Deregulation of Secreted Frizzled-Related Protein 5 in Nonalcoholic Fatty Liver Disease Associated with Obesity

FINAL DEGREE PROJECT

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1. ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. NAFLD spectrum ranges from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH). The mechanisms underlying the development and progression of NAFLD are beginning to be understood. Secreted frizzled-related protein 5 (SFRP5) is an antagonist of noncanonical Wingless-MMTV Integration Site (WNT) pathway, which promotes liver damage; however, SFRP5 role in liver diseases is not clear. The aim of this study was to clarify the involvement of SFRP5 and the noncanonical WNT pathway in NAFLD pathogenesis. Plasma SFRP5 levels were determined by ELISA in women with normal weight (NW; n=20) and morbid obesity (MO; n=69). All women with MO were subclassified according to hepatic histology into normal liver (NL; n=28) and NAFLD (n=41) groups. NAFLD group included 24 women with SS and 17 women with NASH. We used RT-qPCR to evaluate hepatic and jejunal mRNA abundance of SFRP5 and hepatic expression of WNT family member 5a (WNT5A) and Jun N-Terminal Kinase (JNK). Circulating SFRP5 levels were lower in NW than in MO, that undergone a very low-calorie diet before the surgery. Hepatic SFRP5 was higher in SS than in NL or NASH; moreover, patients with hepatic ballooning and inflammation presented lower expression of SFRP5. Jejunal SFRP5 expression did not show differences between groups but were correlated positively with PPARγ and negatively with TLR2. WNT5A and JNK hepatic expressions were enhanced in NAFLD than in NL. In conclusion, we reported that circulating SFRP5 levels increase after a caloric restriction in subjects with MO regardless of NAFLD. However, hepatic SFRP5 seems to have a protective role in the first steps of NAFLD trying to inhibit noncanonical WNT pathway, but in an advanced stage of the disease it is deregulated. Furthermore, jejunal SFRP5 seems to also have a protective role against metabolic dysfunction.

Keywords: SFRP5; NAFLD; inflammation; liver; noncanonical WNT pathway.

2. ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
DBP	Diastolic blood pressure
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGT	Gamma-glutamyltransferase
HbA1c	Glycosylated haemoglobin
HCC	Hepatocellular carcinoma
HDL-C	High density lipoprotein cholesterol
HOMA2-IR	Homeostasis model 2 assessment of IR
HSC	Hepatic stellate cells
IL	Interleukin
IR	Insulin resistance
JNK	Jun N-Terminal Kinase
LDL-C	Low density lipoprotein cholesterol
MO	Morbid obesity
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NL	Normal liver
NW	Normal weight
PPAR	Peroxisome proliferator-activated receptor
SBP	Systolic blood pressure
SFRP5	Secreted frizzled-related protein 5
SS	Simple steatosis
T2DM	Type 2 diabetes mellitus
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
WNT	Wingless-MMTV Integration Site
WNT5A	WNT family member 5a

3. BACKGROUND

3.1 Nonalcoholic fatty liver disease

3.1.1 Definition of nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) has emerged as the most common cause of chronic liver disease [1]. The estimated prevalence of NAFLD worldwide is approximately 25% in adults and 70-90% in adults with obesity. NAFLD is defined by the presence of intrahepatic fat in the absence of secondary cause of hepatic fat accumulation, such as significant alcohol consumption, use of steatogenic medication or other causes of concomitant liver disease [2]. NAFLD is the consequence of excessive accumulation of triglycerides in hepatocytes (>5%) to compensate for the increased cellular content of non-esterified fatty acids (free fatty acids), which can increase endoplasmic reticulum (ER) stress [3,4].

NAFLD is considered a metabolic disorder associated with insulin resistance (IR), type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia [5], and obesity, which is one of the major risk factors [6,7].

The underlying precise mechanisms of disease pathogenesis have just begun to be understood. The current hypothesis is based on a ‘‘parallel, multiple-hit model’’, in which various factors that act in parallel, such as obesity, sedentary lifestyle, high fat diet, IR, hepatic lipid accumulation and gut microbiota, lead to the development of hepatic inflammation and fibrosis [8]. Moreover, progression to NASH is linked to systemic inflammation and it is associated with other pathological processes, like innate immunity alterations, ER stress, intestinal dysbiosis and toll-like receptors (TLRs) activation [9–12].

3.1.2 Classification

NAFLD comprises a variety of hepatic conditions (Figure 1) ranging from nonalcoholic fatty liver or simple steatosis (SS), a relatively benign condition with a low risk of progression; and nonalcoholic steatohepatitis (NASH), which is considered the severe form of NAFLD. NASH is confirmed when the hepatic tissue shows perilobular inflammation, hepatocellular ballooning, Mallory's hyaline, and acidophil bodies with or without fibrosis [8,13,14]. If NASH left untreated, can evolve into liver cirrhosis and hepatocellular carcinoma (HCC) [15].

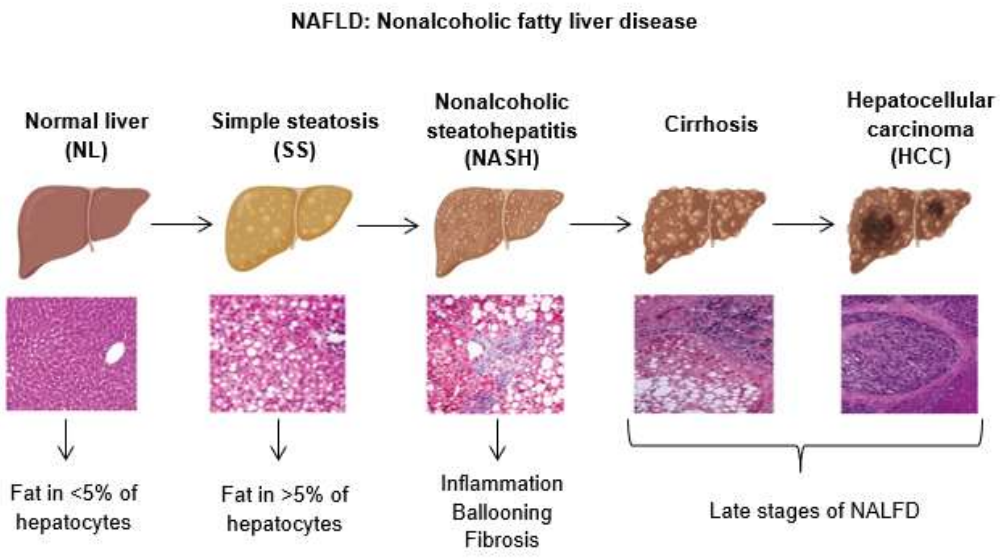

Figure 1: Classification and stages of NAFLD. NAFLD comprises a variety of hepatic conditions ranging from SS to NASH [8,13,14]. Moreover, NASH can evolve to cirrhosis and HCC [15]. *Source: Self-made.*

Almost one third of NAFLD evolves to NASH [3,16] and approximately 30–50% of NASH patients may progress to cirrhosis within 10 years [17].

The development of liver fibrosis is most strongly associated with morbidity; therefore, the population in greatest need of therapeutic interventions is those with NASH and significant fibrosis [18,19]. At this point, liver transplantation may become essential to avoid the death of the patient.

3.1.3 Diagnostic and treatment

The most common noninvasives tools used in clinical practice to detect NAFLD are imaging studies and serological tests [20]. However, liver biopsy has still been the “gold standard” diagnostic method, but it has well-known limitations, including invasiveness, rare but potentially life-threatening complications, sampling variability and high cost [13,21].

Different diagnostic methods used to detect NAFLD were graphically represented in Figure 2.

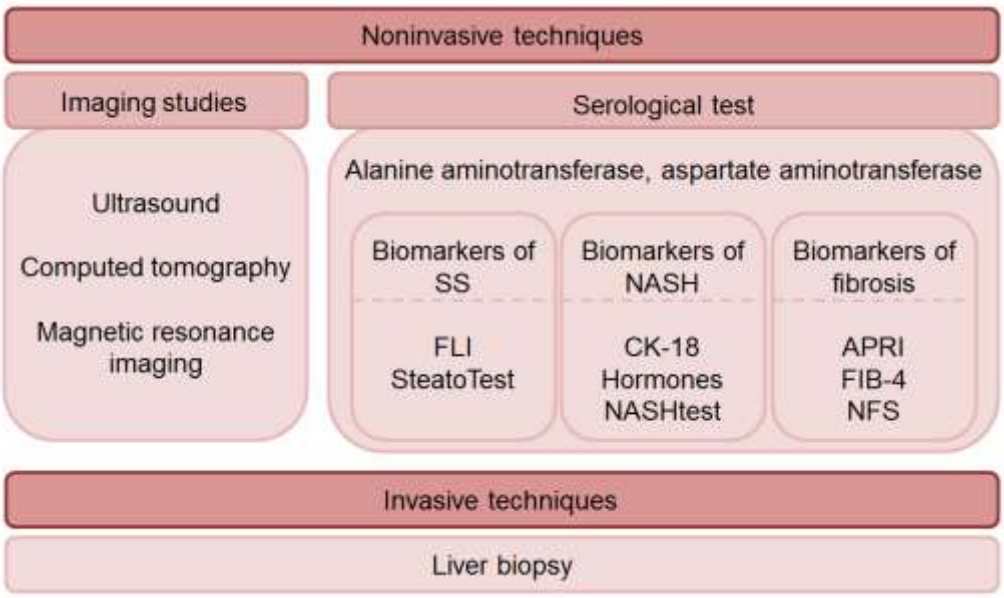

Figure 2: **Main techniques for the diagnosis of NAFLD**. Whereas NAFL can be diagnosed by imaging studies such as ultrasound, computed tomography, or magnetic resonance imaging [22], the diagnostic of NASH still requires liver biopsy [20]. Aspartate aminotransferase (AST)-alanine aminotransferase ratio (ALT) can be used as a first step to detect NAFLD. There are some specifics biomarkers of different states of NAFLD such as CK-18 or APRI [23–26]. NAFL; nonalcoholic fatty liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; FLI, fatty liver index; CK-18, cytokeratin-18; APRI, AST-to platelet ratio index; FIB-4, fibrosis-4 index; NFS, NAFLD fibrosis score. *Source: Self-made.*

There is no fully effective, specific treatment approved by FDA (U.S. Food and Drug Administration) for NAFLD. The main goals of treatment are to improve steatosis and to prevent progression of the disease. Lifestyle modification and specific treatments for NAFLD risk factors (obesity, T2DM, hypertension…) are usually used for the disease management. Some of current and new strategies (still under study) to treat NAFLD are shown in Figure 3 [2,23].

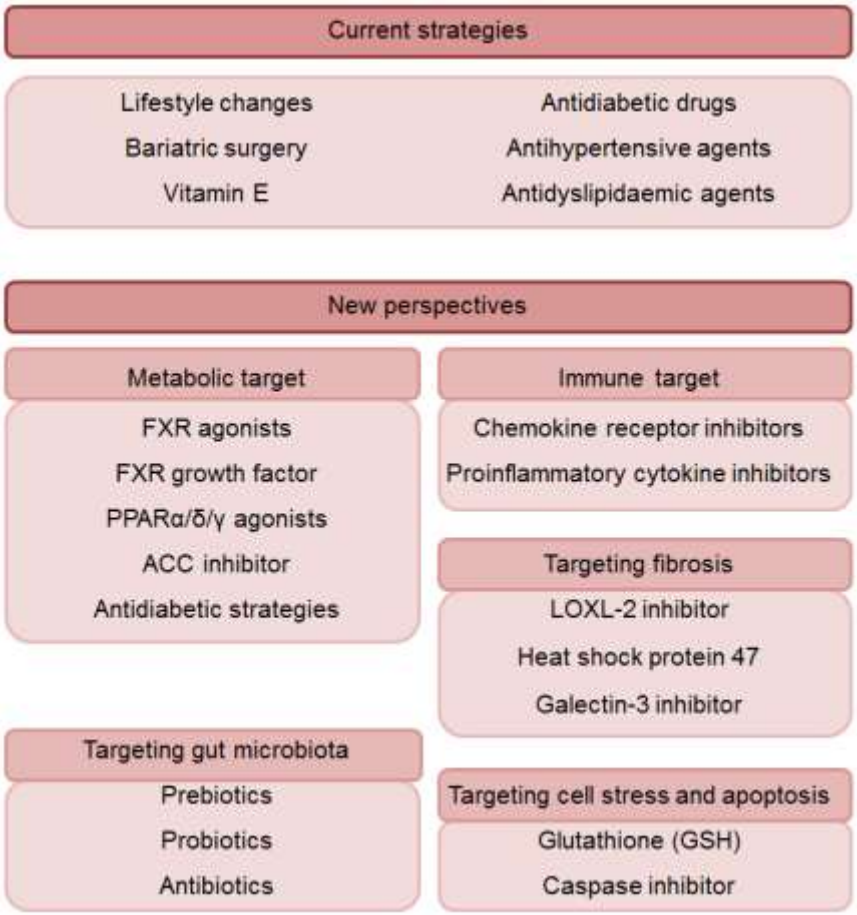

Figure 3: **Therapeutic strategies for the treatment of NAFLD.** FXR, farnesoid X receptor; PPAR, peroxisome proliferator-activated receptor; ACC, acetyl-CoA carboxylase; LOXL-2, Lysyl Oxidase Like 2. *Source: Self-made.*

3.2 Secreted frizzled-related protein 5

3.2.1 Secreted frizzled-related protein 5

Secreted frizzled-related protein 5 (SFRP5) is a secreted glycoprotein produced by adipose tissue [27]. SFRP5 is member of the SFRP family and it is an anti-inflammatory adipocytokine that contains a domain [28] which exhibit a close homology with the frizzled cysteine-rich domain, allowing SFRP5 to block the Wingless-MMTV Integration Site (WNT) signalling pathway [27].

3.2.2 SFRP5 signalling in WNT pathway and its implication in NAFLD

SFRP proteins play key roles inhibiting WNT signalling pathway as shown Figure 4. WNTs comprise a family of 19 secreted glycoproteins that act as extracellular signalling molecules and activate target cells to regulate cell proliferation, differentiation and migration during development and homeostasis [29,30].

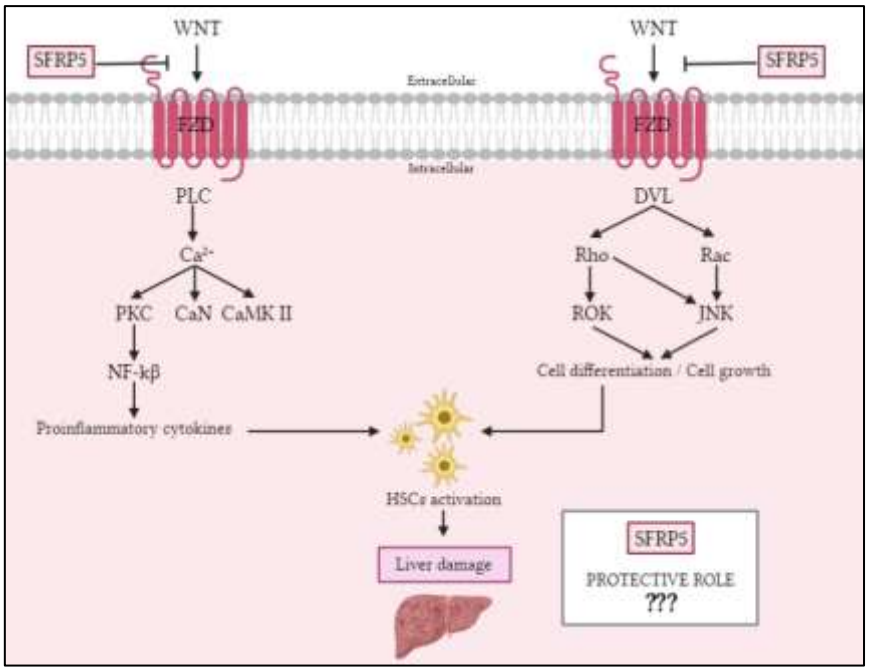

Figure 4. Noncanonical WNT signalling pathway. WNT signalling pathway activates proteins which promote the synthesis of proinflammatory cytokines and cell differentiation. These two processes cause the activation of HSCs, what will cause liver damage if the stimulation persists. However, SFRP5 is able to inhibit WNT signalling pathway suggesting its protective role against liver damage. PLC, phospholipase C; Ca2+, calcium 2+; PKC, protein kinase C; CaN, serine/threonine-protein phosphatase; CaMK II, Ca2+/calmodulin-dependent protein kinase II; NF-kβ, nuclear factor kappa-light-chain-enhancer of activated B cells; DVL, disheveled; ROK, rho-associated protein kinase; JNK, Jun N-terminal kinase; Wnt, Wingless-MMTV Integration Site; Sfrp5, Secreted frizzled-related protein 5; FZD, frizzled receptor; HSC, hepatic stellate cells. *Source: Self-made.*

In WNT signalling pathway there is a canonical and non-canonical pathway. It has been shown that WNT signalling is activated during liver fibrosis, and some elements of the WNT signalling pathway are upregulated and implicated in the process. Although little is known about the role of WNT signalling pathway in liver fibrosis, it seems to play crucial roles in the control of cell growth and liver diseases, such as NASH and HCC [29].

SFRP5 has been shown to act as an antagonist for WNT noncanonical signalling pathway, which involves Jun N-terminal kinase (JNK), through binding with

extracellular WNT or sequestering it [27,30,31]. Moreover, previous studies have shown that SFRP5 is capable of promoting adipocyte differentiation [27] so, it seems to be involved in the regulation of lipid metabolism, but its specific mechanism needs further study [32].

WNT, via JNK, promotes cell differentiation and growth and, it triggers the activation of hepatic stellate cells (HSCs), which are undifferentiated cells that play an important role in liver regeneration. Reported evidence confirms that activated HSCs induce the occurrence and development of liver fibrosis [33].

3.2.3 SFRP5 and metabolic diseases

Some recent studies in mice, have shown that SFRP5 expression is downregulated in models with obesity and T2DM [31] and mRNA was decreased in the liver mice fed a high fat diet compared to those with a standard diet [32]. It seems that SFRP5 is a protective adipokine for glucose tolerance, hepatic steatosis and fibrosis and its anti-inflammatory role is perturbed in models of obesity and T2DM [32,34].

However, the role of SFRP5 in human is not clear. Recent study found no differences in SFRP5 serum concentrations in NAFLD between obese patients and controls [35]. But it seems that, there is a correlation between SFRP5 with markers of oxidative stress and insulin resistance amongst predominantly overweight and obese Caucasian populations, suggesting that the action of SFRP5 in humans may be affected by metabolic and inflammation conditions [27].

Although serum SFRP5 levels have been found to be higher in lean and non-T2DM subjects than in obese and T2DM patients; other studies have reported opposite results [6].

4. HYPOTHESIS AND OBJECTIVES

SFRP5 is a glycoprotein involved in energy metabolism and seems to play an important role in NAFLD. SFRP5 is an anti-inflammatory cytokine, and it is postulate that confers protection against liver damage. Therefore, the effect is not clear, and the literature is very controversial.

In this scenario, we hypothesize that SFRP5 may have a protective role in NAFLD progression in women with morbid obesity.

To explore this idea, this study aims:

1) To define the specific role of SFRP5 in NAFLD: on the one hand analysing serum SFRP5 levels in women with normal weigh (NW) and morbid obesity (MO) with normal liver (NL) histology, SS or NASH; on the other hand, analysing the relative mRNA hepatic and jejunal abundance of SFRP5 in women with MO with different degrees of NAFLD.
2) To study in the same cohort of patients, the mRNA hepatic abundance of WNT5A and JNK, two of the main genes implicated in noncanonical WNT pathway and their involvement in NAFLD.
3) Explore the relationship with lipid metabolism-related genes and TLRs.

5. MATERIALS AND METHODS

5.1 Subjects

The institutional review board (Institut Investigació Sanitària Pere Virgili (IISPV) CEIm; 23c/2015) approved the study, and all participants gave written informed consent. The studied cohort consisted of 20 Caucasian women with NW (body mass index; BMI<25 kg/m^2) and 69 Caucasian women with MO (BMI>40 kg/m^2). Liver biopsies were obtained during planned surgery from patients with MO. All liver biopsies were indicated for clinical diagnosis. The study exclusion criteria were: (1) subjects who had an intake of alcohol or other toxins higher than 10 g/day; (2) patients who had acute or chronic hepatic, inflammatory, infectious or neoplasic diseases; (3) women who were menopausal or undergoing contraceptive treatment; (4) women with diabetes receiving insulin or other medication that can modulate endogenous insulin levels; and (5) patients treated with antibiotics in the previous 4 weeks.

According to the hepatic histopathological classification described elsewhere [36], women with MO who followed the study criteria were includes in the research and subclassified by one experienced pathologist into obese patients with NL histology (n=28) and NAFLD (n=41) [SS (micro/macrovesicular steatosis without inflammation or fibrosis, n=24) and NASH (Brunt Grades 1-2, n=17)]. None of the patients with NASH in our cohort presented fibrosis.

5.2 Sample size

This work is mainly focus on define the specific role of SFRP5 in NAFLD. For that reason, sample size has been calculated using the hepatic relative expression of SFRP5; accepting an α risk of 0.05 and a β risk of less than 0.2 in a bilateral contrast, 24 subjects per group are needed to detect a difference ≥ 0.2 arbitrary units. It is assumed that the common standard deviation is 0.3.

5.3 Biochemical analyses

The studied cohort underwent physical, anthropometric and biochemical assessments. Specialized nurses performed blood extraction through a BD Vacutainer® system, after overnight fasting before bariatric surgery. Venous blood samples were obtained using empty and ethylenediaminetetraacetic acid coated tubes, which were respectively separated in serum and plasma aliquots by centrifugation (3,500 rpm, 4 ºC, 15 min). Biochemical parameters were analysed using a conventional automated analyser. Insulin resistance was estimated using homeostatic model assessment method-insulin resistance (HOMA2-IR).

Interleukin (IL)-1β, IL-6, IL-7, IL-8, IL-10, IL-13, IL-22, tumour necrosis factor α (TNF-α), adiponectin and resistin were determined using multiplex sandwich immunoassays and the MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 1 (HADK1MAG-61K, Millipore, Billerica, MA, USA) and MILLIPLEX MAP Human High-Sensitivity T Cell Panel (HSTCMAG28SK, Millipore, Billerica, MA, USA), and the Bio-Plex 200 instrument at the Center for Omic Sciences (Universitat Rovira i Virgili), according to the manufacturer's instructions. Peripheral SFRP5 levels were analysed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Ref. BA E-8900, Labor Diagnostika Nord, Nordhorn, Germany).

5.4 Gene expression in the liver and jejunum

Hepatic and jejunal samples were collected in tubes with RNAlater (Qiagen, Hilden, Germany) after bariatric surgery and were conserved at 4ºC and then, processed and stored at -80ºC. The RNeasy mini kit (Qiagen, Barcelona, Spain) was used to extract total RNA from both tissues. Reverse transcription to cDNA was

performed with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Madrid, Spain). Real-time quantitative PCR was performed with the TaqMan Assay predesigned by Applied Biosystems for the detection of SFRP5 (Hs00169366_m1), JNK (Hs01548508_m1), WNNT5A (Hs00998537_m1), TLR2 (Hs02621280_s1), TLR4 (Hs00152939_m1), TLR5 (Hs05021301_s1), TLR9 (Hs00370913_s1), SREBP1c (Hs01088691_m1), LXRα (Hs00173195_m1), FAS (Hs00188012_m1), FXR (Hs01026590_m1), peroxisome proliferator-activated receptor (PPAR)α (Hs00947538_m1), PPARγ (Hs01115513_m1), 18S ribosomal RNA (Fn04646250_s1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs02786624_g1). The expression of each gene was calculated relative to the expression of 18S RNA for liver genes and GAPDH for jejunal genes. All reactions were carried in duplicate in 96-well plates using the 7900HT Fast Real-Time PCR system (Applied Biosystem, Madrid, Spain).

5.5 Statistical analysis

The data was analysed using the SPSS/PC+ for Windows statistical package (version 23.0; SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to assess the distribution of variables. Parametric variables were reported as the mean±SD**;** nonparametric variables were reported as the median and 25-75th percentile. The different comparative analyses were performed using a nonparametric Mann-Whitney U test or Kruskal-Wallis test, according to the presence of two or more groups. The strength of the association between variables was calculated using Pearson's method (parametric variables) and Spearman's ρ correlation test (nonparametric variables). P-values<0.05 were statistically significant.

6. RESULTS

6.1 Baseline characteristics of subjects

The clinical characteristics and biochemical measurements of the population are shown in Table 1. We first classified our cohort depending on their BMI as NW (n=20) and MO (n=69) which were comparable in terms of systolic blood pressure (SBP), diastolic blood pressure (DBP), HOMA2-IR, glucose and low density lipoprotein cholesterol (LDL-C). Then, those with MO were subclassified according to hepatic

histology as NL (n=28), SS (n=24) and NASH (n=17) which were comparable in terms of weight, BMI, SBP, DBP, HOMA2-IR, insulin, glycosylated haemoglobin (HbA1c), TG, cholesterol, high density lipoprotein cholesterol (HDL-C), LDL-C, aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT),

Table 1. Anthropometric and biochemical variables of women in the studied cohort.

		MO (N=69)		
	NW	**NL**	**SS**	**NASH**
Variables	**(N=20)**	**(N=28)**	**(N=24)**	**(N=17)**
Weight (kg)	54.20(51.00-64.25)	**116.50(107.25-130.50)***	**113.20(108.33-128.00)***	**112.00(104.65-125.00)***
BMI (kg/m^2)	21.97(20.47-23.90)	**43.30(40.94-46.47)***	**44.35(40.82-46.83)***	**44.46(40.76-46.03)***
SBP (mmHg)	118.56±10.92	119.00±18.26	120.09±13.41	113.44±13.96
DBP (mmHg)	72.00(68.50-75.00)	63.00(57.75-75.75)	62.00(59.00-72.50)	65.50(56.75-70.75)
HOMA2-IR	1.05(0.60-1.30)	1.23(0.75-2.05)	1.49(0.95-2.18)	0.86(0.61-3.00)
Glucose (mg/dL)	90.00(84.00-97.00)	**85.50(76.25-93.00)^**	93.50(85.75-107.00)	91.00(82.50-101.20)
Insulin (mUI/L)	7.80(4.80-9.90)	9.43(5.59-16.21)	**11.27(7.81-14.51)***	6.57(5.09-23.04)
HbA1c (%)	4.75(4.50-5.03)	**5.40(5.30-5.70)***	**5.60(5.25-6.03)***	**5.50(5.20-6.10)***
TG (mg/dL)	79.50(49.50-149.25)	**106.00(89.00-136.00)***	**129.50(85.75- 175.50)***	**140.00(106.00-247.00)***
Cholesterol (mg/dL)	193.98±30.66	171.88±36.20	**174.42±35.41***	185.28±43.39
HDL-C (mg/dL)	63.75±16.03	40.84±9.89	**42.56±12.38***	**38.89±8.47***
LDL-C (mg/dL)	109.99±30.67	108.16±27.94	104.39±31.21	104.62±31.58
AST (UI/L)	19.50(15.75-23.00)	20.50(15.75-36.25)	23.00(17.00-35.00)	**24.00(17.00-43.00)***
ALT (UI/L)	15.00(12.00-21.00)	**22.00(16.00-27.00)*^**	**31.00(23.00-35.75)***	**30.00(15.50-40.00)***
GGT (UI/L)	12.00(9.00-20.00)	18.00(16.00-27.00)	**21.00(16.25-30.50)***	**25.00(15.00-27.00)***
ALP (UI/L)	54.44±14.10	**60.42±13.09^**	**75.80±11.66***	**62.77±11.16^**

MO, morbid obesity; NW, normal weight; NL, normal liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; BMI, body mass index; HOMA2-IR, homeostatic model assessment method-insulin resistance; HbA1c, glycosylated haemoglobin; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; SBP, systolic blood pressure; DBP, diastolic blood pressure. Data are expressed as the mean ± standard deviation or median (interquartile range) depends on the distribution of the variables. *Significant differences vs NW group ($p<0.05$). ^Significant differences vs SS group ($p<0.05$).

Biochemical analyses indicated that patients with NW had significantly lower weight, BMI, HbA1c, TG and alanine aminotransferase (ALT) than NL, SS and NASH groups; also, levels of insulin, GGT and alkaline phosphatase (ALP) were lower in NW compared to SS; meanwhile AST and GGT levels of NW subjects were lower compared to NASH patients. On the other hand, our NW subjects presented higher levels of HDL-C than NAFLD patients (NL, SS and NASH), and enhanced levels cholesterol than those with SS. In the MO cohort, we found higher levels of glucose,

ALT and ALP in SS subjects than in NL group, and increased levels of ALP in SS than in NASH group.

6.2 Evaluation of serum SFRP5 levels according to BMI and hepatic histology

To achieve the main objective of this study we evaluated serum SFRP5 levels in a cohort of women with NW and MO (NL, SS and NASH). Our results showed significant lower levels of SFRP5 in NW patients than those with MO, specifically in NL, SS and NASH subjects as shown Figure 5. Unfortunately, we did not find significant differences of SFRP5 levels between NL, SS and NASH groups.

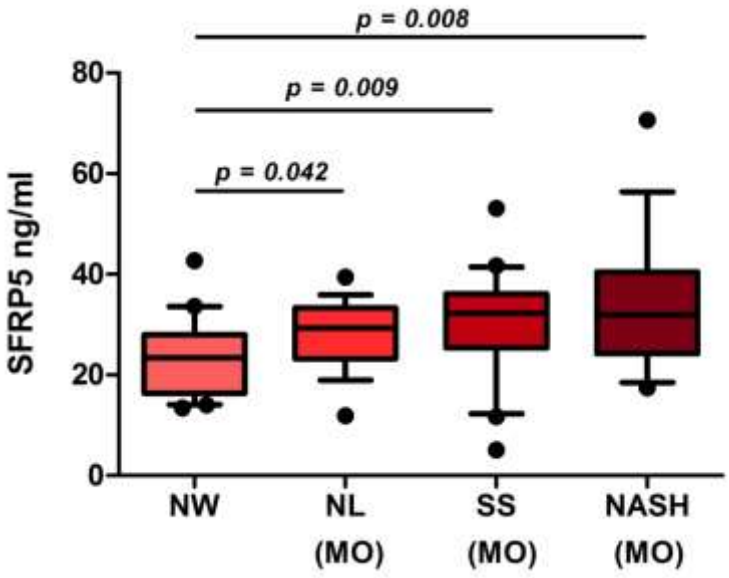

Figure 5: **Differential serum SFRP5 levels between groups.** Serum levels of SFRP5 of women with NW and women with MO subclassified accordingly into NL, SS and NASH. NW, normal weight; MO, morbidly obesity; NL, normal liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; SFRP5, secreted frizzled-related protein 5. ($p<0.05$ was considered statistically significant).

6.3 Evaluation of relative mRNA abundance of SFRP5 in liver and jejunum

Apart from determining SFRP5 levels in our study cohort, to achieve the main objective of this study, we also evaluated the relative mRNA hepatic abundance of SFRP5 in a cohort of women with MO without or with NAFLD (SS and NASH). First, we classified our patients into NL and NAFLD, and we observed that the hepatic relative mRNA expression of SFRP5 was higher in NAFLD group than in NL subjects (Figure

6A). Then, when we analysed hepatic relative mRNA expression of SFRP5 according to different degrees of NAFLD, we found that SFRP5 expression was significantly higher in patients with SS than those with NL or NASH. However, hepatic relative mRNA abundance of SFRP5 did not show significant differences between NL and NASH subjects (Figure 6B).

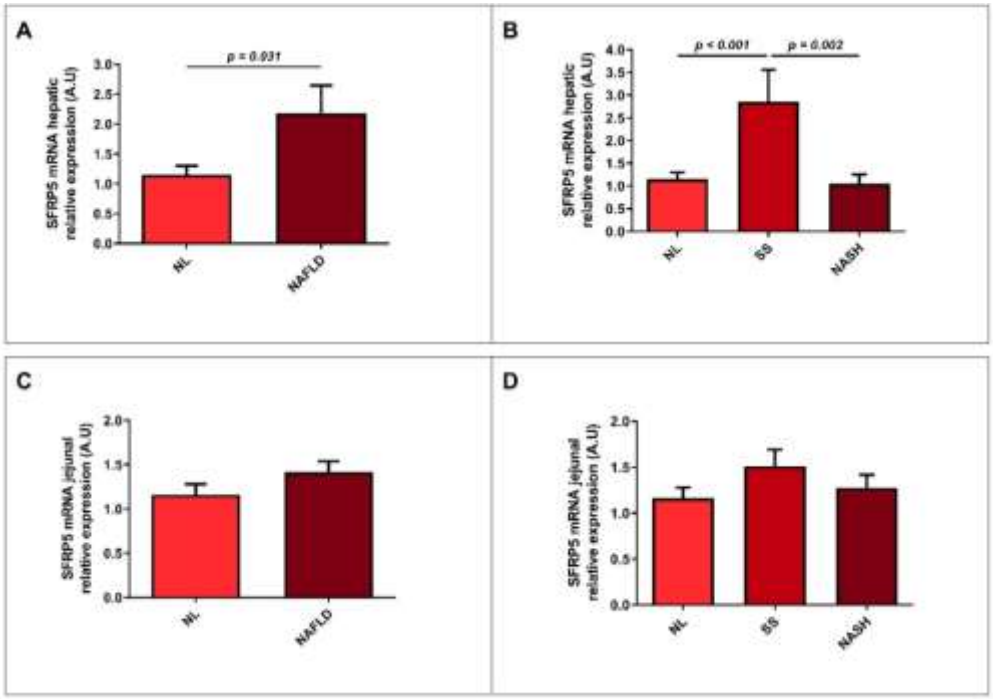

Figure 6: **Differential relative mRNA abundance of SFRP5 in liver and jejunum.** Differential relative mRNA abundance of SFRP5 in liver between (A) women with MO with NL histology and women with MO with NAFLD; (B) women with MO with NL histology, women with MO with SS and women with MO with NASH. Differential relative mRNA abundance of SFRP5 in jejunum between (C) women with MO with NL histology and women with MO with NAFLD; (D) women with MO with NL histology, women with MO with SS and women with MO with NASH. A.U, arbitrary units; MO, morbidly obesity; NAFLD, nonalcoholic fatty liver disease; NL, normal liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; SFRP5; secreted frizzled-related protein 5. ($p<0.05$ was considered statistically significant).

Moreover, given that the importance of gut-liver axis in NAFLD [37,38], and the fact that SFRP5 expression was reported in the gastrointestinal tract, we wanted to analyse jejunal relative mRNA abundance of SFRP5 in our cohort of patients (Figure 6C and D). There were no significant differences in jejunal SFRP5 expression according to the presence of NAFLD or the hepatic histopathological classification.

6.4 Evaluation of relative mRNA abundance of SFRP5 in liver according to liver inflammation-related parameters

Since the relative mRNA hepatic abundance of SFRP5 was higher in NAFLD, we wanted to explore its implication in hepatic inflammation. Therefore, we studied SFRP5 mRNA hepatic relative expression in our cohort classified according to some liver inflammation-related parameters. In this regard, we observed decreased levels of mRNA abundance of SFRP5 in patients with liver inflammation than those without it (Figure 7A). We also found lower levels of mRNA relative abundance of SFRP5 in subjects with hepatic ballooning than those in absence of it (Figure 7B). Finally, we observed low levels of hepatic mRNA SFRP5 abundance in patients with lobular inflammation than subjects without lobular inflammation (Figure 7C). There were no significant differences in SFRP5 mRNA hepatic relative expression according to portal inflammation presence or absence (Figure 7D).

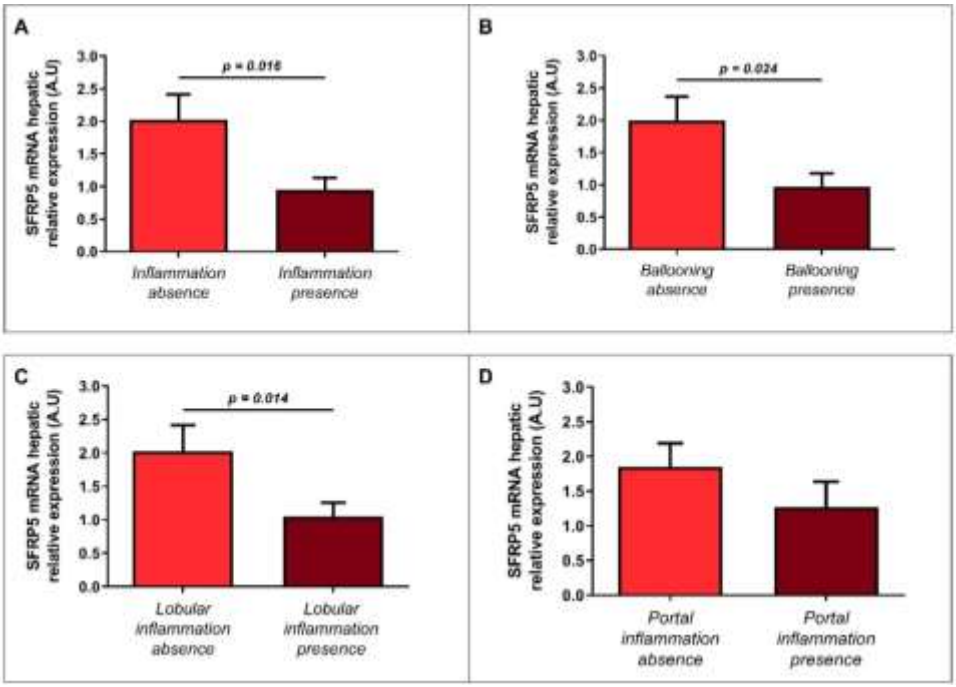

Figure 7: **Differential relative expression of SFRP5 in liver between different parameters related to inflammation.** Differential relative mRNA abundance of SFRP5 in liver between (A) inflammation absence and inflammation presence; (B) ballooning absence and ballooning presence; (C) lobular inflammation absence and presence and (D) portal inflammation absence and presence. A.U, arbitrary units; SFRP5; secreted frizzled-related protein 5. ($p<0.05$ was considered statistically significant).

6.5 Correlations of relative hepatic and jejunal mRNA abundance of SFRP5 with different NAFLD-related parameters

To deepen the knowledge of the role of SFRP5 in NAFLD pathogenesis, we wanted to analyse correlations between SFRP5 relative expression in liver with different parameters related to NAFLD such as weight, BMI, glucose, insulin, liver transaminases, different adipocytokines, etc (Table 2).

Table 2. Significant correlations between SFRP5 relative hepatic expression and other genes.

Genes	SFRP5 mRNA hepatic R.E.	
	rho	P value
ALP (UI/L)	0.404	0.016
WNT5A hepatic R.E.	0.535	<0.001
JNK hepatic R.E.	0.513	0.001

SFRP5, secreted frizzled-related protein 5; JNK, Jun N-Terminal Kinase; ALP, alkaline phosphatase; R.E., relative expression. Data are expressed as the correlation coefficient rho of Spearman and p-value ($p<0.05$ was considered statistically significant).

When we analysed correlations between SFRP5 relative expressions in jejunum with other parameters (previous mentioned and some jejunal genes related to NAFLD such as PPAR genes and TLR), we observed a negative correlation between jejunal SFRP5 abundance and jejunal TLR2 relative expression (rho=-0.336, p=0.015). On the other hand, we found a positive association between jejunal SFRP5 mRNA and PPARγ jejunal relative expression (rho=0.383, p=0.005).

6.6 Evaluation of relative mRNA abundance of WNT5A and JNK in liver

To explore the implication of WNT signalling pathway in NAFLD pathogenesis, we also wanted to analyse in our study cohort, the hepatic mRNA abundance of WNT5A and JNK, two of main genes involved in the WNT pathway together with SFRP5. On one hand, we observed significantly higher mRNA relative expression of WNT5A in the liver of NAFLD patients than those with NL histology as shown Figure 8A. Moreover, when we analysed hepatic relative mRNA abundance of WNT5A according to different degrees of NAFLD, we found that WNT5A hepatic expression was significantly enhanced in patients with SS than those with NL or NASH (Figure 8B). On the other hand, we observed higher levels of hepatic JNK relative expression in NAFLD patients than NL subjects (Figure 8C). Additionally, we also found increased

levels of hepatic mRNA abundance of JNK in SS patients than those with NL as was graphically represented in Figure 8D.

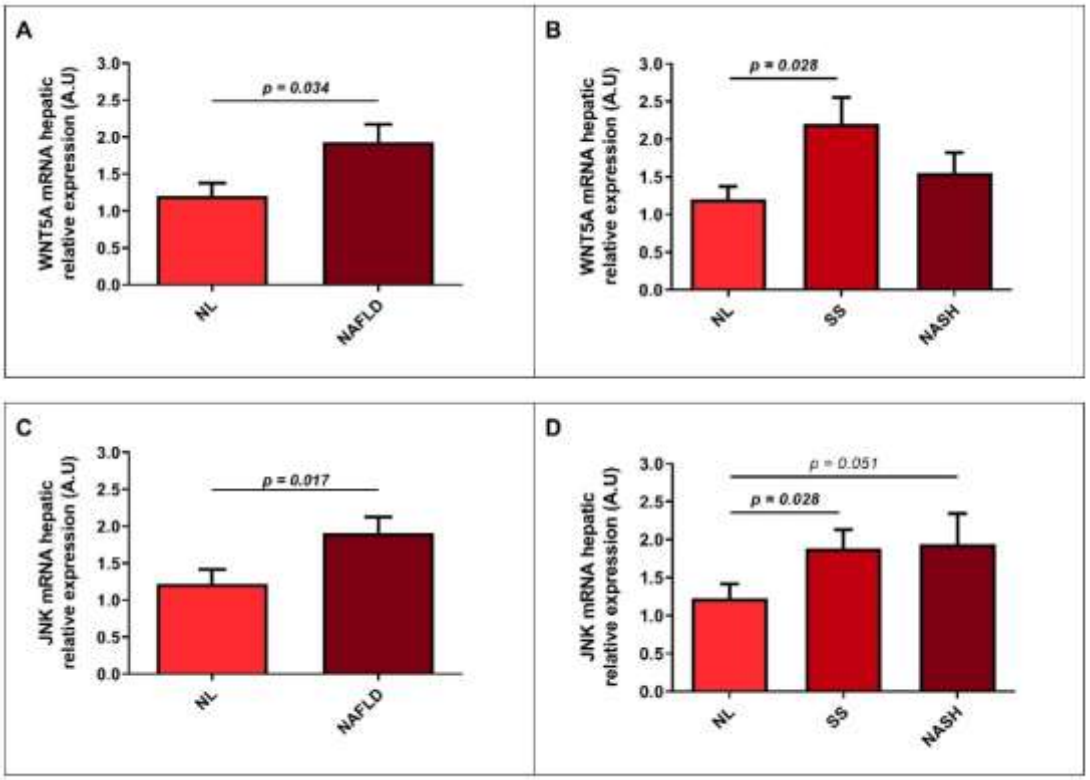

Figure 8: **Differential relative mRNA abundance of WNT5A and JNK in liver.** Differential relative mRNA abundance of WNT5A in liver between (A) women with MO with NL histology and women with MO with NAFLD; (B) women with MO with NL histology, women with MO with SS and women with MO with NASH. Differential relative mRNA abundance of JNK in liver between (C) women with MO with NL histology and women with MO with NAFLD; (D) women with MO with NL histology, women with MO with SS and women with MO with NASH. A.U, arbitrary units; MO, morbidly obesity; NAFLD, nonalcoholic fatty liver disease; NL, normal liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; WNT5A, WNT family member 5a; JNK, Jun N-Terminal Kinase. ($p<0.05$ was considered statistically significant).

6.7 Correlations of relative hepatic mRNA abundance of WNT5A and JNK with different parameters

On the one hand, we found significant correlations between WNT5A relative hepatic expression and other variables, as shown Table 3.

Table 3. Significant correlations between WNT5A relative hepatic expression and other genes.

Genes	WNT5A mRNA hepatic R.E.	
	rho	P value
GGT (UI/L)	0.318	0.033
SFRP5 hepatic R.E.	0.535	<0.001
JNK hepatic R.E.	0.846	<0.001

SFRP5, secreted frizzled-related protein 5; JNK, Jun N-Terminal Kinase; GGT, gamma-glutamyltransferase; R.E., relative expression. Data are expressed as the correlation coefficient rho of Spearman and p-value ($p<0.05$ was considered statistically significant).

On the other hand, when we analysed correlations between JNK relative expression in liver and other parameters, we found that hepatic JNK correlated positively with hepatic SFRP5 expression (rho=0.513, p=0.001) and with hepatic WNT5A relative abundance (rho=0.846, p=<0.001).

6.8 Evaluation of relative mRNA abundance of SFRP5, WNT5A and JNK in liver according to hepatic histology

Given that SFRP5, WNT5A and JNK mRNA hepatic abundance have shown a differential expression in NAFLD compared to NL subjects, we wanted to compare these expressions among them, according to the histopathological classification of the liver, as was represented in Figure 9. In this sense, the relative mRNA abundance of SFRP5, WNT5A and JNK of NL subjects showed similar expression levels. Nevertheless, in SS group, we found increased mRNA expressions of SFRP5, WNT5A and JNK than NL, but SFRP5 showed a higher increase compared to the other genes. In contrast, when we analysed NASH cohort, we observed that the mRNA expression was decreased again in SFRP5 and WNT5A, but this reduction was only significant in SFRP5.

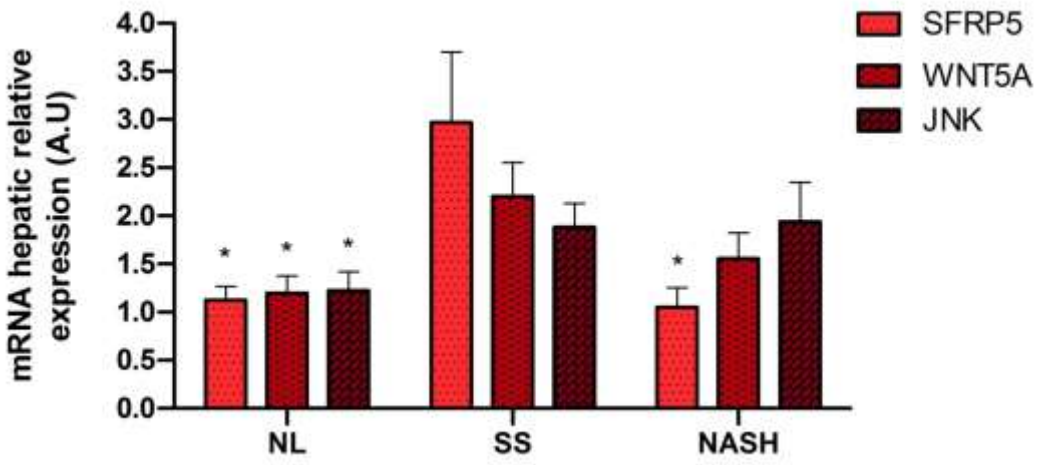

Figure 9: **Differential relative mRNA abundance of SFRP5, WNT5A and JNK in liver.** Differential relative mRNA abundance of SFRP5, WNT5A and JNK in liver of women with MO subclassified by NL, SS and NASH. A.U, arbitrary units; MO, morbidly obesity; NL, normal liver; SS, simple steatosis; NASH, nonalcoholic steatohepatitis; SFRP5, Secreted frizzled-related protein 5; WNT5A, WNT family member 5a, JNK Jun N-Terminal Kinase. *Significant differences vs SS group ($p<0.05$).

7. DISCUSSION

The main novelty of the present study is that we analysed the involvement of SFRP5 and the noncanonical WNT pathway in NAFLD pathogenesis in a well-characterized cohort of women with MO and NAFLD. Although we did not find significant differences in the serum SFRP5 levels in patients with MO with or without NAFLD, we reported an increased hepatic mRNA abundance of SFRP5 in patients with SS relative to those with NL or NASH. Moreover, we found enhanced hepatic mRNA expression of WNT5A and JNK, two of the main genes involved in the noncanonical WNT pathway, in SS compared to NL.

In the first instance, we reported higher serum SFRP5 levels in our patients with MO than in those with NW. These results are contrary to previous studies; for example, Hu et al. showed that subjects with obesity had lower circulating SFRP5 levels than subjects at a NW [39] while Tan et al. demonstrated low SFRP5 levels in children with obesity [40]. This contradiction can be explained because our MO patients underwent a very low-calorie diet 3 months before bariatric surgery while our NW subjects followed a normal diet before blood extraction. However, the patients analysed by Tan et al. and Hu et al. did not follow this type of strict diet. Additionally, some children with obesity in the Tan et al. study underwent a reduction in caloric intake and reported an increase in SFRP5 levels after this lifestyle intervention [40], which is consistent with our study

results. In addition, it should be taken into account that in those articles, male subjects and children were used, whereas we studied adult women.

The higher serum SFRP5 levels in patients with MO is supported by a previous human study that demonstrated that SFRP5 can be used as a biomarker of the anti-inflammatory effect after caloric restriction [41].

Regarding our cohort with NAFLD, we did not find significant differences in the SFRP5 serum levels among the NL, SS and NASH patients. Our results are consistent with that of Gutiérrez-Vidal et al., who reported no significant differences between NAFLD groups or between the first steps of fibrosis [6]. Regarding fibrosis, we could not perform this analysis because none of the patients with NASH presented hepatic fibrosis in our study.

Later, we analysed the relative mRNA abundance of SFRP5 in livers from MO patients with or without NAFLD. We reported increased expression of hepatic mRNA SFRP5 in NAFLD patients compared with NL patients; then, when we classified our NAFLD subjects according to SS and NASH, we observed higher expression in the SS group than the NL and NASH groups. Moreover, only one previous human study analysed the hepatic mRNA expression of SFRP5 in NAFLD, and they reported a decrease in SFRP5 expression in the liver as NAFLD progressed. However, when the data were deeply analysed, this reduction was not significant between the controls and SS subjects but was significant between the controls and NASH group [6]. These discrepancies can be attributed to the use of actin-β expression to standardize the hepatic abundance of SFPR5 in Gutierrez-Vidal et al. [6], and the use of 18S expression and the normalization of expression in relation to the control group in our study.

Given that inflammation and ballooning are two of the main findings of NASH [42], we wanted to explore the SFRP5 abundance differences according to the presence or absence of inflammation (portal and lobular) and hepatic ballooning. Our results showed that SFRP5 was lower in patients with general inflammation, lobular inflammation and ballooning. This result can be explained by the fact that SFRP5 is an anti-inflammatory molecule with a protective role in the first steps of hepatic steatosis; however, inflammation seems to deregulate SFRP5 signalling, thereby blocking its inhibition of the noncanonical WNT pathway, which promotes NAFLD progression.

Some authors have reported the importance of gut-liver axis in NAFLD pathogenesis [37,38], in this sense, we wanted to evaluate the mRNA jejunal SFRP5

expression to add more knogledge about the role of SFRP5 in NAFLD disease. Regarding jejunal SFRP5 expression, we did not found significant differences between groups. However, we observed a negative correlation between jejunal SFRP5 abundance and jejunal TLR2 relative expression, which agree with Aragonès et al. [12] who demonstrated that jejunal TLR2 expression was enhanced in NAFLD patients triggering hepatic inflammation. Moreover, we found a positive association between jejunal mRNA SFRP5 expression and PPARγ jejunal relative expression, a gene involved in lipid metabolism that improves insulin sentitivity,which has been described that it is downregulated in obese mouse models [43]. Therefore, SFRP5 jejunal expression seems to also have a protective role against inflammation and lipogenesis.

Since it was reported that the noncanonical WNT pathway involving JNK activation has been implicated in fatty liver disease [29,34], we wanted to study the relative hepatic mRNA expression of WNT5A and JNK to add new knowledge about the role of this pathway in NAFLD pathogenesis.

On the one hand, we found higher relative mRNA WNT5A expression in patients with NAFLD than in those with NL histology and observed significantly enhanced expression in the SS subjects compared with the NL subjects. On the other hand, we reported increased expression of JNK in the NAFLD patients and found higher levels in the SS group than the NL group. Significant differences in hepatic WNT5A and JNK expression were not observed between the SS and NASH patients. Our results support the fact that WNT5A and JNK are upregulated in NAFLD, thereby triggering noncanonical WNT signalling that promotes liver damage [44], although we could not demonstrate that this pathway is increased in an advanced stage of NAFLD, such as NASH.

Analysing the expression of WNT5A and JNK hepatic in NAFLD human subjects is a novelty, although our results seemed to agree with other studies that assessed animal models, such as Ji-nian Wang et al., who observed that the WNT pathway could play a key role in hepatic stellate cell activation and proliferation that trigger liver regeneration [29]. Moreover, Shuxia Wang et al. postulated an association between the noncanonical WNT pathway and NAFLD, liver inflammation and fibrosis [44]; Kodama et al. indicated that blocking JNK may prevent the development of steatosis in mouse models [45], which could represent a therapeutic target for this disease; and Hirosumi et al. observed the activation of JNK in the livers of obese mice, and Jnk1 knockout mice were protected from the development of obesity and insulin resistance [46].

Finally, we observed a correlation between hepatic mRNA expression of WNT5A and GGT. Coccia et al. demonstrated that the GGT levels significantly increased with steatosis and fibrosis grade [47]. This fact agrees with our results, since WNT5A has been described as a promoter of fibrosis [29]. Moreover, we observed that the hepatic mRNA expression of SFRP5, WNT5A and JNK was correlated, which was supported by the significant increase of the three genes in steatosis state, whereas a significant decrease of only SFRP5 was observed in NASH while WNT5A and JNK were maintained. Thus, it seems that in the initial states of NAFLD, SFRP5 competes against the activation of the WNT pathway, perhaps as a protective molecule. However, in an advanced stage of liver damage and inflammation, the effects of SFRP5 seem to be deregulated and noncanonical WNT pathway signalling could be activated, thereby triggering NAFLD progression.

In this study, our cohort of women with MO made it possible to establish some relationships between the WNT pathway (SFRP5, WNT5A and JNK) and NAFLD without the interference of confounding factors, such as sex or age. However, these results cannot be extrapolated to other obesity groups or patients who do not follow a caloric restriction. Further studies, including studies using these cohorts, would be useful to validate our findings.

8. CONCLUSIONS

Circulating SFRP5 levels increase after caloric restriction in subjects with MO regardless of NAFLD status. However, hepatic SFRP5 could have a protective role in the first steps of NAFLD in an attempt to inhibit the noncanonical WNT pathway but could be deregulated at the advanced stage of the disease while WNT5A and JNK are activated, thus promoting liver damage. Additionally, SFRP5 jejunal expression seems to also have a protective role against metabolic dysfunction which can have an impact on liver health through gut-liver axis.

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