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1 **Plasma BCAA changes in Patients with NAFLD are Sex Dependent**

2 Guillaume Grzych^{1,2}, Luisa Vonghia^{3,4}, Marie-Adélaïde Bout², Jonas Weyler^{3,4}, An Verrijken^{4,5},
3 Eveline Dirinck^{4,5}, Marie Joncquel², Luc Van Gaal^{4,5}, Réjane Paumelle¹, Sven Francque^{3,4*},
4 Anne Tailleux^{1*}, Joel T. Haas^{1*}, Bart Staels^{1,2*}

5
6 **Affiliations:**

7 ¹ Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011- EGID, F-59000 Lille, France.

8 ² CHU Lille, Service d'Hormonologie, Métabolisme, Nutrition, Oncologie, F-59000 Lille,
9 France

10 ³ Department of Gastroenterology and Hepatology, Antwerp University Hospital, 2650
11 Edegem/Antwerp, Belgium

12 ⁴ Laboratory of Experimental Medicine and Pediatrics, Faculty of Medicine and Health
13 Sciences, University of Antwerp, 2610 Wilrijk/Antwerp, Belgium

14 ⁵ Department of Endocrinology, Diabetology and Metabolism, Antwerp University Hospital,
15 2650 Edegem/Antwerp, Belgium

16 * These authors co-supervised the study

17

18 **Corresponding Author:** Joel T. Haas

19 Institut Pasteur de Lille, 1 rue du professeur Calmette, BP245, 59019 LILLE - France

20 Tel: +33320877171 - Fax: +33320877360

21 e-mail: joel.haas@pasteur-lille.fr

22

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31 **Abstract**

32 **Context:** Plasma branched chain amino acid (BCAA) concentrations correlate positively with
33 body mass index (BMI), measures of insulin resistance (IR) and severity of non-alcoholic
34 fatty liver disease (NAFLD). Moreover, plasma BCAA concentrations also differ between the
35 sexes, which display different susceptibilities to cardio-metabolic diseases.

36 **Objective:** Assess whether plasma BCAA concentrations associate with NAFLD severity
37 independently of BMI, IR and sex.

38 **Patients:** Patients visiting the obesity clinic of the Antwerp University Hospital were
39 consecutively recruited from 2006 to 2014.

40 **Design and Setting:** A cross-sectional study cohort of 112 obese patients (59 women and 53
41 men) was divided into four groups according to NAFLD severity. Groups were matched for
42 sex, age, BMI, HOMA-IR and HbA_{1c}.

43 **Main Outcome Measures:** Fasting plasma BCAA concentrations were measured by tandem
44 mass spectrometry using the aTRAQ™ method.

45 **Results:** In the study cohort, a modest positive correlation was observed between plasma
46 BCAA concentrations and NAFLD severity, as well as a strong effect of sex on plasma BCAA
47 levels. Subgroup analysis by sex revealed that while plasma BCAA concentrations increased
48 with severity of NAFLD in women, they tended to decrease in men. Additionally, only
49 women displayed significantly increased plasma BCAAs with increasing fibrosis.

50 **Conclusion:** Plasma BCAA concentrations display sex-dimorphic changes with increasing
51 severity of NAFLD, independently of BMI, IR and age. Additionally, plasma BCAA are
52 associated with significant fibrosis in women, but not in men. These results highlight the
53 importance of a careful consideration of sex as a major confounding factor in cross-sectional
54 studies of NAFLD.

55 **Introduction**

56
57 Non-Alcoholic Fatty Liver Disease (NAFLD) is a major global public health problem. NAFLD
58 covers a spectrum of conditions from simple hepatic triglyceride accumulation, termed non-
59 alcoholic fatty liver (NAFL), to more severe non-alcoholic steatohepatitis (NASH) which
60 combines NAFL with hepatic necroinflammation (1). Patients with NASH are at high risk for
61 progressive liver fibrosis, hepatocellular carcinoma and cardiovascular disease (2). Recent
62 studies indicate that NAFLD progression and incidence are significantly influenced by sex,
63 suggesting NAFLD may be a sexually dimorphic disease (3,4).

64 Development of non-invasive diagnostic markers for NAFLD remains a major challenge.
65 Current guidelines for accurate NAFLD diagnosis require liver biopsy (5,6), which is invasive
66 and susceptible to inter-observer discrepancies (7,8). Non-invasive imaging, individual
67 biochemical markers and combined clinical and biochemical score-based methods are being
68 developed, but have not yet been accepted as a valid substitute for histological diagnosis.
69 Thus, there is an urgent need to identify accurate markers of NAFLD stages, especially NASH
70 and fibrosis, to simplify disease staging for diagnosis and monitoring treatment response.

71 Moreover, as biological plausibility is an important feature of these non-invasive markers,
72 their identification often yields insight into disease mechanisms.

73 The branched chain amino acids (BCAA) leucine, valine and isoleucine are essential amino
74 acids whose plasma concentrations are increased in metabolic diseases such as obesity and
75 type 2 diabetes (T2D) (9–11). Recently, plasma BCAA were also found to be increased in
76 NAFLD patients compared to patients without NAFLD (12–14). Interestingly, a recent large-
77 scale population study demonstrated that plasma BCAA and their degradation products
78 were elevated in men compared to women (15). Given the known discrepancies between
79 men and women in metabolic disease susceptibility and the interaction between obesity, IR
80 and NAFLD (9,11,16–20), it is currently unclear whether changes in plasma BCAA are
81 specifically related to NAFLD progression or rather to accompanying metabolic alterations.

82 Consequently, the aim of this study was to determine whether changes in plasma BCAA
83 concentrations are specifically associated with severity of NAFLD in humans. We measured
84 plasma BCAA concentrations in a well-characterized patient cohort with histologically
85 assessed NAFLD. Our results show that plasma BCAA levels vary with increasing severity
86 from NAFL to NASH in a sex-dependent manner, independently of alterations in glucose
87 metabolism.

88

89 **Methods**

90 **Description of the patients**

91 Patients were consecutively recruited at the metabolic unit of the obesity clinic of the
92 Antwerp University Hospital (a tertiary referral facility) from October 2006 to May 2014. The
93 study protocol, part of the HEPADIP protocol (Belgian registration number B30020071389),
94 was approved by the Ethical Committee of the Antwerp University Hospital (file 6/25/125)
95 and required written informed consent of the patient. Inclusion criteria were previously
96 described (21). Briefly, patients underwent extensive metabolic characterization and
97 systematic non-invasive screening for the presence of NAFLD (*e.g.* plasma alanine
98 transaminase (ALT), aspartate transaminase (AST) and gamma glutamyltransferase (GGT)
99 measurements, and ultrasound features) and proposed for liver biopsy upon suspicion of
100 NAFLD. Liver biopsy was performed percutaneously (16G Menghini), transjugularly (16G
101 transjugular liver biopsy needle) or perioperatively (16G Trucut needle) for patients who
102 subsequently underwent bariatric surgery. The herein described assessment, including liver
103 biopsy, was performed before any treatment initiation or consideration.

104

105 **Clinical assessment and biological assay**

106 Fasting plasma glucose and insulin were measured as previously described (22). Insulin
107 resistance (IR) was estimated using both the homeostatic model assessment of insulin
108 resistance (HOMA-IR) calculated by $[\text{insulin (mU/L)} \times \text{glucose (mmol/L)}] / 22.5$ and using the
109 updated homeostasis model assessment HOMA2 from Levy *et al.* (23) that estimates insulin
110 resistance (HOMA2-IR), steady state beta cell function (HOMA2-B) and insulin sensitivity
111 (HOMA2-S), as percentages of a normal reference population.

112

113 **Liver histology**

114 Hematoxylin and eosin, Sirius red, reticulin and Perls iron stains were routinely performed
115 on all liver biopsies and analyzed in batch by two experienced pathologists blinded to clinical
116 data. The histological features of NAFLD (steatosis, ballooning, lobular inflammation and
117 fibrosis) were assessed using the NASH Clinical Research Network Scoring System (5).
118 According to biopsy evaluation, patients were divided in 4 groups: Healthy, NAFL, NASH and
119 NASH with significant fibrosis. The healthy group was defined as steatosis=0, ballooning=0,
120 lobular inflammation=0 and fibrosis=0. The NAFL group included patients with steatosis > 0
121 and allowing some presence of either ballooning or lobular inflammation, but not meeting
122 the criteria for NASH. NASH diagnosis required the combined presence of steatosis,
123 ballooning and lobular inflammation, according to current guidelines (24,25). Patients with
124 NASH were subsequently divided according to fibrosis stage: NASH without significant
125 fibrosis (fibrosis ≤ 1) and NASH with significant fibrosis (NASH-F, fibrosis ≥ 2).

126

127 **Plasma amino acid measurements**

128 Blood samples were collected in EDTA-coated tubes after an overnight fast. Isolated plasma
129 was aliquoted, immediately frozen and stored at -80°C until analysis. Targeted
130 metabolomics was performed to quantify plasma amino acids using the aTRAQ™ Kit (Sciex,
131 Framingham, MA, USA) as described previously (26). Briefly, plasma amino acids were
132 amine-modified using the aTRAQ™ labeling reagent, which provides a specific mass tag for
133 tandem mass spectrometry (MS). The tag is identified by MS/MS fragmentation of samples
134 and standards while in multiple reaction monitoring (MRM) mode. An internal standard (IS)
135 set of aTRAQ™ labeled amino acids was used for detection and quantification by high
136 performance liquid chromatography (LC) (Shimadzu C18 column, Kyoto, Japan) associated
137 with MS/MS (Sciex 3200 Qtrap, Framingham, MA). Quantification was performed dividing

138 the peak area for each analyte by the peak area of the corresponding IS and multiplying by
139 the IS concentration.

140

141 **Rationale of study group composition**

142 As outlined above, BCAA metabolism and concentrations are influenced by BMI, diabetes
143 and sex. These factors are poorly accounted for when studying the relationship between
144 BCAA concentrations and presence and severity of NAFLD. In order to unequivocally study
145 the relation between plasma BCAA concentrations and NAFLD severity, the following
146 approach was used. Patients were first classified into clearly distinct groups (“NAFLD
147 stages”) based on their liver histological phenotype, as follows: healthy liver, NAFL, NASH
148 and NASH-F (see histology section above) or unclassified (i.e. patients not clearly falling into
149 one of the previously defined categories). Patients were subsequently selected from the 4
150 well defined NAFLD stages to compose groups of equal size that were further matched for
151 the following parameters: BMI, fasting plasma glucose, HOMA-IR, HbA1c and age. Finally, an
152 equal number of men and women was included in each study group when possible.

153

154 **Statistical analysis**

155 Patient selection was performed using regression-based matching in R (*matchit package* R-
156 project) considering clinical parameters as described above. Values are expressed as mean \pm
157 standard deviation (mean \pm SD) or median \pm Inter Quartile Range (IQR) as indicated in the
158 figure legends. Statistical differences in clinical and biological parameters were assessed by
159 Mann-Whitney or ANOVA test for quantitative values and Chi-squared test for categorical
160 values. The Spearman rank test was used to determine correlation between variables. All

161 univariate correlations were then verified in a linear model adjusting for BMI, HOMA-IR and
162 sex. P-values less than 0.05 were considered statistically significant.

163 **Results**

164 **Patient selection and matching**

165 Among all consecutively recruited patients with available clinical information (n=408) and
166 fasting plasma samples, four study groups corresponding to different stages of NAFLD were
167 established (study cohort) as described in the methods section. This process resulted in 4
168 clearly distinct liver phenotypical groups of about 30 patients, matched for BMI, fasting
169 plasma glucose (FPG), HOMA-IR, HbA_{1c} and age. For the group of healthy liver, a lower
170 number of patients who met both the histological phenotype and all matching criteria was
171 available, n = 23. As outlined in the methods section, an equal number of men and women
172 was included in each study group when possible. The clinical, biological and liver histological
173 characteristics of the patients are shown in **Table 1**. Overall, 112 patients were divided over
174 the 4 groups as follows: 23 healthy liver (15 women and 8 men), 30 with NAFL (15 women
175 and 15 men), 30 with NASH (15 women and 15 men) and 29 with NASH and significant
176 fibrosis (NASH-F; 14 women and 15 men). As per the matching protocol, the four groups did
177 not show significant differences in sex, age, BMI, FPG, HOMA-IR and HbA_{1c} (**Table 1**).

178

179 **Plasma BCAA concentrations highly associate with sex, but not with NAFLD stages**

180 Plasma concentrations of the three BCAA in the study cohort were measured and
181 correlations between plasma BCAA concentrations and NAFLD stage were assessed. In
182 agreement with previous studies (12–14), plasma leucine and, to a greater extent, plasma
183 isoleucine correlated with NAFLD stage (Spearman coefficient: rho=0.17 and rho=0.25,

184 p=0.06 and p<0.01, respectively) (**Figure 1A, 1C**). However, plasma valine concentrations did
185 not significantly correlate with NAFLD stage (**Figure 1B**).

186 To assess which factor most contributed to plasma BCAA variation in the study cohort, we
187 calculated the correlations between BCAA and several known confounding factors (i.e. age,
188 BMI, HOMA-IR and sex). Interestingly, sex showed the strongest association with plasma
189 BCAA concentrations when compared to age, BMI, HOMA-IR and NAFLD stage (**Figure 2**). As
190 expected based on the patient stratification strategy, no or minor correlations were
191 observed between BCAA and age, BMI and HOMA-IR. Moreover, we calculated the
192 Spearman correlation coefficient between plasma BCAA and HOMA2 parameters (HOMA2-
193 IR) and no significant correlations were observed (**Figure 2**).

194 To confirm this specific correlation of plasma BCAA with sex, independently of BMI, age and
195 glucose homeostasis parameters, a linear model including age, BMI, HOMA-IR, NAFLD stage
196 and sex was developed. This model confirmed an independent association between the 3
197 BCAA plasma concentrations and sex (p<0.001). We consequently analysed the BCAA
198 concentrations in the study cohort separating men and women, irrespective of NAFLD
199 status. The plasma concentrations of the 3 BCAA were higher in men compared to women
200 (p<0.001) (**Figure 3A, 3B, 3C**), indicating that sex is a major determinant of plasma BCAA
201 concentrations.

202

203 **Sex differentially influences the correlation of plasma BCAA with NAFLD severity**

204 Due to the strong effect of sex on plasma BCAA concentrations, men and women were
205 subsequently analysed separately. Clinical, biological and liver histological characteristics of
206 the patients separated according to sex (53 men and 59 women) and NAFLD stage are
207 shown in **Table 2**. The NAFLD subgroups stratified by sex remained matched according to

208 age, BMI and glucose homeostasis parameters (**Table 2**), as was the case for the 4 initial
209 groups (**Table 1**). Moreover, average age, BMI, FPG, HOMA-IR, HbA1c and liver histological
210 characteristics were not different between men and women (**Table 3**). For women,
211 menopause status was collected, and the proportion of women post-menopause did not
212 differ with NAFLD severity (**Table 2**). Moreover, for women with NASH (n=29), we observed
213 no significant differences in the plasma BCAA concentrations between pre- (n=18) and post-
214 menopausal (n=11) women (**data not shown**). The low numbers of post-menopausal
215 patients in the Healthy and NAFL groups did not permit the same comparison. Still, these
216 results suggest that menopause alone is unlikely to lead to a major difference in plasma
217 BCAA levels in the context of NAFLD.

218 Interestingly, plasma BCAA showed an inverse relation to NAFLD depending on patient sex.
219 In men, plasma leucine and valine negatively correlated with increasing NAFLD stage (**Figure**
220 **4A, 4B**), whereas plasma isoleucine did not correlate with NAFLD stage (**Figure 4C**).
221 Conversely, in women, plasma leucine, valine and isoleucine concentrations all positively
222 correlated with NAFLD severity (plasma leucine: Spearman rho=0.43, p<0.01; plasma valine:
223 Spearman rho=0.39, p<0.01; plasma isoleucine: Spearman rho=0.43, p<0.01; **Figure 4D, 4E,**
224 **4F**). These results indicate a sex-specific signature of plasma BCAA in NAFLD.

225

226 **Sex-dependent association of plasma BCAA with NAFLD features**

227 To better understand the specific disease features associated with plasma BCAA changes,
228 plasma BCAA concentrations were correlated with each NAFLD histological characteristic
229 separately in men and women. In men (n=53), with the exception of a modest inverse
230 correlation of plasma valine with lobular inflammation (rho=-0.33, p=0.02), plasma BCAA
231 concentrations did not significantly associate with any of the parameters tested (**Figure 5**).

232 Conversely, in women, the 3 plasma BCAA correlated positively with steatosis and fibrosis.
233 Additionally, plasma leucine and isoleucine concentrations significantly correlated with the
234 histological ballooning score ($\rho=0.36$, $p=0.004$ for leucine and $\rho=0.33$, $p=0.009$ for
235 isoleucine) (**Figure 5**).
236 Interestingly, all 3 plasma BCAA most strongly correlated with fibrosis in women only (**Figure**
237 **5**). To investigate whether BCAA could discriminate early from more advanced stages of
238 fibrosis in patients with NASH, women and men were subgrouped in 2 fibrosis stages: no or
239 low fibrosis (F0 to F1; F0-1) and significant fibrosis ($F\geq 2$). In men, no significant association
240 was found between plasma BCAA and fibrosis group (**Figure 6A-C**). Conversely, women
241 displayed a significant increase of all three plasma BCAAs with the presence of significant
242 fibrosis (leucine: 117 ± 21 $\mu\text{mol/L}$ vs 147 ± 31 $\mu\text{mol/L}$; valine: 221 ± 34 $\mu\text{mol/L}$ vs 278 ± 50
243 $\mu\text{mol/L}$; isoleucine: 61 ± 10 $\mu\text{mol/L}$ vs 81 ± 16 $\mu\text{mol/L}$; F0-1 vs $F\geq 2$, **Figure 6D-F**). Moreover, as
244 expected from the initial matching strategy, HOMA-IR, BMI, age and glucose homeostasis
245 parameters were not significantly different between the fibrosis subgroups.

246

247 **Discussion**

248 Here, we investigated the correlation of plasma BCAA levels with NAFLD severity and
249 fibrosis in patient groups from a cross-sectional NAFLD cohort. To account for their potential
250 confounding effects, groups were matched for age, BMI, glucose metabolism parameters
251 and sex. Our results indicate that plasma BCAA correlate with severity of NAFLD
252 independently of known confounders, but in a sex-dependent manner, increasing from
253 NAFL to fibrotic NASH in women, while decreasing in NASH in men.

254 Our results extend recent findings from Gaggini *et al.* (14), who found a positive association
255 of plasma BCAA with increasing NAFLD severity and fibrosis in a cohort of both obese and

256 non-obese NAFLD patients. However, in this study, sex ratio imbalances were present
257 between the disease groups (14). Indeed, there was a higher proportion of men in the obese
258 NAFLD group, whereas in the control group there was a higher proportion of women. Our
259 results suggest this may have exaggerated the association of elevated BCAA with NAFLD.
260 Lake *et al.* also found a positive association between plasma BCAA and NAFLD stages with
261 limited numbers of patients (12). However, non-standard NAFLD classification criteria were
262 used, and no data on sex distribution nor insulin-resistance was provided. Finally, our
263 findings in women are in agreement with a third study reporting a positive association of
264 plasma BCAA with increasing NAFLD severity in a cohort of obese, non-diabetic women (13).

265

266 There are numerous sources of seeming discordance in the literature among cohort studies
267 of metabolite changes in NAFLD. These can arise from the definition of experimental groups
268 (i.e. NAFLD vs Healthy or NASH versus NAFL, etc.) or differences in confounding parameters
269 between experimental and control groups. For example, we found that alterations in
270 circulating bile acids, previously reported to be elevated in NAFLD (27,28), may be due to
271 differences in insulin resistance rather than NASH *per se* in a cohort of non-diabetic NASH
272 patients (29). The present study, with clearly distinct groups based on their liver phenotype,
273 but matched for several potential confounders, allowed specific assessment of the
274 association of histological liver alterations with plasma metabolites, i.e. BCAA. Therefore,
275 studies designed to identify specific associations with NAFLD presence and severity should,
276 in our opinion, always be controlled for known confounding factors, especially sex, as shown
277 in the present study.

278 The effect of sex in cohort studies of NAFLD has recently been highlighted, as prevalence
279 and severity of NAFLD is higher in men than in pre-menopausal women (3,30,31). Hence, a

280 specific metabolic signature dependent on patient sex may be expected in NAFLD. To our
281 knowledge, the present study is the first to reveal sex-dependent associations of plasma
282 metabolites with NAFLD severity. Indeed, plasma BCAA concentrations increased with
283 NAFLD severity in women whereas they decreased in men, independently of glucose
284 metabolism parameters. With respect to other clinical variables, we observed no significant
285 differences between men and women for plasma triglycerides, plasma total cholesterol,
286 ASAT or ALAT (not shown). As expected, plasma HDL-C was lower in men than in women,
287 and tended to decrease in both sexes with increasing NAFLD severity (not shown). Based on
288 the strong influence of patient sex in the determination of plasma metabolite composition
289 (15), we believe that untargeted metabolomic studies may reveal further examples of such
290 metabolic changes that may be key to explanations of divergent disease phenotypes
291 between men and women. The fact that NAFLD is associated with changes in sex hormone
292 concentrations such as sex hormone binding globulin or testosterone, may provide a
293 potential explanation for sex susceptibility differences in NAFLD (32,33). Our data suggest
294 that BCAA measurement could be useful to evaluate disease progression in women and thus
295 be integrated in future non-invasive scores for NAFLD screening. However, large-scale
296 prospective studies with paired biopsies, such as currently ongoing clinical trials, will be
297 necessary to fully test the applicability of such a strategy.

298

299 Whether BCAA are markers or actors in NAFLD progression remains unclear. BCAA
300 metabolism plays an important role in many cellular functions and signaling (34). Plasma
301 BCAA levels reflect net differences in BCAA intake from the diet and their catabolism. The
302 first two steps of BCAA catabolism, catalyzed by BCAT (branched-chain amino acid
303 transaminase, in peripheral tissues) and BCKDH (branched-chain α -ketoacid dehydrogenase,

304 mainly in liver), respectively, are reduced in insulin resistance and obesity (35). The
305 reductions in these enzymatic activities limit the availability of BCAA-derived succinyl-CoA
306 and acetyl-CoA, which would otherwise feed the TCA cycle. The combination of these
307 changes could lead to lower hepatic mitochondrial activity. Interestingly, BCAA (especially
308 leucine) are well-known activators of the mTOR pathway (36–38), whose activation may
309 further drive insulin resistance and abnormal lipid accumulation. In line, feeding obese mice
310 with BCAA was associated with chronic phosphorylation of mTOR and enhanced liver injury
311 in this model (39). However, the involvement of BCAA in liver injury in humans appears to
312 be inconsistent with these preclinical models. For example, in patients with cirrhosis, long-
313 term oral BCAA supplementation reduced liver injury and related complications (40–42).
314 Consequently, elevated plasma BCAA in humans may be a protective mechanism to reduce
315 further hepatic injury in a chronic setting.

316

317 Although our findings do not prove causality of the observed association, they highlight that
318 patient sex must be considered when developing metabolite-based diagnostic/evaluation
319 scores for NAFLD.

320

321

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325

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327

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482 **Figure 1. Plasma leucine and isoleucine but not valine are positively correlated to NAFLD stage.**

483 Plasma BCAA concentrations according to NAFLD stage in Healthy (n=23), NAFL (n=30), NASH (n=30)
484 and NASH-F (n = 29) patients. Data are expressed as median \pm IQR. Rho coefficients and p-values
485 are obtained using the Spearman Rank test.

486

487 **Figure 2. Plasma BCAA concentrations are strongly associated with sex.**

488 Spearman correlations between plasma BCAA concentrations and NAFLD stage, sex, age, BMI and
489 HOMA-IR and HOMA2-IR in the study cohort (n=112). Colors and area of circles reflect the
490 Spearman rho values (red for positive, blue for inverse correlations). Only correlations with
491 significant p-value ($p < 0.05$) were represented.

492

493 **Figure 3. Plasma BCAA are affected by sex.**

494 BCAA concentrations in women (n=59) and men (n=53). Data are expressed as median \pm IQR. P-
495 values are obtained using the Mann-Whitney test.

496

497 **Figure 4. Plasma BCAA variations related to NAFLD stage are sex dependent.**

498 **A to C:** BCAA concentrations according to NAFLD stage for men in Healthy (n=8), NAFL (n=15), NASH
499 (n=15) and NASH-F (n=15) patients. **D to F:** BCAA concentrations according to NAFLD stage for
500 women in Healthy (n=15), NAFL (n=15), NASH (n=15) and NASH-F (n=14) patients. Data are
501 expressed as median \pm IQR. Rho coefficients and p-values are obtained using the Spearman Rank
502 test.

503

504 **Figure 5. Association between plasma BCAA concentrations and liver histological characteristics**
505 **are sex dependent.**

506 Spearman correlations between plasma BCAA concentrations and liver histological characteristics in
507 the men (n=53) and the women (n=59) patients. Colors and area of circles reflect the

508 Spearman rho values (red for positive, blue for inverse correlations). P-values are obtained by
509 Spearman rank test, *: p-value<0.05, **, p-value < 0.01, ***P < 0.001, ****<p-value<0.0001.

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511 **Figure 6. The association of plasma BCAA concentrations with fibrosis is sex dependent.**

512 **A to C:** BCAA concentrations according to fibrosis stage (low fibrosis, F0-1, n=36 and high fibrosis
513 F≥2, n=17) in men group. **D to F:** BCAA concentrations according to fibrosis stage (low fibrosis, F0-1,
514 n=36 and high fibrosis F≥2, n=17) in women group. Data are expressed as median ± IQR. P-values are
515 obtained using the Mann-Whitney test.

516

517 **Table 1. Clinical, biological and liver histological characteristics of patients according to NAFLD**
518 **stage.**

519 Data are expressed as mean ± standard deviation. P-values are obtained using the Chi-square
520 test for qualitative data and the ANOVA test for quantitative data. P-values <0.05 are considered
521 statistically significant (bold).

522

523 **Table 2. Clinical, biological and liver histological characteristics of the patients according to sex and**
524 **NAFLD stage.**

525 Data are expressed as mean ± standard deviation. P-values are obtained using the Chi-square test
526 for qualitative data and the ANOVA test for quantitative data. P-values <0.05 are considered
527 statistically significant (bold).

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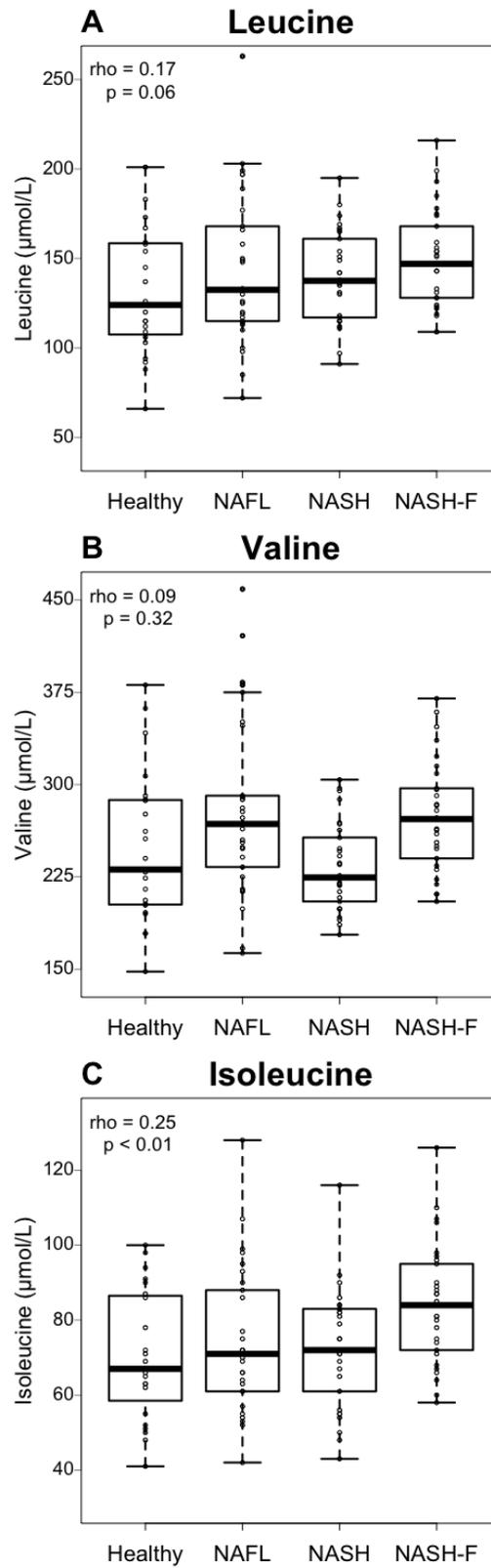
529 **Table 3. Clinical, biological and liver histological characteristics of the patients according to sex.**

530 Data are expressed as mean ± standard deviation. P-values are obtained using the Chi-square test
531 for qualitative data and the Mann-Whitney test for quantitative data. P-values <0.05 are considered
532 statistically significant (bold).

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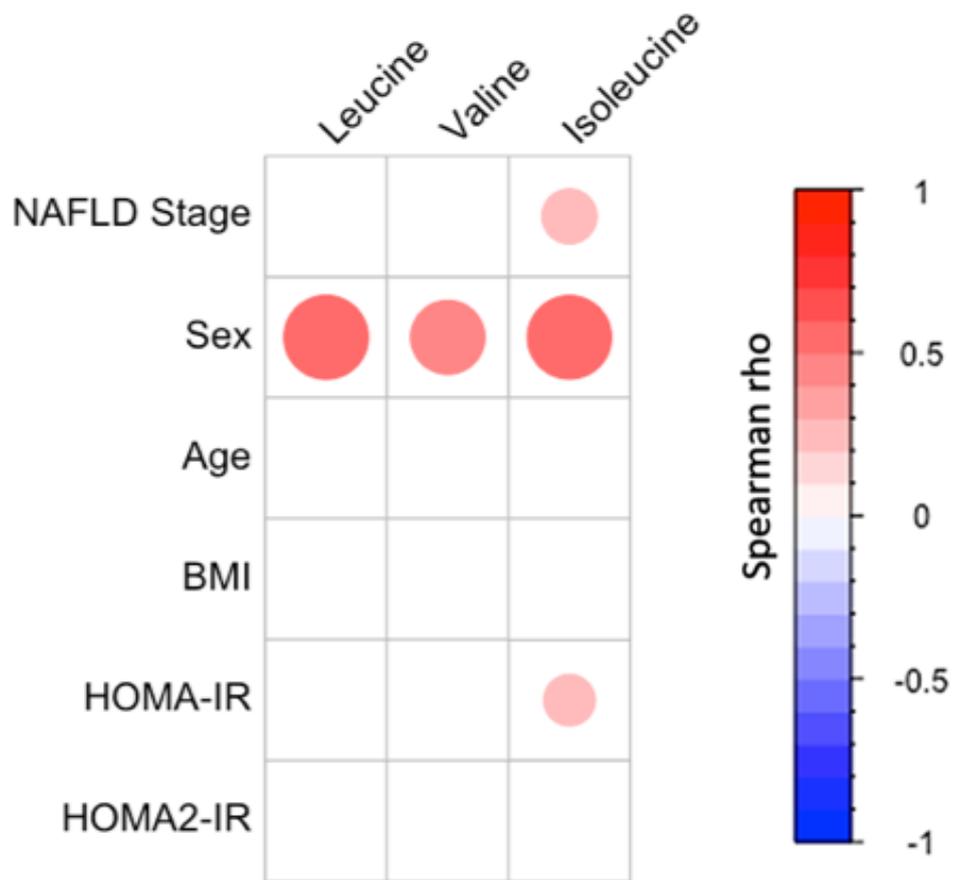
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Figure 1. Plasma leucine and isoleucine but not valine are positively correlated to NAFLD stage.



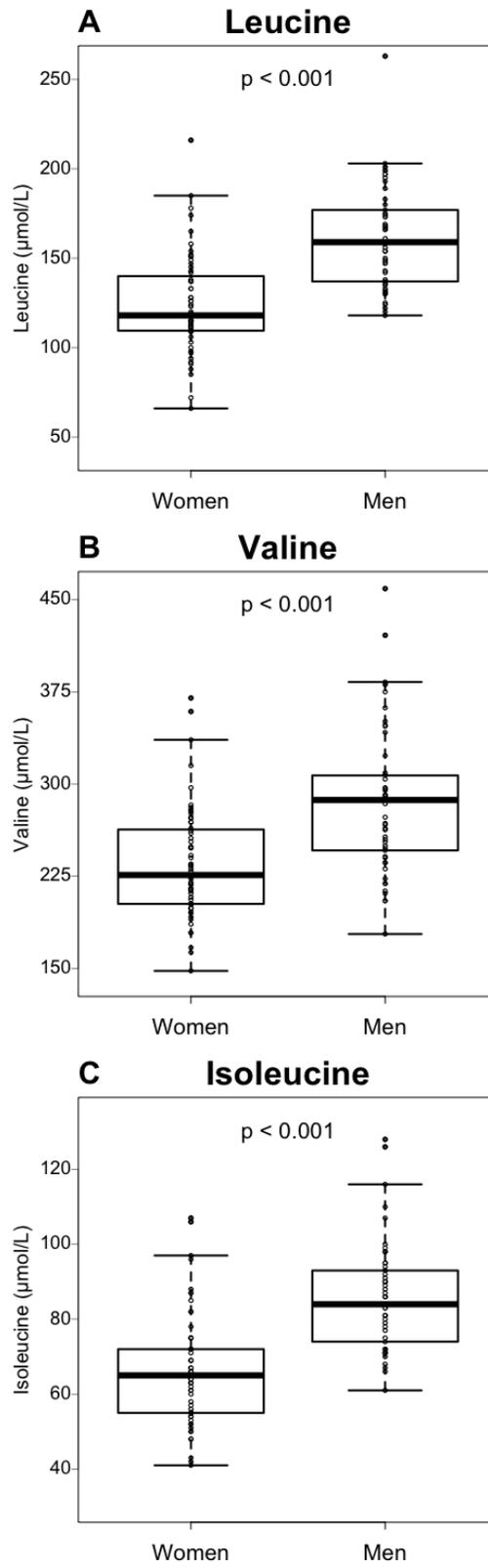
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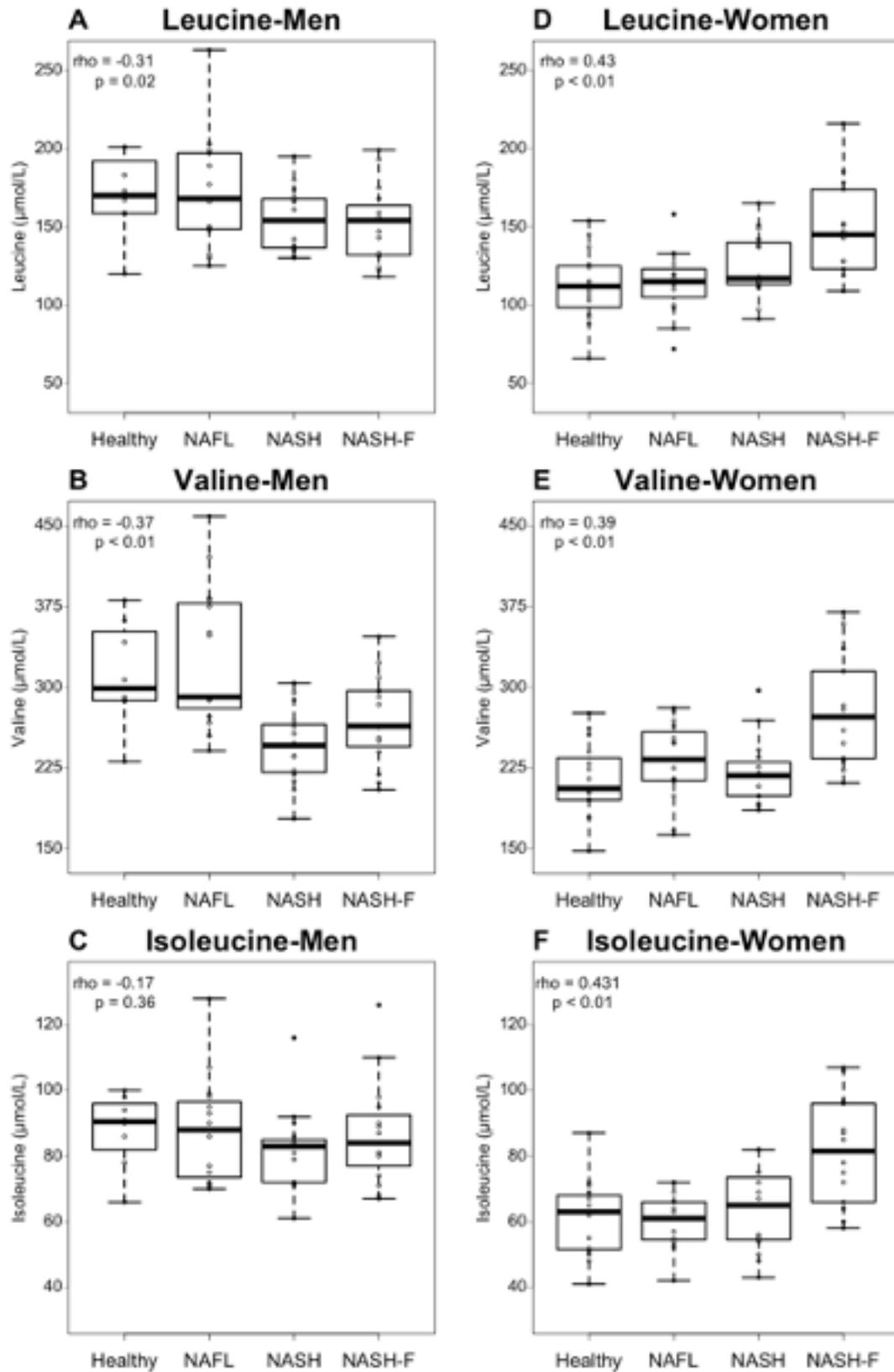
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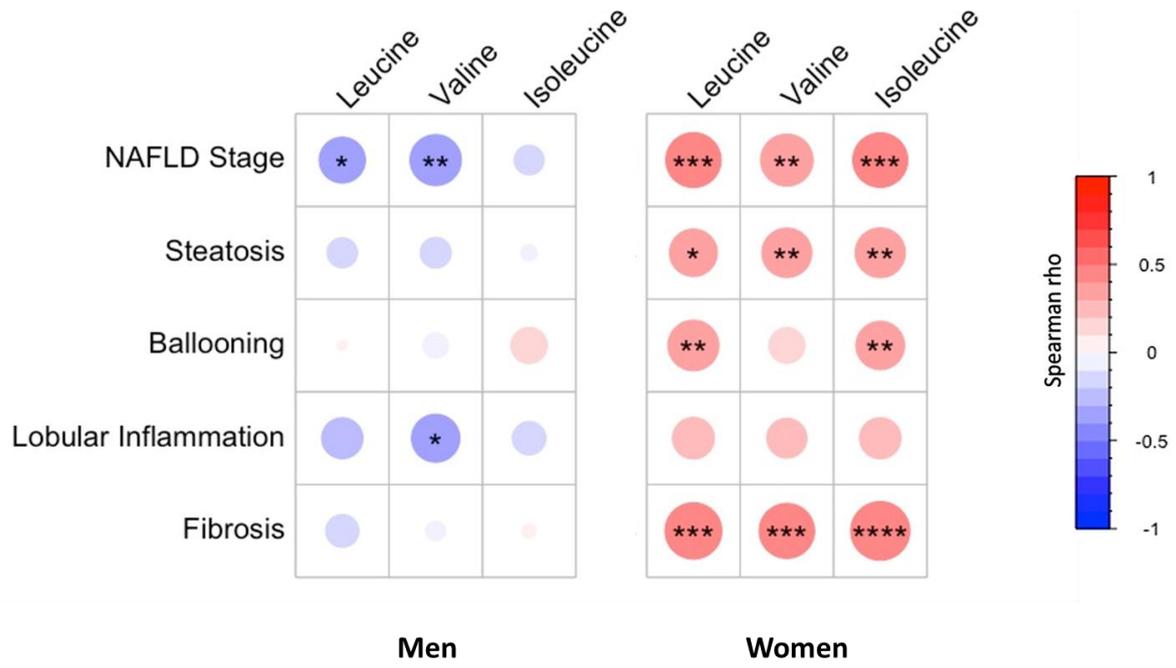
551 Figure 4. Plasma BCAA variations related to NAFLD stage are sex dependent.
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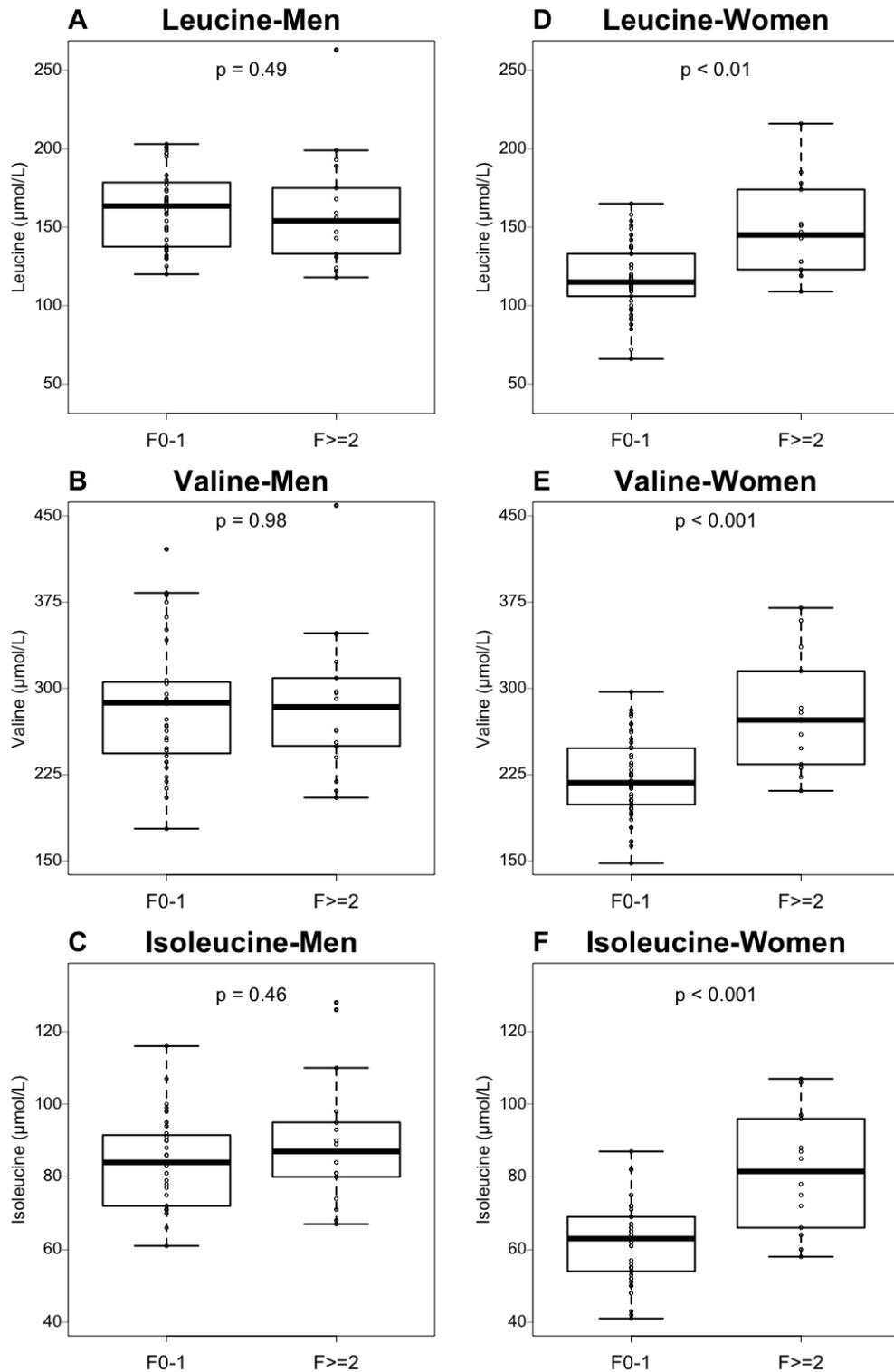
556 **Figure 5. Association between plasma BCAA concentrations and liver histological characteristics**
 557 **are sex dependent.**



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Figure 6. The association of plasma BCAA concentrations with fibrosis is sex dependent.



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Table 1. Clinical, biological and liver histological characteristics of patients according to NAFLD stage.

NAFLD Stage	All patients	Healthy	NAFL	NASH	NASH-F	p-value
n	112	23	30	30	29	
Sex (Women/Men)	59/53	15/8	15/15	15/15	14/15	0.61
Age (years)	42.6 ± 12.5	40 ± 12	43 ± 11	43 ± 13	43 ± 13	0.75
BMI (kg/m²)	40.5 ± 6.5	38 ± 4.9	42 ± 6.5	39 ± 4.5	41 ± 5.7	0.11
Fasting Plasma Glucose (mg/dL)	86 ± 10	85 ± 9.9	86 ± 13.6	87 ± 11.9	89 ± 8.8	0.23
HbA_{1c} (%)	5.6 ± 0.5	5.4 ± 0.3	5.6 ± 0.4	5.6 ± 0.4	5.5 ± 0.6	0.32
HOMA-IR	4.1 ± 2.8	4.2 ± 4.7	3.5 ± 1.8	4.0 ± 2.4	4.3 ± 1.5	0.32
HOMA2-B (%)	181 ± 69	177 ± 65	186 ± 80	185 ± 73	174 ± 58	0.82
HOMA2-S (%)	57 ± 36	64 ± 46	48 ± 24	58 ± 28	60 ± 37	0.95
HOMA2-IR	2.3 ± 1.4	1.9 ± 0.9	2.8 ± 2	2.3 ± 1.2	2.1 ± 0.8	0.64
Steatosis Grade (0,1,2,3)	23/39/25/25	23/0/0/0	0/22/8/0	0/12/6/12	0/5/11/13	<0.0001
Ballooning (0,1,2)	43/46/23	23/0/0	20/7/3	0/18/12	0/21/8	<0.0001
Lobular Inflammation (0,1,2,3)	41/44/21/6	23/0/0/0	18/10/2/0	0/20/8/2	0/14/11/4	<0.0001
Fibrosis Stage (0,1,2,3,4)	69/12/23/8/0	23/0/0/0/0	26/2/2/0/0	20/10/0/0/0	0/0/21/8/0	<0.0001

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Table 2. Clinical, biological and liver histological characteristics of the patients according to sex and NAFLD stage.

Men	Healthy	NAFL	NASH	NASH-F	p-value
n	8	15	15	15	
Age	38 ± 15	45 ± 11	41 ± 13	40 ± 11	0.48
BMI (kg/m ²)	39 ± 4.6	41 ± 8.5	38 ± 4.6	39 ± 4.1	0.38
Fasting Plasma Glucose (mg/dL)	89 ± 12	88 ± 14	85 ± 6	89 ± 9	0.99
HbA_{1c} (%)	5.5 ± 0.3	5.6 ± 0.5	5.6 ± 0.3	4.9 ± 0.3	0.99
HOMA-IR	5.8 ± 7.3	3.4 ± 1.2	3.6 ± 2.2	4 ± 1.3	0.95
HOMA2-B (%)	169 ± 33	162 ± 59	216 ± 79	171 ± 59	0.51
HOMA2-S (%)	56 ± 21	55 ± 26	51 ± 35	56 ± 23	0.94
HOMA2-IR	1.9 ± 0.6	2.3 ± 4.6	2.6 ± 1.2	2.1 ± 0.8	0.91
Steatosis Grade (0,1,2,3)	8/0/0/0	0/11/4/0	0/7/3/5	0/1/6/8	<0.0001
Ballooning (0,1,2)	8/0/0	7/6/2	0/10/5	0/11/4	<0.0001
Lobular Inflammation (0,1,2,3)	8/0/0/0	11/3/1/0	0/10/4/1	0/8/5/2	<0.0001
Fibrosis Stage (0,1,2,3,4)	8/0/0/0/0	12/1/2/0/0	10/5/0/0/0	0/0/11/4/0	<0.0001
Women	Healthy	NAFL	NASH	NASH-F	p-value
n	15	15	15	14	
Age	41 ± 11	42 ± 13	46 ± 14	45 ± 15	0.28
BMI (kg/m ²)	38 ± 5.2	42 ± 4.8	39 ± 4.4	42 ± 6.8	0.13
Fasting Plasma Glucose (mg/dL)	83 ± 8	86 ± 13	89 ± 16	90 ± 9	0.16
HbA_{1c} (%)	5.4 ± 0.4	5.6 ± 0.4	5.6 ± 0.6	5.6 ± 0.8	0.25
HOMA-IR	3.2 ± 1.6	3.6 ± 2.1	4.4 ± 2.7	4.7 ± 1.8	0.06
HOMA-2B (%)	181 ± 78	211 ± 93	154 ± 52	177 ± 59	0.42
HOMA-2S (%)	68 ± 56	41 ± 21	65 ± 41	64 ± 48	0.85
HOMA2-IR	2.0 ± 1.1	3.3 ± 2.4	2 ± 1.1	2.1 ± 0.8	0.54
Menopause (no/yes)	13/2	12/3	9/6	9/5	0.31
Steatosis Grade (0,1,2,3)	15/0/0/0	0/11/4/0	0/5/3/7	0/4/5/5	<0.0001
Ballooning (0,1,2)	15/0/0	13/1/1	0/8/7	0/10/4	<0.0001
Lobular Inflammation (0,1,2,3)	15/0/0/0	7/7/1/0	0/10/4/1	0/6/6/2	<0.0001
Fibrosis Stage (0,1,2,3,4)	15/0/0/0/0	14/1/0/0/0	10/5/0/0/0	0/0/10/4/0	<0.0001

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593 **Table 3. Clinical, biological and liver histological characteristics of the patients according to sex**
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	Men	Women	p-value
n	53	59	
Age	41 ± 12	43 ± 12	0.89
BMI (kg/m²)	39 ± 5.5	40 ± 5.5	0.24
Fasting Plasma Glucose (mg/dL)	87 ± 9	86 ± 10	0.29
HbA_{1c} (%)	5.5 ± 0.4	5.5 ± 0.5	0.58
HOMA-IR	4.2 ± 3.5	4.0 ± 2.1	0.77
HOMA2-B (%)	181 ± 65	181 ± 73	0.87
HOMA2-S (%)	54 ± 26	60 ± 44	0.93
HOMA2-IR	2.2 ± 1.2	2.3 ± 1.5	0.93
Steatosis Grade (0,1,2,3)	8/19/13/13	15/20/12/12	0.58
Ballooning (0,1,2)	15/27/11	28/19/12	0.08
Lobular Inflammation (0,1,2,3)	19/21/10/3	22/23/11/3	0.99
Fibrosis Stage (0,1,2,3,4)	30/6/13/4/0	39/6/10/4/0	0.74

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