1   **Title: Liver FoxO1 overexpression is positively associated with the degree of liver injury**

2   **in cirrhotic patients**

3   **Short title: FoxO1, intracellular marker of liver injury**

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21 **ABSTRACT:**

22 **Introduction:** Chronic liver disease is associated with high mortality. Liver transplantation is

23 the definitive treatment for patients with end-stage liver disease, improving their survival and

24 quality of life. However, chronic rejection of the graft and the imbalance between the demand

25 and the availability of organs limit its applicability. Therefore, finding therapeutic and/or

26 diagnostic alternatives for these patients is a priority. In this context, preclinical studies in

27 rodents have demonstrated that Akt plays a key role in liver dysfunction. Even with all this

28 evidence, the activation status of Akt and its downstream targets in the liver of patients with

29 chronic hepatopathy is still unknown. Hence, the present study aims to determine the

30 activation status of the molecules involved in the Akt signaling pathway in livers of cirrhotic

31 patients. **Materials and Methods:** In this study, 36 liver tissue samples from a cohort of 27

32 cirrhotic patients and 9 patients without cirrhosis were included. A total of 10 proteins

33 involved in Akt/mTOR pathway (GSK3β, IGF1R, IRS1, mTOR, p70S6K, IR, PTEN,

34 GSK3α, TSC2, and RPS6) were analyzed using a multiplex immunoassay based on

35 Luminex® technology. **Results:** Significant differences were found in several Akt/mTOR

36 target proteins between the groups of cirrhotic patients vs. non-cirrhotic: FoxO1 (9.5 vs. 4.4;

37 $p<0.01$), p-Akt (2.1 vs. 1.0; $p<0.01$), PTEN (3.061 vs. 1.877; $p<0.05$) and p70S6K (196.3 vs.

38 270.5; $p<0.001$). FoxO1 showed the best correlation with biochemical markers of liver injury

39 aspartate aminotransferase and serum alanine aminotransferase (ASAT: r=0.51, $p<0.05$;

40 ALAT: r=0.49, $p<0.05$). Moreover, the individual influence of FoxO1 on these parameters

41 was confirmed by multiple regression analysis. It was the only enzyme in the Akt signaling

42 pathway identified as a positive independent predictor of increased ASAT and ALAT levels.

43 **Conclusion:** FoxO1 is overexpressed in the liver of cirrhotic patients after partial

44 hepatectomy. FoxO1 levels are also associated with the degree of liver injury, showing a

45 positive correlation with current biomarkers used in clinical practice to detect liver injury.

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47 **Keywords:** Akt, FoxO1, cirrhosis, liver regeneration.

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Abbreviations:

Akt: Protein kinase B

HCC: Hepatocellular carcinoma

eNOS: Endothelial nitric oxide synthase

FoxO1: Forkhead box protein O1

GSK3α: Glycogen synthase kinase 3 alpha

GSK3β: Glycogen synthase kinase 3 beta

GRK2: G protein-coupled receptor kinase 2

IR: Insulin receptor

IRS1: Insulin receptor substrate 1

MFI: Median Fluorescence Intensity

mTOR: Mammalian target of rapamycin.

PDK1: Phosphoinositide-dependent protein kinase 1

PI3-K: Phosphoinositide 3-kinase

PTEN: Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase

RPS6: Ribosomal protein S6

TNFα: Tumor necrosis factor alpha

TSC2: Tuberous sclerosis complex 2

p70S6K: Ribosomal protein S6 kinase beta-1

DRU: Densitometric relative units

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1. INTRODUCTION

In Spain, most of the centers with more than 500 beds offer specific consultations for chronic liver disease complications such as hepatocellular carcinoma (HCC) (57%), cirrhosis (46%), and liver transplantation (46%). Moreover, this condition is the diagnostic category that has experienced the second-highest growth in the last five years, based on existing data (2007-2011)(1). Medical care related to liver disease is complex and burdens outpatient consultations, hospitalizations and high mortality rates. One of the most remarkable facts is that this illness affects the economically active population, and therefore impacts on the productivity of society. Besides, between 7,000 and 10,000 people die each year in Spain as a result of cirrhotic disease or its associated complications. Liver transplantation is the definitive treatment for patients with end-stage liver disease, improving their survival and quality of life. However, chronic rejection of the graft, impaired liver regeneration in these patients, and the imbalance between the demand and the availability of organs compromise the effectiveness of this therapeutic strategy.

As a result of these challenges, there is an urgent need to find new diagnostic and therapeutic alternatives for these patients, to block disease progression and prevent organ failure and associated comorbidities with the subsequent socioeconomic costs.

In this context, a significant number of studies have demonstrated that protein kinase b (Akt) is a crucial element for the control of liver function. For instance, liver cirrhosis has been associated with impaired Akt activity in experimental rat models, while the restoration of its function, using gene therapy, improved liver hemodynamics (2). Several mechanisms contribute to Akt dysregulation in liver dysfunction. Among them, the existence of an interaction between GRK2, an inhibitor of G-protein-coupled receptor signaling, and Akt-mediated eNOS activation has been reported in experimental models of liver fibrosis (3).

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95   The beneficial effect of Akt activation on liver function was corroborated by another study

96   conducted in an experimental model of orthotopic liver transplantation in pigs. In this case,

97   the ischemia-reperfusion injury during transplantation is the leading cause of oxidative stress

98   and cellular death, and this fact is clinically associated with loss of graft functionality and

99   increased risk of graft rejection. Constitutive activation of the Akt pathway, through the

100   forced expression of the myr-Akt variant in transduced liver grafts, restored the liver function

101   and counteracted the apoptotic processes, increasing the viability of the graft (4). This result

102   supports previous findings about the importance of Akt signaling in the maintenance of

103   fundamental physiological processes of the liver.

104   Recent studies also suggested that Akt could have an essential function in the liver

105   regeneration process. Several publications agree that during liver regeneration, Akt is

106   phosphorylated (active) and prevents the apoptosis of hepatocytes by induction of cell

107   survival signaling (5)(6). The deficiency of Akt or its activating molecules, PDK1 and PI3-K,

108   results in decreased liver regeneration and increased mortality after partial hepatectomy (7).

109   This is because Akt and some of its targets or activators are positive regulators of cell

110   survival, as in the case of IR, IRS, PI3-K, and PTEN.

111   Additionally, Akt is a crucial mediator of cellular growth through direct regulation of mTOR.

112   Ultimately, a study carried out using an experimental model of partial hepatectomy in

113   FoxO1-deficient mice, demonstrated that specific inhibition of FoxO1, mediated by Akt, in

114   hepatocytes is essential for liver regeneration (8).

115   Notwithstanding all this evidence supporting the fact that Akt activity has an impact on the

116   physiology and pathophysiology of the liver; most of the studies have been conducted in

117   experimental models in rodents. Thus, the activation status of Akt and its downstream targets

118   in the liver of patients with chronic hepatopathy is still unknown.

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119   This fact limits the transfer of acquired knowledge in pre-clinical studies to the development

120   of new therapies or biomarkers. For this reason, the evaluation of the activation status of Akt

121   and its molecular targets in patients with chronic liver disease is clinically relevant.

122   **OBJECTIVES**

123   To assess the activation status of proteins involved in Akt/mTOR pathway in the liver of

124   patients with cirrhosis. Additionally, we want to evaluate the association between Akt

125   proteins and liver injury and loss of functionality, as well as to determine their diagnostic and

126   prognostic value.

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2. MATERIALS AND METHODS

2.1. Study design and subjects.

In this retrospective study, we included specimens from patients with or without biopsy-confirmed cirrhosis who underwent therapeutic partial hepatectomy. A total of 36 liver tissue samples were analyzed, of which 27 were cirrhotic tissue samples and 9 samples of healthy tissue (non-cirrhotic group). Cirrhotic liver samples were obtained by liver resection from patients with cirrhosis associated with hepatitis C virus infection (HCV). The samples used as "non-cirrhotic group" were fragments of healthy liver tissue obtained from resections of colorectal metastasis before vascular clamping. Samples with the histological presence of tumor tissue were excluded for further analysis. The demographic characteristics of the patients included in the study are shown in **Table 1**.

The present study was approved by the Hospital Clinic Ethics Committee, and informed consent was obtained from all patients. Likewise, this study was conducted in accordance with the ethical principles of the 1975 Declaration of Helsinki for medical research involving human subjects.

2.2. Analytical parameters of liver injury and function

In the group of cirrhotic patients, several biomarkers of liver injury and functionality were evaluated, to study their possible correlation with target proteins involved in the Akt pathway. For this purpose, biochemical markers were determined following the established clinical protocol: aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma-glutamyl transferase (GGT); albumin and bilirubin. The tumor marker, alpha-fetoprotein (AFP), was also determined, among other parameters included in the hepatic panel: glucose, cholesterol, and triglycerides. All these parameters were determined in serum

153   prior to the liver resection surgery. All the assays were carried out at the CORE laboratory of

154   the Hospital Clínic of Barcelona, using the autoanalyzers ADVIA 2400 Chemistry System

155   and ADVIA Centaur XP (Siemens Healthineers, Tarrytown, NY).

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157   **2.3.Luminex® Immunoassay: analysis of proteins involved in the Akt/mTOR**

158   **pathway**

159   We determined the levels of 10 phosphoproteins involved in the Akt / mTOR signaling

160   pathway in a total of 36 liver tissue samples. The following analytes: GSK3β, IGF1R, IRS1,

161   mTOR, p70S6K, IR, PTEN, GSK3α, TSC2, and RPS6 were quantified using a multiplex

162   immunoassay based on Luminex®xMAP®technology, the Akt/mTOR phosphoprotein

163   magnetic bead panel 11-plex 96-well plate assay (Merck Millipore, Darmstadt, Germany).

164   This assay uses paramagnetic beads (6.5 μm diameter) to fix analytes and reaction substrates,

165   allowing the quantification of multiple analytes in a single test. Assays were performed

166   according to manufacturer's instructions, standards and samples were run in duplicate, the

167   incubation step was performed overnight with shaking at 4ºC (18 h, 750 rpm), and a hand-

168   held magnetic block was used for the washing steps. Data was acquired on a Luminex

169   MagPix 200 system (Luminex, Molecular Diagnostics, Toronto, Canada) and analyzed with

170   the XPonent software (Luminex, Molecular Diagnostics, Toronto, Canada).

171   For the liver tissue analysis, it was necessary to perform a sample pre-treatment, which

172   included the homogenization of 100 mg of tissue in a Polytron Homogenizer (Thomas

173   Scientific). Subsequently, the total protein concentration was quantified and normalized in all

174   samples using the Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA,

175   USA). Results were expressed as median fluorescence intensity (MFI).

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177   **2.4.Western Blot: expression levels of Akt and FoxO1 proteins**

178   The tissue abundance of Akt and FoxO1 proteins, as well as their degree of phosphorylation,

179   was determined by Western Blot. Tissue lysates were prepared in a lysis buffer (Tris-HCl 20

180   mM [pH 7,4]) containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50mM

181   NaCl, 2.5 mM ethylenediaminetetraacetic acid, 1 mM $Na_4P_2O_7 \cdot 10H_2O$, 20 mM NaF, 1 mM

182   Na_3VO_4, 2 mM Pefabloc and protease inhibitor (Mini Complete, Roche, Switzerland).

183   Proteins were separated on a polyacrylamide gel with 10% of sodium dodecyl sulfate(SDS)

184   (Mini Protean III; Bio-Rad, Richmond, CA), and they were transferred for 2 hours at 48ºC to

185   nitrocellulose membranes of 0.45 μm. After blocking, the membranes were incubated at 48°C

186   overnight with the following antibodies: rabbit anti-FoxO1 (1:1000; Cell Signaling), rabbit

187   anti-Akt (1:5000; Cell Signaling), rabbit anti-phospho-Akt (Ser473) (1:5000; Cell Signaling)

188   and anti-tubulin antibody (1:5000 Cell Signaling). Next, the membranes were incubated with

189   peroxidase-conjugated secondary antibodies at 1: 5000 dilution (GE Healthcare) for 1 hour at

190   room temperature. The respective bands were visualized using Luminata Forte Western HRP

191   Substrate (Millipore) e Image-Quant LAS 4000 (GE Healthcare). Densitometry analysis of

192   the gels was performed using the Image J software (version 1.37). Results were expressed in

193   densitometric relative units (DRU), as a result of the densitometric ratios of p-Akt/Akt and

194   FoxO1/tubulin.

195   **2.5.Statistical analysis**

196   Continuous variables were expressed as medians ± interquartile range (IQR). Differences in

197   quantitative variables among groups were evaluated using the Mann-Whitney U test. The

198   degree of correlation between variables was studied using the Pearson or the Spearman

199   coefficient, depending on the variable distribution. For linear correlation and linear regression

200   analysis, the FoxO1 variable was logarithmically transformed (LnFoxO1) to correct the

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201   skewness of the distribution. The parameters that showed a significant or near significant

202   correlation with analytical parameters of liver injury and function, were selected for the

203   multiple regression analysis. The multivariate statistical analysis performed was linear

204   regression. The validity of the resulting models was evaluated plotting residuals or Pearson

205   residual against adjusted values and plotting the influence of each observation on the adjusted

206   response according to Cook's distance. All statistical analyses were performed using public

207   libraries from "The Comprehensive R Archive Network" (CRAN; http://CRAN.R-

208   project.org) rooted in the open-source statistical computing environment R, version 3.1

209   (http://www.R -project.org/). The software "GraphPad Prism version 8.0.2" (GraphPad

210   Software, La Jolla California USA) was used for graphic representations. p-value ≤ 0.05 was

211   considered significant.

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213   ## 3. RESULTS

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215   ### 3.1.Analytical parameters of liver injury and function

216   All cirrhotic patients showed aminotransferases (ALAT and ASAT) and GGT levels

217   compatible with liver cirrhosis, as shown in **Table 2**. These increases in biochemical markers

218   of liver injury were only observed in the serum samples from cirrhotic patients and not in the

219   samples from the non-cirrhotic patients (data not shown).

220   ### 3.2.Comparative study: Intrahepatic expression of Akt pathway proteins in cirrhotic

221   ### patients vs. non-cirrhotic.

222   Determination of protein expression levels (FoxO1 and Akt) in liver tissues, were carried out

223   by Western blot analysis. The results obtained showed increased levels of p-Akt (active form)

224   in the group of cirrhotic patients compared to the non-cirrhotic group. These differences were

225   statistically significant ($p<0.01$), see **Figure 1A**. By contrast, no statistically significant

226   differences were found in the Akt levels between both studied groups. Levels of FoxO1,

227   which is negatively regulated by Akt, were represented in **Figure 1B**. The median of FoxO1

228   levels was significantly higher in the cirrhotic group compared to the non-cirrhotic group (p

229   <0.01).

230   The other target proteins from the Akt pathway were quantified using a multiple

231   immunoassay panel based on the technological platform Luminex®. Among all the proteins

232   analyzed --GSK3β, IGF1R, IRS1, mTOR, p70S6K, IR, PTEN, GSK3α, TSC2 y RPS6--

233   statistically significant differences were observed in 2 proteins: p70S6K and PTEN. The

234   PTEN median levels were increased in the cirrhotic group compared with the non-cirrhotic

235   ($p<0.05$). On the contrary, p70S6K levels were decreased in cirrhotic patients compared to

236   the group of patients without cirrhosis ($p <0.01$), as represented in **Figure 2**. For the rest of

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237   the variables studied, no significant differences were found when comparing the median

238   between both groups (**Table 3**).

239   **3.3.Correlation between Akt pathway proteins and analytical parameters of liver**

240   **injury and function**

241   The association between all the studied proteins from the Akt pathway and the analytical

242   parameters of functionality and liver injury has been studied. **Figure 3** shows the correlation

243   matrix resulting from this analysis. The degrees of linear correlation, as well as its statistical

244   significance, were calculated according to the Pearson correlation coefficient. Variables that

245   did not fit a normal distribution were excluded from this matrix, and their relationship was

246   evaluated individually using the Spearman correlation coefficient. The results obtained

247   revealed that FoxO1 presented the best correlation with liver injury tests. The FoxO1 protein

248   expression levels and serum concentration of both transaminases showed a positive

249   (moderate-high) and statistically significant correlation: ASAT (r = 0.51, p=0.036) and

250   ALAT (r = 0.49, p =0.049). The linear correlation and the corresponding fitted regression line

251   between FoxO1 and the transaminases are detailed in **Figure 3B**.

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3.4. Regression analysis to explain the influence of FoxO1 on biochemical markers of liver injury

FoxO1 and GSK3α proteins showed a statistically significant correlation with the concentration of both transaminases in serum: FoxO1 with ASAT (r=0.51; $p = 0.036$) and ALAT (r=0.49; $p=0.049$) and GSKA with ASAT (r=0.42; $p=0.039$). For this reason, both Akt targets were included in the multivariate analysis, which showed that both parameters were positive independent predictors of ASAT, explaining 37% of the variance (R^2-adj=0.37; F=5.75; $p=0.015$). In the case of multivariate linear regression analysis for the dependent variable ALAT, only FoxO1 was identified as a positive independent predictor explaining 18% of the variance (R^2-adj=0.18; F=4.61; $p=0.049$) (**Table 1 Supplementary**).

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267   **4. DISCUSSION**

268   The PI3K/AKT signaling pathway is crucial for energy homeostasis, cell growth, and

269   survival. To date, studies in rodents have extensively shown that Akt plays a key role in liver

270   regeneration as well as in normal liver function (9). The actions of the Akt pathway on the

271   cell cycle are closely linked to FoxO1 inhibition, a member of the Foxo (forkheadbox)

272   transcription factors. These factors are necessary for multiple functions in the liver: they

273   regulate the stress response, adaptation to fasting, and cell proliferation (10). It is well known

274   that Akt inactivates FoxO1, by phosphorylation, which leads to its nuclear exclusion and thus

275   inhibits its pro-apoptotic signals (11). For this reason, the Akt-FoxO1 interaction has been

276   identified as the most important mechanism in liver regeneration. In summary, the

277   maintenance of Akt activity and consequent inactivation of FoxO1 is associated with better

278   functionality and regenerative capacity of the liver. Therefore Akt and FoxO1 have been

279   proposed as promising therapeutic targets for cirrhosis and other chronic liver diseases.

280   However, there is currently insufficient evidence on the degree of activation of these

281   molecules in cirrhotic patients, which hinders their possible translation to the clinic.

282   Our study is the first to comprehensively assess the activation status of Akt signaling pathway

283   molecules in livers of cirrhotic patients. Previously, we demonstrated in experimental models

284   of cirrhotic rats that the enzymatic activity of Akt is decreased in the dysfunctional liver (2).

285   However, in the present study carried out in cirrhotic patients after partial hepatectomy, we

286   have shown that Akt and its target FoxO1 are overexpressed in liver cirrhosis. These

287   conflicting results should be interpreted cautiously since these discrepancies could be

288   attributed to other factors such as comorbidities present in cirrhotic patients or the etiology of

289   the disease. The latter is not present in the animal model, in which cirrhosis is induced by

290   carbon tetrachloride inhalation. In our cohort of patients with cirrhosis, the baseline etiology

291   in all cases was HCV infection. The relationship between the Akt pathway and viral

292   replication processes in chronic HCV infection is still unknown. In vitro studies suggest that

293   the NS5A viral protein can activate the PI3K-Akt pathway, thus inhibiting cellular apoptosis

294   (12). Some authors have described this relationship as an evolutionary mechanism used by

295   most DNA mammalian viruses to promote cell survival and ensure their replication (13). The

296   results of this study highlight the importance of validating the pre-clinical results obtained for

297   the molecules involved in the Akt pathway in humans. The most relevant finding of the

298   present study is that FoxO1 is overexpressed in the liver of cirrhotic patients. This finding is

299   in line with previous research showing FoxO1 overexpression in liver tissue from patients

300   with another chronic liver disease of different etiology, non-alcoholic steatohepatitis (14).

301   FoxO1 overexpression is clinically relevant, considering that most of the patients diagnosed

302   with HCC present underlying cirrhosis (15). Currently, surgical resections of these tumors or

303   metastatic lesions in cirrhotic patients remain a significant clinical challenge due to the

304   associated morbidity and mortality (16). In cirrhotic patients, the regeneration capacity of

305   hepatocytes after surgery is reduced, limiting the applicability of therapeutic resection in

306   situations such as the treatment of HCC (17). This observation is consistent with the

307   hypothesis that the higher the expression of FoxO1, the lower the liver's regenerative

308   capacity, as previously demonstrated in a murine model of liver regeneration caused by

309   partial hepatectomy (8). Furthermore, it is coherent with the fact that in our study, FoxO1

310   levels were significantly correlated with the degree of liver damage. Our statistical model

311   revealed that FoxO1 is the only, of all molecules studied, that has proven to be an

312   independent predictor of ALAT and ASAT levels in serum. Therefore, considering both these

313   results and our previous preclinical studies, FoxO1 overexpression could be one of the

314   underlying mechanisms that limit liver regeneration in cirrhotic tissue. The demonstration of

315   this hypothesis would be of great importance since the intrahepatic levels of FoxO1 could be

316   an indicative factor of individual liver regenerative capacity and, therefore, a marker with

317 prognostic value for patients with cirrhosis and HCC. In this context, understanding the

318 mechanisms of induction and regulation of liver regeneration is needed to open the way for

319 the identification of new therapeutic targets. This would favor the development of therapies

320 based on the restoration of liver functionality, improving the prognosis of patients with

321 advanced liver disease.

322 Our results support the need to continue this line of research to achieve a better understanding

323 of the role of Akt-FoxO1 in cirrhosis. Carrying out prospective observational studies would

324 be an appropriate strategy to determine the status of this pathway in the cirrhotic population

325 with different etiologies. These future studies would provide evidence to support the view

326 that selective modulation of Akt or its targets is a viable strategy with beneficial effects for

327 the management of patients with liver disease.

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384 **Table 1.** Demographic characteristics of the patients included in this study.

Group	N	Age Median (IQR)	Sex % male
Non-cirrhotic	9	51.0 (36.0-66.0)	44.4%
Cirrhotic	27	66.5 (57.0-69.0)	84.6%
Total	**36**	**65.5 (51.5-68.5)**	**74.3%**

385 **Notes:**IQR:interquatilic range

386

387 **Table 2.** Analytical parameters determined in the serum of cirrhotic patients.

Parameters	N	Median (IQR)	Reference range
Markers of liver injury and function			
ASAT	25	**71.0 (52.0-114.0)**	5-40 IU/L
ALAT	25	**113.0 (72.0-165.0)**	5-40 IU/L
GGT	25	**71.0 (38.0-140.0)**	5-40 IU/L
Albumin	24	43.0 (39.5-45.0)	34-48 g/L
Bilirubin	22	0.8(0.6-1.0)	< 1.2 mg/ dL
Others			
Glucose	25	99.0 (87.0-119.0)	65-110 mg/dL
Cholesterol	24	164.5 (149.5-180.5)	≤ 200 mg/dL
Triglycerides	24	99.5 (86.5-135.0)	< 150 mg/dL
Alfa-fetoprotein	20	**12.5 (7.5-49.0)**	< 10 ng/mL

388 **Notes:** Variables are expressed as median and interquartile range (IQR). Values outside the range are in bold.
389 IU: International Units, ALAT: alanine aminotransferase, ASAT: aspartate aminotransferase, GGT: gamma-
390 glutamyl transferase. N: number of patients in which it was possible to determine the parameter.

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**Table 3.** Comparative study of 12 target proteins from the Akt pathway.

	Non-cirrhotic N=9			Cirrhotic N=27				Total N=36		
Parameter	N	Median	IQR	N	Median	IQR	P	N	Median	IQR
*FoxO1	6	4.4	[1.0-6.4]	18	9.5	[6.6-13.0]	**<0.01**	24	7.4	[4.3-11.5]
p-Akt	6	1.0	[0.6-1.1]	17	2.1	[1.3-3.5]	**<0.01**	23	1.5	[1.0-2.4]
+GSK3α	9	123.0	[25.5-144.8]	27	158.5	[102.8-232.3]	0.67	36	144.6	[83.6-210.9]
GSK3β	9	73.5	[66.8-155]	27	83.5	[64.0-112.5]	0.66	36	83.0	[65.4-119.3]
IGF1R	9	2.5	[0-6.5]	27	5.0	[0-10.5]	0.62	36	3.8	[0-10.0]
IR	9	8.5	[0-11.0]	27	9.0	[3.8-14.5]	0.51	36	8.8	[3.2-13.5]
IRS1	9	57.5	[32.5-73.5]	27	66.8	[44.5-96.5]	0.27	36	62.8	[41.1-91.5]
mTOR	9	43.5	[34.5-60.3]	27	52.0	[36.3-83.0]	0.53	36	49.5	[35.4-79.3]
p70S6K	9	270.5	[243.8-299.0]	27	196.3	[174.9-262.0]	**<0.01**	36	217.5	[178.5-269.5]
PTEN	9	1,876.5	[1,245.0-2,479.8]	27	3,060.8	[1,676.5-4,142.3]	**0.04**	36	2,840.9	[1,469.0-3,903.0]
RPS6	9	103.3	[64.0-127.5]	27	97.3	[44.5-268.5]	0.44	36	100.3	[49.0-206.8]
TSC2	9	105.5	[49.5-193.5]	27	162.5	[105.3-260.0]	0.34	36	149.8	[81.6-223.3]

Notes: IQR, interquartile range. Differences between groups were evaluated with the Mann-Whitney test. P-value ≤0.05 was considered significant. * FoxO1 and p-Akt values correspond to densitometric relative units (DRU), calculated as the densitometric ratio between FoxO1/tubulin and p-Akt/Akt. + The rest of the proteins are expressed in median fluorescence intensity (MFI) units.

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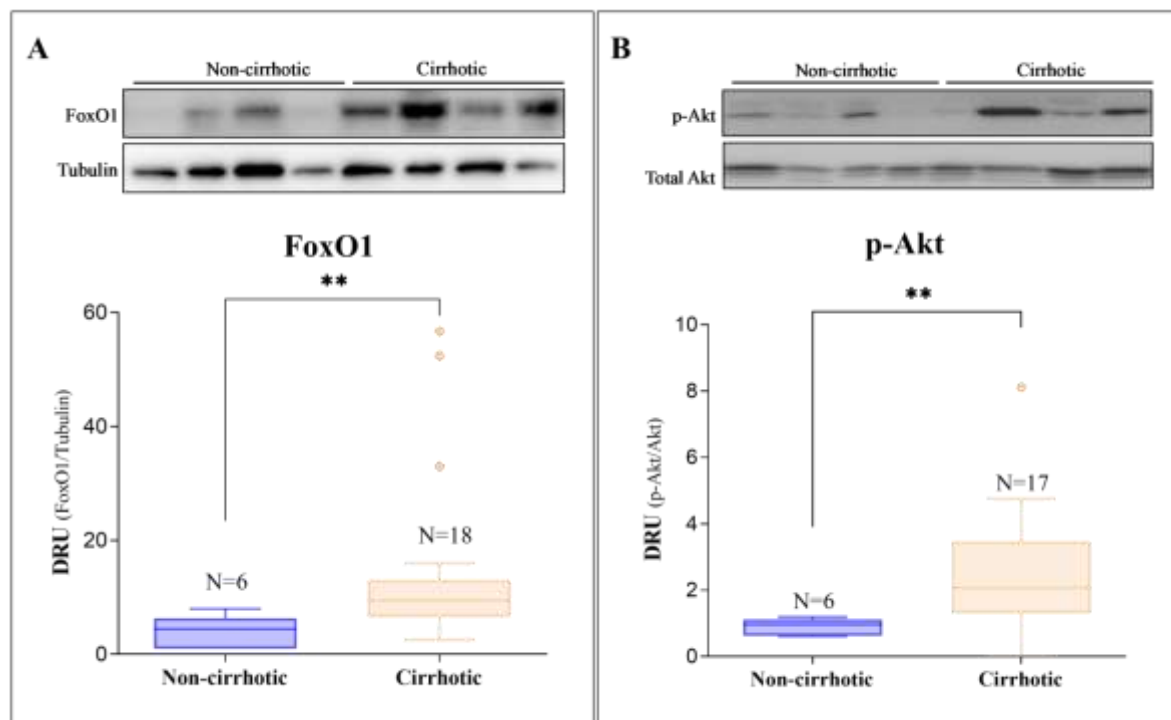
399 **Figure 1.** Expression levels of FoxO1 and p-Akt proteins in liver of cirrhotic patients vs.
400 non-cirrhotic.

401 Box plot comparing FoxO1 (A) and p-Akt (B) levels expressed as densitometric relative units (DRU). DRU
402 were calculated as the ratio between the specific bands FoxO1/tubulin and p-Akt/Akt quantified with the
403 software ImageJ A representative image of Western blot analyses is shown at the top of each figure.Statistically
404 significant differences (**$p < 0.01$) were observed when comparing the median of both proteins between groups:
405 **FoxO1**: median = 4.44 (non-cirrhotic) vs. 9.50 (cirrhotic), **p-Akt**: median = 0.97 (non-cirrhotic) .vs. 2.07
406 (cirrhotic).

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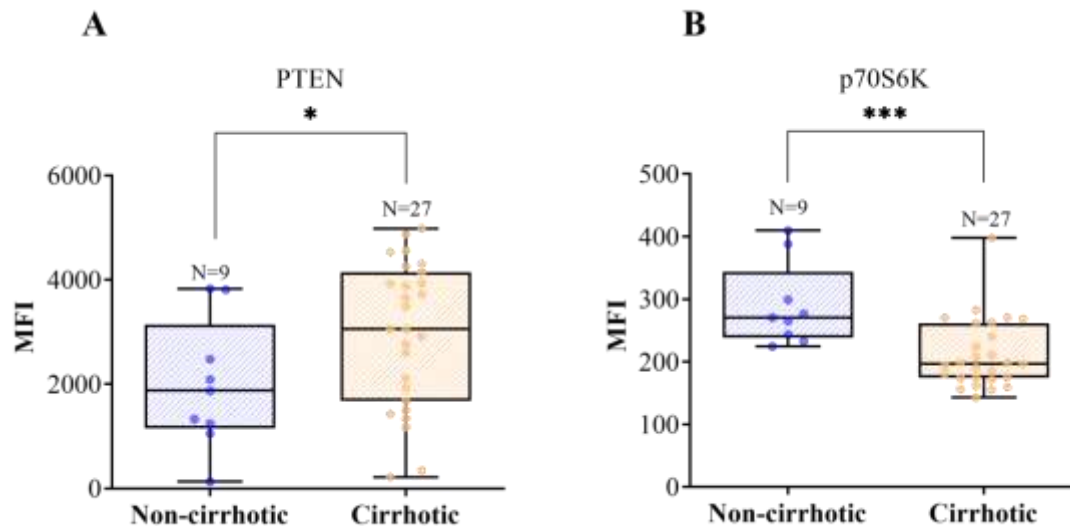
Figure 2. Box plot representative of p70S6K (A) and PTEN (B) levels in both groups.

The levels of each protein in liver tissue are expressed in MFI (median fluorescence intensity). Box-plot and also the individual values for each patient are represented.

A. PTEN levels are higher in cirrhotic patients (median=3061, N=27) compared with patients without cirrhosis (median=1877, N=9). These differences are statistically significant (* $p < 0.05$).

B. The group of cirrhotic patients presents lower levels of p70S6K (196.3; N=27) compared with the group of non-cirrhotic patients (270.5; N=9). These differences are statistically significant (*** $p < 0.001$).

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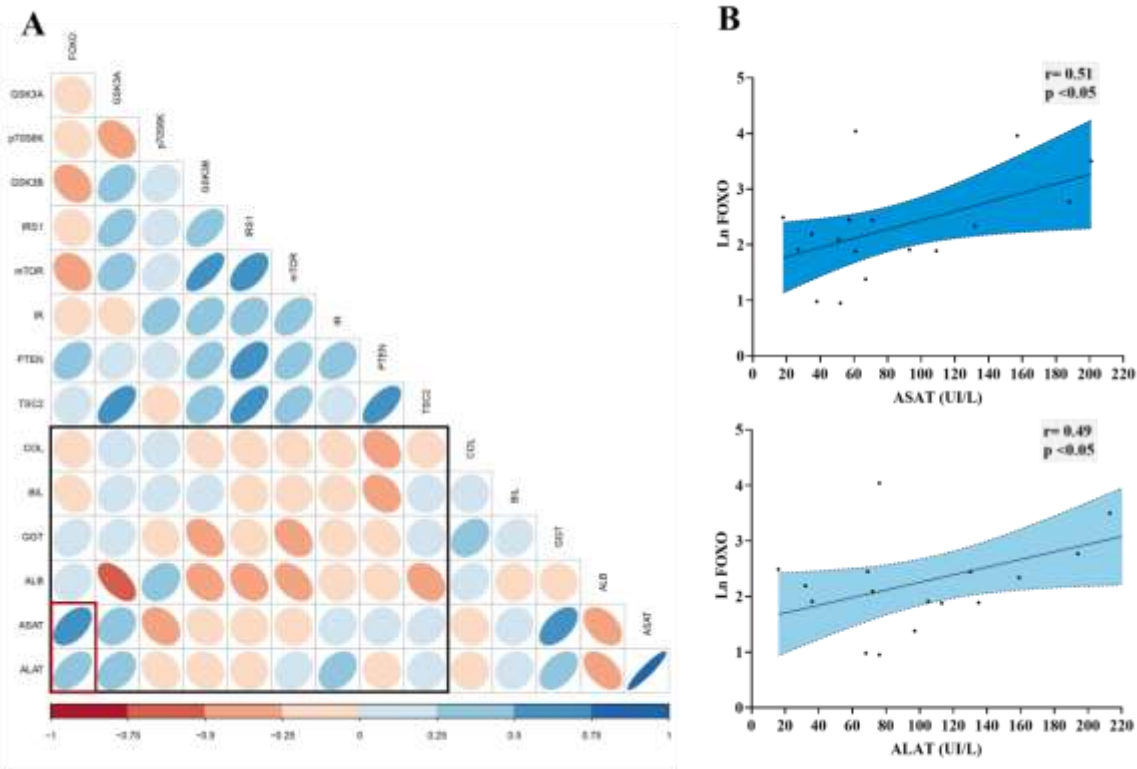
Figure 3. Correlation matrix between target proteins involved in the Akt pathway and analytical parameters of liver injury and function.

A: All possible correlations between the variables studied are shown**.** The black rectangle highlights the correlations of interest: The most relevant correlations between FoxO1 and transaminases are inside the red box. The color scale indicates whether it is a positive (blue) or negative (red) correlation. Color intensity and the size of the ellipses are proportional to the correlation coefficients (Pearson).

B: The scatter dot plot indicates that there is a positive correlation between levels of FoxO1 protein in liver tissue and the concentration of transaminases (ASAT and ALAT) in serum. These correlations are statistically significant, with Pearson's correlation coefficients: r=0.51 for ASAT and r=0.49 for ALAT. The corresponding regression line is also represented; the shaded area indicates the 95% confidence intervals.

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