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Bone morphogenetic protein 2 is a new molecular target linked to non-alcoholic fatty liver disease with potential value as non-invasive screening tool

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is the commonest cause of chronic liver disease worldwide, being non-alcoholic steatohepatitis (NASH) its most clinically relevant form. Given the risks associated with taking a liver biopsy, the design of accurate non-invasive methods to identify NASH patients is of upmost importance. BMP2 plays a key role in metabolic homeostasis; however, little is known about its involvement in NAFLD onset and progression. This study aimed to elucidate the impact of BMP2 in NAFLD pathophysiology.

Methods: Hepatic and circulating levels of BMP2 were quantified in serum and liver specimens from 115 biopsy-proven NAFLD patients and 75 subjects with histologically normal liver (NL). In addition, BMP2 content and release was determined in cultured human hepatocytes upon palmitic acid (PA) overload.

Results: We found that BMP2 expression was abnormally increased in livers from NAFLD patients than in subjects with NL and this was reflected in higher serum BMP2 levels. Notably, we observed that PA upregulated BMP2 expression and secretion by human hepatocytes. An algorithm based on serum BMP2 levels and clinically relevant variables to NAFLD showed an AUROC of 0.886 (95%CI, 0.83–0.94) to discriminate NASH. We used this algorithm to develop SAN (Screening Algorithm for NASH): a SAN < 0.2 implied a low risk and a SAN ≥ 0.6 indicated high risk of NASH diagnosis.

Conclusion: This proof-of-concept study shows BMP2 as a new molecular target linked to NAFLD and introduces SAN as a simple and efficient algorithm to screen individuals at risk for NASH.

Keywords: Non-alcoholic fatty liver disease, Bone morphogenetic proteins, BMP2, Non-invasive diagnosis, Hepatocytes

Introduction

Non-alcoholic fatty liver disease (NAFLD), is becoming the most prevalent chronic liver disease worldwide and is considered the hepatic manifestation of the metabolic syndrome; consequently, it is highly linked with the most relevant features of this syndrome, as obesity, hypertension, dyslipidemia, central adiposity and insulin

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resistance or type 2 diabetes (T2D) [1, 2]. Indeed, the prevalence of NAFLD in obese and diabetic patients can be over 50%, whereas, the global prevalence of NAFLD is around 25% in adults, being higher in Western countries [3, 4].

NAFLD encompasses a range of liver diseases from simple fatty liver (NAFL), mostly a benign non-progressive clinical entity defined as the presence of fat in > 5% of hepatocytes, to steatohepatitis (NASH), a more severe condition featured by steatosis, lobular and portal inflammation, and degeneration (ballooning) of hepatocytes, with or without fibrosis, which in turn can lead to more severe conditions of liver disease such as cirrhosis, portal hypertension and even hepatocellular carcinoma (HCC) [5, 6]. In this regard, while most of NAFLD patients are usually asymptomatic and only present a simple accumulation of fat in the hepatocyte (NAFL), a 44–59% of patients end up developing NASH with variable fibrosis stages. Up to 25% of patients with NAFLD progress to cirrhosis and approximately 7% to end-stage liver disease, needing for liver transplantation [3].

The prompt diagnosis and treatment of the early stages of NAFLD are important to prevent the progression to advanced stages of the liver disease. The determination of the lipid content of the liver can be an important challenge in terms of identification, treatment and control of the progression of the disease [7]. Nowadays, there are no validated non-invasive diagnostic methods for quantifying NAFLD histological evolution, and liver biopsy is still the gold standard to differentiate fatty liver from NASH and/or fibrosis stages [1]. However, liver biopsy is not recommended for routine use due to increased risk of bleeding and complications, and intra and inter-observer variations. In the last years, many diagnostic non-invasive tools (mainly serological tests and imaging techniques) have been described, but they are not ready yet for widespread use, or to replace histological examination [8, 9].

Bone morphogenetic proteins (BMPs) are secreted glycoproteins belonging to the transforming growth factor β (TGFβ) superfamily. More than 20 BMPs have been described and originally identified as growth factors involved in the differentiation of osteogenic cells; however, it has been demonstrated that these proteins also play a critical role in the development of many cells types in various tissues, acting in cell proliferation and differentiation, tooth morphogenesis, organogenesis, embryonic development, apoptosis, chemotaxis and tissue regeneration [10, 11].

Regarding BMPs and liver physiology, these proteins are known to have a crucial role in different stages of hepatic development and also are implicated in adult liver homeostasis, as iron and glucose homeostasis [12]. In this regard, while it has been demonstrated the pathophysiological role of some BMPs, such as BMP3B, BMP4, BMP6, BMP8B and BMP9, in animal models of NASH and NASH patients [13–17], little is known about the involvement of BMP2 in experimental models of NAFLD [18], and no data exist so far directly linking BMP2 to human NAFLD. Therefore, we sought to explore whether BMP2 could be related to NAFLD by assessing the expression of BMP2 in liver and serum from patients with biopsy-proven NAFLD as well as in an in vitro model of hepatic steatosis.

Methods

Study population

This study comprises 115 patients with biopsy-proven NAFLD, 56 with simple steatosis (NAFL) and 59 with steatohepatitis (NASH), to whom a liver biopsy were performed for diagnosis purposes or had histological features of NAFLD in a liver biopsy carried out during programmed laparoscopic cholecystectomy, and 75 patients with histologically normal liver (NL) to whom a liver biopsy was performed during laparoscopic cholecystectomy. All patients were recruited from Hospital Universitario Santa Cristina (Madrid, Spain) and Hospital Universitario Virgen del Rocío (Sevilla, Spain). Inclusion criteria were: age between 18 and 75 years old, alcohol consumption lower than 20 g/day, no history of potentially hepatotoxic drugs and no analytical evidence of iron overload, autoimmunity and hepatitis B, hepatitis C and human immunodeficiency virus infection.

This study was performed in agreement with the Declaration of Helsinki, and with local and national laws. The Human Ethics Committee of the Hospital Universitario Santa Cristina (reference, PI-688A) and Hospital Universitario Virgen del Rocío (reference, C.I. 0359-N-15) approved the study procedures, and all participants signed an informed written consent before inclusion in the study.

Clinical, biochemical and metabolic assessment

A complete clinical examination was performed to all patients studied. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. At the time of liver biopsy, blood samples of each participant were obtained to determine serum levels of liver enzymes, metabolic parameters and serologic tests using routine laboratory methods. Insulin resistance was calculated by the homeostasis model assessment (HOMA-IR) method [19].

Histopathological assessment

Hematoxylin-eosin and Masson's trichrome-stained paraffin-embedded liver biopsy sections showing more

than 10 complete portal tracts were examined and interpreted by experienced hepatopathologists of each clinical site using the same internationally accepted criteria. Briefly, steatosis was assessed as outlined by Brunt et al. [20] grading percentage involvement by steatotic hepatocytes as follows: grade 0, < 5%; grade 1, 5–33%; grade 2, > 33–66%; and grade 3, > 66%. Simple steatosis or NAFL was defined as the presence of at least 5% of steatotic hepatocytes with or without mild lobular or portal inflammation but in the absence of features of hepatocellular injury (ballooning, apoptosis or necrosis) or fibrosis. On the other hand, minimal criteria for the histological diagnosis of NASH included the combined presence of grade 1 steatosis, hepatocellular injury and lobular inflammation with or without fibrosis. In addition, NAFLD activity score was also calculated to each liver biopsy according to Kleiner's criteria [21].

Cell culture and treatment

Human hepatoma cell line Huh7 (ATCC, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM, Cytiva, USA) containing high glucose, antibiotics and supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. For the steatogenic protocol, cells were treated overnight with 750 μM of palmitic acid (PA, Merk Millipore, Darmstadt, Germany), dissolved in isopropyl alcohol, in serum free DMEM with 1% BSA. In all experiments, control cells (C) were treated with the corresponding vehicle (isopropanol).

Gene expression analysis

Total RNA from liver samples was extracted using the NucleoSpin RNA purification kit (Macherey-Nagel, GmbH & Co. KG, Dueren, Germany) following manufacturer's instruction. For cell samples, plates were washed with phosphate buffered saline (PBS) and total RNA was extracted with TRIzol Reagent (Vitro, Sevilla, Spain). Reverse transcription was performed with ImProm-II™ Reverse transcription kit (Promega Inc., Madison, WI, USA) in a T100TM Thermal Cycler (BioRad Inc., Madrid, Spain). Quantitative Real time PCR (RTqPCR) was performed using Syber Green method and quantified with ΔΔCt method. All samples were run in duplicate and normalized by *36B4*.

Primer sequences used were: *BMP2*, 5′ CAACACTGT CGCAGCTTC 3′ (forward) and 5′ GAAGAATCTCCG GGTTGTTTTC 3′ (reverse); *CD36*, 5′ ATGTGTGTG GAGAGCGTCAACC 3′ (forward) and 5′ TGAGCAGAG TCTTCAGAGACAGCC 3′ (reverse); *36B4*, 5′ CAGGCG TCCTCGTGGAAGTGAC 3′ (forward) 5′ CCAGGT CGCCCTGTCTTCCCT 3′ (reverse).

ELISA assay

BMP2 concentration in serum samples from patients or supernatants from cell culture was measured using an ELISA kit (CSB-E04507h, Cusabio Technology LLC, Hubei, China) following manufacturer's instructions. Absorbance from samples was interpolated to a standard curve using a four parameter logistic (4-PL) equation.

Intracellular lipid content detection by oil red O staining

Cells were cultured in 12 mm coverslips and treated as described above. Then, cells were washed with PBS and stained with an Oil red O (ORO, Merk Millipore, Darmstadt, Germany) working solution (60% ORO/isopropanol) and counter-stained with hematoxylin. Images were taken using an optical microscope Nikon Eclipse E400 (Nikon, Tokyo, Japan) equipped with a plan Apocromatic 20x objective (Nikon). Intensity of red stain was quantified using the FIJI software (NIH, Bethesda, MD).

Statistical analysis

Kolmogorov-Smirnoff test was applied to evaluate if the variables were adjusted or not to a normal distribution. Qualitative variables are presented as relative frequencies. Quantitative variables are expressed as measures of central tendency (mean) and dispersion (standard deviation -SD- for patients' data or standard error of mean -SEM- for experimental data). Data between groups were compared with Student's t test for variables following a normal distribution and Mann-Whitney U test for continuous variables following a non-parametric distribution. Binary and multinomial logistic regression analysis adjusted for confounders were used to test the independence of the associations of dependent variable (NASH) with its significant correlates in univariate models and with those considered of clinical interest. In addition, multivariate regression models were constructed and parameters selected by stepwise method based on likelihood ratio test. Box-Tidwell procedure was used for testing linearity of logit and to obtain a linear logit the appropriate transformation of variables was used. The goodness of fit of the model was evaluated using the Hosmer-Lemeshow statistic and Pearson's Chi-squared test as appropriated. In order to assess diagnostic accuracy of distinct regression models constructed, receiver operating characteristics (ROC) curves and area under the ROC curve (AUROC) were carried out. All statistical analyses were performed using the GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA) and the IBM SPSS Statistics 24.0 (SPSS Inc., IBM, Armonk, NY) software with two-sided tests, with a p value of < 0.05 considered as statistically significant.

Results

Characteristics of the study population

Characteristics of all patients included in this study based on the histological diagnosis are shown in Table 1. Briefly, women were predominant in all the histological groups studied and NAFLD patients were significantly older than NL subjects. As expected, BMI was significantly higher in NASH patients compared to both NL and NAFL groups, and variables regarding glucose metabolism (glucose, insulin, HOMA-IR) and liver enzymes (aspartate aminotransferase -AST-, alanine aminotransferase -AST-, gamma-glutamyltransferase -GGT-) were significantly different between all groups.

Hepatic BMP2 expression is increased in patients with NAFLD

We found that BMP2 gene expression was significantly increased in the liver tissue of NAFLD patients with respect to NL subjects (2-fold, $p < 0.0002$; Fig. 1A). Indeed, when compared with NL, hepatic mRNA levels of BMP2 were significantly higher in patients with NAFL (1.7-fold, $p = 0.0323$) and NASH (2.6-fold, $p < 0.0001$) (Fig. 1B), in parallel with the increase of the liver injury. It was noteworthy that hepatic BMP2 levels were significantly more elevated in NASH than in NAFL patients ($p = 0.0047$). Notably, a significant positive correlation in all study population was observed ($p < 0.0001$, Fig. 1C) between the hepatic mRNA levels of BMP2 and CD36, a fatty acid transporter which contributes to liver fat accumulation in patients with NAFLD [22].

Serum levels of BMP2 are elevated in NAFLD patients

Next, we measured the concentration of BMP2 in serum samples from all NAFLD patients and NL subjects included in our study. Mean circulating BMP2 levels were significantly higher in NAFLD patients than in NL subjects ($p = 0.0033$; Fig. 2A). Notably, serum BMP2 concentrations were more elevated in both NAFL and

Table 1 Characteristics of the study population

	NL ($n = 75$)	NAFL ($n = 56$)	NASH ($n = 59$)
Age (years)	46.58 ± 14.11	52.51 ± 14.15*	55.42 ± 11.1***
Gender (female/male, %)	13.33 / 86.67	33.9 / 66.1	44.07 / 55.93
BMI (kg/m^2)	27.57 ± 5.1	28.46 ± 3.96	34.14 ± 6.56***###
Glucose (mg/dL)	92.6 ± 19.46	99.23 ± 13.92***	119.35 ± 48.5***
Insulin (μU/L)	7.67 ± 4.44	10.06 ± 5.36**	21.38 ± 15.11***###
HOMA-IR	1.83 ± 1.31	2.52 ± 1.53***	6.19 ± 4.92***###
Triglycerides (mg/dL)	122.33 ± 66.78	135.52 ± 56.47*	176.22 ± 120.84***##
Total cholesterol (mg/dL)	191.17 ± 38.48	203.96 ± 39.13	192.44 ± 45.29
AST (IU/L)	17.96 ± 5.69	21.59 ± 8.65*	42.12 ± 25.95***###
ALT (IU/L)	18.52 ± 10.1	27.12 ± 17.27***	54.73 ± 33.02***###
GGT (IU/L)	32.36 ± 29.51	48.82 ± 52.26**	117.24 ± 194.33***###
Steatosis (%)			
Grade 0	100%		8.5%
Grade 1		73.21%	28.8%
Grade 2		17.86%	33.9%
Grade 3		8.93%	28.8%
Ballooning and lobular inflammation (%)			
Grade 0	100%	87.5%	1.85%
Grade 1		12.5%	37.04%
Grade 2			51.85%
Grade 3			9.26%
Fibrosis (%)			
Grade 0	100%	100%	13.56%
Grade 1			35.6%
Grade 2			18.64%
Grade 3			20.34%
Grade 4			11.86%

NL Normal liver, *NAFL* Nonalcoholic fatty liver, *NASH* Nonalcoholic steatohepatitis, *BMI* Body mass index, *HOMA-IR* Homeostatic model assessment-insulin resistance, *AST* Aspartate aminotransferase, *ALT* Alanine aminotransferase, *GGT* Gamma-glutamyltransferase

Data are shown as mean ± SD or as number of cases (%)

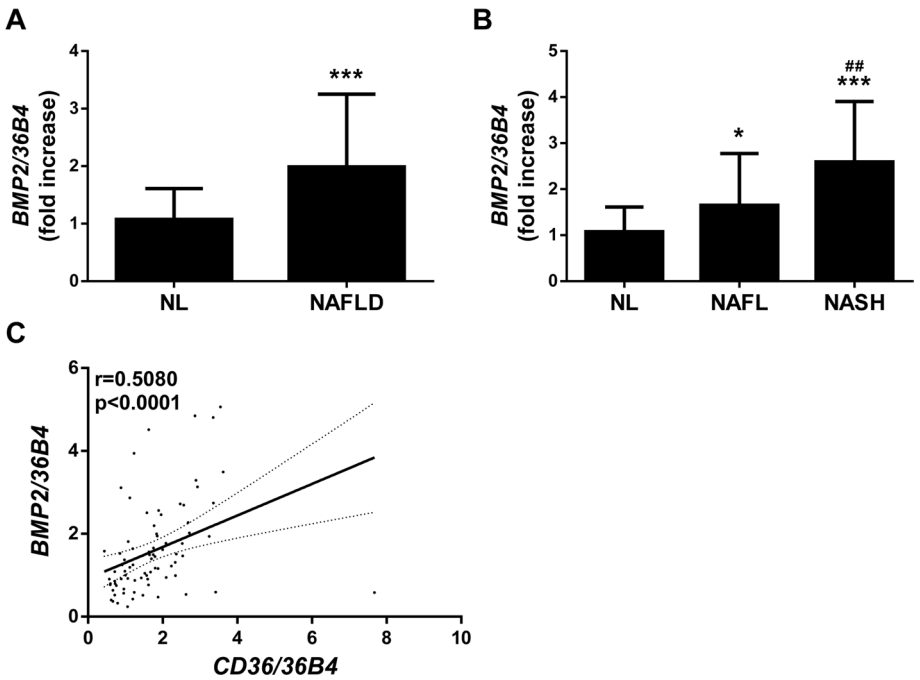

Fig. 1 Expression of BMP2 is increased within the liver of NAFLD patients. **A** and **B** Hepatic *BMP2* mRNA levels determined by RT-qPCR and normalized to *36B4* gene expression. Data are expressed as fold increase relative to control condition [1] and presented as mean ± SD. **C**. Correlation in the study population of matched mRNA expression levels (*BMP2* and *CD36*). Study population: Normal liver (NL) individuals ($n = 75$), NAFLD patients ($n = 115$: 56 NAFL and 59 NASH). *$p < 0.05$ and ***$p < 0.005$, NAFLD, NAFL or NASH vs. NL; $^{\#\#}p < 0.01$, NASH vs. NAFL

NASH patients compared to NL subjects ($p = 0.0552$ and $p = 0.0025$, respectively; Fig. 2B). Interestingly, no significant differences were found between patients with NAFL and NASH. In addition, a significant and progressive increase in serum BMP2 levels was found in NAFLD patients in relation to the histological grade of steatosis ($p = 0.0032$, Fig. 2C) and NAFLD activity score ($p = 0.0081$, Fig. 2D). However, circulating BMP2 levels did not correlate with either BMI ($p = 0.9162$, Fig. 2E) or HOMA-IR ($p = 0.4420$, Fig. 2F), indicating that serum BMP2 levels in patients with NAFLD are not influenced by adiposity or insulin resistant state.

BMP2 expression in human hepatocytes

In order to clarify which liver cells can be the cellular source of BMP2, we performed some in vitro experiments using human hepatocytes (Huh7 cells) treated with different doses of palmitic acid (PA) for 16 hours, a known protocol for inducing intracellular lipid accumulation [23]. After the exposure to PA, results obtained revealed an elevation of intracellular lipid content (Fig. 3A) in parallel to an increase of mRNA levels of BMP2 in Huh7 hepatocytes (Fig. 3B). Moreover, an increase of supernatant BMP2 concentration was also observed upon PA treatment (Fig. 3C).

Circulating BMP2 is an independent predictor of NASH and the algorithm SAN is able to discriminate NASH

Since serum BMP2 levels were significantly higher in NASH than in NL subjects, we performed logistic regression analysis in the entire study population in order to determine whether serum BMP2 levels are associated with NASH. In order to find the best sensitivity and specificity threshold for NASH diagnosis, the best cut-off value for serum BMP2 concentrations was established by using the Youden index at 49.21 pg/mL. Circulating BMP2 was identified as an independent predictor of NASH in univariate analysis (OR [95% CI], 3.5 [1.47–8.37], $p = 0.005$; Table 2). Multivariate analysis, adjusted for potential confounders (age, gender and BMI) and variables clinically relevant to NAFLD (glucose and GGT) showed that serum BMP2 concentrations persisted significantly associated with NASH (OR [95% CI], 5.07 [1.55–16.6], $p = 0.007$; Table 2).

Thus, we performed ROC curve analysis to determine the accuracy of the algorithm termed SAN (Screening Algorithm for NASH), based in a formula derived from the previously mentioned logistic regression model (Fig. 4), which revealed a good accuracy to discriminate NASH (AUROC, 0.886). Next, 0.1 unit intervals of SAN were analyzed to establish best cut off points (Table 3). A SAN ≤ 0.2 can be used to exclude NASH (SN = 0.84;

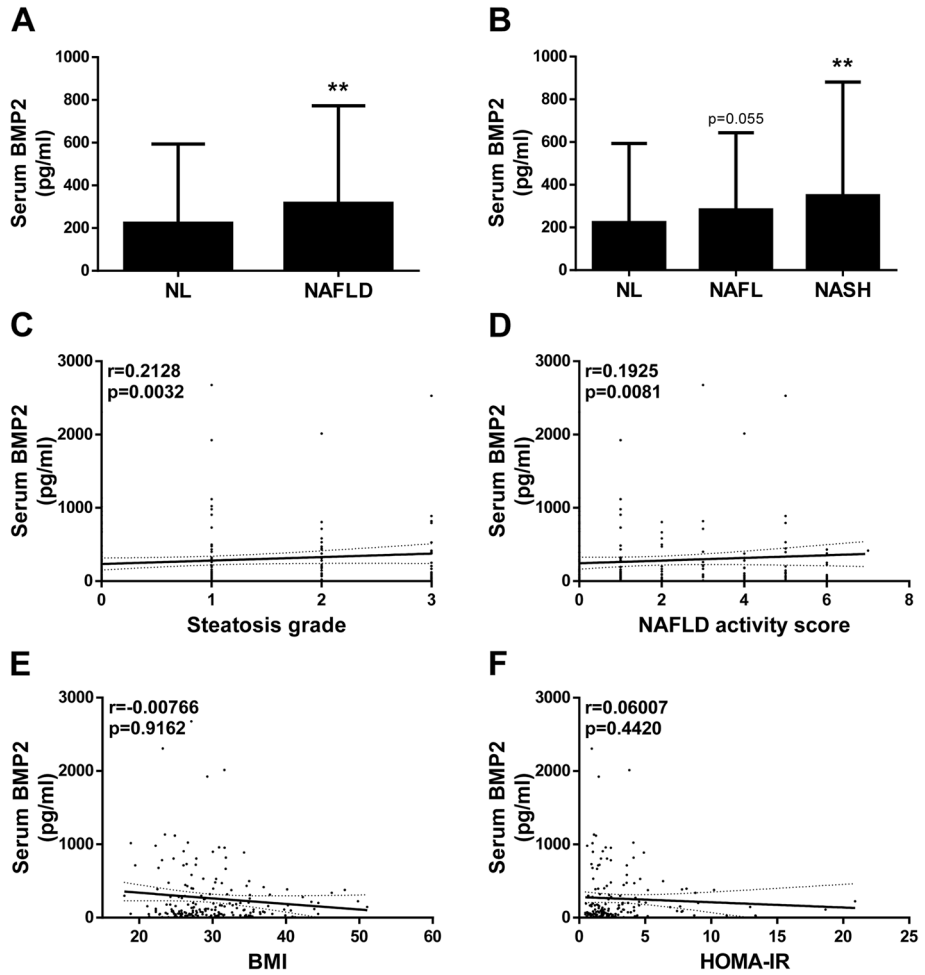

Fig. 2 Serum levels of BMP2 are increased in NAFLD patients. **A** and **B** Serum levels of BMP2 determined by ELISA. Data are expressed as pg/ml and presented as mean ± SD. **C**. Correlation in the study population of matched serum BMP2 levels with the steatosis grade. **D** Correlation in the study population of matched serum BMP2 levels with the NAFLD activity score. **E** Correlation in the study population of matched serum BMP2 levels with the BMI. **F** Correlation in the study population of matched serum BMP2 levels with the HOMA-IR. Study population: Normal liver (NL) individuals ($n = 75$), NAFLD patients ($n = 115$: 56 NAFL and 59 NASH). $^{**}p < 0.01$, NAFLD, or NASH vs. NL

NPV = 0.909) and a SAN ≥ 0.6 to rule it in (SP = 0.96; PPV = 0.86).

Discussion

Non-alcoholic fatty liver disease (NAFLD), is the major cause of chronic liver disease worldwide, with a diverse histopathological spectrum ranging from simple steatosis without significant inflammation, also termed fatty liver (NAFL), to steatohepatitis (NASH) with varying stages of fibrosis and, ultimately, cirrhosis and hepatocellular carcinoma [24].

Given that NAFLD is usually asymptomatic at the time of diagnosis, the determination of the lipid content within the liver is an important clinical challenge in terms of early identification to allow appropriate therapeutic interventions in order to prevent the progression of liver disease to more advanced fibrotic states or, even, to the end-stage liver disease. In the last decade, there has been an explosion in the discovery of biomarkers for NAFLD diagnosis and/or prognosis. Considering the wide range of their potential applications in clinical practice, the growing interest of the academic and industrial community in biomarkers is not surprising. Indeed, lack of awareness about risk factors for NAFLD and its progression, combined with insufficient or unreliable screening and surveillance modalities, may contribute to a delay in diagnosis and may explain why many patients are diagnosed in the later stages of the disease [7].

A novel finding of the present study is that BMP2 expression is increased in the liver from NAFLD

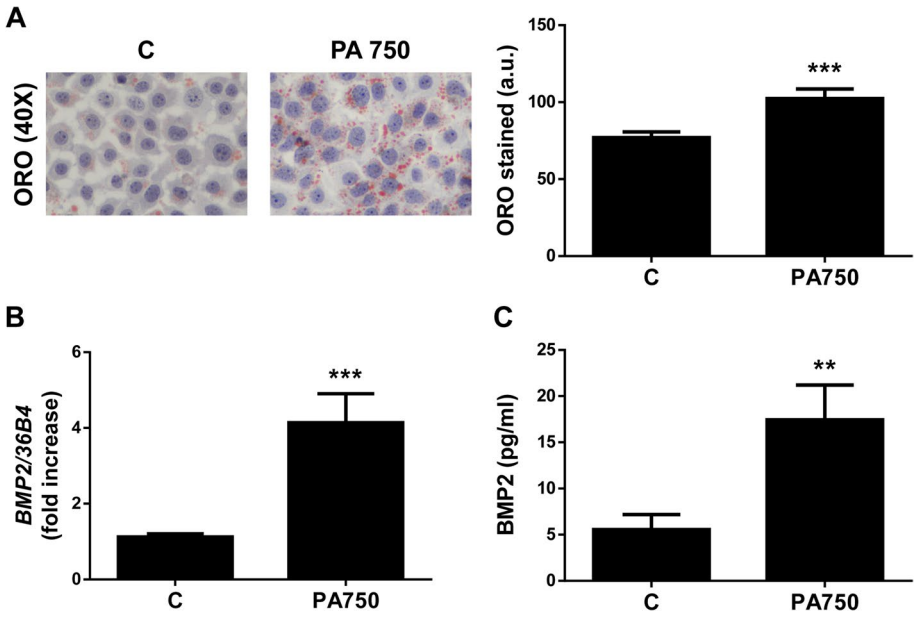

Fig. 3 Palmitic acid overload upregulates mRNA levels of BMP2 in Huh7 hepatocytes. **A** Representative images of Oil Red O (ORO) staining and its quantification. **B** Hepatic *BMP2* mRNA levels were determined by RT-qPCR and normalized to *36B4* gene expression. Data are expressed as fold increase and presented as mean ± SEM relative to control condition [1]. **C** BMP2 levels in the cell supernatant determined by ELISA. Data are expressed as pg/ml and presented as mean ± SEM. Experimental conditions: Huh7 cells treated with 750 µM palmitic acid (PA750) for 16 h ($n = 3$ independent experiments performed by duplicate). $^{**}p < 0.01$ and $^{***}p < 0.005$, PA750 vs. Control (C)

Table 2 Univariate and multivariate analysis of the independent variables associated with NASH in the study population

Independent variables	Univariate analysis			Multivariate analysis		
	OR	95% CI	*p* value	OR	95% CI	*p* value
BMP2 (pg/ml) Cut-off = 49.21	3.5	[1.47–8.37]	0.005	5.07	[1.55–16.6]	0.007
Gender (female/male)	2.77	[1.43–5.36]	0.002	2.96	[1.22–7.14]	0.016
Age (years)	1.04	[1.01–1.06]	0.004	1.03	[0.99–1.06]	0.06
BMI (kg/m^2)	1.22	[1.14–1.31]	< 0.001	1.28	[1.16–1.41]	< 0.001
Glucose (mg/dL)	1.03	[1.01–1.04]	< 0.001	1.02	[1–1.04]	0.018
GGT (IU/L)	1.01	[1.01–1.02]	0.001	1.01	[1–1.02]	0.016

OR Odds ratio, *CI* Confidence interval, *BMI* Body mass index, *GGT* Gamma-glutamyltransferase

patients as well as that serum BMP2 levels were also higher in these patients than in subjects with histologically normal liver, positioning this protein as a new molecular target linked to NAFLD. One limitation of this study is the limited number of individuals taking into account that women were predominant in all the histological groups studied, especially within patients with histological NL, and NAFLD patients were significantly older than NL subjects. In fact, BMP2 expression might be affected by age and sex. However, no correlation was found between circulating BMP2 levels and age ($r = -0.05484$ $p = 0.4524$), and there were no differences when separating the population by sex ($p = 0.2035$). In addition, we have also shown herein that human hepatocytes are able to produce and secrete BMP2 upon stimuli with PA mimicking steatotic conditions [23], indicating that BMP2 might be involved in hepatosteatosis development, not only in the fibrogenic process as some studies have previously reported [25, 26].

Interestingly, there are only two studies so far showing that the expression of some BMPs, specifically BMP6 and BMP8B, is increased in the liver of NAFLD patients [13, 27]. These studies, along with our findings, indicate that BMPs might play a role in NAFLD pathophysiology. In this regard, it has been demonstrated that the

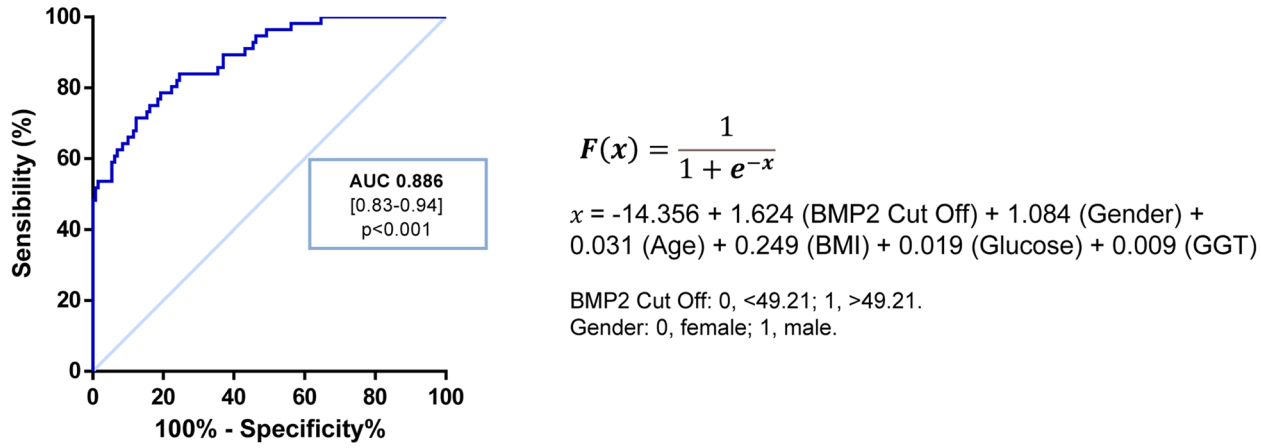

Fig. 4 Receiver operating characteristic (ROC) curve showing diagnostic accuracy of SAN algorithm for detecting NASH. AUROC, area under the ROC curve; BMI, body mass index; GGT, gamma-glutamyltransferase

Table 3 Diagnostic accuracy of SAN

SAN cut point	Accuracy	%	SN	SP	PPV	NPV	LR+	LR-
≥ 0.10	0.66	60.2	0.93	0.54	0.46	0.95	2.02	0.13
≥ 0.20	**0.71**	**48.9**	**0.84**	**0.66**	**0.52**	**0.90**	**2.47**	**0.24**
≥ 0.30	0.80	36.6	0.77	0.81	0.63	0.89	4.05	0.28
≥ 0.40	0.81	32.8	0.73	0.85	0.67	0.88	4.87	0.32
≥ 0.50	0.83	24.2	0.62	0.92	0.78	0.85	7.75	0.41
≥ 0.60	**0.83**	**18.8**	**0.54**	**0.96**	**0.86**	**0.83**	**13.5**	**0.48**
≥ 0.70	0.84	15.1	0.48	0.99	0.96	0.82	48.0	0.53
≥ 0.80	0.82	12.4	0.41	1.00	1.00	0.80	N/A	0.59
≥ 0.90	0.78	8.1	0.27	1.00	1.00	0.76	N/A	0.73

SAN Screening Algorithm for NASH, *SN* Sensitivity, *SP* Specificity, *PPV* Positive predictive value, *NPV* Negative predictive value, LR+, positive likelihood ratio; LR-, negative likelihood ratio

pharmacological inhibition of BMP signaling reduced diet-induced hepatic steatosis in obese mice by targeting the production of triglycerides in the liver [18]; but also, this study has also shown that this effect is specific for the inhibition of BMP type I receptor ALK3, proposing a role of BMP2 and BMP4 in the establishment of NAFLD [18].

Going further, our study is the first which reports circulating BMP2 concentrations in NAFLD patients. Other authors have previously reported that serum levels of distinct BMPs varied widely in the context of other metabolic disorders. For instance, elevated serum BMP4 levels have been correlated with carotid atherosclerosis in T2D patients [28]. Conversely, various studies have associated BMP9 with glucose and lipid metabolism because a decrease in the circulating concentrations of BMP9 have been observed in patients who displayed features of metabolic syndrome such as insulin resistance, T2D or arterial hypertension [29–31]. BMP9 has also been related to other diseases, such as portopulmonary hypertension, with lowered levels of this protein acting as a potential risk factor [32]. Regarding liver diseases, while BMP7 has been proposed as a marker for hepatic carcinogenesis because its serum levels were found to be decreased in patients with liver cancer [33], other authors have reported that circulating BMP7 levels are augmented in patients with viral chronic liver diseases [34]. Considering BMP2 reports, some studies have associated increased serum levels of this BMP with coronary artery disease in diabetic patients [35]. Moreover, an elevation of serum BMP2 concentrations was observed in overweight and obese middle-aged and elderly women [36], whereas in the present study, a correlation between circulating levels of BMP2 and BMI was not observed in agreement with results recently reported by Lopes et al. [37]. Nevertheless, further investigations are warranted to elucidate the role of BMP2 regulating glucose and lipid homeostasis and its impact in the pathogenesis of metabolic diseases such as obesity, diabetes and NAFLD.

One of the most striking findings of the present study is that serum BMP2 levels were significantly associated with biopsy-proven NASH in multivariate logistic regression model, suggesting that BMP2 might have utility as biomarker to screen NASH in different clinical settings. Using serum BMP2 levels combined with age, gender, BMI, glucose and GGT, an algorithm termed SAN (Screening Algorithm for NASH) was derived from a logistic regression model which discriminated NASH patients from subjects with histologically normal liver or NAFL. This is a breakthrough because there are no validated non-invasive tools for predicting NASH so far, the most clinically relevant form of NAFLD which can only be diagnosed by liver biopsy. In our population, a value of SAN ≥ 0.6 indicated a high risk for biopsy-proven NASH with a good accuracy (AUROC, 0.86; SP, 0.96) showing that SAN may be useful to screen populations at risk for NAFLD.

Conclusion

In conclusion, this proof-of-concept study provides the first scientific evidence that hepatic and serum levels of BMP2 are abnormally elevated in NAFLD patients, indicating that BMP2 is a new molecular target linked to human NAFLD. In addition, we describe a simple and efficient algorithm termed SAN which was able to discriminate NASH with a good accuracy in our study population. Further studies, however, in distinct and larger cohorts of NAFLD patients are needed to validate the potential utility of SAN as a non-invasive algorithm for NAFLD evaluation.

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Authors' contributions
AGR conceived and supervised the study. CGM, JRdC, JA and MRG carried out and analyzed the clinical parameters. PM, ER, RGD and RMV were involved in data generation. PM, CEFG, CGM and AGR analyzed and discussed data. PM, AGR and CGM prepared the manuscript. All authors critically revised and approved the manuscript.

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Availability of data and materials
Authors declared that all and the other data supporting the findings of this study are available within the paper. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was performed in agreement with the Declaration of Helsinki, and with local and national laws. The Human Ethics Committee of the Hospital Universitario Santa Cristina (reference, PI-688A) and Hospital Universitario Virgen del Rocío (reference, C.I. 0359-N-15) approved the study procedures, and all participants signed an informed written consent before inclusion in the study.

Consent for publication
Not applicable.

Competing interests
All authors have declared that no competing interest exists.

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References

1. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism. 2016;65(8):1038–48 PubMed PMID: 26823198.
2. Goh GB, McCullough AJ. Natural history of nonalcoholic fatty liver disease. Dig Dis Sci. 2016;61(5):1226–33 PubMed PMID: 27003142.
3. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64(1):73–84 PubMed PMID: 26707365.
4. Younossi ZM, Stepanova M, Younossi Y, Golabi P, Mishra A, Rafiq N, et al. Epidemiology of chronic liver diseases in the USA in the past three decades. Gut. 2020;69(3):564–8 PubMed PMID: 31366455.
5. Svegliati-Baroni G, Ridolfi F, Di Sario A, Casini A, Marucci L, Gaggiotti G, et al. Insulin and insulin-like growth factor-1 stimulate proliferation and type I collagen accumulation by human hepatic stellate cells: differential effects on signal transduction pathways. Hepatology. 1999;29(6):1743–51 PubMed PMID: 10347117. Epub 1999/05/29. eng.
6. Sokol RJ, McKim JM Jr, Goff MC, Ruyle SZ, Devereaux MW, Han D, et al. Vitamin E reduces oxidant injury to mitochondria and the hepatotoxicity of taurochenodeoxycholic acid in the rat. Gastroenterology. 1998;114(1):164–74 PubMed PMID: 9428230.
7. Lindenmeyer CC, McCullough AJ. The natural history of nonalcoholic fatty liver disease-an evolving view. Clin Liver Dis. 2018;22(1):11–21 PubMed PMID: 29128051. Pubmed Central PMCID: PMC6130315.
8. Castera L, Friedrich-Rust M, Loomba R. Noninvasive assessment of liver disease in patients with nonalcoholic fatty liver disease. Gastroenterology. 2019;156(5):1264–81 e4 PubMed PMID: 30660725. Pubmed Central PMCID: 7505052.
9. Arthur MJ, Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. Am J Physiol Gastrointest Liver Physiol. 2000;279(2):G245–9 PubMed PMID: 10915630.
10. Carreira AC, Alves GG, Zambuzzi WF, Sogayar MC, Granjeiro JM. Bone morphogenetic proteins: structure, biological function and therapeutic applications. Arch Biochem Biophys. 2014;561:64–73 PubMed PMID: 25043976.
11. Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. J Biochem. 2010;147(1):35–51 PubMed PMID: 19762341.
12. Herrera B, Sanchez A, Fabregat I. BMPS and liver: more questions than answers. Curr Pharm Des. 2012;18(27):4114–25 PubMed PMID: 22630083.
13. Arndt S, Wacker E, Dorn C, Koch A, Saugspier M, Thasler WE, et al. Enhanced expression of BMP6 inhibits hepatic fibrosis in non-alcoholic fatty liver disease. Gut. 2015;64(6):973–81 PubMed PMID: 25011936.

14. Mahli A, Seitz T, Beckroge T, Freese K, Thasler WE, Benkert M, et al. Bone morphogenetic protein-8B expression is induced in Steatotic hepatocytes and promotes hepatic steatosis and inflammation in vitro. Cells. 2019;8(5):457. PubMed PMID: 31096638. Pubmed Central PMCID: 6562647.
15. Peng Q, Chen B, Wang H, Zhu Y, Wu J, Luo Y, et al. Bone morphogenetic protein 4 (BMP4) alleviates hepatic steatosis by increasing hepatic lipid turnover and inhibiting the mTORC1 signaling axis in hepatocytes. Aging (Albany NY). 2019;11(23):11520–40 PubMed PMID: 31831718. Pubmed Central PMCID: 6932923.
16. Platko K, Lebeau PF, Byun JH, Poon SV, Day EA, MacDonald ME, et al. GDF10 blocks hepatic PPARgamma activation to protect against diet-induced liver injury. Mol Metab. 2019;27:62–74 PubMed PMID: 31288993. Pubmed Central PMCID: 6717799.
17. Yang M, Liang Z, Yang M, Jia Y, Yang G, He Y, et al. Role of bone morphogenetic protein-9 in the regulation of glucose and lipid metabolism. FASEB J. 2019;33(9):10077–88 PubMed PMID: 31237775.
18. Thayer TE, Lino Cardenas CL, Martyn T, Nicholson CJ, Traeger L, Wunderer F, et al. The role of bone morphogenetic protein signaling in non-alcoholic fatty liver disease. Sci Rep. 2020;10(1):9831 PubMed PMID: 32561790. Pubmed Central PMCID: 7305229.
19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28(7):412–9 PubMed PMID: 3899825. eng.
20. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94(9):2467–74 PubMed PMID: 10484010. Epub 1999/09/14. eng.
21. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313–21 PubMed PMID: 15915461. Epub 2005/05/26. eng.
22. Miquilena-Colina ME, Lima-Cabello E, Sanchez-Campos S, Garcia-Mediavilla MV, Fernandez-Bermejo M, Lozano-Rodriguez T, et al. Hepatic fatty acid translocase CD36 upregulation is associated with insulin resistance, hyperinsulinaemia and increased steatosis in non-alcoholic steatohepatitis and chronic hepatitis C. Gut. 2011;60(10):1394–402 PubMed PMID: 21270117. Epub 2011/01/29.
23. Gonzalez-Rodriguez A, Mayoral R, Agra N, Valdecantos MP, Pardo V, Miquilena-Colina ME, et al. Impaired autophagic flux is associated with increased endoplasmic reticulum stress during the development of NAFLD. Cell Death Dis. 2014;5:e1179 PubMed PMID: 24743734. Pubmed Central PMCID: 4001315. Epub 2014/04/20. eng.
24. Brunt EM, Kleiner DE, Carpenter DH, Rinella M, Harrison SA, Loomba R, et al. NAFLD: reporting histologic findings in clinical practice. Hepatology. 2021;73(5):2028–38 PubMed PMID: 33111374.
25. Chung YH, Huang YH, Chu TH, Chen CL, Lin PR, Huang SC, et al. BMP-2 restoration aids in recovery from liver fibrosis by attenuating TGF-beta1 signaling. Lab Investig. 2018;98(8):999–1013 PubMed PMID: 29789683.
26. Shen H, Huang GJ, Gong YW. Effect of transforming growth factor beta and bone morphogenetic proteins on rat hepatic stellate cell proliferation and trans-differentiation. World J Gastroenterol. 2003;9(4):784–7 PubMed PMID: 12679932. Pubmed Central PMCID: PMC4611450. Epub 2003/04/08.
27. Sanz S, Pucilowska JB, Liu S, Rodriguez-Ortigosa CM, Lund PK, Brenner DA, et al. Expression of insulin-like growth factor I by activated hepatic stellate cells reduces fibrogenesis and enhances regeneration after liver injury. Gut. 2005;54(1):134–41 PubMed PMID: 15591519. Pubmed Central PMCID: 1774353.
28. Son JW, Jang EH, Kim MK, Baek KH, Song KH, Yoon KH, et al. Serum BMP-4 levels in relation to arterial stiffness and carotid atherosclerosis in patients with type 2 diabetes. Biomark Med. 2011;5(6):827–35 PubMed PMID: 22103619.
29. Huang H, Wang W, Yang G, Zhang Y, Li X, Liu H, et al. Circulating bone morphogenetic protein-9 levels are associated with hypertension and insulin resistance in humans. J Am Soc Hypertens. 2018;12(5):372–80 PubMed PMID: 29550458.
30. Luo Y, Li L, Xu X, Wu T, Yang M, Zhang C, et al. Decreased circulating BMP-9 levels in patients with type 2 diabetes is a signature of insulin resistance. Clin Sci. 2017;131(3):239–46 PubMed PMID: 27940998.
31. Xu X, Li X, Yang G, Li L, Hu W, Zhang L, et al. Circulating bone morphogenetic protein-9 in relation to metabolic syndrome and insulin resistance. Sci Rep. 2017;7(1):17529 PubMed PMID: 29235531. Pubmed Central PMCID: 5727514.
32. Nikolic I, Yung LM, Yang P, Malhotra R, Paskin-Flerlage SD, Dinter T, et al. Bone morphogenetic protein 9 is a mechanistic biomarker of Portopulmonary hypertension. Am J Respir Crit Care Med. 2019;199(7):891–902 PubMed PMID: 30312106. Pubmed Central PMCID: 6444661.
33. Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol. 2007;8(12):970–82 PubMed PMID: 18000526.
34. Tacke F, Gabele E, Bataille F, Schwabe RF, Hellerbrand C, Klebl F, et al. Bone morphogenetic protein 7 is elevated in patients with chronic liver disease and exerts fibrogenic effects on human hepatic stellate cells. Dig Dis Sci. 2007;52(12):3404–15 PubMed PMID: 17415633.
35. Zhang M, Sara JD, Wang FL, Liu LP, Su LX, Zhe J, et al. Increased plasma BMP-2 levels are associated with atherosclerosis burden and coronary calcification in type 2 diabetic patients. Cardiovasc Diabetol. 2015;14:64 PubMed PMID: 26003174. Pubmed Central PMCID: 4450848.
36. Ribeiro S, Lopes LR, Paula Costa G, Figueiredo VP, Shrestha D, Batista AP, et al. CXCL-16, IL-17, and bone morphogenetic protein 2 (BMP-2) are associated with overweight and obesity conditions in middle-aged and elderly women. Immun Ageing. 2017;14:6 PubMed PMID: 28293269. Pubmed Central PMCID: PMC5346187. Epub 2017/03/16.
37. Lopes LR, Ribeiro S, Figueiredo VP, Leite ALJ, Nicolato RLC, Gomes JAE, et al. The overweight increases circulating inflammatory mediators commonly associated with obesity in young individuals. Cytokine. 2018;110:169–73 PubMed PMID: 29763838. Epub 2018/05/16.

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