

# Ficolin-2 Plasma Levels Assesses Liver Fibrosis in Non-Alcoholic Fatty Liver Disease

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## Research Article

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## **Ficolin-2 plasma levels assesses liver fibrosis in non-alcoholic fatty liver disease**

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List of Abbreviations:

NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; T2DM type 2 diabetes mellitus, PPI, protein-protein interactions; MO, morbidly obese; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT,  $\gamma$ -glutamyltransferase; TAG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; FIB-4, fibrosis-4 score; APRI, AST to platelet ratio index; FCN-2, Ficolin-2 protein; FCNscore, Ficolin-2 score; MyoPheNet, myofibroblast phenotype acquisition PPI network; SM, supplementary materials.

## **Abstract**

**Background & Aims:** In the next 20 years, non-alcoholic fatty liver disease (NAFLD) is expected to become the leading cause of liver transplantation. Fibrosis is the most important prognostic factor for liver-related outcomes and mortality, but the need for liver biopsy limits diagnosis. We assessed the performance of plasma ficolin-2 (FCN-2) as a biomarker of fibrosis. FCN-2 candidate was selected by an *in silico* discovery strategy.

**Methods:** We enrolled 235 morbidly obese (MO) subjects with biopsy-proven NAFLD stratified by fibrosis stage (F0, n=44; F1, n=134; F2, n=46; F3/F4, n=11) and 40 cirrhotic patients as positive controls. The cohort was subdivided into discovery (n=76) and validation groups (n=159). The plasma level of FCN-2 and other biomarkers was determined by enzyme-linked immunosorbent assay.

**Results:** Plasma level of FCN-2 correlated inversely with the stage of liver fibrosis ( $\rho = -0.49$ ,  $p < 0.0001$ ), independently of steatosis ( $p = 0.90$ ), inflammation ( $p = 0.57$ ), and ballooning ( $p = 0.59$ ). In the global cohort, FCN-2 level decreased significantly in a stepwise fashion from F0/F1 (median 4753 ng/mL) to F2-F3-F4 (2760 ng/mL) and in cirrhotic subjects (1418 ng/mL). The diagnostic performance of FCN-2 in detecting  $F \geq 2$  fibrosis was higher than that of other fibrosis indexes (APRI, FIB-4, NFS) (AUROC 0.82, 0.68, 0.67, and 0.68, respectively), showing a substantially improved accuracy when combined with APRI score and HDL plasma values in a diagnostic index (FCNscore, AUROC 0.85).

**Conclusion:** FCN-2 plasma level can accurately discriminate liver fibrosis status (minimal vs. moderate/advanced). Its inclusion in the diagnostic workup significantly improves the fibrosis diagnostic algorithms.

**Keywords:** biomarkers, *in silico*, non-alcoholic fatty liver disease, discovery strategy, omics, blood-based tests, ELISA, obesity, metabolic syndrome, protein-protein interactions

**Graphical Abstract**



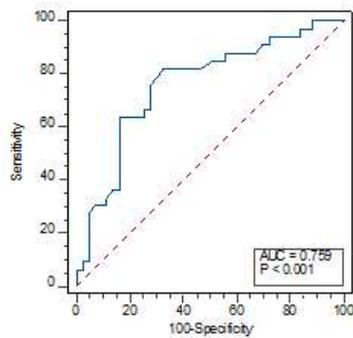
N = 235

Discovery MO Cohort  
N = 76 (42 F0-F1 / 34 F2-F3-F4)

Validation MO Cohort  
N = 159 (135 F0-F1/24 F2-F3-F4)

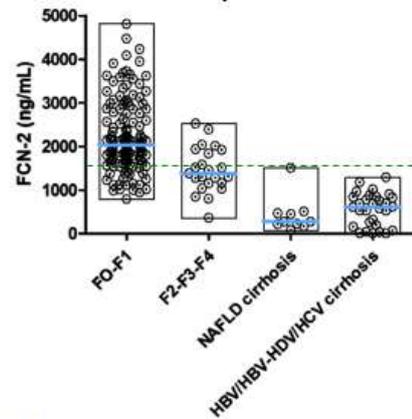
Validation Cirrhosis Cohort  
N = 40 (10 NAFLD cirrhosis + 30 viral cirrhosis)

**Enriched Fibrosis Prevalence = 44%**



Cut-off 1590 ng/mL

**Fibrosis prevalence = 15%**



**Well-classified:** TN + TP = 107 + 16 = 123 (77%)  
**Misclassified:** FP + FN = 28 + 8 = 36 (23%)  
**FCN-2 -> AUC = 0.74**

**Text word limit: 4000 max (including refs)**

## **Introduction**

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder in Western countries, expected to become the leading cause of liver transplantation by 2030 [1] [2]. Metabolic factors such as type 2 diabetes mellitus (T2DM) and obesity increase the risk of developing severe liver disease in NAFLD [3].

NAFLD is divided into the clinical-histological entities “simple steatosis” (NAFL) and “non-alcoholic steatohepatitis” (NASH), the progressive phenotype involving hepatocyte injury (ballooning), presence of inflammatory infiltrates, and fibrogenesis – with an elevated risk of cirrhosis and liver cancer [4]. Several reports indicate fibrosis as the strongest predictor of long-term clinical outcomes in NAFLD patients [5].

Liver biopsy, the gold standard for its diagnosis, was established almost a century ago [6]. Despite the invasiveness, costs, and sampling error limitations, there are still no reliable non-invasive diagnostic tests for fibrosis in NAFLD. Two pathways have been exploited in approaching alternatives to the gold standard. Blood-based non-invasive indirect tests have been combined in indexes such as the fibrosis-4 (FIB-4) score [7], the AST to platelet ratio index (APRI) or individual markers indirect tests; it is the case of type III collagen neo-epitopes (PRO-C3) [8].

Imaging technologies (magnetic resonance elastography, shear wave elastography, or acoustic radial force imaging) have been tested for the non-invasive diagnosis of NAFL/NASH and fibrosis. However, none of these modalities satisfies the desired clinical accuracy and practicability [9].

Interestingly, in the big-data era, new approaches contribute to the discovery of promising biomarkers. The fast development of omics technologies has enormously favoured biomedical sciences. Specifically, the improvements in data acquisition and analysis through high-throughput

technologies (such as microarrays and RNA-seq) have the power to evolve biomedical science from a static to a more dynamic form. [10]. Thus, the availability of harmonized datasets in many public repositories allows *in silico* strategies to develop biomarker discovery pipelines.

In this context, Page S. *et al.*, using enrichment analysis of phosphoproteomic datasets, proposed C-C motif chemokine (CCL2) and tumor necrosis factor ligand superfamily member 6 (sFasL) as biomarkers in NAFLD pathogenesis [11]. Hotta K. *et al.* [12] described a transcriptomic study identifying core gene networks associated with NAFLD progression. Ryaboshapkina M. *et al.* reported a transcriptomic meta-analysis identifying several genes involved in NAFLD progression and others as biomarkers for disease stratification [13].

In the present study, we assessed the performance of Ficolin-2 protein (FCN-2) as a putative novel biomarker of liver fibrosis and tested its utility combined in a blood-based score test. We also described the *in silico* strategy used to identify FCN-2 and other protein candidates potentially functional in fibrosis diagnosis.

## **Materials and Methods**

### **Study design and participants**

The assessment of our candidate biomarkers was performed retrospectively in a cohort of morbidly obese (MO) subjects enrolled in a bariatric surgery program. The liver biopsy was performed at the time of the surgical procedure. All subjects gave their written consent to participate in the study. Sensitive data were protected through anonymization. The local Ethical Committee has approved the study under protocol N. 22979 (Comitato Etico Regionale Unico, FVG, SSN, Italy). Enrolled subjects were  $\geq 18$  years, with a body mass index (BMI)  $> 40 \text{ kg/m}^2$  (or  $> 35 \text{ kg/m}^2$  if obesity-related comorbidities were already present), with acceptable operative risks, failure of nonsurgical treatments,

and declared compliance to follow lifelong medical surveillance. Subjects were excluded if they had coexistent chronic liver disease, including suspected/confirmed hepatocellular carcinoma, alcoholic liver disease (>25 g/day alcohol consumption), known HBV, HCV, HIV positivity, and therapy with drugs that could affect the liver.

Blood samples from subjects with cirrhosis attributable to either NAFLD (n=10) as well as chronic viral infection (HBV, HDV, and HCV) (n=30) were collected and considered positive controls of advanced fibrosis (F4).

### **Clinical assessment**

Anthropometric parameters including age, gender, body weight and height, BMI calculation, and waist circumference were recorded in the baseline visit. After overnight fasting, blood samples were collected before surgery to determine glucose, liver biochemistry (AST, ALT, GGT), albumin, platelets, lipid profile (TG, T-Chol, HDL), and others. Diabetes was diagnosed according to the ESC-EASD guidelines [14]. Blood-based tests of liver fibrosis such as FIB-4, APRI, FORNS, and NFS were calculated as described [15].

### **Liver biopsy, histopathology, diagnosis of fibrosis and cirrhosis**

Liver specimens collected during the surgical procedure (wedge biopsy) were histologically analyzed by an expert pathologist blinded to all clinical data. Steatosis was graded according to the amount of fat (as lipid droplets in hepatocytes) on hematoxylin and eosin staining. Biopsies showing no or minimal (<5%) steatosis and absent injury or fibrosis were considered normal. The samples showing more than 5% steatosis were labelled as NAFLD. The histological diagnosis of NASH and fibrosis was

made according to Kleiner-Brunt criteria [16]. In most cases, cirrhosis in positive controls was diagnosed by ultrasound and three *via* needle biopsy.

### ***In silico* strategy for protein candidates' discovery**

We used a systematic discovery strategy in which the human proteome (originated from ~20,000 protein-coding genes [17]) was visualized at the top of a giant funnel, and several *in silico* filters were cross-placed at different heights (Figure 1). Proteins moving down in the funnel reach a thicker grade of selectivity and specificity. In the approach, the *in silico* filters were represented by Venn diagrams used to apply selection criteria at the bio-datasets, consequently obtaining the most selective sub-bio-datasets. The full description of the strategy is presented in Supplementary Materials – SM1.

### **Plasma Ficolin-2 and other candidates' assessment**

Plasma levels of five of the total thirty-five candidates were measured by ELISA commercial kits: Fibrillin 1 (RayBio® Human FBN1 ELISA Kit, E-EL-H2266, Elabscience), Insulin-like growth factor-binding protein 5 (RayBio® Human IGFBP-5 ELISA Kit, ELH-IGFBP5, RayBiotech), Noelin-2 (RayBio® Human Olfactomedin 2, ELH-OLFM2, RayBiotech), Urokinase-type plasminogen activator (Human U-Plasminogen Activator Simple Step ELISA® Kit, ab226904, Abcam) and Ficolin-2 (RayBio® Human Ficolin-2 ELISA Kit, RayBiotech).

### **Statistical analysis**

The MO cohort (n=235) was divided into two subsequent cohorts, the discovery MO cohort including 76 MO subjects in which the prevalence of fibrosis was adjusted to 44% (enrichment of the moderate/advanced fibrosis proportion) and the validation MO cohort in which 159 MO subjects were included, maintaining the fibrosis prevalence (15%) close to those of the global MO population (24%). In both MO discovery and validation cohorts, the subjects were stratified according to fibrosis stage (F0-F1, minimal fibrosis; F2-F3-F4, moderate/advanced fibrosis) to assess the best candidate based on diagnostic performance analysis (accuracy and determination of optimal cut-off values). Continuous variables were expressed as mean  $\pm$  standard deviation and categorical as numbers or percentages. Categorical variables were analyzed using chi-square tests with correction, when appropriate. Independent *t*-test and ANOVA were used for normally distributed continuous variables. Non-parametric tests (Mann–Whitney, and Kruskal–Wallis) were applied for ordinal or continuous variables that failed to pass D’Agostino & Pearson omnibus normality test. Correlation analyses were performed using Pearson or Spearman’s correlation coefficients to estimate the association of plasma levels and several factors of interest. Statistical analysis was performed by using GraphPad Prism version 5.01 software. Logistic regression analysis was used to identify independent factors associated with fibrosis. The predictive model was built, including the four best-associated variables (independent factors) selected after using the hierarchical forward selection algorithm in the subset selection test modality performed in NCSS statistical software (version 12.0.16).

The diagnostic performance of the selected candidate FCN-2 was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUROC) using DeLong method was used to compare the accuracy among the different fibrosis diagnostic tests. The sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) for relevant cut-offs (according to Youden’s index) were also calculated using MedCalc statistical software version 16.4.3.

## **Results:**

### **Identification by the *in silico* funnel strategy of candidate biomarkers for liver fibrosis**

We identified some proteins as candidate biomarkers for liver fibrosis after applying an *in silico* discovery strategy. Briefly, we collected data of the human proteome from several open databases (see SM1 – Figure 1A, Table 1 and 2) and filtered them by applying several *in silico* sieves to select the markers with the desired characteristics. Selection criteria were the following: 1) candidates must participate in the acquisition of myofibroblast phenotype; 2) be expressed in liver tissue; 3) the expression must be modified during fibrosis; and 4) must be secreted and measurable in plasma (Figure 1A and 1B). From this analysis, 35 proteins were identified as putative candidates, excluding collagen proteins or other extracellular matrix components, since they might be stabilized in the surrounding area of liver cells without release into the bloodstream, obtaining 29 candidates (SM2 – Table S1). Five randomly selected proteins of the total candidates were tested in this study (Figure 1).

### **Demographics and biochemical data, and association with liver fibrosis**

Table 1 summarizes the clinical-demographic details of the patients included in the discovery, validation, and combined MO cohorts. Since the primary endpoint of this study was to predict fibrosis, we stratified the cohorts accordingly to the fibrosis stage (F0-F1 vs F2-F3-F4). We used the discovery cohort to qualify the candidates and the validation cohort for their verification, testing the reproducibility of the diagnostic performances of the best candidate.

In Table 2 the clinic-demographic characteristics of the discovery cohort are presented. Significant differences between both fibrotic groups were observed in gender (30% female higher in the F0-F1 group) and blood parameters. AST and GGT levels were higher in the moderate/advanced fibrosis group ( $32 \pm 18$  IU/L vs.  $23 \pm 12$  IU/L,  $p = 0.02$ , and  $49 \pm 41$  IU/L vs.  $32 \pm 27$  IU/L,  $p = 0.04$ ,

respectively) while blood platelets were reduced ( $220 \pm 57 \times 10^3/\mu\text{L}$  vs.  $266 \pm 60 \times 10^3/\mu\text{L}$ ,  $p = 0.002$ ). The differences were also reflected by the blood-based indexes, such as FIB-4 ( $1.27 \pm 1.1$  F2/F3-F4 vs.  $0.72 \pm 0.3$  F0-F1,  $p = 0.007$ ), FORNS ( $4.2 \pm 1.9$  F2/F3-F4 vs.  $3.0 \pm 1.4$  F0-F1,  $p = 0.003$ ) and NFS ( $-0.06 \pm 1.3$  F2/F3-F4 vs.  $-1.2 \pm 1.2$  F0-F1,  $p = 0.0003$ ).

The clinic-demographic characteristics of the validation cohorts are reported in Table S2 (SM2). MO and cirrhotic cohorts showed significant differences in several parameters, particularly GGT, platelets, total cholesterol, triglycerides, and among others in the blood-based indexes (APRI, FIB-4, NFS).

### **FCN-2 plasma levels correlate with the fibrosis stage**

The plasma level of the five candidates was assessed in all MO discovery samples ( $n=76$ ). No significant differences were found except for FCN-2 (SM2, Fig. S1). FCN-2 levels' significantly decrease when liver fibrosis progresses from minimal to moderate/advanced stage (Figure 2A). FCN-2 was able to distinguish F0/F1 from F2/F3/F4 with level decreasing from 4313 ng/mL (interquartile range: 3295 - 5849) to 2676 ng/mL (1983 - 3482), independently of gender and steatosis grade (Figure 2B and 2C).

Having shown an association between the plasma level of FCN-2 and the stage of fibrosis, we investigated the relationships with other biochemical and histological parameters. FCN-2 plasma level had a significant positive correlation with platelets ( $\rho = 0.37$ ,  $P = 0.001$ ) and negative with fibrosis stage ( $\rho = -0.49$ ,  $P < 0.001$ ), FIB-4 ( $\rho = -0.32$ ,  $P = 0.006$ ) and NFS ( $\rho = -0.30$ ,  $P = 0.01$ ) (Table S3 – SM2). The correlations indicate that changes in FCN-2 level reflect liver fibrosis but not steatosis, inflammation and ballooning, as observed when the cohort was stratified by NAFLD stages and the grade of the different histological characteristics. (SM2 – Figure S2 and S3).

## Diagnosis of liver fibrosis using FCN plasma level

In the discovery cohort (n = 76) an optimal FCN-2 cut-off level for the detection of moderate-advanced fibrosis was determined. FCN-2  $\leq$  3650 ng/mL had an AUROC of 0.79 for moderate-advanced fibrosis detection (sensitivity 84%, specificity 72%). This was replicated in the validation cohort (n = 159, AUROC = 0.74, sensitivity 71%, specificity 84%) and also in the overall combined cohort (n = 235, AUROC = 0.82, sensitivity 78%, specificity 81%) (Figure 3A-C and Table S4 – SM2). The FCN-2 plasma concentration in all analyzed samples stratified by liver injury is showed in Figure 3E.

Next, we compared the diagnostic performance of FCN-2 in detecting fibrosis with those from APRI, FIB-4, FORNS, and NFS indexes. FCN-2 marker has the best diagnostic accuracy for significant fibrosis diagnosis in the discovery, validation, and combined cohorts. Comparison of AUROCs using DeLong's method demonstrated that FCN-2 was superior to APRI (p < 0.013), FIB-4 (p < 0.002), FORNS (p < 0.005) and NFS (p < 0.005). AUROCs, the sensitivity, specificity, PPVs, NPVs and significance comparisons for optimal cut-off values in the three cohorts are summarized in Table S4.

Since several blood parameters or their combination in diagnostic scores displayed differences with the fibrosis stage (Table S3), we performed a logistic regression analysis with a subset selection method, individuating the appropriate variables to be included in the regression model, improving the diagnostic performance. AUROC of the combined model designed as FCNscore (FCN-2, APRI, and HDL) was 0.85 (95% confidence interval: 0.80 to 0.89) for the diagnosis of significant fibrosis (cut-off value >0.35, specificity 84% and sensitivity 72%) in the combined cohort (Figure 3D). The diagnostic performances of FCNscore and each component are presented in Figure S4-SM2. The number of

variables included in the analysis and the estimated equation for the combined-diagnostic model are detailed in Table S5. AUROC comparison analysis demonstrated that FCNscore was superior to all the included simple non-invasive scores APRI, FIB-4, FORNS and NFS, with accuracies of 68% ( $p = 0.0001$ ), 67% ( $p < 0.0001$ ), 68% ( $p = 0.0001$ ) and 68% ( $p = 0.0002$ ), respectively (Table S6). Using the FCNscore model, the optimal threshold (Table 3) correctly staged 189 out of the 235 patients (80%) in the combined cohort, as compared to 179 patients (76%) with APRI, to 142 patients (60%) with FIB-4, to 148 (63%) with FORNS and 136 (58%) with NFS. Considering the negative predictive value (NPV) for each model, of 178 patients with non-significant fibrosis, 148 (83%) were staged correctly using FCNscore and 152 (85%), 100 (56%), 114 (64%) and 91 (51%) using APRI, FIB-4, FORNS, and NFS, respectively (Table 3).

## **Discussion**

NAFLD affects a quarter of the global population, and its prevalence increases in parallel with the increasing prevalence of obesity, MetS, and T2DM [1] [18]. In obese people, NAFLD prevalence varies from 60 to 95% [19], and notably, NASH and fibrosis have been reported with prevalence ranges of 18 -60% and 6 - 90% in severely obese subjects [20] [21] [22]. Although not all NAFLD patients, including those with NASH, develop liver fibrosis, it is crucial to assess the fibrosis severity since it is one of the strongest predictors of liver-related complications and mortality [5].

The standard gold method for diagnosing NAFLD and fibrosis stages is histological analysis. Considering the well-known limitations of the liver biopsy (invasiveness, observer variability, sampling errors, among others), the development of alternatives is challenging in clinical management.

In the present study, we report the determination of FCN-2 in the plasma of a well-histologically characterized NAFLD obese cohort and evidenced its potential use for fibrosis diagnosis.

FCN-2 was one of a series of 29 candidates individuated by a systematic *in silico* strategy. These candidates fulfil the desired selection criteria as relevant in the fibrotic process, secreted, and traceable in plasma. Specifically, nineteen were differentially expressed in fibrotic liver and part of the myofibroblast phenotype acquisition PPI network (MyoPheNet); nine were enriched proteins with elevated expression in healthy liver and MyoPheNet components. Ficolin-2 was the only candidate to accomplish the three main selection criteria. It was part of the MyoPheNet, showed reduced expression with fibrosis, and was expressed in a healthy liver.

The main finding of this study is that FCN-2 plasma level was strongly associated with the fibrosis stage assessed by the histological analysis. Besides fibrosis, no association between histology and FCN-2 levels was shown, also when the MO cohort was stratified in No NAFL, NAFL, and NASH. We found a reduction of FCN-2 level in MO subjects with significant fibrosis ( $\geq 2$ ), further reduced in cirrhosis, regardless of origin. To our knowledge, this is the first study reporting the potential use of plasma FCN-2 as a marker of fibrosis in morbidly obese patients. Our data agree with those reported by Dai where FCN-2 and CPB2 proteins were shown as biomarkers of liver fibrosis through serum proteomics analysis and quantified using ELISA, in a cohort of 46 CHB subjects [23]. Furthermore, Chen observed that intrahepatic expression and serum levels of FCN-2 were much lower in HCC and cirrhosis than in healthy controls [24]. On the contrary, Liu reported an increase of serum FCN-2 associated with the severity of fibrosis and the activity of HCV infection [25].

FCN-2 is a serum protein expressed by hepatocytes and secreted into the circulation [26]. The serum/plasma median concentration in healthy people is approximately 5000 ng/mL; values below

1000 ng/mL have not been found in healthy adults. FCN2 plays a significant role in host innate immunity, appears to bind human DNA, attaches to apoptotic/necrotic cells, promoting their removal [27]. Relative FCN-2 deficiencies have been found associated with prematurity, low birth weight, and infections in neonates <sup>38</sup>.

The plasma level of FCN-2 allowed a good discriminative power to categorize hepatic fibrosis. The AUROC for diagnosis of significant fibrosis was 0.82 ( $F \geq 2$ ) and was superior to any other index tested (FIB-4, FORNS, and NFS). Moreover, the plasma FCN-2, when combined with APRI and HDL in FCN score, yields an excellent discriminative power with AUC of 0.85 and identifies correctly 80 % of patients included in the study.

Beyond the limitations of this study as the relatively small cohort size and the low number of subjects with advanced fibrosis or cirrhosis, we believe that FCN-2 has potential as a biomarker and should be included in future non-invasive indexes of hepatic fibrosis. Our observation needs to be validated in large independent cohorts such as the RESOLVE-IT [28] or the European NAFLD registry longitudinal cohort [29], in which candidates like PRO-C3, YKL-40, A2M have been recently tested [8] [28].

In conclusion, we have developed an *in silico* strategy to detect putative proteins as biomarkers for fibrosis and demonstrated that FCN-2 either alone or in combination with APRI and HDL is a good non-invasive diagnostic index for significant fibrosis.

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## **Statements & Declarations**

### **Data availability**

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

### **Animal Research (Ethics)**

Not applicable

### **Consent to Participate (Ethics)**

The study was approved by the ethics committee of the and the local ethics committees under protocol N. 22979 (Comitato Etico Regionale Unico, FVG, SSN, Italy) and performed in accordance with the current version of the Helsinki Declaration. All patients signed an informed consent form prior to study inclusion.

### **Consent to Publish (Ethics)**

Informed consent for publication was obtained from all authors.

### **Plant reproducibility**

Not applicable

### **Clinical Trials Registration**

Not applicable

### **Author Contribution**

Enrollment and clinical assessment of the patients' cohort: SP, CS, DL, BC, NdM, TP, GR. Performed bariatric surgery, blood samples and liver biopsy collection: SP, NdM, CS, DL, TP, GR. Conceive and designed the experiments: PJG, NS, NR. Conceived and perform the *in silico* discovery strategy: PJG. Performed experiments: PJG, NS, NR. Analyzed and interpreted the experimental data: PJG, NR, NS, CT. Clinical data and histological analysis: DB, PJG, NR, SP, GR, CT. Contributed reagents/materials/analysis tools: NR, SP, NM, GR, CT. Wrote the paper: PJG, NR, CT. All authors approved the final manuscript.

### **Conflict of Interest:**

No authors report conflicts of interest/financial disclosures.

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**Table 1. Clinical characteristics of all patients**

BMI. body mass index. ALT. alanine aminotransferase; AST. aspartate aminotransferase. GGT. gamma-glutamyl transferase. T2DM. type 2 diabetes mellitus. HDL. high density cholesterol. APRI. FIB4. FORNS. NFS. Data are shown as mean  $\pm$  SD for continuous variables. number (%) for binary variables. and number per group for categorical variables. ANOVA was used to test for significant differences within continuous variables were normally distributed while Kruskal-Wallis with Dunn post-test when non normally distributed. Chi-Squares test was used for categorical variables. \* $p < 0.05$  was considered statistically significant.

**Table 2. Demographic and clinical characteristics of the discovery cohort**

MO. Morbidly obese cohort; BMI. body mass index. ALT. alanine aminotransferase; AST. aspartate aminotransferase. GGT. gamma-glutamyl transferase. T2DM. type 2 diabetes mellitus. HDL. high-density cholesterol. APRI. FIB4. FORNS. NFS. NA. Not available. \* $p < 0.05$  was considered statistically significant vs. respective controls.

Data are shown as mean  $\pm$  SD for continuous variables. number (%) for binary variables. and number per group for categorical variables. ANOVA and t-test were used to test for significant differences within continuous variables were normally distributed while Mann-Whitney and Kruskal-Wallis when non normally distributed. Chi-Squares test was used for categorical variables.

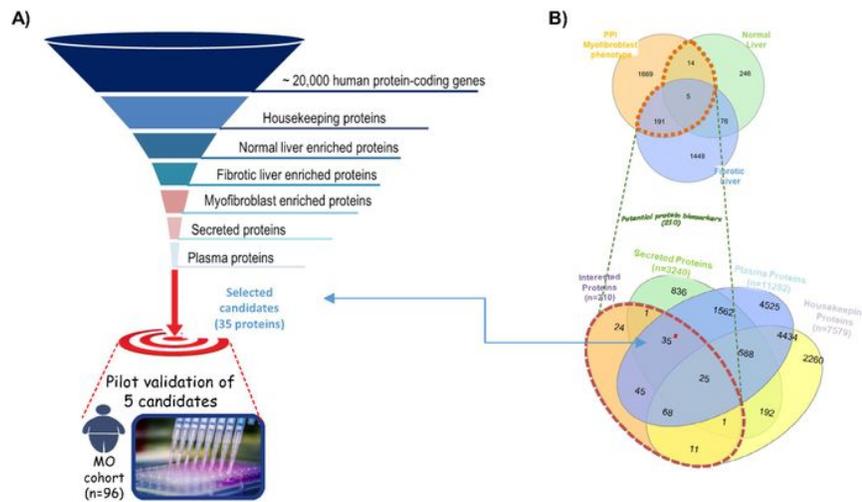
**Table 3. Classification of subjects in the combined cohort according to moderate/advanced fibrosis (prevalence 24%, n= 235)**

**Fig 1. Summary of the *in silico* biomarkers discovery strategy used in the study.** A) Layout of the *in silico* funnel strategy. B) Venn diagrams illustrating the different datasets used to identify candidates satisfying our selection criteria.

**Fig. 2. Boxplot for FCN-2 measurements in the morbidly obese discovery cohort.** A) Plasma abundances of FCN-2 determined by ELISA in MO (n = 76) subjects stratified by fibrosis B) MO stratified by gender and fibrosis' stage and C) MO stratified by steatosis' grade.\*\*\*Significant at  $p < 0.001$ ; \*\*significant at  $p < 0.01$  and \*significant at  $p < 0.05$ .

**Fig. 3. Receiver operating characteristics (ROC) curves for diagnosis of significant fibrosis.** FCN-2 vs blood-based tests (APRI, FIB-4, NFS, FORNS) AUROCs in A) Combined cohort (n=235, disease prevalence 24%), B) Discovery MO cohort (N=76, disease prevalence 43%) and C) Validation MO cohort (N=159, disease prevalence 15%). D) FCNscore (FCN-2 levels, APRI and HDL combined in a diagnostic model) vs common blood-based tests (FIB-4, NFS, APRI, FORNS) AUROCs in the combined cohort. E) Full picture for FCN-2 plasma levels in all samples included in the study. Significant fibrosis (F2, F3, and F4 stages).

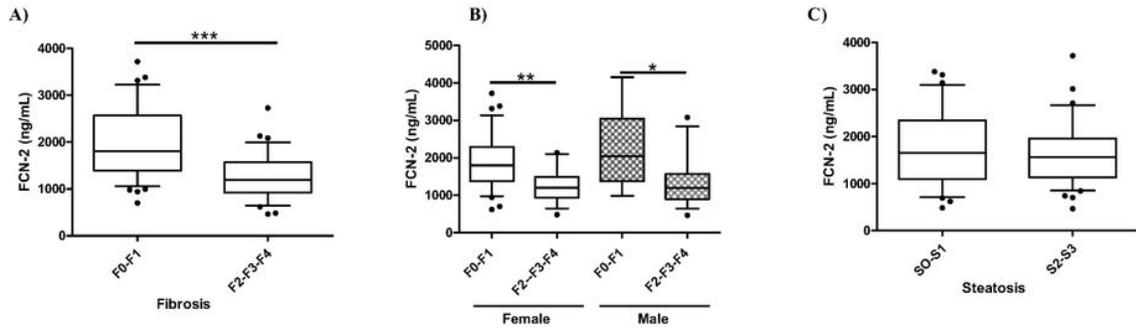
# Figures



**Fig 1. Summary of the *in silico* biomarkers discovery strategy used in the study.** A) Layout of the *in silico* funnel strategy. B) Venn diagrams illustrating the different datasets used to identify candidates satisfying our selection criteria.

## Figure 1

*Summary of the in silico biomarkers discovery strategy used in the study.* A) Layout of the *in silico* funnel strategy. B) Venn diagrams illustrating the different datasets used to identify candidates satisfying our selection criteria.

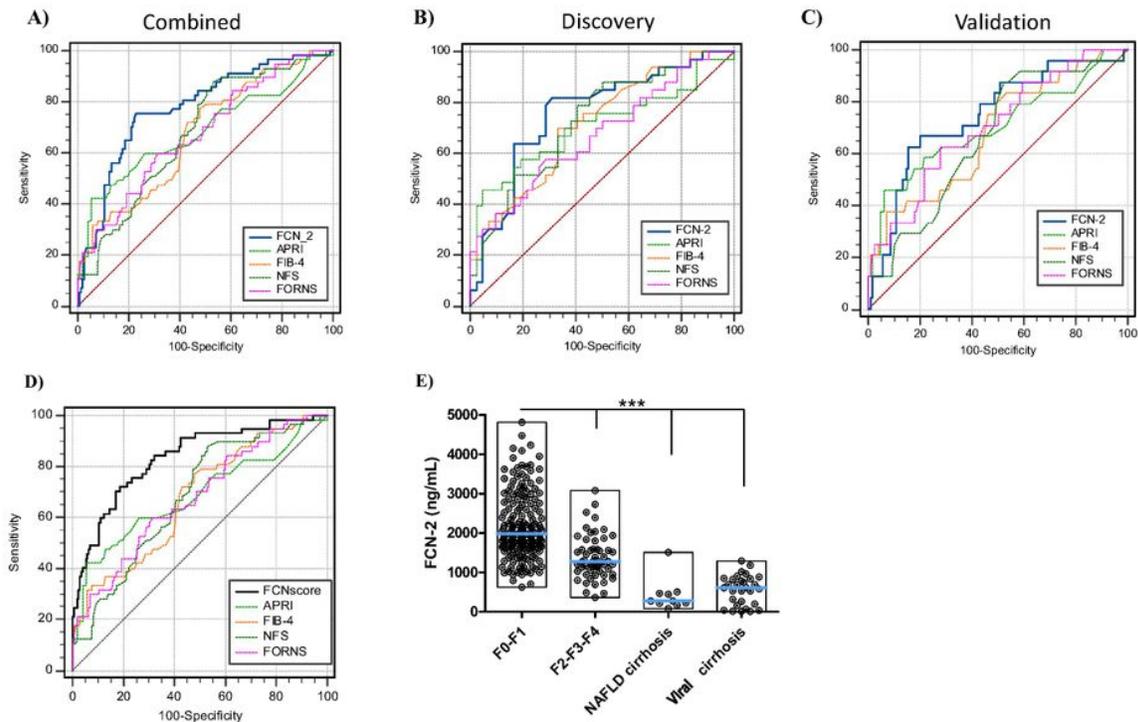


**Fig. 2. Boxplot for FCN-2 measurements in the morbidly obese discovery cohort (MO, n=96).** A) Plasma abundances of FCN-2 determined by ELISA in MO subjects stratified by fibrosis' stage. B) MO stratified by gender and fibrosis' stage and C) MO stratified by steatosis' grade.\*\*\*Significant at  $p < 0.001$ ; \*\*significant at  $p < 0.01$  and \*significant at  $p < 0.05$ .

## Figure 2

*Boxplot for FCN-2 measurements in the morbidly obese discovery cohort. A) Plasma*

*abundances of FCN-2 determined by ELISA in MO (n = 76) subjects stratified by fibrosis B) MO stratified by gender and fibrosis' stage and C) MO stratified by steatosis' grade.\*\*\*Significant at  $p < 0.001$ ; \*\*significant at  $p < 0.01$  and \*significant at  $p < 0.05$ .*



**Fig. 3. Receiver operating characteristics (ROC) curves for diagnosis of significant fibrosis.** A) Combined cohort (n=235, disease prevalence 25%), B) Discovery MO cohort (N=76, disease prevalence 43%) and C) Validation MO cohort (N=159, disease prevalence 15%). FCN-2 vs common blood-based tests (APRI, FIB-4, NFS, FORNS). D) FCNscore (FCN-2 levels, APRI and HDL combined in a diagnostic model) vs common blood-based tests (FIB-4, NFS, APRI, FORNS) AUROCs in the combined cohort. E) Full picture for FCN-2 plasma levels in all samples included in the study. Significant fibrosis (F2, F3, and F4 stages). \*\*\*Significant at  $p < 0.001$ .

## Figure 3

*Receiver operating characteristics (ROC) curves for diagnosis of significant fibrosis.* FCN-2 vs blood-based tests (APRI, FIB-4, NFS, FORNS) AUROCs in A) Combined cohort (n=235, disease prevalence 24%), B) Discovery MO cohort (N=76, disease prevalence 43%) and C) Validation MO cohort (N=159, disease prevalence 15%). D) FCNscore (FCN-2 levels, APRI and HDL combined in a diagnostic model) vs common blood-based tests (FIB-4, NFS, APRI, FORNS) AUROCs in the combined cohort. E) Full picture for FCN-2 plasma levels in all samples included in the study. Significant fibrosis (F2, F3, and F4 stages).

## Supplementary Files

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