimmune hepatitis, primary sclerosing cholangitis, hemochromatosis, Wilson's disease,  $\alpha$ 1-antitrypsin deficiency, coeliac disease, and drug-induced liver injury), and alcohol consumption  $\geq$ 20 g of EtOH per day in females, and  $\geq$ 30 g of EtOH per day in males. Daily alcohol intake was quantified by the standardized questionnaire Systematic Inventory of Alcohol Consumption (SIAC).<sup>27</sup> A reported alcohol consumption of  $\geq$ 20 g of EtOH/day or >7 units/week for women, and  $\geq$ 30 g/day or >14 units/week for men were used to distinguish NAFLD from ALD as previously recommended by EASL.<sup>26,28,29</sup> Liver biopsy was performed in unclear cases to rule out chronic liver diseases other than ALD/NAFLD or to stage NAFLD/NASH. Patients with ALD served as a comparative group. In addition, patients were retrospectively reclassified applying the recently proposed MAFLD criteria, including MAFLD-related cirrhosis (MAFLDC), and dual etiology fatty liver disease (DEFLD).<sup>18</sup> In contrast to NAFLD criteria, the diagnosis of MAFLD is based upon “positive” criteria. These criteria are defined as hepatic steatosis detected either by imaging techniques, blood biomarkers/scores, or liver biopsy with either BMI  $\geq$ 25 kg/m<sup>2</sup> or T2DM. For patients with a BMI <25 kg/m<sup>2</sup> at least 2 metabolic risk abnormalities need to be present in addition to hepatic steatosis.<sup>18</sup> Of note, MAFLD criteria have been applied regardless of alcohol consumption due to the absence of data.

This study was part of a larger Austrian project aimed at developing biomarkers to differentiate NAFLD from other chronic liver diseases, while performing a detailed metabolic characterization (BioPersMed). The study was performed in accordance with the guidelines of the Declaration of Helsinki (1964, including current revisions) and Good Clinical Practice Guidelines. The study protocol including amendments (#747/2011) was approved by the Ethics Committee of the Medical University of Vienna. Prior to any study-related procedures all patients signed a written informed consent.

### Assessment of alcohol intake

Study participants were screened for hazardous drinking and active alcohol use disorders using the Alcohol Use Disorders Identification Test - Consumption (AUDIT-C).<sup>30</sup> In men an AUDIT-C score of  $\geq$ 4, in women a score of  $\geq$ 3 was considered indicative of an alcohol use disorder. AUDIT-C and SIAC were applied during the same visit by the treating hepatologist, who was part of the study personnel. Patients in whom an alcohol use disorder was suspected, were referred to an addiction specialist.

Additionally, the semiquantitative amount of alcohol intake was assessed by objective alcohol parameters in hair, urine and blood: hEtG, the fatty acid ethyl ester (FAEE) ethyl palmitate in hair (hEtP), uEtG, carbohydrate deficient transferrin (CDT), mean corpuscular volume (MCV), and gamma glutamyltransferase (GGT). The findings were additionally compared to ANI score.<sup>25</sup> This score is based upon an equation that incorporates MCV, AST/ALT ratio, BMI and male sex:

$$-58.5 + 0.637 (\text{MCV}) + 3.91 (\text{AST/ALT}) - 0.406 (\text{BMI}) + 6.35 \text{ for male sex.}$$

Based upon this equation, an ANI >0 was reported to incrementally favour a diagnosis of ALD, while ANI <0 meant a higher likelihood of NAFLD.

According to the recommendations of the Society of Hair Testing (SoHT), hEtG was used as the first choice for abstinence assessment.<sup>31</sup> A negative hEtG result was confirmed by a negative hEtP result, yet a positive hEtG result could not be overruled by a positive hEtP test.<sup>32</sup>

In case alcohol marker test results were unexpectedly positive or high, thus contradictory to the quantitative EtOH amount reported and quantified by SIAC, the results were discussed with the patient. Only biomarkers that were positive, as confirmed by the patient, were counted as true positive.

Patients originally classified as NAFLD (= presumed NAFLD) were reclassified according to alcohol markers as patients with moderate risk of alcohol-related liver damage (hEtG >7 pg/mg and ≤30 pg/mg corresponding to ≥10 and <60 g EtOH/day = repeated moderate alcohol consumption), and as patients at high risk of alcohol-related liver damage (hEtG >30 pg/mg corresponding to >60 g EtOH/day = excessive alcohol consumption).

### Clinical characterization of the study population

Data on BMI, co-morbidities, and routine laboratory markers were collected. Insulin resistance was assessed through calculation of the homeostasis model assessment of insulin resistance (HOMA-IR).<sup>33</sup> Renal function was assessed by glomerular filtration rate calculated according to the Modification of Diet in Renal Disease formula.<sup>34</sup> Chronic kidney disease (CKD) was classified according to KDIGO guidelines<sup>35</sup> in order to avoid misclassification of patients as heavy drinkers due to false positive uEtG and hEtG levels.<sup>36–38</sup>

### Technical methods

hEtG was measured in hair samples collected from the vertex posterior of the head. The 2 segments 0–3 cm, reflecting alcohol consumption within the last 3 months, and 3–6 cm, reflecting the last 3–6 months, were analysed separately, when available. hEtG concentrations were measured with gas chromatography-mass spectrometry using a validated method as reported earlier.<sup>39</sup> The following cut-offs were used as recommended by the SoHT<sup>40</sup>: >30 pg/mg corresponds to excessive EtOH consumption of >60 g/day, 7 to 30 pg/mg to moderate consumption of ≥10 g/d to <60 g/d, and <7 pg/mg to rare or no alcohol consumption.

Further, concentrations of the FAEEs ethyl-mystirate (E14:0), ethyl-palmitate (E16:0; hEtP), ethyl-oleate (E18:1), and ethyl-stearate (E18:0) were measured through headspace solid phase microextraction and gas chromatography-mass spectrometry as described previously.<sup>41</sup> We used the concentration of the single FAEE hEtP for analysis instead of the sum of FAEE, based on the SoHT Consensus Revision 2016.<sup>31,32,42</sup> For 0–3 cm scalp hair the recommended cut-off concentrations of <0.12 ng/mg for low or no alcohol, 0.12–0.35 ng/mg for moderate, and >0.35 ng/mg for excessive alcohol consumption were applied. For 3–6 cm scalp hair the cut-off concentrations of <0.15 ng/mg for low to no alcohol, 0.15–0.45 ng/mg for moderate and >0.45 ng/mg for excessive alcohol consumption were applied.<sup>31,32,42</sup> The remaining FAEE ethyl-mystirate, -oleate and -stearate were used for confirmation of hEtP.

uEtG was measured in cooled urine by a standardized ELISA (DRI™ Ethyl Glucuronide CE Drugs of Abuse Assay, Thermo-Fisher Scientific, Waltham, MA, USA) according to the

manufacturer's instructions. uEtG was normalized to creatinine levels in urine measured by Jaffe's method (Roche Diagnostics, Mannheim, Germany) in order to minimize the influence of impaired kidney function on uEtG levels,<sup>38</sup> and to exclude intentionally diluted samples. Both cut-offs, *i.e.*, 0.1 mg/L and 0.5 mg/L, were used for alcohol consumption and compared.<sup>43,44</sup>

CDT was measured by high performance liquid chromatography with anion exchange chromatography (Bio-Rad Laboratories GmbH, Vienna, Austria) calculating the percentage of disialotransferrin. A cut-off of < 2.3% was used according to the International Federation of Clinical Chemistry (IFCC) recommendations.<sup>45</sup>

MCV (normal range for females and males: 78.0–98.0 fL) was measured by Sysmex XE-2100.<sup>46</sup> GGT (normal range for females: <40 U/L, males: <60 U/L) was measured by an enzyme kinetics test according to Szasz.<sup>47</sup>

### Genetic testing

DNA extraction from 200 µl whole blood was performed with a commercial kit (Maxwell 16 Blood DNA Purification Kit, Promega), according to the manufacturer's instructions. The *PNPLA3* rs738409 SNP was analysed by a StepOnePlus Real-Time PCR System and a TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA), using published sequences from the NCBI Entrez SNP Database (<http://www.ncbi.nlm.nih.gov/entrez>): 5'-AAGGAGGGATAAGGCCACTGTA-3' as forward and 5'-CTTTCACAGGCCTTGGTATGTTCC-3' as reverse primer. *TM6SF2* (rs58542926) and *MBOAT7* (rs626283) genotyping was performed by a ViiA7 Real-Time PCR System (Applied Biosystems), using specific TaqMan SNP Genotyping Assays from Applied Biosystems (C–89463510–10 for *TM6SF2* and C—2916337–10 for *MBOAT7*).

### Statistical considerations

Median values, first quartile (Q1) and third quartile (Q3) were used to describe numerical variables. Absolute and relative frequencies were used to describe categorical variables. Wilcoxon-Rank-Sum test was used to compare numerical variables between groups, Chi-square test or Fisher's exact test were used to compare categorical variables as appropriate. The diagnostic accuracy of all direct and indirect markers, *i.e.*, hEtG, uEtG, CDT, MCV, GGT, as well as AUDIT-C was calculated by the AUC for the detection of moderate to excessive, as well as excessive alcohol consumption, in patients with presumed NAFLD. Alcohol marker results were deemed true positive only if patients admitted alcohol consumption after discussing the test results. Sensitivity, specificity, negative (NPV) and positive predictive value (PPV) were calculated for each test. Cut-offs were identified by means of the Youden's index.<sup>48</sup>

Patients were reclassified as having NAFLD with low to no EtOH consumption, NAFLD at moderate, and NAFLD at high risk of alcohol-related liver damage according to alcohol markers, as well as based on MAFLD criteria.

Subgroup analysis was performed for metabolic parameters such as age, sex, BMI, T2DM/impaired glucose tolerance (IGT), and laboratory parameters in order to characterize patients with true NAFLD. Patients with confirmed ALD served as a control population. Independent predictors of repeated moderate to excessive EtOH consumption were analysed by univariate and multivariate binary logistic regression and are indicated as odds ratios (ORs) with 95% CIs.



*P* values of <0.05 were considered statistically significant. Computations were performed using Statistical Package for the Social Sciences (SPSS) version 25.0 and R 3.5.2.

## Results

### Study population

In total, 188 patients were included in the study. Four patients (2.1%) had to be excluded from analysis due to insufficient amounts of hair available for hEtG analysis. Hair testing was generally very well accepted in our hands, and none of the screened patients refused to take part in hair testing.

The final patient cohort consisted of 184 patients and was predominantly male (61.4%) with a median age of 54.0 years, and a median BMI of 30.1 kg/m<sup>2</sup>. The study cohort included 114 (62.0%) patients originally classified as having NAFLD and 70 (38.0%) patients with ALD. No patients with alcoholic steatohepatitis were included. Patient characteristics at study inclusion are presented in Table 1.

In total, 52.6% (60/114) of patients originally classified as having NAFLD reported low to moderate alcohol consumption (<20 g EtOH/day in females, <30 g EtOH/day in males) as quantified by SIAC, whereas 47.4% (54/114) of patients indicated no alcohol consumption. In patients with ALD, 18.6% (13/70) of patients reported active alcohol consumption, whereas 81.4% (57/70) of patients indicated alcohol abstinence within at least 6 months prior to testing.

However, in 63.2% (72/114) of patients classified as having NAFLD, and in 82.9% (58/70) of patients with ALD, any direct or indirect alcohol marker was elevated. The rate of positive alcohol markers is reported in Table 2 and detailed below.

### AUDIT-C

The AUDIT-C score indicated that 24.6% (28/114; 61% males) of patients with NAFLD were at risk of hazardous drinking or active alcohol use (AUDIT-C score ≥3 in females and ≥4 in males), 44.7% (51/114; 45% males) indicated non-hazardous drinking (AUDIT-C

score 1-2 in females and 1-3 in males), and 30.7% (35/114; 34% males) of patients indicated being non-drinkers. In patients with ALD, the AUDIT-C score indicated that 30.0% (21/70; 81% males) of patients were at risk of hazardous drinking, while 70.0% (49/70; 90% males) indicated alcohol abstinence within the last 6 months (Table 2). AUDIT-C scores showed a high correlation with the amount of EtOH units consumed as assessed by SIAC (Pearson correlation coefficient *r* = 0.860, *p* <0.001).

### Hair analysis

In all 184 study participants a hair strand of 0-3 cm length was available for hEtG analysis. An additional 3-6 cm hair strand was available in 65 (35.3%) patients. The median (Q1;Q3) hair length of all participants was 3 cm (3;3). In 16 (8.7%) patients the collected hair was bleached or dyed. None of the patients reported use of alcohol-containing hair products. In total, 29.3% (54/184) of all patients had hEtG levels ≥7 pg/mg and ≤30 mg/pg (corresponding to ≥10 <60 g EtOH/day = repeated moderate alcohol consumption) in 0-3 cm hair strands, and 29.2% (19/65) of all patients had hEtG levels ≥7 pg/mg in 3-6 cm hair strands, indicating repeated moderate to excessive alcohol intake within the last 3-6 months.

In patients originally classified as having NAFLD, hair analysis indicated excessive alcohol consumption (hEtG >30 pg/mg) in 10.5% (12/114), and moderate alcohol consumption (7-30 pg/mg) in 19.3% (22/114) of patients within the last 3-6 months. Overall, hEtG indicated moderate to excessive alcohol consumption in 29.8% (34/114) of patients with presumed NAFLD within the last 3 months, and 23.5% (12/51) within the last 3-6 months. All patients with excessive alcohol consumption according to hEtG admitted to excessive alcohol consumption after discussing the test results. In patients with moderately elevated hEtG, 2 were lost to follow-up, and therefore were excluded from further analysis. In 30% (6/20) of patients, the hEtG results supported the patients' statement on alcohol consumption, but in 70% (14/20)

**Table 1. Patient characteristics at study inclusion.**

	Patient population n = 184	Presumed NAFLD n = 114	ALD n = 70	<i>p</i> value
Age (yr); median (Q1;Q3)	54.0 (45.9;61.4)	50.9 (39.1;59.9)	59.0 (52.2;62.6)	<0.001
Male sex; n (%)	113 (61.4)	52 (45.6)	61 (87.1)	<0.001
BMI (kg/m <sup>2</sup> ); median (Q1;Q3)	30.1 (25.4;38.3)	34.2 (27.8;43.6)	25.9 (22.9;30.4)	<0.001
T2DM/IGT; n (%)	125 (67.9)	87 (76.3)	38 (54.3)	0.003
CKD ≥3a (GFR <60 ml/min); n (%)	13 (7.1)	4 (3.5)	9 (12.9)	0.034
CKD ≥4 (GFR <30 ml/min); n (%)	5 (2.7)	1 (0.9)	4 (5.7)	0.070
Steatosis/fibrosis; n (%)	110 (59.8)	101 (88.6)	9 (12.9)	<0.001
Cirrhosis; n (%)	74 (40.2)	13 (11.4)	61 (87.1)	<0.001
PNPLA3 rs738409; n (%)	n = 138	n = 108	n = 30	
C/C	65 (47.1)	56 (51.9)	9 (30.0)	0.039
C/G	62 (44.9)	46 (42.6)	16 (53.3)	
G/G	11 (8.0)	6 (5.6)	5 (16.7)	
TM6FS2 rs58542926; % (n)	n = 108	n = 85	n = 23	
C/C	87 (80.6)	69 (81.2)	18 (78.3)	0.814
C/T	20 (18.5)	15 (17.6)	5 (21.7)	
T/T	1 (0.9)	1 (1.2)	0 (0)	
MBOAT7 rs626283; % (n)	n = 108	n = 85	n = 23	
C/C	27 (25.0)	21 (24.7)	6 (26.1)	0.795
C/G	46 (42.6)	35 (41.2)	11 (47.8)	
G/G	35 (32.4)	29 (34.1)	6 (26.1)	

Liver disease etiology was based on ruling out other liver diseases than NAFLD/ALD according to EASL-guidelines.<sup>26</sup> Daily ethanol intake was quantified according to Systemic Inventory of Alcohol Consumption questionnaire.<sup>27</sup> Group comparisons were performed by Wilcoxon-Rank-Sum test and Kruskal-Wallis test as applicable for continuous variables and Chi Square test for categorical variables. A *p* value of <0.05 was considered statistically significant.

ALD, alcohol-related liver disease; CKD, chronic kidney disease; IGT, impaired glucose tolerance; NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus.

**Table 2. Results of alcohol marker analyses at study inclusion.**

	Patient population n = 184	Presumed NAFLD n = 114	ALD n = 70	p value
SIAC; n (%)				
≥20 or 30 g EtOH/day	12 (6.5)	0 (0)	12 (17.1)	
<20 or 30 g EtOH/day	61 (33.2)	60 (52.6)	1 (1.4)	
no alcohol within the last 6 months	111 (60.3)	54 (47.4)	57 (81.4)	
AUDIT-C				
≥3 (females)/ ≥4 (males)	49 (26.6)	28 (24.6)	21 (30.0)	0.418
<b>Alcohol marker</b>				
Any alcohol marker pos; n (%)	<b>130 (70.7)</b>	<b>72 (63.2)</b>	<b>58 (82.9)</b>	<b>0.004</b>
hEtG <sub>(0-3 cm)</sub> pos; n (%)	54 (29.3)	34 (29.8)	20 (28.6)	0.856
hEtG <sub>(3-6 cm)</sub> pos; n (%), n = 65	19 (29.2)	12 (23.5), n = 51	7 (50.0), n = 14	0.054
hEtP <sub>(0-3 cm)</sub> pos; n (%), n = 142	72 (50.7)	48 (52.7), n = 91	24 (47.1), n = 51	0.515
hEtP <sub>(3-6 cm)</sub> pos; n (%), n = 48	18 (37.5)	10 (27.8), n = 36	8 (72.7), n = 11	0.006
uEtG pos (cut-off ≥0.1 mg/L); n (%), n = 171	43 (25.1)	28 (26.9), n = 104	15 (22.4), n = 67	0.505
uEtG pos (cut-off ≥0.5 mg/L); n (%), n = 171	22 (12.9)	15 (14.4), n = 104	7 (10.4), n = 67	0.449
MCV pos; n (%)	12 (6.5)	0 (0)	12 (17.1)	<0.001
GGT pos; n (%)	98 (53.3)	47 (41.2)	51 (72.9)	<0.001
CDT pos; n (%), n = 176	4 (2.3)	3 (2.9), n = 102	1 (2.0), n = 51	0.714
Not determinable; n (%)	23 (13.1)	6 (5.6)	17 (26.2)	
MCV + GGT + CDT pos; n (%), n = 153	0 (0)	0 (0), n = 102	0 (0), n = 51	

Patients were classified as presumed NAFLD or ALD based on their self-reported intake of alcohol using the questionnaire SIAC. MCV normal range: 78-98 fL, GGT normal range: <40 U/L for females, <60 U/L for males, CDT cut-off: <2.3%, uEtG cut-off: 0.1 mg/L and 0.5 mg/L. Group comparisons were performed by Chi Square test. A p value of <0.05 was considered statistically significant.

ALD, alcohol-related liver disease; CDT, carbohydrate deficient transferrin; GGT, gamma glutamyltransferase; hEtG, ethyl glucuronide in hair; hEtP, ethyl palmitate in hair; MCV, mean corpuscular volume; NAFLD, non-alcoholic fatty liver disease; SIAC, Systemic Inventory of Alcohol Consumption; uEtG, urinary ethyl glucuronide.

of patients repeated moderate alcohol consumption was admitted only after confrontation with the test results.

Within the ALD cohort, 81.4% (57/70) of patients indicated alcohol abstinence within the 6 months prior to hair collection. Patient information was confirmed by negative hEtG results in 50 patients, however, 28.6% (20/70) showed positive hEtG values indicating either moderate (11.4%, 8/70) or excessive (17.1%, 12/70) alcohol consumption within the last 3-6 months. In 35.0% (7/20) of patients, active alcohol consumption was only detected by hEtG. All patients admitted alcohol consumption after discussing the test results.

In total, 9.8% (n = 18/184) of patients had impaired renal function with a glomerular filtration rate <60 ml/min (Table 1). However, hEtG levels were not significantly different in patients with normal vs. impaired renal function (p = 0.454).

FAEE in hair were available in 77.2% (142/184) of patients (Table S2). hEtP was used to confirm hEtG results as described above. Of 130 patients with a negative hEtG test, 99 hEtP test results were available. Of these, 59.6% (59/99) were negative, 40.4% (40/99) were positive. In these 40 patients no other alcohol test was positive despite uEtG in 6 of 37 (cut-off 0.1 mg/L), and in 2 of 37 (cut-off 0.5 mg/L) patients, respectively. Of 54 patients with positive hEtG results, 45 hEtP results were available. Of these, 73.3% (33/45) were positive.

hEtP values were not significantly different between abstaining patients with NAFLD and ALD (median hEtP 0-3 cm: 0.088 (Q1;Q3: 0.040;0.196) vs. 0.069 (Q1;Q3: 0.0373;0.183); p = 0.596).

### Blood-based alcohol markers

CDT was measured in 95.7% (176/184) of patients (see also Table 2). CDT results were not available in 4.3% (8/184) of patients due to organizational issues. CDT could not be analysed in 13.1% (23/176) of patients due to reduced serum transferrin (14/23), hyperbilirubinemia (5/23), hemolysis (2/23) or suspected

genetic variants (2/23). CDT detected harmful alcohol consumption in 2.3% (4/176) of patients, in 2.8% (3/108) of patients with NAFLD, and in 1.5% (n = 1/68) of patients with ALD (intent-to-diagnose-analysis considering unreliable results). All patients with positive CDT also showed positive hEtG within the last 3 months.

### Urinalysis

uEtG analysis was performed in 92.9% (171/184) of patients (Table 2). uEtG was not available in 2.7% (5/184) of patients due to organizational issues and was not analysed in 4.7% (8/171) of patients due to reduced creatinine concentration in the urine, to avoid false negative results. uEtG detected alcohol consumption in 25.1% (43/171, cut-off 0.1 mg/L) or 12.9% (22/171, cut-off 0.5 mg/L) of patients, depending on the cut-off used (intent-to-diagnose analysis). uEtG detected recent alcohol consumption in 14.4% (15/104, cut-off 0.5 mg/L) of patients with NAFLD, and 10.4% (7/67, cut-off 0.5 mg/L) of patients with ALD (intent-to-diagnose analysis). Sixteen of 22 patients who tested positive for uEtG also tested positive for hEtG. In the 6 remaining patients, all of them classified as having NAFLD, alcohol use was only detected with uEtG.

### Comparison of alcohol markers

We further investigated the diagnostic accuracy of the applied alcohol markers to detect repeated moderate (≥10 g <60 g EtOH/day) and excessive (≥60 mg EtOH/day) alcohol consumption in patients with NAFLD (see Table 3 and Fig. 1).

For the detection of moderate alcohol consumption, CDT, MCV, and GGT showed AUCs of 0.544, 0.500, and 0.572, respectively. uEtG with cut-offs of 0.5 mg/L, and 0.1 mg/L showed AUCs of 0.660, and 0.766, respectively. hEtG showed an AUC of 0.897.

For the detection of excessive alcohol consumption, CDT, MCV, and GGT showed similar AUCs as for moderate alcohol consumption. uEtG with cut-offs of 0.5 mg/L and 0.1 mg/L

**Table 3. Diagnostic accuracy of alcohol markers to detect moderate to excessive ( $\geq 10$  g EtOH/day) and excessive ( $\geq 60$  g EtOH/day) alcohol consumption.**

Alcohol test	AUC (95% CI)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Youden Index
<b>All patients, n = 182</b>						
<b>Moderate to excessive EtOH consumption</b>						
hEtG >7 pg/mg	0.927 (0.867–0.987)	85.4	100	86.7	100	0.854
uEtG $\geq 0.1$ mg/L	0.754 (0.661–0.846)	60.4	90.3	58.2	90.4	0.507
uEtG $\geq 0.5$ mg/L	0.666 (0.564–0.768)	35.4	97.8	36.4	98.2	0.333
CDT $\geq 2.3\%$	0.542 (0.439–0.645)	8.3	100	7.8	100	0.083
MCV >98.0 fl	0.494 (0.393–0.595)	4.2	94.6	6.7	93.4	-0.012
GGT $\geq 60$ U/L (m), $\geq 40$ U/L (f)	0.552 (0.451–0.653)	58.3	49.5	58.3	48.4	0.078
ANI score >-12.82	0.628 (0.535–0.720)	89.6	38.7	25.0	73.8	0.283
AUDIT-C $\geq 4$ (m), $\geq 3$ (f)	0.733 (0.638–0.827)	58.3	88.2	56.7	87.7	0.465
<b>Excessive EtOH consumption</b>						
hEtG >30 pg/mg	0.974 (0.915–1.032)	94.7	100	92.3	100	0.947
uEtG $\geq 0.1$ mg/L	0.740 (0.611–0.869)	68.4	79.5	65.2	80.8	0.479
uEtG $\geq 0.5$ mg/L	0.665 (0.517–0.814)	42.1	91.0	43.5	91.8	0.331
CDT $\geq 2.3\%$	0.544 (0.397–0.692)	10.5	98.4	9.5	98.4	0.089
MCV >98.0 fl	0.502 (0.361–0.642)	5.3	95.1	11.5	94.2	0.003
GGT $\geq 60$ U/L (m), $\geq 40$ U/L (f)	0.727 (0.605–0.849)	78.9	50.8	80.8	50.6	0.298
ANI score >-10.10	0.757 (0.656–0.857)	100	45.1	100	43.6	0.451
AUDIT-C $\geq 4$ (m), $\geq 3$ (f)	0.736 (0.607–0.865)	68.4	78.7	65.4	79.5	0.471
<b>Presumed NAFLD, n = 112</b>						
<b>Moderate to excessive EtOH consumption</b>						
hEtG >7 pg/mg	0.897 (0.814–0.980)	79.4	100	80.0	100	0.794
uEtG $\geq 0.1$ mg/L	0.766 (0.656–0.876)	61.8	91.4	59.5	90.8	0.531
uEtG $\geq 0.5$ mg/L	0.659 (0.536–0.782)	35.3	96.6	35.1	96.9	0.318
CDT $\geq 2.3\%$	0.544 (0.419–0.669)	8.8	100	8.3	100	0.088
MCV >98.0 fl	0.500 (0.377–0.623)	0.0	100	n.a.	n.a.	0.000
GGT $\geq 60$ U/L (m), $\geq 40$ U/L (f)	0.569 (0.447–0.691)	50.0	63.8	45.0	59.7	0.138
ANI score >-12.82	0.781 (0.685–0.878)	85.3	62.1	15.0	95.8	0.474
AUDIT-C $\geq 4$ (m), $\geq 3$ (f)	0.722 (0.606–0.838)	52.9	91.4	50.0	88.9	0.443
<b>Excessive EtOH consumption</b>						
hEtG >30 pg/mg	1.000 (1.000–1.000)	100	100	100	100	1.000
uEtG $\geq 0.1$ mg/L	0.790 (0.637–0.943)	80.0	78.0	72.7	78.0	0.580
uEtG $\geq 0.5$ mg/L	0.751 (0.562–0.940)	60.0	90.2	54.5	90.1	0.502
CDT $\geq 2.3\%$	0.538 (0.338–0.737)	10.0	97.6	9.1	97.7	0.076
MCV >98.0 fl	0.500 (0.309–0.691)	0.0	100	n.a.	n.a.	0.000
GGT $\geq 60$ U/L (m), $\geq 40$ U/L (f)	0.605 (0.418–0.791)	60.0	61.0	58.3	60.0	0.210
ANI score >-10.10	0.861 (0.764–0.958)	100	64.6	100	66.0	0.646
AUDIT-C $\geq 4$ (m), $\geq 3$ (f)	0.752 (0.579–0.926)	70.0	80.5	66.7	80.0	0.505
<b>ALD, n = 70</b>						
<b>Moderate to excessive EtOH consumption</b>						
hEtG >7 pg/mg	1.000 (1.000–1.000)	100	100	100	100	1.000
uEtG $\geq 0.1$ mg/L	0.729 (0.556–0.901)	57.1	88.6	55.6	89.8	0.457
uEtG $\geq 0.5$ mg/L	0.679 (0.492–0.865)	35.7	100	38.9	100	0.357
CDT $\geq 2.3\%$	0.536 (0.350–0.721)	7.1	100	6.7	100	0.071
MCV >98.0 fl	0.500 (0.410–0.783)	14.3	85.7	20.0	84.0	0.000
GGT $\geq 60$ U/L (m), $\geq 40$ U/L (f)	0.521 (0.343–0.700)	78.6	25.7	85.0	32.0	0.043
ANI score >6.52	0.514 (0.308–0.721)	28.6	94.3	25.0	82.0	0.229
AUDIT-C $\geq 4$ (m), $\geq 3$ (f)	0.771 (0.614–0.929)	71.4	82.9	70.0	86.0	0.543
<b>Excessive EtOH consumption</b>						
hEtG >30 pg/mg	0.944 (0.823–1.066)	88.9	100	85.7	100	0.889
uEtG $\geq 0.1$ mg/L	0.690 (0.481–0.899)	55.6	82.5	58.3	85.5	0.381
uEtG $\geq 0.5$ mg/L	0.574 (0.352–0.795)	22.2	92.5	33.3	94.5	0.147
CDT $\geq 2.3\%$	0.556 (0.333–0.778)	11.1	100	10.0	100	0.111
MCV >98.0 fl	0.481 (0.274–0.687)	11.1	85.0	21.4	83.9	-0.039
GGT $\geq 60$ U/L (m), $\geq 40$ U/L (f)	0.650 (0.480–0.820)	100	30.0	100	33.9	0.300
ANI score >3.00	0.600 (0.361–0.839)	55.6	77.5	50.0	66.1	0.331
AUDIT-C $\geq 4$ (m), $\geq 3$ (f)	0.708 (0.512–0.905)	66.7	75.0	64.3	78.6	0.417

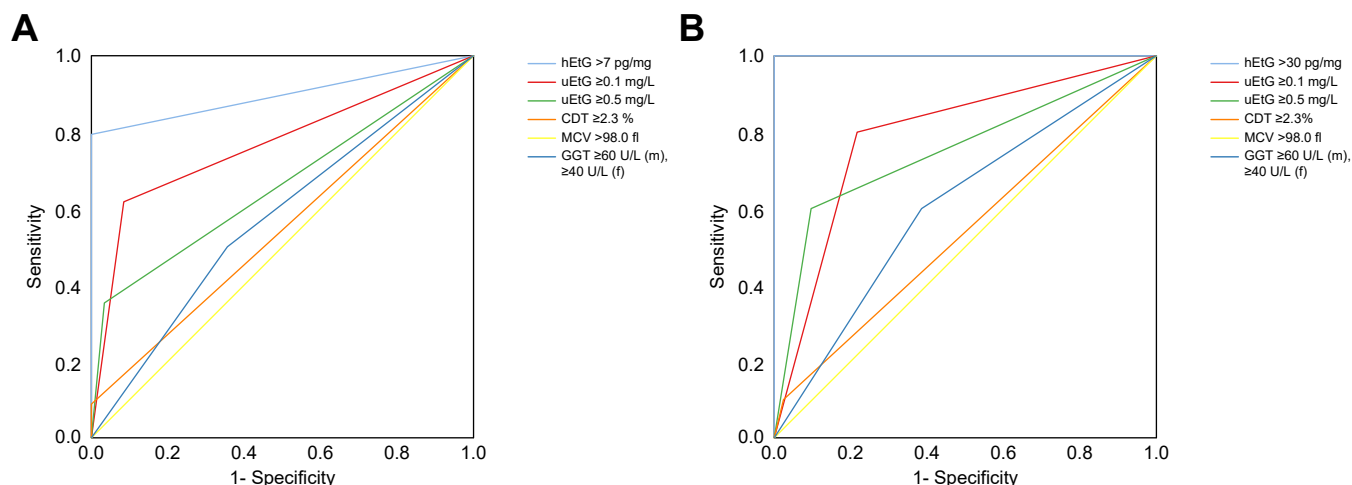
ANI, ALD/NAFLD index; AUDIT-C, Alcohol Use Disorders Identification Test – Consumption; CDT, carbohydrate deficient transferrin; EtOH, ethanol; GGT, gamma glutamyl-transferase; hEtG, ethyl glucuronide in hair; MCV, mean corpuscular volume; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity; uEtG, ethyl glucuronide in urine.

showed AUCs of 0.752, and 0.792, respectively. hEtG showed an AUC of 1.000.

The AUCs of AUDIT-C for the detection of hazardous drinking or active alcohol use were 0.694, and 0.733, respectively (Table 3).

### Clinical characteristics of alcohol consumption

The study participants were reclassified according to the quantity of alcohol they consumed as indicated by hEtG and re-analyzed for their clinical features (Table 4). In total, of 112 patients with presumed NAFLD (2 lost to follow-up and



**Fig. 1. Diagnostic accuracy of objective alcohol parameters in patients with presumed NAFLD.** The AUC was calculated for (A) repeated moderate to excessive EtOH consumption defined as  $\geq 10$  g EtOH/day, (B) excessive alcohol consumption defined as  $\geq 60$  g EtOH/day after confirmation of the test results by the patients ( $n = 112$ ). Both, uEtG and hEtG showed good or excellent accuracy, respectively, whereas CDT, MCV, and GGT were not reliable. CDT, carbohydrate deficient transferrin; GGT, gamma glutamyltransferase; hEtG, ethylglucuronide in hair; MCV, mean corpuscular volume; uEtG, urinary ethyl glucuronide.

therefore unavailable to be confronted and confirm the results of alcohol biomarkers) 71.4% (80/112) consumed no or only low amounts of alcohol ( $< 10$  g EtOH/day), and 28.6% (32/112) of patients presented with repeated moderate to excessive alcohol consumption.

Of note, patients with at least repeated moderate alcohol consumption were significantly more often male, had a significantly lower BMI, and suffered significantly less frequently from T2DM or IGT (Table 4, Fig. 2) than patients with low to no EtOH consumption. No statistical differences were found when patients with presumed NAFLD with moderate alcohol consumption were compared to those with excessive alcohol consumption.

Regarding liver biopsy, which was available in 57 patients, no significant differences were found in terms of NAS, grade of fibrosis or grade of steatosis (Table 4).

#### Genetic risk loci and routine laboratory parameters

Compared to patients with NAFLD, significantly more patients with ALD were found to carry the unfavorable *PNPLA3* SNPs rs738409 hetero- or homozygosity (Table 1). However, this statistical difference disappeared after re-analyses based on alcohol consumption indicated by alcohol markers (Table 4).

The differences in laboratory parameters are displayed in Table S1. Patients with at least repeated moderate alcohol consumption had significantly higher MCV, bilirubin, creatinine, transferrin saturation and ferritin, and significantly lower platelets, HOMA-IR, and hemoglobin A1c (HbA1c).

#### Impact of sex, BMI and deranged glucose homeostasis

The 3 clinical factors of sex, BMI, and T2DM/IGT, as well as the aforementioned laboratory parameters were significantly different in patients with NAFLD with low to no alcohol consumption compared to those with potentially harmful consumption (Fig. 2).

Using binary logistic regression, we identified lower BMI, lower HbA1c, higher transferrin saturation and higher ferritin as univariate predictors of repeated moderate to excessive EtOH consumption in presumed NAFLD and ALD (Table 5). When put

into a multivariate model, BMI remained the only significant predictor of repeated moderate to excessive alcohol consumption (Table 5). The optimal BMI cut-off was  $33.4 \text{ kg/m}^2$  (sensitivity: 46.9%, specificity 85.2; Youden index: 0.321). Patients with a BMI  $> 33.4 \text{ kg/m}^2$  had a significantly reduced risk of repeated moderate to excessive EtOH consumption (OR 0.192; 95% CI 0.077–0.479;  $p < 0.001$ ).

#### ANI score

ANI score identified 74.2% (135/182) of our total patient population as having NAFLD, and 25.8% (47/182) as likely suffering from ALD. The AUCs of the ANI score for identifying repeated moderate to excessive alcohol consumption in this mixed cohort of patients with NAFLD and ALD, within the cohort with presumed NAFLD, and in those with ALD were 0.628, 0.781, and 0.514, respectively (Table S3). The AUCs of the ANI score for identifying patients with excessive alcohol consumption in a mixed cohort of patients with NAFLD and ALD, within the cohort of presumed NAFLD, and in patients with ALD were 0.757, 0.81, and 0.600, respectively (Table S3). The respective cut-offs, sensitivity, specificity, PPV, and NPV are given in Table 3. Of note, the cut-off  $> 0$  as proposed by Dunn *et al.* was not applicable to our patient cohort.

#### Reclassification according to MAFLD criteria

Patients were retrospectively reclassified according to MAFLD criteria, including MAFLDC and DEFLD (Table 6). Most notably, applying the criteria of MAFLD/DEFLD in the presence of cirrhosis limited our total patient cohort to 128 patients. This results from the fact that these criteria, in the presence of cirrhosis, include the documentation of MAFLD by previous liver biopsy or historical documentation of steatosis by hepatic imaging ( $n = 56$ , 30.4% diagnosis based on given criteria not possible). Of 128 patients with available data to make the diagnoses of MAFLD, MAFLDC, or DEFLD, 12 (9.4%) patients did not fulfil such diagnostic criteria, while 116 (90.6%) did (MAFLD  $n = 105$ , MAFLDC  $n = 11$ , DEFLD  $n = 9$ ; Table 6). In total, 107 (92.2%) patients with MAFLD/MAFLDC indicated no alcohol consumption, while 9 (7.8%) patients



**Table 4. Clinical characteristics of patients with presumed NAFLD stratified according to the amount of alcohol consumption based on alcohol markers.**

(A)	Presumed NAFLD overall n = 112	Presumed NAFLD no to low EtOH n = 80	Presumed NAFLD moderate to excessive EtOH n = 32	p value
Age (y); median (Q1;Q3)	50.9 (38.2;59.9)	50.5 (39.1;59.2)	54.5 (35.5;62.3)	0.493
Male sex; n (%)	52 (46.4)	28 (35.0)	24 (75.0)	<0.001
BMI (kg/m <sup>2</sup> ); median (Q1;Q3)	34.2 (27.8;44.1)	37.8 (30.1;45.8)	28.1 (25.9;31.6)	<0.001
T2DM/IGT; n (%)	86 (76.8)	66 (82.5)	20 (62.5)	0.024
CKD ≥3 a (GFR <60 ml/min); n (%)	4 (3.6)	2 (2.5)	2 (6.3)	0.334
CKD ≥4 (GFR <30 ml/min); n (%)	1 (0.9)	1 (1.3)	0 (0)	0.525
Steatosis/fibrosis; n (%)	98 (87.5)	69 (86.3)	29 (90.6)	0.711
Cirrhosis; n (%)	13 (11.6)	8 (10.0)	5 (15.6)	0.402
Liver biopsy, n = 57				
NAS; median (Q1;Q3)	4.0 (2.0;5.0)	4.0 (2.0;5.0)	4.0 (3.0;4.0)	0.583
Fibrosis grade; median (Q1;Q3)	1.0 (0.5;2.0)	1.0 (0.5;1.5)	1.0 (0.25;3.5)	0.529
Steatosis grade; median (Q1;Q3)	2.0 (1.0;3.0)	2.0 (1.0;3.0)	1.5 (1.0;3.0)	0.933
PNPLA3 rs738409; n (%), n = 106				
C/C	55 (51.9)	42 (55.3)	13 (43.3)	0.268
C/G	45 (42.5)	29 (38.2)	16 (53.3)	0.154
G/G	6 (5.7)	5 (6.6)	1 (3.3)	0.673
TM6FS2 rs58542926; % (n), n = 84				
C/C	68 (81.0)	46 (79.3)	22 (84.6)	0.567
C/T	15 (17.9)	12 (20.7)	3 (11.5)	0.311
T/T	1 (1.2)	0 (0)	1 (3.8)	0.310
MBOAT7 rs626283; % (n), n = 84				
C/C	21 (25.0)	13 (22.4)	8 (30.8)	0.414
C/G	34 (40.5)	24 (41.4)	10 (38.5)	0.801
G/G	29 (34.5)	21 (36.2)	8 (30.8)	0.628
(B)	Presumed NAFLD moderate to excessive EtOH, n = 32	Presumed NAFLD moderate EtOH n = 20	Presumed NAFLD excessive EtOH n = 12	p value
Age (y); median (Q1;Q3)	54.5 (35.5;62.3)	51.6 (31.0;59.0)	55.5 (48.0;64.5)	0.275
Male sex; n (%)	24 (75.0)	15 (75.0)	9 (75.0)	1.000
BMI (kg/m <sup>2</sup> ); median (Q1;Q3)	28.1 (25.9;31.6)	28.4 (26.3;33.2)	26.6 (23.8;29.9)	0.134
T2DM/IGT; n (%)	20 (62.5)	13 (65.0)	7 (58.3)	0.706
CKD ≥3a (GFR <60 ml/min); n (%)	2 (6.3)	2 (10.0)	0 (0)	0.516
CKD ≥4 (GFR <30 ml/min); n (%)	0 (0)	0 (0)	0 (0)	
Steatosis/fibrosis; n (%)	29 (90.6)	19 (95.0)	11 (91.7)	0.605
Cirrhosis; n (%)	5 (15.6)	3 (15.0)	2 (16.7)	1.000
Liver biopsy, n = 8				
NAS; median (Q1;Q3)	4.0 (3.0;4.0)	3.50 (2.25;4.25)	4.00 (4.00;4.00)	0.475
Fibrosis grade; median (Q1;Q3)	1.0 (0.25;3.5)	1.00 (0.00;2.50)	2.50 (1.00;-)	0.429
Steatosis grade; median (Q1;Q3)	1.5 (1.0;3.0)	2.00 (0.75;3.00)	1.50 (1.25;1.75)	0.857
PNPLA3 rs738409; n (%), n = 30				
C/C	13 (43.3)	6 (33.3)	7 (58.3)	0.406
C/G	16 (53.3)	11 (61.1)	5 (41.7)	0.457
G/G	1 (3.3)	1 (5.6)	0 (0)	0.264
TM6FS2 rs58542926; % (n), n = 26				
C/C	22 (84.6)	13 (81.3)	9 (90.0)	0.547
C/T	3 (11.5)	2 (12.5)	1 (10.0)	1.000
T/T	1 (3.8)	1 (6.3)	0 (0)	1.000
MBOAT7 rs626283; % (n), n = 26				
C/C	8 (30.8)	4 (25.0)	4 (40.0)	0.664
C/G	10 (38.5)	8 (50.0)	2 (20.0)	0.218
G/G	8 (33.8)	4 (25.0)	4 (40.0)	0.664

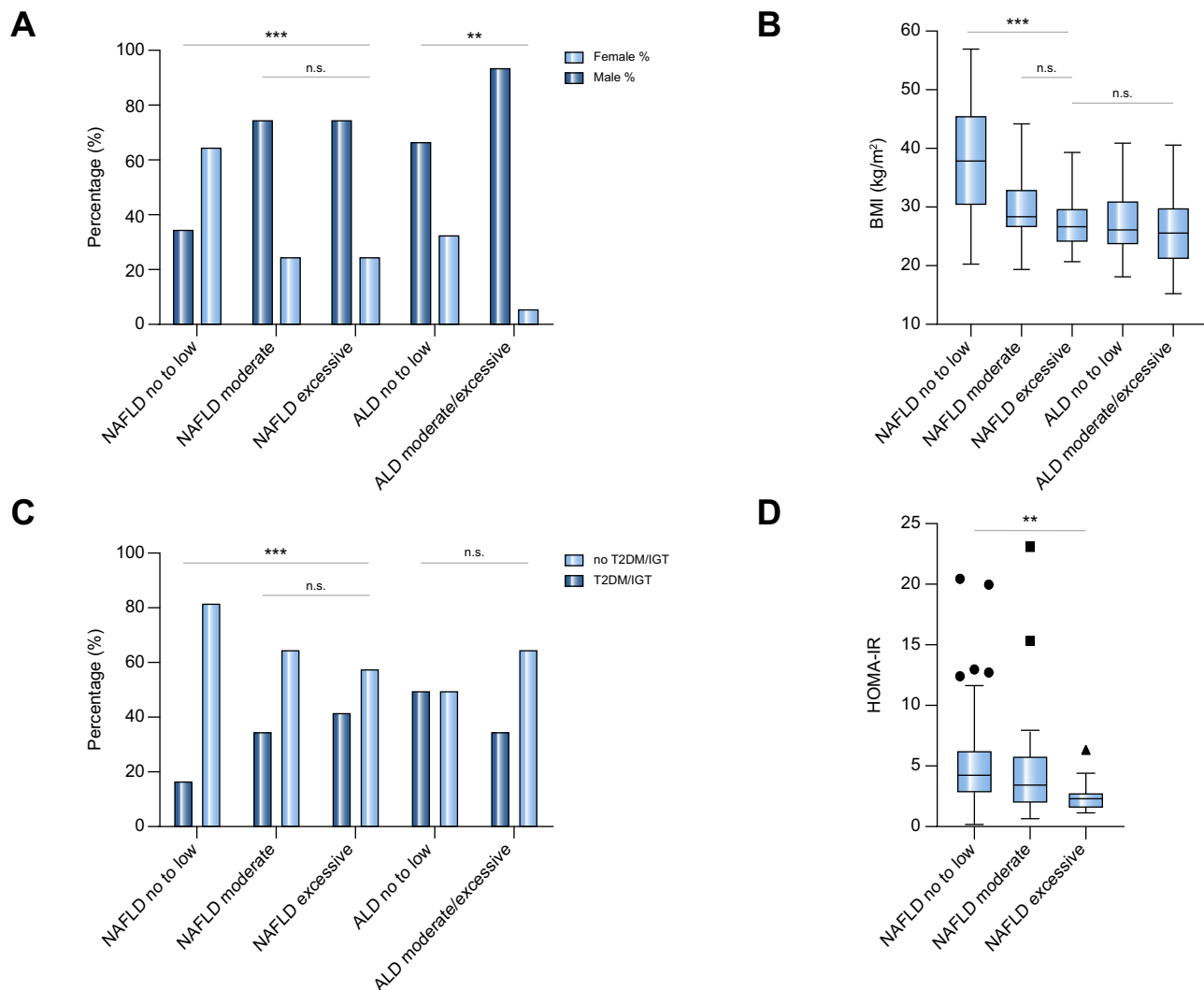
CKD, chronic kidney disease; EtOH, ethanol; IGT, impaired glucose tolerance; NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus. Displayed are characteristics of (A) presumed NAFLD patients without alcohol consumption vs. patients with moderate to excessive alcohol consumption, as well as (B) NAFLD patients with moderate vs. excessive alcohol consumption after confrontation with alcohol marker test results. Only patients who admitted alcohol consumption after confrontation entered the analysis. Two patients were lost to follow-up. Group comparisons were performed by Wilcoxon-Rank-Sum test for continuous variables and Chi Square test for categorical variables. A p value of <0.05 was considered statistically significant.

declared alcohol consumption ≥20 mg (females) or 30 mg (males) of EtOH/day (DEFLD). Of the 12 patients without MAFLD/MAFLDC, 4 indicated no alcohol consumption, 8 reported alcohol consumption above the respective limits.

With the use of objective alcohol markers measured in this study, excessive alcohol consumption was identified in 27 (25%) patients with MAFLD/MAFLDC, and excessive alcohol consumption was identified in 9 (8.2%), over a period of several months.

## Discussion

NAFLD and ALD share several pathomechanistic and clinical features.<sup>49</sup> Since liver histology as the diagnostic gold standard cannot reliably distinguish between NAFLD and ALD,<sup>50</sup> NAFLD has been diagnosed by the exclusion of both secondary causes, and daily alcohol consumption of ≥20 g EtOH for women and ≥30 g for men.<sup>26</sup> Given that >75% of adults in Europe and America consume alcohol,<sup>24</sup> and about 70% of patients are overweight or obese,<sup>51</sup> the co-existence of metabolic risk factors and alcohol



**Fig. 2. Sex distribution, BMI and T2DM/IGT in patients with presumed NAFLD stratified according to the amount of EtOH consumption measured by hEtG.** (A) In patients with presumed NAFLD, as well as in patients with ALD, males significantly more often showed repeated moderate ( $\geq 10$  g <60 g EtOH/day) or excessive ( $\geq 60$  g EtOH/day) EtOH consumption. (B) BMI was significantly higher in patients with presumed NAFLD and no to low EtOH consumption (<10 g EtOH/day) compared to repeated moderate and excessive EtOH consumption, or patients with ALD. (C) T2DM/IGT was significantly more frequent in patients with presumed NAFLD and no to low EtOH consumption, than with repeated moderate or excessive alcohol consumption. This effect was not seen in patients with ALD. (D) HOMA-IR was significantly higher in patients with presumed NAFLD and no to low EtOH consumption than with repeated moderate or excessive EtOH consumption. Significance levels are displayed by asterisks: \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; n.s., non-significant. Group comparisons were performed by Wilcoxon-Rank-Sum test for continuous variables and Chi Square test for categorical variables. A  $p$  value of <0.05 was considered statistically significant. ALD, alcohol-related liver disease; EtOH, ethanol; hEtG, ethyl glucuronide in hair; NAFLD, non-alcoholic fatty liver disease; T2DM/IGT, type 2 diabetes mellitus/impaired glucose tolerance.

consumption renders a clear distinction between NAFLD and ALD challenging. Since the main etiologic factors, alcohol and obesity, frequently coexist, the term “both alcoholic and non-alcoholic fatty liver disease” (BAFLD) has been proposed.<sup>52</sup> More recently, the term metabolic dysfunction-associated fatty liver disease (MAFLD) was proposed to replace the term non-alcoholic fatty liver disease (NAFLD) to more accurately reflect pathogenesis, improve patient stratification for management, and destigmatize.<sup>53</sup> The diagnosis of MAFLD is based on metabolic dysfunction (insulin resistance, abnormal lipid profile), not the absence of other liver diseases or conditions irrespective of the amount of EtOH consumption. MAFLD thus may coexist with ALD, however, this group has not been investigated and characterized further until now.<sup>53</sup>

In this study, we investigated the value of hEtG and uEtG in comparison to the currently established gold standard for alcohol detection (CDT, MCV, GGT and AUDIT-C) in metabolically well characterized patients with NAFLD and ALD. Our data show that up to 29% of patients with NAFLD and 25% of patients with MAFLD are at moderate to high risk of alcohol-related liver damage in the presence of repeated moderate to excessive alcohol consumption. Notably, 11% of patients with presumed NAFLD, and 8% of patients with MAFLD consumed EtOH excessively. The most valuable parameters to detect repeated moderate and excessive EtOH consumption were uEtG at a cut-off of 0.1 mg/L, and hEtG, both showing good to excellent accuracy. Both male sex and lower BMI were univariate predictors of alcohol consumption. When put into a multivariate model, BMI

**Table 5. Univariate and multivariate logistic regression analysis to identify predictors of repeated alcohol consumption in patients with presumed NAFLD and ALD.**

	Univariate analysis, all patients (N = 184)			Multivariate analysis					
	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI	p value
Male sex	1.742	0.882–3.437	0.110						
BMI	0.925	0.887–0.966	<0.001	0.920	0.874–0.969	0.002			
BMI >33.4	0.197	0.086–0.449	<0.001				0.192	0.077–0.479	<0.001
T2DM/IGT	0.729	0.374–1.420	0.353						
HOMA-IR	0.907	0.817–1.007	0.068						
HbA1c	0.473	0.278–0.804	0.006	0.654	0.376–1.139	0.134	0.597	0.345–1.032	0.065
MCV	1.033	0.986–1.082	0.171						
Platelets	1.000	0.997–1.004	0.941						
Bilirubin	1.068	0.981–1.164	0.130						
Creatinine	1.249	0.854–1.825	0.252						
Transferrin saturation	1.019	1.000–1.039	0.047	0.997	0.971–1.023	0.820	1.000	0.975–1.025	0.990
Ferritin	1.001	1.000–1.003	0.036	1.001	0.999–1.003	0.338	1.001	0.999–1.002	0.425

Independent predictors of repeated moderate to excessive alcohol consumption were identified by binary logistic regression analysis. Multivariate analysis was performed including significant univariate predictors, first using BMI as a covariate (left column), and second using BMI >33.4 kg/m<sup>2</sup> as a covariate (right column). BMI remained the only significant predictor after multivariate analysis (*p* <0.001). Patients with a BMI >33.4 kg/m<sup>2</sup> had a significantly reduced risk of repeated moderate to excessive alcohol consumption. A *p* value of <0.05 was considered statistically significant.

HbA1c, hemoglobin A1c; HOMA-IR homeostasis model assessment of insulin resistance; MCV, mean corpuscular volume; OR, odds ratio; T2DM/IGT, type 2 diabetes mellitus/ impaired glucose tolerance.

remained the only significant predictor. Patients with a BMI >33.4 kg/m<sup>2</sup> had a significantly reduced risk of repeated moderate to excessive EtOH consumption (OR = 0.192).

Both hEtG and uEtG have been repeatedly shown to be highly sensitive and specific markers of alcohol consumption in patients with and without significant liver disease.<sup>44,54,55</sup> EtG emerges during EtOH metabolism, as a very small amount (<0.1%) of EtOH undergoes conjugation reactions with glucuronic acid in the presence of (1) membrane-bound mitochondrial uridine diphosphate glucuronyl transferase to produce EtG, (2) sulfotransferase to produce ethyl sulfate (EtS), and FAEE-synthase to produce FAEE.<sup>56</sup> EtG is then excreted in urine (uEtG, EtS), hair (hEtG) or can be detected in hair after metabolization to FAEE.

uEtG is a direct short-time EtOH marker, which can be detected up to 80 hours after complete EtOH elimination from the body, and up to 130 hours after ingestion of high EtOH

amounts, and may also be detectable after the consumption of very small amounts of EtOH.<sup>57,58</sup> Importantly, uEtG is neither affected by the presence of cirrhosis nor by BMI.<sup>59</sup> Therefore, uEtG is a useful marker for binary screening of recent alcohol consumption in patients with liver disease, but it cannot distinguish between recent low alcohol intake or excessive alcohol intake. It is also worth noting that uEtG may test false positive in patients with heavily impaired kidney function, or when urine has been stored above 4°C before measurement.<sup>55</sup> These limitations were accounted for by storing the urine cooled until measurement, and by normalizing to creatinine as mentioned in the Methods section). Samples were excluded from the study if we were unable to control temperature storage or normalize to creatinine (*n* = 8/171, 4.7%).

In contrast to uEtG, hEtG is a long-term EtOH marker, which semi-quantitatively detects alcohol consumption within

**Table 6. Reclassification of patients with presumed NAFLD, and ALD based on criteria for MAFLD.**

	All patients, N = 184	Presumed NAFLD, n = 114	ALD, n = 70
No cirrhosis	n = 110 (59.8%)	n = 101 (88.6%)	n = 9 (12.9%)
MAFLD, n (%)			
No	5 (4.5)	4 (4.0)	1 (11.1)
Yes	105 (95.5)	97 (96.0)	8 (88.9)
Cirrhosis	n = 74 (40.2%)	n = 13 (11.4%)	n = 61 (87.1%)
MAFLD-related cirrhosis			
No	7 (9.5)	0	7 (11.5)
Yes	11 (14.9)	10 (76.9)	1 (1.4)
Classification n.p.*	56 (75.7)	3 (23.1)	53 (6.9)
Dual etiology FLD			
No	119 (64.7)	111 (97.4)	0 (11.4)
Yes	9 (4.9)	3 (2.6)	9 (12.9)
Classification n.p.*	56 (30.4)	0	53 (75.7)

MAFLD was defined according to the EASL international expert consensus statement on MAFLD<sup>18</sup> according to "positive" criteria regardless of alcohol consumption. The criteria were defined as hepatic steatosis detected either by imaging techniques or liver biopsy with either BMI ≥25 kg/m<sup>2</sup>, type 2 diabetes mellitus, or BMI < 25 kg/m<sup>2</sup> if at least 2 metabolic risk abnormalities were present. MAFLD-related cirrhosis was defined as cirrhosis in the absence of typical histology who meet at least one of the following criteria: Past or present evidence of metabolic risk factors that meet the criteria to diagnose MAFLD with at least one of the following: i) Documentation of MAFLD on a previous liver biopsy. ii) Historical documentation of steatosis by hepatic imaging. History of past alcohol consumption was considered ALD. Dual etiology FLD (concomitant MAFLD and other liver disease) was defined as meeting the criteria for MAFLD plus any other cause of liver disease e.g., alcohol use disorder defined as consumption of >3 drinks per day in men and >2 drinks per day in women, or binge drinking (defined as >5 drinks in males and >4 drinks in females, consumed over a 2 hour period), as defined by the National Institute of Alcoholism and Alcohol Abuse.

ALD, alcohol-related liver disease; FLD, fatty liver disease; MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

the last 3 to 6 months and can distinguish between low to no, repeated moderate, and excessive EtOH consumption. As the hair root takes up to 3 weeks to grow out of the scalp, there may be a detection gap comprising of the most recent 3 weeks.<sup>42</sup> hEtG is highly specific and the recommended cut-offs were chosen to avoid false positive test results. While hEtG might be false positive in patients with heavily impaired kidney function, this study found no statistical difference in hEtG levels between patients with normal and impaired renal function.

hEtG has become an established test in the forensic context. At first glance, the costs for the assessment of hEtG per single test are higher than for uEtG or CDT, yet have the important added value of reflecting the last 3-6 months. Since clinical interest and utility have been constantly increasing, reimbursement strategies beyond forensic questions need to be discussed in the future.

Until now, neither hEtG nor uEtG have been investigated explicitly in patients with NAFLD. Against the background that NAFLD pathogenesis has been linked to increased endogenous EtOH levels due to alcohol-producing microbiota,<sup>16</sup> and impairment of alcohol dehydrogenase activity in the presence of insulin resistance,<sup>17</sup> it seems important to consider liver disease etiology as a potential bias for alcohol biomarkers. However, EtOH levels reported in the aforementioned studies in either adult patients with NASH in comparison to patients with obesity and healthy controls (16), or in children with NAFLD (17) were way too low to be detectable by any alcohol marker. Thus, it is unlikely, that hEtG or uEtG results have been influenced by the presence of NAFLD itself. To evaluate a potential bias due to liver disease etiology, we additionally (1) measured FAEE, and particularly hEtP, in both patients with presumed NAFLD and those with ALD, and (2) confronted the study participants with the test results. hEtP values were not significantly different between patients with NAFLD or ALD, who did not consume alcohol within the last 3 to 6 months, making an etiology bias unlikely. Furthermore, although 2 patients with presumed NAFLD were lost to follow-up, all patients admitted alcohol consumption corresponding to the amount measured by hEtG.

Previously, Hagström and colleagues used phosphatidylethanol (PEtH), a marker of excessive alcohol consumption within the last 2 weeks, in a study including 120 patients with NAFLD to assess alcohol consumption. In this study, PEtH was neither confirmed by patient confrontation, nor has it been validated in patients with NAFLD so far. However, 10.8% (13/120) of patients with NAFLD were PEtH positive and therefore likely to have been consuming alcohol excessively within the last 2 weeks. This number of patients is consistent with the number of patients we detected to be drinking excessively within the last 3 to 6 months by hEtG.

A recent systematic review highlights the importance of discussing test results with the patients, as under-reporting of alcohol consumption was found to be the most common type of inconsistency across short-, intermediate- and long-term biomarkers in up to 56% of patients.<sup>60</sup> Patients with alcohol use disorders (including harmful alcohol consumption) are subject to social stigma. Thus, it seems intuitive that patients tend to underreport their alcohol consumption, and neglecting or underreporting alcohol consumption is part of the disease. Alcohol marker testing (irrespective of liver disease origin) helps objectify alcohol consumption. In the presence of a trusting doctor-patient relationship, discussing the results of alcohol marker testing can support discussing actual drinking habits, illness insight can be fostered, and treatment can be offered.

Furthermore, we found lower BMI and lower HbA1c, higher transferrin saturation and higher ferritin as univariate predictors of repeated moderate to excessive EtOH consumption in patients with presumed NAFLD. Using multivariate analysis, lower BMI remained the only significant predictor, and a BMI of  $>33.4 \text{ kg/m}^2$  was associated with a significantly reduced risk of repeated moderate to excessive EtOH consumption (OR 0.192) rendering true NAFLD likely. This finding is in line with Dunn and colleagues' study of 2006 which reported BMI to be an important determinant to separate NAFLD from ALD.<sup>25</sup> Also, Hagström and colleagues included BMI in their analysis. However, cut-off values have not been reported. Our findings shed further light on the concept of lean NAFLD, which is not fully understood, and is believed to include pathophysiological mechanisms such as a dysfunctional adipose tissue, altered body composition, genetic mutations, epigenetic changes occurring early in life and a different pattern of gut microbiota with a similar prognosis as of obesity-related NAFLD.<sup>61,62</sup> Additional systematic studies investigating the impact of alcohol consumption in lean NAFLD/NASH are needed to further investigate this relationship.

T2DM or IGT were present in 76.3% of patients with presumed NAFLD, while T2DM or IGT was significantly more frequent in NAFLD with low to no EtOH consumption compared to patients with presumed NAFLD and repeated moderate to excessive EtOH consumption (82.5% vs. 62.5%,  $p = 0.024$ ). Additionally, lower HbA1c was identified as a univariate predictor of repeated moderate to excessive alcohol consumption, but lost statistical significance in multivariate analysis. This suggests that, as widely known, NAFLD is associated with T2DM/IGT, but its presence is not pathognomonic and may not help in distinguishing NAFLD from ALD. Subsequently, the new term MAFLD, especially emphasizing metabolic dysfunction in the context of fatty liver may require further refinement. Most importantly, the amount or pattern of alcohol consumption requires particular attention in order to choose optimal treatment modalities.

ANI score, a score based upon an equation that incorporates MCV, AST/ALT ratio, BMI and male sex,<sup>25</sup> performed well in identifying patients with excessive alcohol consumption in our mixed cohort, but was unreliable for identifying repeated moderate (to excessive) alcohol consumption. In addition, it was not reliable when applied to patients with ALD. This could be caused by the larger number of patients with cirrhosis in this cohort and is in line with the data of Dunn *et al.*, who reported a lack of accuracy of the ANI score in patients with more advanced liver disease.

Based on our findings we would like to suggest a diagnostic and management strategy for patients with fatty liver disease in order to rule out potentially harmful alcohol consumption contributing to liver disease progression (Fig. S1). We would like to stress that, independently of applying NAFLD or MAFLD criteria, patients with potentially harmful alcohol consumption plus obesity and metabolic dysfunction, alcohol use disorder and metabolic disease have to be adequately treated at the same time.

The strengths of our manuscript include the prospective nature of our study, and the thorough clinical characterization of patients from 3 liver centers. Further, this is the first study to use direct short- and long-term alcohol markers in patients with presumed NAFLD and ALD, and within a cohort of patients with MAFLD. Our findings are limited by the fact that in contrast to NAFLD/ALD diagnostic criteria, MAFLD criteria were applied retrospectively, and that not all patients included in the study had received a liver biopsy. Thereby, it is difficult to translate our



results directly into characteristic histological findings of NAFLD and ALD. Nevertheless, we believe that histological characteristics should be seen in the context of objective alcohol biomarker testing in future studies.

In conclusion, hEtG demonstrated an excellent accuracy to identify repeated moderate to excessive EtOH consumption in patients with fatty liver disease at risk of alcohol-related liver damage. In addition, uEtG and AUDIT-C were found to be helpful screening instruments, whereas the use of CDT had no added value and should be questioned. A BMI <33.4 kg/m<sup>2</sup> was an independent predictor of alcohol-related liver damage. Accurate diagnosis and identification of patients with additive risk of liver disease progression due to alcohol consumption is pertinent.

### Abbreviations

ALD, alcohol-related liver disease; ANI, ALD/NAFLD index; ALT, alanine amino transferase; AST, aspartate amino transferase; AUDIT-C, Alcohol Use Disorders Identification Test – Consumption; BAFLD, both alcoholic and non-alcoholic fatty liver disease; CDT, carbohydrate deficient transferrin; CKD, chronic kidney disease; EtOH, ethanol; EtS, ethyl sulfate; FAEE, fatty acid ethyl ester; GGT, gamma-glutamyltransferase; hEtG, ethyl glucuronide in hair; hEtP, ethyl palmitate in hair; HOMA-IR, homeostasis model assessment of insulin resistance; HbA1c, hemoglobin A1c; IGT, impaired glucose tolerance; MAFLD, metabolic dysfunction-associated liver disease; MBOAT7, membrane-bound O-acyltransferase domain-containing protein 7; MCV, mean corpuscular volume; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; PEtH, phosphatidyl ethanol; PNPLA3, patatin-like phospholipase 3; SIAC, Systematic Inventory of Alcohol Consumption; SoHT, Society of Hair Testing; TM6SF2, Transmembrane 6 Superfamily Member 2; T2DM, type 2 diabetes mellitus; uEtG, ethyl glucuronide in urine.

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### Conflict of interest

GS, UHS, SS, PP, TMS, BP, PF, TS, MY, and CD have no conflict to declare with respect to this work. KS discloses speaker fees as well as travel support from Vifor Pharma, employee of Versantis AG. MT discloses grant support from Albireo, Alnylam, Cymabay, Falk, Gilead, Intercept, MSD, Takeda and UltraGenyx, honoraria for consulting from BiomX, Boehringer Ingelheim, Falk, Genfit, Gilead, Intercept, Janssen, MSD, Novartis, Phenex, Regulus and Shire, speaker fees from BMS, Falk, Gilead, Intercept and MSD, as well as travel support from Abbvie, Falk, Gilead and Intercept.

Please refer to the accompanying ICMJE disclosure forms for further details.

### Authors' contributions

K Stauffer: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis; U Huber-Schönauer: acquisition of data; G Strebinger: acquisition of data; P Pimistingstorfer: acquisition of data; S Suesse: technical and material support, acquisition of data; TM Scherzer: acquisition of data; B Paulweber: technical and material support, acquisition of data; P Ferenci: technical and material support, acquisition of data; T Stimpfl: technical and material support, acquisition of data; M Yegles: technical and material support,

acquisition of data; C Datz: acquisition of data, interpretation of data, critical revision of the manuscript for important intellectual content; M Trauner: interpretation of data, critical revision of the manuscript for important intellectual content.

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### Data availability statement

The data that have been used are confidential. They can only be shared for research questions after personal request and approval of the respective Ethics Committee.

### Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2022.04.040>.

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