1 *Title:* **Novel urinary protein panels for NAFLD and fibrosis stages diagnosis**

2

3 **Short Title:** Urinary NITs for NAFLD diagnosis

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Conflicts of Interest

The authors disclose no conflicts of interest.

Abbreviation list: *AUC, area under the curve; ELISA, enzyme-linked immunosorbent assay; LASSO, least absolute shrinkage and selector operation; Limma, linear models for microarray data; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score*; *NASH, non-alcoholic steatohepatitis; ROC, receiver operator characteristic; OPLS-DA, orthogonal partial least square discriminant analysis; UPLC-MS/MS, ultra-performance liquid chromatography-mass spectrometry.*

Abstract

Background & Aims: There is an unmet clinical need for simple non-invasive tests to diagnose nonalcoholic fatty liver disease (NAFLD) and fibrosis stages. We aimed to test whether urine samples could be used to diagnose NAFLD, NAFLD with fibrosis (fibrosis stage F≥1), and NAFLD with significant fibrosis (fibrosis stage F≥2).

Methods: We collected urine samples from 100 patients with biopsy-proven NAFLD and 40 healthy volunteers and proteomics and bioinformatics analyses were performed in this derivation cohort. Diagnostic models were developed for detecting NAFLD (UP_{NAFLD} model), NAFLD with fibrosis ($UP_{fibrosis}$ model), or NAFLD with significant fibrosis ($UP_{significant\ fibrosis}$ model). Subsequently, the derivation cohort was divided into training and testing sets to evaluate the diagnostic efficacy of these diagnostic models. In a separate independent validation cohort of 100 patients with biopsy-proven NAFLD and 45 healthy controls, urinary enzyme-linked immunosorbent assay (ELISA) analyses were undertaken to validate the accuracy of these newly developed diagnostic models.

Results: The $UP_{fibrosis}$ model and the $UP_{significant\ fibrosis}$ model showed an AUROC of 0.863 (95% CI: 0.725-1.000) and 0.858 (95% CI: 0.712-1.000) in the training set; 0.837 (95% CI: 0.711-0.963) and 0.916 (95% CI: 0.825-1.000) in the testing set. The UP_{NAFLD} model showed excellent diagnostic performance and the area under the receiver operator characteristic curve (AUROC) exceeded 0.90 in the derivation cohort. In the independent validation cohort, the AUROC for all three of the above diagnostic models exceeded 0.80.

109 **Conclusions:** Our newly developed models constructed from urine protein
110 biomarkers have good accuracy for non-invasively diagnosing early liver fibrosis in
111 NAFLD.
112
113 **Keywords:** Fibrosis, NAFLD, Urinary proteomics, Diagnosis, Liver biopsy
114

115 **Introduction**

116 Nonalcoholic fatty liver disease (NAFLD) has become the most common chronic
117 liver disease, affecting up to ~30% of the world's population.[1,2] NAFLD includes a
118 spectrum of progressive liver conditions ranging from nonalcoholic fatty liver (NAFL)
119 to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma.[3-5]
120 Patients with NAFLD may develop varying amounts of liver fibrosis**,** and previous
121 studies have shown that the severity of liver fibrosis is the strongest histologic risk
122 factor of liver-related complications and mortality.[6,7] Recent studies have also shown
123 that even NAFLD with fibrosis (fibrosis stage F⩾1) or NAFLD with significant
124 fibrosis (fibrosis stage F⩾2) is a predictor of overall and liver-related mortality in
125 patients with NAFLD.[8-11] The assessment of liver fibrosis extends beyond the realm
126 of NAFLD and holds significance in terms of evaluating cardiovascular risk.[12]
127 Noninvasive assessment of hepatic fibrosis may predict cardiovascular events and
128 overall mortality in patients with NAFLD.[12] While the diagnostic performance of
129 traditional non- invasive tests are satisfactory for ruling out advanced fibrosis (fibrosis
130 stage F⩾3), their performance is not adequate for diagnosing fibrosis or significant
131 liver fibrosis.[13,14] Therefore, the detection of NAFLD with fibrosis and NAFLD with
132 significant fibrosis can facilitate a more accurate assessment of the patient's condition,
133 treatment response, and prognostic outcomes.

134

135 To date, liver biopsy remains the 'gold standard' for staging the severity of liver
136 disease in NAFLD. However, liver biopsy is an invasive method that is expensive,

137 potentially risky, has possible sampling errors, and patients may not be available to
138 undergoing repeated liver biopsies over time for a benign condition.[13,15,16]
139 Consequently, it is essential to find pragmatic, less expensive and safer non-invasive
140 methods for diagnosis, staging and monitoring liver fibrosis. Non-invasive diagnosis
141 of each of the stages of NAFLD has been an unmet clinical need.[17,18] Urine tests are
142 simple and also more easily accessible than blood tests; thus, urine has been proposed
143 as a source of potential biomarkers for diagnosis of human diseases in several
144 research fields.[19] Moreover, urine changes may reflect dynamic changes in disease
145 status, which is potentially important for liver disease in NAFLD, especially when
146 clinicians need to know whether the disease is improving or not with a pharmacologic
147 treatment.[20]
148
149 Recently, proteomic analyses of liver tissue and blood have been used to discover new
150 biomarkers and therapeutic targets in NAFLD.[21,22] Urine proteomics has also become
151 a focus of research in other chronic diseases, such as diabetic nephropathy, type 1
152 diabetes, and adult-onset Still's disease.[23,24,25] To date, however, proteomic studies on
153 urine in individuals with NAFLD are scarce, and there is still a lack of any relevant
154 evidence as to whether urine can be used to diagnose NAFLD and the severity of liver
155 fibrosis. Thus, the aim of our study was to explore the urinary protein panels to
156 diagnose NAFLD, NAFLD with fibrosis, and NAFLD with significant fibrosis in a
157 liver biopsy-based derivation cohort of patients with NAFLD.
158

159 **Methods**

160 ***Patients and Urine Sample Collection***

161 Patients with NAFLD were diagnosed at the First Affiliated Hospital of Wenzhou
162 Medical University from December 2016 to December 2018, as part of the
163 Prospective Epidemiologic Study of Significantly Characterized Non-Alcoholic
164 Steatohepatitis (PERSONS) cohort study.[14] The inclusion criteria for the study were
165 as follows: (1) individuals aged 18-75 years; (2) individuals with fatty liver diagnosed
166 by imaging and/or elevated serum liver enzymes who were willing to undergo a liver
167 biopsy; (3) individuals with availability of urine samples for protein quantification
168 using label-free quantitative proteomics technology; and (4) individuals who were
169 willing to provide written informed consent. We excluded from the study: (1)
170 individuals with significant alcohol consumption (≥140 g/week in men or ≥70 g/week
171 in women); (2) those who were taking potentially hepatotoxic medications; (3)
172 individuals with viral hepatitis, autoimmune hepatitis, or other known chronic liver
173 diseases; (4) those with pathological liver biopsy suggesting fat content < 5%; and (5)
174 those with incomplete data. Since urine values can vary considerably during a 24-h
175 period, the first-morning urine samples were collected for all participants. Healthy
176 controls were derived from a physical examination population, who was free of fatty
177 liver, as confirmed by ultrasonography. Finally, in the derivation cohort, we obtained
178 100 patients with biopsy-confirmed NAFLD who had completed urine proteomic
179 abundance measurements from the Wenzhou center and 40 non-steatotic healthy
180 controls from the Southwest Hospital of the Army Medical University, who also

181 completed urine proteomic abundance measurements. The derivation cohort was then
182 divided into a training set and a testing set (in a 1:1 ratio) to examine the diagnostic
183 efficacy of early patterns diagnosis models for NAFLD. In a separate and independent
184 validation cohort, we included 45 healthy controls from the Southwest Hospital of the
185 Army Medical University and 100 patients with biopsy-confirmed NAFLD from the
186 First Affiliated Hospital of Wenzhou Medical University. The flowchart of the study is
187 summarized in **Figure 1**. We undertook proteomics and bioinformatics analyses to
188 uncover potential diagnostic biomarkers and develop diagnostic models for
189 identifying NAFLD (UP_{NAFLD} model), NAFLD with fibrosis stage F≥1 ($UP_{fibrosis}$
190 model), or NAFLD with significant fibrosis stage F≥2 ($UP_{significant\ fibrosis}$ model),
191 respectively in the derivation cohort. Furthermore, we also validated the diagnostic
192 efficacy of urinary diagnostic panels utilizing enzyme-linked immunosorbent assay
193 (ELISA) in an external validation cohort of patients with biopsy-confirmed NAFLD.
194 The study was conducted in accordance with the ethical guidelines of the Declaration
195 of Helsinki and the International Conference on Harmonization Guidelines for Good
196 Clinical Practice. Written informed consent was obtained from all participants.
197
198 ***Liver histology***
199 Ultrasound-guided percutaneous liver biopsy was performed using a 16-gauge
200 Hepafix needle. Liver biopsy samples were stained with hematoxylin, Masson's
201 trichrome as well as eosin and subsequently assessed by an experienced liver
202 pathologist, who was blinded to patients' clinical and laboratory data. Liver biopsy

specimens were required to be >1 cm and the number of portal areas was >6. The histological scoring of NAFLD was assessed by using the NAFLD activity score (NAS), as proposed by the NASH Clinical Research Network.[26] The NAS score includes three histologic features including the presence of steatosis, lobular inflammation and hepatocellular ballooning. Liver fibrosis was staged from zero to 4 as follows: 0 = no fibrosis, 1 = perisinusoidal or portal fibrosis; 2 = perisinusoidal and portal/periportal fibrosis; 3 = bridging fibrosis; and 4 = highly suspicious or definite cirrhosis, respectively. The presence of liver fibrosis was defined as fibrosis stage ≥F1, while significant fibrosis was defined as fibrosis stage ≥F2, which is clinically relevant for prognostic liver-related outcomes.[27]

Label-free quantitative proteomics technology

Label-free proteomic quantification was based on the use of the ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS). Mass spectrometry can obtain the mass-to-charge ratio and the signal intensity of peptides in a sample, as well as the mass-to-charge ratio and the signal intensity of fragment ions after peptide fragmentation.[28] Usually, the information at the peptide level is referred to as a first-level spectrum and the peptide fragment ion information a second-level spectrum. The information contained in the spectra is very complex, and a database is created to resolve the peptide sequences contained in the spectra. Before searching the database, a theoretical secondary spectrum database is constructed from the protein sequences in the database. Then the secondary spectrum generated by mass spectrometry is

225 analysed and compared with the theoretical secondary spectrum, and the correct
226 matching theoretical peptide sequences obtained after algorithm scoring and filtering.
227 The protein information contained can be identified by the protein-specific peptides
228 identified. The secondary mass spectrometry data in this experiment was searched
229 using Maxquant (v1.6.15.0).[29] To obtain high-quality data , the search library analysis
230 results required further data filtering. We set the false discovery rate at 1% for the
231 three levels of the spectrum, peptide and protein identification; and the identified
232 proteins had to contain at least one specific (unique) peptide.
233
234 ***Measurement of specific protein levels in urine samples***
235 The levels of ACE2 (CAT#F10272-A), CILP2 (CAT#F111328-A), ENPP7
236 (CAT#F111336-A), MPST (CAT#F111323-A), OGFOD3 (CAT#F111319-A), P2RX4
237 (CAT#F111332-A), TMEM256 (CAT#F111315-A), TMEM25 (CAT#F111311-A), or
238 ICAM-1/CD54 (CAT#F0034-A) proteins in the urine samples from patients (n=45 for
239 healthy volunteers and n=100 for biopsy-proven NAFLD patients) were quantified
240 using the corresponding commercial ELISA Kit (Fankew, Shanghai FANKEL
241 Industrial Co., Ltd, Shanghai, China), according to their manufacturer's instructions.
242
243 ***Statistical analysis***
244 Reporting of clinical data was according to the most frequently obtained data from
245 each participant, with the mean ± SD presented for normal continuous variables and
246 the median (interquartile range) for non-normal continuous variables. Otherwise, the

247 frequency was used for categorical variables. For proteomic abundances, proteins
248 with a missing value ratio higher than 50% in all samples were removed, and the k-
249 nearest neighbor algorithm was used to fill protein abundances, and log2
250 transformation was performed for data at the same time. The methods for screening
251 differential proteins include orthogonal partial least square discriminant analysis
252 (OPLS-DA) or linear models for microarray data (limma). OPLS-DA has been widely
253 used in the multi-omics analysis, including proteomic analysis and metabolomics
254 analysis.[30,31] Limma is a differential expression screening method based on
255 generalized linear equations. The R software package limma (version 3.40.6) was
256 used for differential analysis to obtain the differential proteins among different groups.
257 This method has also been used in proteomic analysis.[32,33] Furthermore, we used
258 LASSO regression to pick out the more important proteins.[34] The R package glmnet
259 was used for the LASSO regression analysis and the 10 folds cross-validation method
260 was used to filter the lambda.[35,36] The cross-validation method divided the data into
261 10 equal parts, first fitting the full data to generate the lambda sequence, then
262 excluding one part of the data at a time and using the remaining 9 parts for validation.
263 The mean and standard deviation of the errors obtained from the 10 validations were
264 calculated. The diagnostic efficacy of each key protein was assessed by the area under
265 the receiver operating characteristics curves (AUROC). The top four ranked proteins
266 of AUROC were considered for inclusion in the pooled analysis of the indicators. The
267 Variance inflation factor (VIF) was calculated to detect the presence of
268 multicollinearity between identified proteins. A cut-off value of 5 was used when

269 applying the VIF in the study.[37] We used a stepwise algorithm to find the best
270 combined model including key proteins, which was based on the Akaike information
271 criterion (AIC) principle that involves removing the variables that were not
272 statistically significant.
273

274 **Results**

275 ***Characteristics of NAFLD patients and urine proteomes in the derivation cohort***

276 Patients with NAFLD were divided into two subgroups: patients with liver fibrosis
277 (defined as stage ≥F1; n=81) and those without fibrosis (n=19) or, alternatively,
278 patients with significant liver fibrosis (defined as stage ≥F2; n=16) and those without
279 significant fibrosis (n=84). **Figure 2** shows the workflow in the derivation cohort.
280 Baseline characteristics of patients with NAFLD and different fibrosis stages in the
281 derivation cohort are presented in **Table 1** and **Supplementary Table 1.** Label-free
282 proteomic analyses identified a total of 4206 proteins among healthy controls and
283 NAFLD patients with different fibrosis stages.
284

285 ***Proteomic biomarkers for non-invasive identification of NAFLD in the derivation***
286 ***cohort***

287 The OPLS-DA method is more sensitive to variables with lower levels of correlation
288 and helps maximize the difference between NAFLD patients and healthy controls
289 based on differential proteins compared with PCA and PLS-DA (**Figure 2e** and
290 **Supplementary Figure 1**). The R2Y represents the interpretation rate of the model to

291 the Y matrix, and the Q2 represents the prediction ability of the model. The closer
292 these three indexes are to 1, the more stable and reliable the model is. Generally, the
293 model is considered effective with a Q2 value above 0.5. In our modeling results, the
294 parameters of R2Y and Q2 were 0.956 and 0.775, respectively, thereby suggesting
295 that the model is reliable and has good prediction ability, and there was also no
296 overfitting in our model **(Supplementary Figure 2)**. The OPLS-DA S-plot clearly
297 shows the distribution of all urinary proteins based on their variable importance in
298 projection (VIP) values. Subsequently, 37 differential proteins were selected by using
299 the following three selection criteria: VIP > 1, $p < 0.05$ and FC > 2 (or FC < 0.5,
300 **Figure 3a)**. We further used the LASSO regression to screen for important differential
301 proteins and found 15 urinary key proteins (**Figure 3b and 3c**). The clustering of
302 these 15 key proteins is shown in **Supplementary Figure 3a,** and their inter-
303 correlations are reported in **Supplementary Figure 4a.** By enrichment analysis, we
304 found that these key proteins are associated with ferroptosis, oxidoreductase activity,
305 and coenzyme A biosynthesis (**Supplementary Figure 3b).** Then, using ROC curve
306 analyses, we found that the top four urinary proteins among these 15 proteins were
307 EPHA10, CILP2, TMEM25, and MPST, respectively (**Figure 4a)**. The correlation
308 analyses of these four key proteins showed that EPHA10 correlated with CILP2,
309 TMEM25 and MPST **(Supplementary Figure 4b; Supplementary Table 2**), but
310 none of these proteins showed evidence of collinearity (VIF < 5). Further, we found
311 by stepwise regression analysis that the combined diagnostic model (UP_{NAFLD} model)
312 of the three urinary key proteins (CILP2, TMEM25, and MPST) had an AUROC of

313 0.983 (95% CI: 0.961-1.000) in the training set and 0.968 (95% CI: 0.932-1.000) in
314 the testing set from derivation cohort, with excellent diagnostic efficacy (**Figure 4b**).
315 We also found that the UP_{NAFLD} model had an AUROC of 0.961 (95% CI: 0.915-
316 1.000) in males and 0.993 (0.979-1.000) in females.
317

318 ***Proteomic biomarkers for non-invasive identification of fibrosis* stage ≥F1 *in the***
319 ***derivation cohort***

320 The R software package limma was used to obtain urinary differential proteins
321 between patients with liver fibrosis ⩾F1 and those without fibrosis. A total of 56
322 differential urinary proteins were subsequently selected by using the criteria of $p <$
323 0.05 and FC > 1.5 (or FC < 0.7, **Figure 3d).** In addition, 22 key proteins were
324 identified using the LASSO regression (**Figures 3e and 3f)**. The clustering results of
325 these 22 key proteins are shown in **Supplementary Figure 3c,** and the correlations
326 among them are shown in **Supplementary Figure 4c**. Our enrichment analysis
327 revealed that these key proteins were associated with the renin-angiotensin system,
328 cornified envelope, humoral immune response, and regulation of inflammatory
329 response (**Supplementary Figure 3d**). After analyzing these 22 key proteins using
330 ROC curves, we found that OGFOD3, ACE2, ENPP7, and P2RX4 were the top four
331 key proteins (**Figure 4c**). Correlation analysis of these four key proteins revealed that
332 ACE2 correlated with OGFOD3, ENPP7, and P2RX4 **(Supplementary Figure 4d;**
333 **Supplementary Table 3**), but none of these variables showed evidence of collinearity
334 (VIF < 5). Moreover, we discovered that the combined diagnostic model ($UP_{fibrosis}$

335 model) of the 3 key proteins (OGFOD3, ENPP7, and P2RX4) had an AUROC of
336 0.863 (95% CI: 0.725-1.000) in the training set and 0.858 (95% CI: 0.712-1.000) in
337 the testing set using stepwise regression analysis **(Figure 4d).** We also discovered that
338 $UP_{fibrosis}$ model had an AUROC of 0.877 (95% CI: 0.765-0.989) in males and 0.857
339 (95% CI: 0.669-1.000) in females.
340

341 ***Proteomic biomarkers for non-invasive identification of fibrosis* stage ≥F2 *in the***
342 ***derivation cohort***

343 By limma analysis, we identified 44 differential urinary proteins with the selection
344 criteria of $p < 0.05$ and FC > 1.5 (or FC < 0.7, **Figure 3g).** We also identified 12 key
345 proteins using LASSO regression (**Figure 3h and 3i**). **Supplementary Figure 3e**
346 shows the clustering results of these 12 key proteins**,** and the correlations among them
347 are reported in **Supplementary Figure 4e**. Using enrichment analysis, we identified
348 these key proteins as being associated with dense platelet granule lumens and
349 collagen-containing extracellular matrix (**Supplementary Figure 3f**). Using the
350 AUROC analyses, we found PECAM1, TMEM256, MSRA, and ICAM1 to be the top
351 four key proteins (**Figure 4e**). TMEM256 had the highest AUROC of 0.772 (95% CI:
352 0.658-0.886), with a sensitivity of 0.750 and specificity of 0.786 for significant
353 fibrosis in NAFLD. PECAM1 was correlated with TMEM256, MSRA, and ICAM1
354 (**Supplementary Figure 4f; Supplementary Table 4**), but none of these four key
355 proteins showed evidence of collinearity (VIF < 5). Furthermore, using a stepwise
356 regression analysis, we found that in the combined diagnostic model ($UP_{significant\ fibrosis}$

357 model) two key proteins (TMEM256 and ICAM1) had an AUROC of 0.837 (95% CI:
358 0.711-0.963) in the training set and 0.916 (95% CI: 0.825-1.000) in the testing set,
359 respectively **(Figure 4f)**. We also found $UP_{significant\ fibrosis}$ model had an AUROC of
360 0.829 (95% CI: 0.698-0.960) in men and 0.833 (95% CI: 0.690-0.977) in women.
361
362 ***Validation by ELISA analyses***
363 ELISA analyses were performed to validate the combined diagnostic models of the
364 key proteins in above urine samples. In the independent validation cohort, we
365 included 45 healthy controls and 100 patients with biopsy-confirmed NAFLD. The
366 baseline characteristics of this validation cohort are presented in **Supplementary**
367 **Table 5**. Of these 100 patients with NAFLD, 57 had fibrosis stage ≥F1 and 13 had
368 significant fibrosis (stage ≥F2). Baseline characteristics of NAFLD patients with
369 different fibrosis stages belonging to the validation cohort are presented in
370 **Supplementary Table 6** and **Supplementary Table 7**. It was found that the model
371 consisting of three urinary proteins, CILP2, TMEM25 and MPST, achieved an
372 AUROC of 0.850 (95% CI: 0.784-0.915) for differentiating NAFLD patients from
373 healthy controls (**Supplementary Figure 5a**). The combined model consisting of
374 three urinary proteins, OGFOD3, ENPP7 and P2RX4, showed an AUROC of 0.804
375 (95% CI: 0.718-0.889) for differentiating patients with fibrosis (stage F ≥1) from
376 those without fibrosis (**Supplementary Figure 5b**). The combined model comprised
377 of two urinary proteins, TMEM256 and ICAM-1, was found to have an AUROC of
378 0.807 (95% CI: 0.715-0.899) for differentiating between patients with significant

379 fibrosis (stage F ≥2) and those without significant fibrosis (F0 + F1 stages)

380 (**Supplementary Figure 5c**).

381

382 **Discussion**

383 Our urine proteomics profiling showed for the first time that there is a pattern of

384 urinary proteins in patients with biopsy-proven NAFLD and liver fibrosis in a Han

385 Chinese population. We discovered for the first time that TMEM256 had an AUROC

386 of 0.772 (95% CI: 0.658-0.886), with a sensitivity of 0.750 and specificity of 0.786

387 for significant fibrosis in NAFLD. The diagnostic model consisting of CILP2,

388 TMEM25, and MPST was useful to distinguish NAFLD patients from healthy

389 controls. The diagnostic models composed of OGFOD3, ENPP7, and P2RX4 for

390 NAFLD with fibrosis, and composed of TMEM256 and ICAM-1 for NAFLD with

391 significant fibrosis provide promising results for future clinical investigation in other

392 ethnic groups. Although Liu et al. identified some potential urinary biomarkers for

393 NAFLD diagnosis, these authors did not use liver biopsy (i.e., the gold standard) for

394 verifying each of the stages of liver disease in NAFLD.[38] In addition, the sample size

395 of the Liu study was small in both the discovery and validation cohorts (neither

396 exceeded 30 cases).[38]

397

398 The utilization of urinary protein panels in personalized medicine for the management

399 of NAFLD and metabolic disorders has the potential to greatly impact current

400 approaches to patient care. Urinary protein panels can help identify individuals who

401 are at risk of developing NAFLD, NAFLD with fibrosis, or NAFLD with significant
402 fibrosis, even before the onset of symptoms. This early detection can aid in the
403 prompt initiation of lifestyle changes and therapeutic interventions that can prevent or
404 slow the progression of the disease. Urinary protein panels can also help monitor the
405 effectiveness of therapeutic interventions and track the progression of the disease over
406 time by changes in model scores. Meanwhile, NAFLD is closely associated with
407 metabolic disorders and may exacerbate the conditions of metabolic disorders.[39]
408 Therefore, utilizing these models to manage NAFLD can help patients improve their
409 metabolic status and reduce the risk of metabolic disorder-related diseases such as
410 cardiovascular disease, stroke, and kidney disease, on the basis of effectively
411 managing NAFLD.
412
413 We found that the model composed of MPST, CILP2 and TMEM25 was a reliable
414 non-invasive diagnostic tool for identifying NAFLD. Li et al. suggested that free fatty
415 acids increased hepatic MPST expression and inhibited the CSE/H_2S pathway, thus
416 leading to NAFLD.[40] MPST might be a potential therapeutic target for NAFLD.
417 Genome-wide association studies have reported multiple loci associated with NAFLD,
418 including CILP2.[41] Meanwhile, we discovered that the diagnostic model composed of
419 OGFOD3, ENPP7 and P2RX4 was useful for identifying NAFLD with fibrosis. Xie
420 et al. showed that disrupting HIF-2**α** in the intestine specifically reduced liver steatosis,
421 along with diminished HIF-2α signaling in the small intestine, several mRNAs
422 encoded by ceramide-synthesis-related genes, were significantly downregulated,

423 including ENPP7.[42]

424

425 We also found that TMEM256 and ICAM-1 could be used as reliable diagnostic
426 biomarkers for significant fibrosis (stage F ≥2) in NAFLD in the future. TMEM256,
427 which localized within the mitochondrial membrane, belongs to mitochondrial
428 proteins and is involved in oxidative phosphorylation.[43] More than 40% of the
429 mitochondrial proteome is associated with human diseases, including NAFLD.[44]
430 Mitochondrial dysfunction and endoplasmic reticulum stress are strongly implicated
431 in the development and progression of NAFLD. As an important member of the
432 immunoglobulin superfamily, the increased expression of ICAM-1 on the cell surface
433 is associated with development of several diseases, and can be used as a predictor of
434 liver fibrosis.[45] The results of animal experiments showed that in liver fibrosis tissues,
435 ICAM1 is expressed in hepatocytes, mostly in the portal zone, inflammatory zone,
436 and focal necrotic zone, and the intensity of its expression increases progressively
437 with the severity of liver inflammation and fibrosis.[46]

438

439 Urine is a readily available and highly accepted test analyte by patients and has
440 become a specimen for monitoring and detection of many diseases or physiological
441 characteristics.[47-49] In our study, GO and KEGG analyses showed that urine proteins
442 in NAFLD patients were mainly associated with ferroptosis, oxidoreductase activity,
443 and coenzyme A biosynthesis. As a newly discovered form of iron-dependent
444 programmed cell death, ferroptosis is involved in the development and progression of

445 NAFLD.[50] A typical feature of NAFLD is development of oxidative stress and redox
446 imbalance.[51] In recent years, an increasing number of studies have found that
447 coenzyme A is closely related to NAFLD from different perspectives. Zhou et al.
448 revealed that inhibition of stearoyl-coenzyme A desaturases-1 ameliorated hepatic
449 steatosis.[52] Huang et al. suggested that enoyl coenzyme A hydratase-1 protected
450 against high-fat-diet-induced hepatic steatosis and insulin resistance.[53] In our study,
451 urine proteins in NAFLD with fibrosis were associated with the renin-angiotensin
452 system, cornified envelope, humoral immune system, and regulation of inflammatory
453 response. A prior report has suggested that ACE2/Ang-(1-7)/Mas may also contribute
454 to NAFLD development.[54] NAFLD is characterized by a dysregulated immune
455 response.[55] It is believed that inflammation and upregulation of inflammatory
456 mediators play a secondary role in the pathogenesis of NAFLD.[56] In our study, urine
457 proteins in NAFLD with significant fibrosis were associated with dense platelet
458 granule lumens and collagen-conjugate lumens. Yang et al. discovered that counting
459 platelets may help to determine the severity of liver injury and liver fibrosis.[57] The
460 above enrichment analysis suggests that urine proteomic data may reflect NAFLD
461 disease characteristics.
462
463 In the field of proteomics research, UPLC-MS/MS based techniques for the detection
464 and relative quantification of thousands of proteins in biological samples have been
465 widely used, and two mainstream methods have been developed for labeled
466 quantification and unlabeled quantification (label-free), both of which are based on

data-dependent acquisition.[48] These methods are based on the data-dependent acquisition (DDA) model to acquire protein spectral data. The label-free proteomic quantification was used in this study. Label-free proteomic quantification is a new protein quantification technique that does not rely on isotope labeling and analyzes enzymatic peptides by liquid-liquid mass spectrometry.[58] This technique simply analyzes the mass spectrometry data generated during the large-scale identification of proteins and compares the signal intensities of the corresponding peptides in different samples for the relative quantification of the corresponding proteins. It facilitates the pursuit of more precise qualitative and quantitative results and avoids experimental errors introduced by labeling and liquid-phase grouping.[59] In this study, a series of frontier technologies such as protein extraction, enzymatic digestion, liquid chromatography-mass spectrometry tandem analysis, and bioinformatics analysis were combined to finally achieve quantitative proteomic data from the samples.

There were some important limitations in the present study that should be mentioned. First, the number of patients with NAFLD included in our study was relatively small. Second, this study only explored the diagnostic biomarkers of NAFLD and associated fibrosis from the perspective of high-throughput omics, but the underlying mechanisms between these biomarkers and the disease were not addressed in depth.

In conclusion, the diagnostic model consisting of CILP2, TMEM25, and MPST was useful for distinguishing NAFLD patients from healthy controls. The diagnostic

models comprising OGFOD3, ENPP7 and P2RX4 (for NAFLD with fibrosis), and comprising TMEM256 and ICAM-1 (for NAFLD with significant fibrosis) now need to be further tested in other ethnic groups. Urine-based biomarkers are more accessible and less invasive than blood tests; and may circumvent the need to invasive liver biopsy to refine the staging of liver disease severity in people with NAFLD.

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647

648 **Table legend**

649 **Table 1.** Baseline characteristics of patients with biopsy-proven NAFLD in the

650 derivation cohort grouped by the severity of fibrosis (fibrosis stages < 1 vs. fibrosis

651 stages ≥ 1).

652

653 **Figure legends**

654 **Figure 1.** Flow-chart of the study design, including the derivation cohort

655 (subsequently divided into the training set and testing set) and the validation cohort.

656 *Note:* Diagnostic models were developed for detecting NAFLD (UP_{NAFLD} model),

657 NAFLD with fibrosis stage F≥1 ($UP_{fibrosis}$ model), or NAFLD with significant fibrosis

658 stage F≥2 ($UP_{significant\ fibrosis}$ model), respectively.

659 **(a)** Derivation cohort. **(b)** Validation cohort.

660

661 **Figure 2.** The diagram illustration of discovering biomarkers of NAFLD and related

662 fibrosis from the perspective of urine proteomics.

663 **(a)** Grouping of patients with liver biopsy-proven NAFLD in the derivation cohort. **(b)**

664 Label-free quantitative proteomics technology data analysis process. **(c)** Proteomics

665 identification results. The x-axis represents the identified substances and the y-axis

666 represents the number of substances. (d) Molecular weight of the identified proteins.

667 The X-axis represents the size of the molecular weight and the Y-axis represents the

668 number of molecular weights in this range. **(e)** OPLS−DA plots of urine proteomics

669 from NAFLD and healthy individuals (R2Y, 0.956; Q2, 0.775).

670

671 **Figure 3.** Volcano plots and LASSO regression of differential proteins.

672 **(a)** Volcano map of differential proteins between NAFLD patients and healthy

673 controls. **(b, c)** LASSO regression screening for important differential key proteins

674 between NAFLD patients and healthy controls. **(d)** Volcano map of differential key

675 proteins between NAFLD patients with fibrosis stage F≥1 and those without fibrosis.

676 **(e, f)** LASSO regression screening for important differential proteins between

677 NAFLD with fibrosis and NAFLD without fibrosis. **(g)** Volcano plot of differential

678 key proteins between NAFLD patients with significant fibrosis stage F≥2 and those

679 without significant fibrosis. **(h, i)** LASSO regression screening for important

680 differential proteins between NAFLD patients with significant fibrosis and those

681 without significant fibrosis.

682

683 **Figure 4.** ROC curve analyses of key proteins and combined models in the derivation

684 cohort.

685 **(a)** ROC curves of EPHA10, CILP2, TMEM25, and MPST. **(b)** The combined

686 diagnostic model (UP_{NAFLD} model) of three key proteins (CILP2, TMEM25, and

687 MPST) for NAFLD patients. **(c)** ROC curves of ACE2, OGFOD3, ENPP7, and

688 P2RX4. **(d)** The combined diagnostic model ($UP_{fibrosis}$ model) of three key proteins

689 (OGFOD3, ENPP7, and P2RX4) for NAFLD patients with fibrosis. **(e)** ROC curves

690 of PECAM1, TMEM256, MSRA, and ICAM1. **(f)** The combined diagnostic model

691 ($UP_{significant\ fibrosis}$) of the two key proteins (TMEM256, and ICAM1) for NAFLD

692 patients with significant fibrosis.

693 D set, training set; V set: testing set.

694

ONLINE-ONLY SUPPLEMENTARY MATERIAL

Supplementary Table 1. Baseline characteristics of patients with biopsy-proven NAFLD in the derivation cohort grouped by stages of fibrosis (fibrosis stage $F < 2$ and fibrosis stage $F \geq 2$).

Supplementary Table 2. Correlation analyses of four key proteins (MPST, EPHA10, TMEM25 and CILP2) between NAFLD patients and healthy controls.

Supplementary Table 3. Correlation analyses of four key proteins (OGFOD3, ENPP7, ACE2 and P2RX4) between NAFLD patients with fibrosis and those without fibrosis.

Supplementary Table 4. Correlation analyses of four key proteins (PECAM1, TMEM256, MSRA and ICAM1) between NAFLD patients with significant fibrosis and those without significant fibrosis.

Supplementary Table 5. Baseline characteristics of healthy controls and NAFLD patients in the validation cohort.

Supplementary Table 6. Baseline characteristics of patients with biopsy-proven NAFLD in the validation cohort grouped by stages of fibrosis (fibrosis stage $F < 1$ and fibrosis stage $F \geq 1$)

Supplementary Table 7. Baseline characteristics of patients with biopsy-proven NAFLD in the validation cohort grouped by stages of fibrosis (fibrosis stage $F < 2$ and fibrosis stage $F \geq 2$)

Supplementary Figure 1. Plots of PCA **(a)** and PLS-DA **(b).**

Supplementary Figure 2. Permutation test of OPLS-DA.

718 (a) The parameters of R2Y, and Q2 in the permutation test of OPLS-DA**. (b)**

719 Permutation analysis to verify whether the model has overfitting.

720 **Supplementary Figure 3.** Heatmaps and enrichment analysis.

721 **(a)** Heatmap of hierarchical cluster analysis of differential key proteins in the NAFLD

722 patient group (A) and in the healthy control group (B). **(b)** GO and KEGG enrichment

723 analysis of 15 key proteins in NAFLD patients. **(c)** Heatmap of hierarchical cluster

724 analysis of differential key proteins in NAFLD patients with fibrosis stage F≥1 (C)

725 and in those without fibrosis (D). **(d)** Enrichment analysis of 22 key proteins in

726 NAFLD patients with fibrosis. **(e)** Heatmap of hierarchical cluster analysis of

727 differential key proteins in NAFLD patients with significant fibrosis stage F≥2 (E)

728 and those without significant fibrosis (F) groups. **(f)** Enrichment analysis of 12 key

729 proteins in NAFLD patients with significant fibrosis.

730 **Supplementary Figure 4.** Correlation analyses between urinary key proteins.

731 **(a)** Correlation analyses among 15 differential proteins selected to distinguish

732 NAFLD patients from healthy controls. **(b)** Correlation analyses of four key proteins

733 (EPHA10, CILP2, TMEM25, and MPST). **(c)** Correlation analyses among 22

734 differential proteins selected to distinguish NAFLD patients with fibrosis from those

735 without fibrosis. **(d)** Correlation analyses of four key proteins (OGFOD3, ACE2,

736 ENPP7, and P2RX4). **(e)** Correlation analyses among 12 differential proteins selected

737 to distinguish NAFLD patients with significant fibrosis from those without significant

738 fibrosis. **(f)** Correlation analyses of four key proteins (PECAM1, TMEM256, MSRA,

739 and ICAM1).

740 **Supplementary Figure 5.** ROC curves of combined models in the independent

741 validation cohort.

742 **(a)** The model consisting of three indicators, CILP2, TMEM25, and MPST, for

743 differentiating NAFLD patients from healthy controls (UP_{NAFLD} model). **(b)** The

744 combined model consisting of three indicators, OGFOD3, ENPP7, and P2RX4, for

745 differentiating NAFLD patients with fibrosis from those without fibrosis ($UP_{fibrosis}$

746 model). **(c)** The combined model comprised of two indicators, TMEM256 and ICAM-

747 1, for differentiating NAFLD patients with significant fibrosis from those without

748 significant fibrosis ($UP_{significant\ fibrosis}$ model).

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