A simple in silico strategy identifies candidate biomarkers for the diagnosis of liver fibrosis in morbidly obese subjects

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disorder, tightly associated with obesity. The histological spectrum of the disease ranges from simple steatosis to steatohepatitis, with different stages of fibrosis, and fibrosis stage is the most significant predictor of mortality in NAFLD. Liver biopsy continues to be the gold standard for its diagnosis and reliable non-invasive diagnostic tools are unavailable. We investigated the accuracy of candidate proteins, identified by an in silico approach, as biomarkers for diagnosis of fibrosis.

Methods: Seventy-one morbidly obese (MO) subjects with biopsy-proven NAFLD were enrolled, and the cohort was subdivided according to minimal (F0/F1) or moderate (F2/F3) fibrosis. The plasmatic level of CD44 antigen (CD44), secreted protein acidic and rich in cysteine (SPARC), epidermal growth factor receptor (EGFR) and insulin-like growth factor 2 (IGF2) were determined by ELISA. Significant associations between plasmatic levels and histological fibrosis were determined by correlation analysis and the diagnostic accuracy by the area under receiver operating characteristic curves (AUROC).

Results: Eighty-two percentage of the subjects had F0/F1 and 18% with F2/F3 fibrosis. Plasmatic levels of IGF2, EGFR and their ratio (EGFR/IGF2) were associated with liver fibrosis, correlating inversely for IGF2 (P < .006) and directly (P < .018; P < .0001) for EGFR and EGFR/IGF2 respectively. The IGF2 marker had the best diagnostic accuracy for moderate fibrosis (AUROC 0.83), followed by EGFR/IGF2 ratio (AUROC 0.79) and EGFR (AUROC 0.71).

Conclusions: Our study supports the potential utility of IGF2 and EGFR as non-invasive diagnostic biomarkers for liver fibrosis in morbidly obese subjects.

Keywords: biomarkers, in silico strategy, liver fibrosis, morbidly obese

Abbreviations: ALT, alanine aminotransferase; APRI, AST to platelet-ratio-index; AST, aspartate aminotransferase; BMI, body mass index; CD44, CD44-antigen; EGFR, epidermal growth factor receptor; FIB-4, fibrosis-4 score; GGT, γ-glutamyltransferase; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; IGF2, insulin-like growth factor 2; LDL, low-density lipoprotein; MO, morbidly obese; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PPI, protein-protein interactions; SPARC, secreted protein acidic and rich in cysteine; TAG, triglyceride.

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1 | INTRODUCTION

According to European Health Interview Survey (2016), almost 1 adult in 6 in the EU is considered obese.1 Morbidly obese (MO) subjects are at particular risk for the development of non-alcoholic fatty liver disease (NAFLD).2,3 Several epidemiological studies have linked NAFLD to unhealthy diet and sedentary behaviours.4,5

NAFLD includes different stages, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). The latter is characterized by steatosis plus necroinflammation and can have different stages of fibrosis ranging from absent to cirrhosis.6 Unfortunately, despite the increase in awareness of this disease, there are still no reliable non-invasive diagnostic tests and liver biopsy remains the gold standard. However, it is invasive, complications may occur and require hospitalization. For these reasons, there is an urgent clinical need to develop non-invasive assays for the staging of liver fibrosis in NAFLD/NASH.

The discovery of new serum biomarkers to be used either separately/combined in a panel of markers could contribute not only to the diagnosis but also to follow-up the progression/remission of the disease. Nevertheless, the identification of novel biomarkers for liver fibrosis can be a daunting work owing to the multiple factors involved in disease progression. Currently, the study of the interactome at gene/protein level is possible through the use of high-throughput and bioinformatic tools, such as Cytoscape software.6 This software allows analysing the biological information about protein-protein interactions (PPI) stored in different molecular databases, such as IntAct, MINT, UniProt, etc. Thus, in silico analysis of biological networks represents an alternative option to elucidate novel biomarkers, as previously described by Page7 and Abdul-Hameed.8

Considering the aforementioned issues, we applied an in silico strategy to identify new effective biomarkers for the diagnosis of moderate/advanced liver fibrosis stages. We then assessed their accuracy in a cohort of MO subjects with different stages of fibrosis.

2 | MATERIALS AND METHODS

2.1 | Patient cohort and study protocol

Seventy-one MO subjects undergoing bariatric surgery were prospectively and consecutively enrolled by a multidisciplinary team (surgeons, dieticians, hepatologists and psychiatrists). All consenting patients were included in accordance with the international guidelines: age 18 to 65 years, a body mass index (BMI) of 40 kg/m² or between 35 and 40 kg/m² with obesity-related co-morbidities, well-informed and motivated patients with acceptable operative risks, failure of non-surgical treatments, declared compliance to follow lifelong medical surveillance.9 Liver biopsy was performed in all subjects at the time of the surgical procedure. The exclusion criteria were as follows: previous diagnosis of others forms of chronic liver disease, including suspected/confirmed hepatocellular carcinoma; alcoholic liver disease (>25 g/day alcohol consumption) or known HBV, HCV and HIV positivity. MO subjects gave their written informed consent before participating in this study, approved by protocol N. 22979 Local Ethical Committee (Comitato Etico Regionale Unico, FVG, SSN).

In addition, blood samples from informed consenting healthy lean subjects and from patients with F3 NASH and advanced metabolic-related cirrhosis were included in the study, and considered as negative and positive controls respectively.

2.2 | Clinical-biochemical assessment

Anthropometric parameters, such as age, sex (M/F) and BMI (kg/m²) were scored during the baseline visit. Blood samples were collected after overnight fasting for the further assessment of liver biochemistry, glucose, glycated haemoglobin (HbA1c) and lipids. The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as described by Matthews.10 Diabetes was diagnosed according to the ESC-EASD guidelines.11 Surrogate markers’ scores of liver fibrosis were calculated as described by Sumida for FIB-4,12 Calès for APRI and FibroMeter13 and Harrison for BARD index.14

2.3 | Liver biopsy and histopathology

Liver wedge biopsies were performed on the left lobe and two pathologists interpreted them. Steatosis was graded according to the amount of fat present in the hepatocytes on haematoxylin/eosin staining. Biopsies showing no or minimal (<5%) steatosis and absent injury or fibrosis were considered as normal. The samples that showed more than 5% steatosis were labelled as NAFLD. The histological diagnosis of NASH and fibrosis was made in accordance with Kleiner-Brunt criteria.15,16

2.4 | In silico biological network analysis

To obtain biological networks for each gene/protein of interest involved in fibrogenesis, we used Cytoscape.6 UniProtKB identifiers and protein-protein interaction (PPI) data from curated databases were used in network creation.17 Each biological network is constituted...
by a central node (e.g., cytokeratin-18 [CK-18]) that interacts with protein partners. Central nodes correspond to the proteins involved in the fibrotic process, and their relevant information is reported in Table S1 (Supporting information). In summary, the selected proteins, used to obtain each biological network, can be categorized into three different groups based on their role in NAFLD: (a) those reported as putative markers for the progression of the disease, such as CK-18, adipocyte fatty acid binding protein (AFABP), fibroblast growth factor 21 (FGF21), insulin-like growth factor-binding protein 3 (IGF2), and lymphocyte cytosolic protein 1 (LCP1); (b) those reported to be involved in phenotype modulation (activation/reversion) of hepatic stellate cells (HSC), such as galectin-1 (GALSA), ubiquitin conjugation factor E4B (UBE4B), vitronectin (VTN) and alpha smooth muscle actin (α-SMA), laminin subunit beta 1 (LAMB1); and (c) those involved in extracellular matrix remodelling such as osteopontin (SPP1), collagen alpha-1 (III) chain (COL1A3), matrix-metalloproteinase-2 (MMP2) and tissue inhibitor of metalloproteinase-2 (TIMP2).

Once defined the network for each protein the total network was generated by the integration of each singular one, the layout is presented in Figure 1.

2.5 | Plasma CD44, SPARC, IGF-2 and EGFR

Plasmatic levels of candidate biomarkers were measured by ELISA commercial kits (further details in Supporting information).

2.6 | Statistical analysis

Continuous variables were expressed as mean ± (standard deviation) or median (interquartile range), and categorical as numbers or percentages. Categorical variables were analysed 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 with post hoc analysis) were applied for continuous variables that failed to pass D’Agostino & Pearson omnibus normality test.

Correlation analysis was performed using Pearson or Spearman’s correlation coefficients to estimate the association of plasmatic candidates’ levels and several factors of interest. Statistical analysis was performed using GraphPad Prism 5.01. Multivariable analysis using multiple linear regression models were performed to determine the independent factors associated with candidates plasmatic levels, using GraphPad Instat 3.

The candidates diagnostic performance was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUROC) was used to compare the accuracy between different fibrosis diagnostic tests. The sensitivity, specificity, positive predictive values (PPVs) and negative predictive values (NPVs) for relevant cut-offs were also calculated. ROC analysis was performed using MedCalc Statistical Software 16.4.3.
The main demographic, clinical and biochemical features of the cohort are reported in Table 1. The MO cohort presented alterations mainly in glucose homeostasis with abnormal values for fasting glucose, glycated haemoglobin and HOMA-IR (113 ± 25 mg/dL, 6.3 ± 1 μU/mL and 5.3 ± 4 respectively) respect to CTRLs. Approximately, 20% of the subjects had type 2 diabetes. No differences in cholesterol and triglycerides were observed among groups. As expected, cirrhosis group had altered levels of GGT, and altered glucose homeostasis. The hepatic histological features of MO cohort are shown in Table 1. Briefly, 58 (82%) MO subjects had no significant/minimal (F0/F1) and 13 (18%) significant/moderate (F2/F3) fibrosis.

From the in silico analysis, four candidates were selected: insulin growth factor 2 (IGF2), secreted protein acidic and rich in cysteine (SPARC), CD44-antigen (CD44) and epidermal growth factor receptor (EGFR). Candidates were chosen since they are soluble proteins, supposed to be released in the plasma from the liver, and because they link several central nodes/proteins involved in fibrogenesis. Specifically, IGF2 interacts with VTN (involved in the modulation of HSC phenotype) and IBP-3 (reported as a potential biomarker for NAFLD progression). EGFR links directly three proteins: IBP3 (mentioned above), LGALS1 and UBE4B, the last two involved in the activation of HSC. Thus, following IGF2/EGFR candidates, we are also able to consider eventual variations of the others connected proteins (like VTN, IBP-3, etc.). These other proteins, in turn, regulate several biological processes associated with the progression of the fibrotic process. Thus, the selection of these candidates improved our probability of obtaining reliable markers to follow liver fibrosis (evidenced in red, Figure 1 and Table S1). To further validate the association of our candidates with fibrosis, gene expression analysis were performed in liver biopsies (Table S2, Figure S1A and B).

### RESULTS

#### 3.1 Subjects' demographic

The plasmatic concentration of our candidate was determined by ELISA (Figure 3). IGF2 levels were significantly decreased as fibrosis progress (Figure 3A). The median IGF2 level in CTRLs and cirrhosis was 6.80 (interquartile range, 4.82-9.40) ng/mL and 0.92 (0.79-1.20) ng/mL respectively (P < .001). Interestingly, IGF2 was able to distinguish F0/F1 from F2/F3 in the MO cohort (2.20 (1.70-2.80) and 1.45 (0.45-1.82) respectively; P < .05).

EGFR levels were significantly increased with the progression of hepatic fibrosis compared to CTRLs (Figure 3B). The median EGFR levels in subjects with F0/F1, F2/F3 and CTRLs were 110 ng/mL (81.5-125.5), 115 ng/mL (107.4-143.0) and 48.5 ng/mL (44.7-63.0) respectively (F0/F1 and F2/F3 vs CTRLs, P < .001). Surprisingly, cirrhosis group showed similar EGFR levels to MO subjects (116.2 ng/mL (90.9-127), been only significantly different from lean controls (P < .01).

Regarding the plasmatic levels of CD44, a trend of increase (not statistically significant) was observed with the progression of liver
To enhance the sensitivity of the two informative candidate biomarkers (IGF2 and EGFR), we calculated their ratio (Figure 3C). The EGFR/IGF2 ratio showed a positive association with the stage of fibrosis and allowed to differentiate subjects with fibrosis (F0/F1, F2/F3) and cirrhosis from CTRLs. The median values were 47.0 (30.5-69.2) for F0/F1, 82.0 (54.0-332.0) for F2/F3, 122.2 (91.6-137.6) for cirrhosis and 5.9 (3.8-9.5) for CTRLs (F0/F1 vs CTRLs, P < .01; F2/F3 and Cirrhosis vs CTRLs, P < .001). Moreover, EGFR/IGF2 values were significantly different between subjects with cirrhosis or significant fibrosis (F2/F3) from those with minimal fibrosis (F0/F1) (Cirrhosis vs F0/F1, P < .05; F2/F3 vs F0/F1, P < .05). As expected, candidate biomarkers in plasma did not show any correlation with steatosis in the MO cohort (Figure S3-Supporting information).

**3.5 | Diagnosis of liver fibrosis using plasmatic biomarkers in MO subjects**

Pearson’s or Spearman’s correlation analysis was performed to evaluate the association between the plasmatic levels of our candidates with BMI, lipid profile, HOMA-IR, FLI and liver histological scores for steatosis, inflammation and fibrosis. IGF2 plasmatic level had a significant negative correlation with both lobular inflammation (P = .024) and fibrosis (P = .006) (Table 2 and Supporting information-Figure S4A and B). On the other hand, EGFR correlates negatively with total cholesterol (P = 0.032) and positively with lobular inflammation (P = .021) and fibrosis (p=0.018) (Table 2 and Supporting information-Figure S4C, D and E). EGFR/IGF2 ratio showed a positive correlation with lobular inflammation (P = .027) and fibrosis (P < .0001) (Table 2 and Supporting information-Figure S4F and G). CD44 and SPARC plasmatic levels showed no correlation with the parameters under analysis (Table S3-Supporting information).

When multivariable analysis using a multiple linear regression model was applied, fibrosis was the main contributor associated with IGF2 plasmatic levels and with the EGFR/IGF2 ratio, whereas, all factors equally contributed in the case of EGFR (Table S4-Supporting information).

The diagnostic accuracy for liver fibrosis of our candidates was compared with those of scoring systems such as FIB-4 and Fibrometer using AUROC analysis (Figure 4). IGF2 showed the best diagnostic accuracy for significant/moderate fibrosis (AUROC 0.83), followed by the EGFR/IGF2 ratio (AUROC 0.79), EGFR (AUROC 0.71), FibroMeter score (AUROC 0.64) and FIB-4 score (0.63). The sensitivity, specificity, PPVs and NPVs of each test for optimal cut-off values are reported in Table 3. Overall, IGF2 had the highest accuracy in detecting significant fibrosis, whereas FIB-4 score had the lowest. At the optimal threshold of 1.9 ng/mL, IGF2 had a 86% sensitivity and 74% specificity in our MO cohort. Thus, IGF2 and EGFR or their ratio had a higher specificity and sensitivity than the currently used surrogate indexes based on routine laboratory tests.

**4 | DISCUSSION**

Since the available non-invasive tools for the diagnosis of NAFLD/NASH are still inconclusive, we prospectively explored the reliability...
FIGURE 3  Boxplot of candidate biomarkers plasmatic levels versus the stage of fibrosis. (A) IGF2; (B) EGFR; (C) EGFR/IGF2 ratio, (D) CD44 and (E) SPARC. Data were expressed as Median (interquartile range [IQR]) and statistical analysis using ANOVA test. ***Significant at $P < .001$; **significant at $P < .01$ and *significant at $P < .05$

| Parameter          | IGF2 Rho  | IGF2 P value* | EGFR Rho | EGFR P value* | EGFR/IGF2 Rho | EGFR/IGF2 P value* |
|--------------------|-----------|---------------|----------|---------------|---------------|---------------------|
| BMI                | -0.15     | .249          | 0.01     | .914          | 0.15          | .240                |
| Triglycerides      | 0.06      | .637          | -0.05    | .698          | -0.08         | .550                |
| Total cholesterol  | -0.04     | .760          | -0.36    | .004**        | 0.02          | .865                |
| HOMA-IR            | -0.16     | .249          | 0.19     | .149          | 0.21          | .135                |
| FLI                | -0.16     | .246          | -0.0006  | .996          | 0.17          | .210                |
| Steatosis          | -0.12     | .387          | -0.06    | .619          | 0.14          | .275                |
| NAS                | -0.18     | .173          | 0.08     | .540          | 0.18          | .178                |
| Lobular inflammation | -0.30   | .024*         | 0.28     | .021*         | 0.30          | .027*               |
| Ballooning         | -0.06     | .660          | -0.01    | .921          | -0.20         | .150                |
| AST/ALT ratio      | 0.02      | .875          | -0.14    | .238          | -0.03         | .840                |
| GGT                | -0.03     | .844          | 0.03     | .809          | 0.04          | .764                |
| FIB-4              | 0.05      | .734          | -0.13    | .297          | 0.05          | .717                |
| Fibrometer         | -0.03     | .819          | 0.004    | .974          | 0.10          | .488                |
| Fibrosis           | -0.36     | .006**        | 0.29     | .018*         | 0.63          | <.0001***           |

Steatosis and fibrosis scores were according to Kleiner-Brunt histological classification. $P$ value corresponds to $H_0: \rho = 0$ (the two variables do not vary together at all).

***$P < .001$, **$P < .01$ and *$P < .05$ were considered statistically significant.
The most relevant finding of this study is that the plasmatic level of two of our candidates (IGF2 and EGFR) is closely associated with the stage of liver fibrosis. IGF2 is inversely correlated with the degree of lobular inflammation and fibrosis and previous studies showed a reduction in IGF2 plasmatic level in subjects with cirrhosis, inversely correlated with the hepatic damage. Moreover, evidence about the association of lower IGF2 levels with the stage of fibrosis was provided in a paediatric NAFLD cohort\textsuperscript{31} and, more recently, in a larger adults NAFLD population. Our study confirms these findings and extends them to a cohort of severely obese adults. Regarding fatty liver, and contrarily to the study of Ajmera,\textsuperscript{32} we did not show any association between plasmatic levels of IGF2 and the degree of liver steatosis.

Beyond the modest PPVs, the accuracy of our candidates

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4}
\caption{Receiver operating characteristics (ROC) curves for the noninvasive markers for the diagnosis of significant fibrosis (Kleiner-Brunt fibrosis stage 2-3).}
\end{figure}

of putative biomarkers for the detection of liver fibrosis in a cohort of severely obese individuals. In line with the range reported by other studies in bariatric subjects,\textsuperscript{24,25} we observed a high prevalence of NASH (62%), 82% with minimal fibrosis and 18% with moderate fibrosis.

Fibrosis stage was recently established to be the most important prognostic factor for liver-related outcomes and mortality.\textsuperscript{26} Even though several simple, non-invasive clinical indexes have been proposed to diagnose fibrosis in subjects with NAFLD (extensively reviewed by Kaswala),\textsuperscript{27} they are neither accurate nor reliable enough to substitute the diagnostic gold standard (liver biopsy). Several studies suggested elastography techniques (transient ultrasound elastography, acoustic radiation force impulse imaging or supersonic shear wave elastography) as the most effective, safe, quick and cheapest imaging tests. Unfortunately, their use is actually limited by the characteristic of the patient and in severely obese subjects, these techniques are not applicable even using the XL probe.\textsuperscript{28} Magnetic resonance elastography (MRI), provide a highly accurate measurement of fibrosis, inflammation and steatosis (recently reviewed by Han), however, its application in clinical practice is limited by the scarce availability (academic centres) and its high cost.\textsuperscript{29} Thus, our study aimed to contribute to providing accurate serum biomarkers which in combination with imaging techniques, would be accurate, safe and reliable in the diagnosis and monitor fibrosis.

\begin{table}[h]
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\caption{Comparison of the performance of each test for the diagnosis of significant fibrosis in the MO cohort}
\begin{tabular}{|l|c|c|c|c|c|c|c|}
\hline
Biomarker/Test & AUROC (95\% CI) & Cut-off & Sens (\%) & Spec (\%) & PPV & NPV \\
\hline
IGF2 & 0.83 (0.70-0.92) & 1.9 & 85.7 & 73.7 & 58.3 & 92.3 \\
EGFR & 0.71 (0.59-0.82) & 102.5 & 94.4 & 52.9 & 46.2 & 95.7 \\
EGFR/IGF2 & 0.79 (0.67-0.88) & 58 & 73.3 & 73.6 & 54.3 & 86.6 \\
Fibrometer & 0.64 (0.51-0.75) & 51 & 76.9 & 64.0 & 47.8 & 86.6 \\
FIB-4 & 0.63 (0.50-0.74) & 0.78 & 66.7 & 64.1 & 44.4 & 81.8 \\
\hline
\end{tabular}
\end{table}

AUROC, Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.
as tests for moderate fibrosis could be improved if prevalence is higher than 20% (reported range in MO subjects -from 8% to 60%). Information on diagnostic accuracy of surrogate markers for liver fibrosis in severely obese subjects is scarce. Cleva reported AUROC data of 0.52, 0.88 and 0.99 for AST/ALT, Age-platelet and APRI, respectively, when used for the diagnosis of advanced fibrosis (≥F3). ALT and HbA1c were combined in a ROC statistical model and used to predict the presence of fibrosis (≥F1) with AUROC of 0.90. Using FIB-4, NFS and Fibrotest; a diagnostic performance for advanced fibrosis with an AUROC of 0.77, 0.75 and 0.72, respectively, was reported.

The main strength of this study is the well-characterized morbidly obese cohort with the biopsy-proven liver disease. Among the limitations, it should be mentioned; the relatively small sample size, the low prevalence of advanced/severe fibrosis or cirrhosis in the MO cohort and the lack of histological data (for ethical issues) in lean controls. In conclusion, this study proposes IGF2 and EGFR as accurate biomarkers for the diagnosis of significant to moderate fibrosis in MO subjects. The introduction of these biomarkers in clinical practice, either alone or combined with others serum markers in a score, may reduce the need for liver biopsy. Larger prospective studies are needed to confirm this conclusion.

CONFLICTS OF INTEREST

No authors report conflicts of interest/financial-disclosures.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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