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Laparoscopic sleeve gastrectomy reverses non-alcoholic fatty liver disease modulating oxidative stress and inflammation

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ABSTRACT

Background & aims: Hepatic alterations, such as in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are frequently associated with obesity. To investigate the molecular mechanisms of these alterations and to identify molecules that could be used as potential therapeutic targets, we investigated the modulation of hepatic indices of oxidative stress and inflammation in obese patients undergoing laparoscopic sleeve gastrectomy (LSG).

Methods: Patients (n = 436) attending our obesity clinic underwent LSG for weight loss. We obtained a diagnostic intraoperative liver biopsy, and a sub-cohort (n = 120) agreed to a 1-year follow-up that included donation of blood samples and additional liver biopsies. Selected key molecules in blood and liver tissue were used to investigate the hepatic alterations in obesity, and their response to LSG.

Results: One year post-surgery, the prevalence of diabetes, dyslipidemia and hypertension decreased significantly. LSG improved liver histology features in all patients. Improvement was greater in severe cases of NAFLD including those with steatohepatitis, bridging fibrosis or cirrhosis. Significant pre-surgery differences in plasma, and liver markers of oxidative stress and inflammation (including chemokine C-C motif ligand 2, paraoxonase-1, galectin-3, and sonic hedgehog) were observed between patients with, and those without, NASH; post-surgery indicated consistent improvements in these parameters.

Conclusion: Our study shows that the histology and liver function of patients with morbid obesity are significantly improved after LSG via mechanisms that involve the reduction of oxidative stress and inflammatory processes. These data encourage the use of LSG as a therapeutic option to improve, or resolve, NAFLD.

1. Introduction

Risks of hepatic disease and metabolic abnormalities increase with higher body mass index (BMI) [1]. In the liver, accumulation of fat causes multiple alterations, such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) which, if untreated or undetected, may subsequently result in life-threatening diseases such as cirrhosis or hepatocellular carcinoma [2]. Management of liver impairment associated with severe obesity presents unique challenges. Intensive changes in lifestyle remain the primary treatment options but which, over the long term, are frequently unsuccessful. Bariatric surgery appears to be a safe and efficient procedure to reduce weight, but data

Abbreviations: BMI, body mass index; CD, cluster of differentiation; CCL2, chemokine (C-C motif) ligand 2; CCR2, C-C chemokine receptor type 2; DAB, 3,3′-diaminobenzidine; FAA, fumarylacetoacetase; HOMA-IR, homeostasis model assessment-insulin resistance; HDL, high-density lipoproteins; IL-10, interleukin-10; NAFLD, non-alcoholic fatty liver disease; NAS, non-alcoholic fatty liver activity score; NASH, non-alcoholic steatohepatitis; PON1, paraoxonase-1; pSTAT3, phospho signal transducer and activator of transcription 3; Shh, sonic hedgehog; α-SMA, α-smooth muscle actin; STAT3, signal transducer and activator of transcription 3; T2DM, type 2 diabetes mellitus; TBBL, 5-thiobutyl butyrolactone; TNF-α, tumor necrosis factor-α.

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are sparse regarding its effectiveness in treating the hepatic alterations [1].

Oxidative stress and inflammation are related to the onset and development of liver diseases [3]. Excessive nutrient intake impairs the redox status in the liver which stimulates inflammation [3]. The molecular mechanisms accounting for these alterations involve modifications of enzyme activity, post-translational modifications of proteins, and activation of nuclear receptors; the consequence is a global modification of metabolic networks [4]. Several biomarkers of oxidative stress and inflammation have been associated with liver diseases. Paraoxonase-1 (PON1) is a lipolactonase and esterase with antioxidant activity present in the hepatocytes, as well as bound to high-density lipoproteins (HDL) in the circulation [5]. Serum PON1 activity is decreased in liver diseases and in several other non-communicable diseases in which there is an increase in free radical production [6]. Oxidative stress and decreased PON1 activity result in an increase in the production of pro-inflammatory cytokines such as chemokine (C-C motif) ligand 2 (CCL2) and tumor necrosis factor-α (TNF-α) [6]. In patients with liver impairment, the circulating levels of these cytokines correlate with the severity of the hepatic inflammation [7,8], while the pharmacological inhibition of CCL2 results in improved liver function [9]. In addition, oxidative stress and inflammation increase the synthesis of galectin-3, and activate the sonic hedgehog (Shh) pathway, both of which stimulate fibrogenesis [10,11]. The inflammatory processes are counteracted by

Table 1
Selected characteristics in patients with severe obesity and in the control group.

	Control group (n = 404)	Obese patients (n = 436)		
		Non-NASH (n = 191)	Probable NASH (n = 151)	Definite NASH (n = 94)
Male, n (%)	175 (43.1)	41 (21.5)[a]	41 (27.2)[b]	25 (26.6)[c]
Age, years	46 (35–59)	46 (39–56)	49 (42–57)	48 (42.25–56.75)
BMI, kg/m^2	26.78 (23.34–28.12)	44.6 (41.3–49.2)[a]	46.6 (43.0–51.4)[b,d]	46.3 (42.3–51.5)[c]
T2DM, n (%)	26 (6.3)	60 (31.6)[a]	66 (44.0)[b,d]	48 (51.1)[c,e]
Hypertension, n (%)	62 (15)	104 (54.5)[a]	83 (55.0)[b]	62 (66.0)[c,e]
Dyslipidemia, n (%)	36 (8.7)	55 (28.8)[a]	58 (38.4)[b,d]	40 (42.6)[c,e]
Medication, n (%)				
Metformin	6 (1.4)	33 (17.3)[a]	45 (30.0)[b,d]	36 (38.3)[c,e]
Insulin	–	10 (5.2)	16 (10.6)[d]	10 (10.6)
Sulfonylureas	6 (1.4)	8 (4.2)[a]	11 (7.3)[b]	9 (9.6)[c]
ACEIs+ARA II	15 (3.6)	55 (28.8)[a]	51 (33.8)[b]	41 (43.6)[c,e]
Diuretics	20 (4.8)	15 (7.9)	14 (9.3)[b]	12 (12.8)[c]
Statins	8 (1.9)	31 (16.3)[a]	34 (22.5)[b]	19 (20.4)[c]
Biochemical variables				
Total cholesterol, mmol/L	5.2 (4.6–5.9)	4.1 (3.5–4.8)[a]	4.4 (3.6–5.1)[b]	4.4 (3.8–5.0)[c]
HDL-cholesterol, mmol/L	1.4 (1.2–1.7)	1.2 (0.9–1.5)[a]	1.1 (0.85–1.4)[b]	1.1 (0.88–1.3)[c]
LDL-cholesterol, mmol/L	3.1 (2.6–3.8)	2.7 (2.1–3.2)[a]	2.7 (2.1–3.3)[b]	2.8 (2.4–3.4)[c]
Triglycerides, mmol/L	1.1 (0.7–1.5)	1.5 (1.1–2.0)[a]	1.7 (1.3–2.4)[b,d]	1.8 (1.2–2.4)[c,e]
Glucose, mmol/L	4.7 (4.3–5.2)	6.7 (5.6–8.3)[a]	7.4 (5.9–9.4)[b,d]	7.6 (6.2–10.9)[c,e]
Insulin, pmol/L	49.4 (31.9–70.0)	78.8 (39.2–131.1)[a]	82.6 (49.1–135.0)[b]	82.6 (53.4–145.1)[c]
HOMA-IR	1.5 (0.9–2.3)	3.6 (1.7–5.6)[a]	4.3 (2.1–7.1)[b,d]	5.0 (2.4–7.6)[c,e]
AST, μKat/L	0.35 (0.30–0.41)	0.45 (0.3–0.6)[a]	0.50 (0.39–0.81)[b]	0.87 (0.56–1.3)[c,e,f]
ALT, μKat/L	0.32 (0.23–0.44)	0.4 (0.3–0.6)[a]	0.53 (0.38–0.86)[b,d]	0.88 (0.56–1.3)[c,e,f]
CRP, mg/L	1.2 (0.5–2.7)	1.3 (0.5–4.3)	2.5 (0.70–9.4)[b,d]	1.83 (0.80–10.90)[c,e]
Steatosis grade				
≤5%	–	132 (69.1)	27 (17.9)	–
5–33%	–	54 (28.3)	74 (49.0)	9 (9.6)
33–66%	–	5 (2.6)	47 (31.1)	50 (53.2)
>66%	–	–	3 (2.0)[d]	35 (37.2)[e,f]
Lobular inflammation				
No foci	–	65 (34.2)	8 (5.3)	–
<2 foci	–	100 (52.6)	54 (36.0)	18 (19.1)
2–4 foci	–	26 (13.2)	64 (42.0)	52 (55.3)
> 4 foci	–	–	25 (16.7)[d]	24 (25.5)[e,f]
Hepatocellular ballooning				
No	–	163 (85.3)	75 (49.6)	7 (7.4)
Few cells		24 (12.7)	67 (44.4)[d]	60 (63.8)[e,f]
Many cells	–	4 (2.0)	9 (6.0)	27 (28.7)[e,f]
Fibrosis				
None (F0)	–	74 (38.7)	28 (18.5)	23 (24.4)
Perisinusoidal or periportal (F1)	–	78 (40.8)	67 (44.3)	21 (22.3)
Perisinusoidal and portal (F2)	–	32 (16.7)	41 (27.1)	29 (30.8)
Bridging fibrosis (F3)	–	7 (3.6)	15 (9.9)	20 (21.3)[e,f]
Cirrhosis (F4)	–	–	–	1(1.0)

Values are shown as number of cases and percentages or medians and interquartile ranges. ACEIs: Angiotensin-converting-enzyme inhibitor; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ARA-II: Angiotensin II receptor antagonists; BMI: Body mass index; CRP: C-reactive protein; HDL: High-density lipoprotein; HOMA-IR: Homeostatic model assessment of insulin resistance; HTG: Hypertriglyceridemia; LDL: Low-density lipoprotein; NASH: Non-alcoholic steatohepatitis; T2DM: Type 2 diabetes mellitus. Significant differences ($p \leq 0.05$ or lower) in comparisons are indicated by [a]Control vs non-NASH. [b]Control vs Probable NASH. [c]Control vs Definite NASH. [d]Non-NASH vs Probable NASH. [e]Non-NASH vs Definite NASH. [f]Probable NASH vs Definite NASH.

Table 2
Selected variables in patients with severe obesity and paired liver biopsies at baseline and 12 months after laparoscopic sleeve gastrectomy.

	Baseline (n = 120)	12 months after surgery (n = 120)	*p*-Value
BMI, kg/m^2	46.4 (42.8)	31.2 (29.1–34.7)3	<0.001
Total cholesterol, mmol/L	4.3 (3.7–5.3)	4.7 (4.2–5.4)	<0.001
HDL-cholesterol, mmol/L	1.0 (0.8–1.4)	1.4 (1.2–1.7)	<0.001
LDL-cholesterol, mmol/L	3.1 (2.5–3.9)	3.0 (2.5–3.3)	0.127
Triglycerides, mmol/L	1.5 (1.1–2.3)	0.9 (0.8–1.3)	<0.001
Glucose, mmol/L	7.0 (6.0–9.1)	4.7 (4.5–5.)	<0.001
Insulin, pmol/L	100.8 (54.3–162.2)	39.6 (24.0–60.1)	<0.001
HOMA-IR	4.4 (2.8–7.5)	1.3 (0.4–2.5)	<0.001
AST, μKat/L	0.6 (0.4–0.8)	0.3 (0.2–0.3)	<0.001
ALT, μKat/L	0.5 (0.4–0.8)	0.2 (0.2–0.3)	<0.001
CRP, mg/L	3.0 (0.82–8.6)	1.5 (0.5–4.2)	<0.001
Steatosis grade			
<5%	25 (20.8)	116 (96.6)	
5–33%	46 (38.3)	4 (3.3)	
>33–66%	37 (30.8)	–	
>66%	12 (10)	–	<0.001
Lobular inflammation			
No foci	25 (20.8)	98 (81.6)	
<2 foci	38 (31.6)	22 (18.4)	
2–4 foci	41 (34.2)	–	
>4 foci	16 (13.3)	–	<0.001
Hepatocellular ballooning			
No	49 (40.8)	98 (81.6)	
Few cells	65 (54.1)	19 (15.8)	
Many cells	6 (5.0)	3 (2.5)	<0.001
Fibrosis			
None (F0)	20 (16.6)	55 (45.8)	
Perisinusoidal or periportal (F1)	51 (42.8)	60 (50.0)	
Perisinusoidal and portal (F2)	39 (32.5)	5 (4.1)	
Bridging fibrosis (F3)	9 (7.5)	–	
Cirrhosis (F4)	1 (0.8)	–	<0.001

Values are shown as number of cases and percentages or medians and interquartile ranges. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein.

anti-inflammatory cytokines such as interleukin-10 (IL-10), a molecule that some studies have found increased in liver diseases as an attempt to attenuate hepatic injury [12].

The aim of the present study was to investigate the molecular mechanisms underlying hepatic alterations in patients with morbid obesity. Analyses included changes in the circulating levels and hepatic expression of markers of oxidative stress and inflammation pre- and post-bariatric surgery.

2. Materials and methods

2.1. Study design and participants

This was a prospective, 12 month follow-up, longitudinal study including 436 patients with severe obesity who underwent laparoscopic sleeve gastrectomy (LSG) at the *Hospital Universitari de Sant Joan de Reus*. Based on previous studies [13], and assuming an alpha risk of 5% and a beta risk of 10%, the minimum sample size needed was 97 patients. All subjects provided 12-hours fasting blood samples immediately before surgery together with an intraoperative wedge-liver biopsy. Written informed consent was obtained according to the procedures approved by our Institutional Review Board (OBESPAD/14-07-31proj3 project) and the ethical guidelines of the 1975 Declaration of Helsinki. Exclusion criteria were age <25 years, alcohol abuse, infectious diseases, primary sclerosing cholangitis, autoimmune diseases, and cancer. One hundred and twenty patients agreed to have a second blood extraction and a liver biopsy at 12 months post-surgery, and signed fully informed consent (OM-NAFLD, ESO3/18012013 project). Biopsies were performed by ultrasound-guided, percutaneous needle puncture. Patients were classified according to the non-alcoholic fatty liver score (NAS) system. The scales included the unweighted sum of steatosis (0–3), lobular inflammation (0–3) and ballooning (0–2) scores. Values assigned were ≤2 for non-NASH, >2 and ≤4 for probable NASH, and ≥5 for definite NASH. Information for fibrosis included the absence of fibrosis (F0), mild to moderate fibrosis (F1 and F2), bridging fibrosis (F3) and cirrhosis (F4) [14]. Liver biopsies were assessed by a single experienced pathologist who was blinded with respect to the provenance of the samples.

For comparisons, we used sera of healthy non-obese controls (n = 404) in which NAFLD diagnosis was discarded using imaging procedures (INFLAMET/15-04/4proj7 project). These subjects were participants in a population-based study conducted in our geographical area. They had no clinical or analytical evidence of renal insufficiency, hepatic damage, or neoplasia. The samples (stored at −80 °C) were obtained from the Biological Samples Bank of our Institution. A detailed description of this population has been published [15].

2.2. Measurement of circulating levels of selected biochemical parameters

Serum and EDTA-plasma samples were collected after centrifugation and stored at −80 °C for batched analyses. Serum PON1 concentrations were determined using an in-house ELISA with antibodies specific of PON1 [5]. Serum PON1 lactonase and esterase activities were determined using synthetic substrates. Lactonase activity was measured as the hydrolysis of 5-thiobutyl butyrolactone (TBBL), and paraoxonase (esterase) activity was determined as the rate of hydrolysis of paraoxon [5]. Plasma concentrations of CCL2, IL-10, TNF-α and galectin-3 were measured by ELISA (PeproTech, London, UK, and R&D Systems, Minneapolis, MN, USA). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, and cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, glucose, C-reactive protein (CRP), and insulin concentrations were analyzed using standard tests in a Roche Modular Analytics P800 system (Roche Diagnostics, Basel, Switzerland).

2.3. Immunohistochemical analyses in hepatic biopsies

Procedures were performed essentially as previously reported [16]. To assess differences in oxidation and inflammation, we analyzed the hepatic immunohistochemical expression of 4-hydroxy-2-nonenal (a marker of lipid peroxidation), cluster of differentiation 68 (CD68, a marker of macrophages), PON1, CCL2, C-C chemokine receptor type 2 (CCR2), IL-10, TNF-α, and galectin-3. The appropriate primary and secondary antibodies and other reagents are described in Supplementary Table S1. Positive staining was quantified using the Image J software (National Institutes of Health, Bethesda, MD, USA).

2.4. Western blotting of liver tissue

Denatured proteins (50 μg) from frozen liver tissues were subjected to 8%–14% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene difluoride membranes (Thermo Fisher, Barcelona, Spain) using bovine serum albumin at 5% in Tris-buffered saline, 0.1% Tween-20 (pH = 7.4) as blocking agent. Membranes were incubated with the corresponding primary and secondary antibodies for PON1, galectin-3, TNF-α, IL-10, CD163 (a marker of anti-inflammatory macrophages) [17], signal transducer and activator of transcription 3 (STAT-3) and its phosphorylated form (pSTAT-3), which regulate multiple metabolic processes [17], α-smooth muscle actin (α-SMA), and sonic hedgehog (Shh); these last two proteins being associated with liver fibrosis. Technical details and reagents are reported in Supplementary Table S1. Fumarylacetoacetate

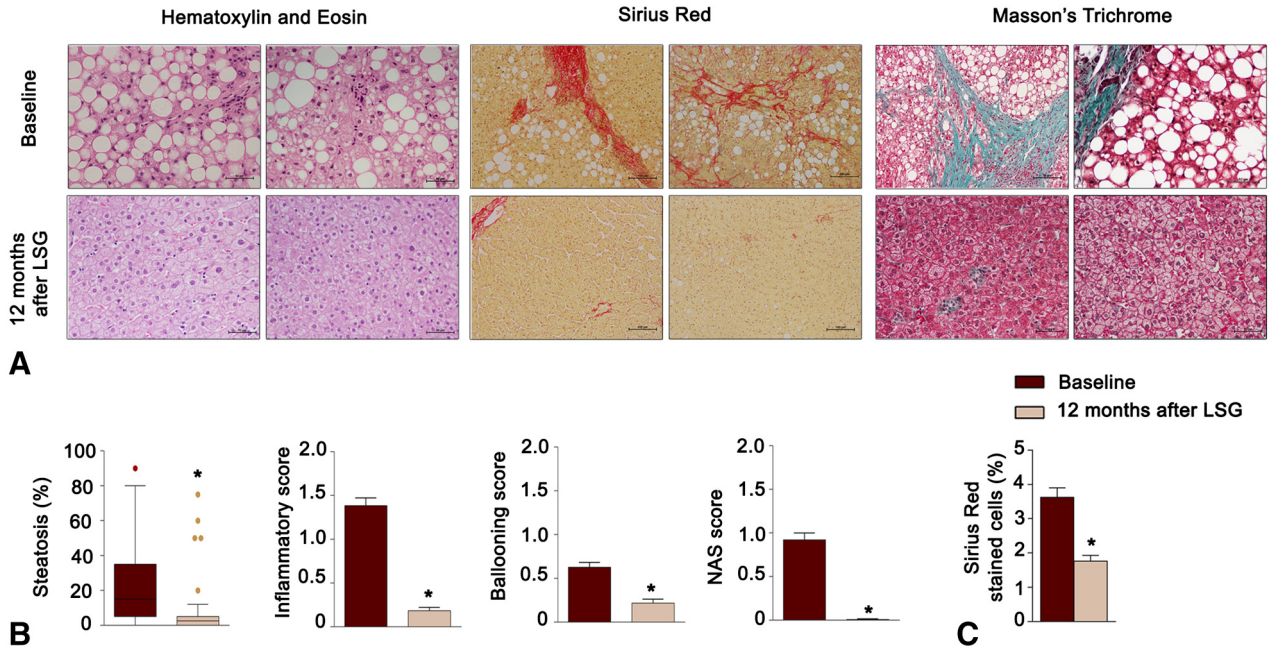

Fig. 1. Post-laparoscopic sleeve gastrectomy (LSG) improvement in liver histological features of patients with non-alcoholic fatty liver disease. (A) Representative microphotographs (bars indicate 100× magnification) of baseline and 12 months post-surgery hepatic biopsies stained with Hematoxylin and Eosin, Sirius Red and Masson's Trichrome. (B) Steatosis, inflammation ballooning and NAS score were quantified according to the non-alcoholic fatty liver activity score (NAS) system. (C) Sirius Red was quantified as percentage of positively-stained areas. Results are shown as means±SEM. *$p < 0.001$ by the Mann-Whitney *U* test.

hydrolase (FAH) was used as a reference (control) protein. Protein bands were visualized using SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL, USA) and analyzed with a ChemiDoc system using Image Lab 2.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Statistical analyses

Results are shown as proportions (qualitative data) and as means ± SEM or medians and interquartile range (quantitative data), as indicated in the tables and figure legends. Kolmogorov-Smirnov test was used to assess the normal distribution of variables. Student's *t*-test (parametric) and Mann-Whitney's *U* test (nonparametric) were used to assess differences between groups. All statistical analyses were carried out by using the SPSS 22.0 package (IBM Corp., Armonk, NY, USA). Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Metabolic outcomes and remission of hepatic alterations post-LSG

Pre-LSG, patients with severe obesity had decreased insulin sensitivity, increased chronic low-grade inflammation, higher prevalence of type 2 diabetes mellitus (T2DM), dyslipemia and hypertension, compared to the healthy population. We observed a high ratio of women to men in the obese cohort. Data presented here are without sex segregation because of the longitudinal nature of the study and, as well, because logistic regression analyses discarded sex as a determinant factor in diagnosis and/or disease outcomes. According to the NAS score, non-NASH, probable NASH and definite NASH were recorded in 43.8%, 34.6% and 21.6% of patients, respectively (Table 1).

One year post-LSG, most clinical and biological metabolic outcomes significantly improved, together with a general amelioration of histological features of NAFLD; improvement was more evident in the most severe cases. Mild steatosis was observed in 4 patients (3%), mild lobular inflammation (<2 foci) in 22 patients (18.4%) and hepatocyte ballooning in 21 patients (17.5%). Fibrosis also improved, especially in the few patients with bridging fibrosis (Table 2 and Fig. 1). Of note, one patient with pre-surgery liver cirrhosis presented only periportal/perisinusoidal fibrosis one year post-surgery (Supplementary Fig. 1).

3.2. Oxidation and inflammation and their association with NASH

We found a significantly higher proportion of PON1, 4-hydroxy-2-nonenal and CD68 stained cells in liver biopsies of patients with definite NASH (n = 94), compared to non-NASH patients (n = 191). Sirius-red-positive areas were also significantly higher (Fig. 2A). CD68 stained cells were more frequent in areas with inflammation and PON1 staining was stronger in hepatocytes with ballooning degeneration. Fat accumulation and 4-hydroxy-2-nonenal staining were more intense in fibrous areas (Supplementary Fig. 2).

We observed significant alterations in the pre-surgery circulating levels of molecules that tracked with oxidation and inflammation. Serum paraoxonase and lactonase activities were significantly decreased in obese patients, but serum PON-1 concentration remained unaltered. Low PON-1 activities were associated with high plasma CCL2, but these measurements did not track with patients through the different stages of NAFLD (Fig. 2B). Circulating levels of TNF-α and IL-10 were also significantly different from those found in control subjects, but differences between non-NASH and definite NASH patients were either minor or negligible. Plasma galectin-3 levels were significantly higher in patients with definite NASH when compared with non-NASH patients (Fig. 2B).

3.3. LSG outcomes promote remission of hepatic alterations through multiple cellular responses

Using selected key markers we compared oxidation, inflammation and fibrosis in liver tissues at baseline and 12 months post-LSG. There were significant reductions in the hepatic immunochemical expressions of PON-1, 4-hydroxy-2-nonenal, CD68, CCL2, CCR2, TNF-α, and galectin-3; but IL-10 staining remained unaltered (Fig. 3). For cross validation we used western blot analysis. We observed a significant reduction in the expression of TNF-α and galectin-3, with minor changes in

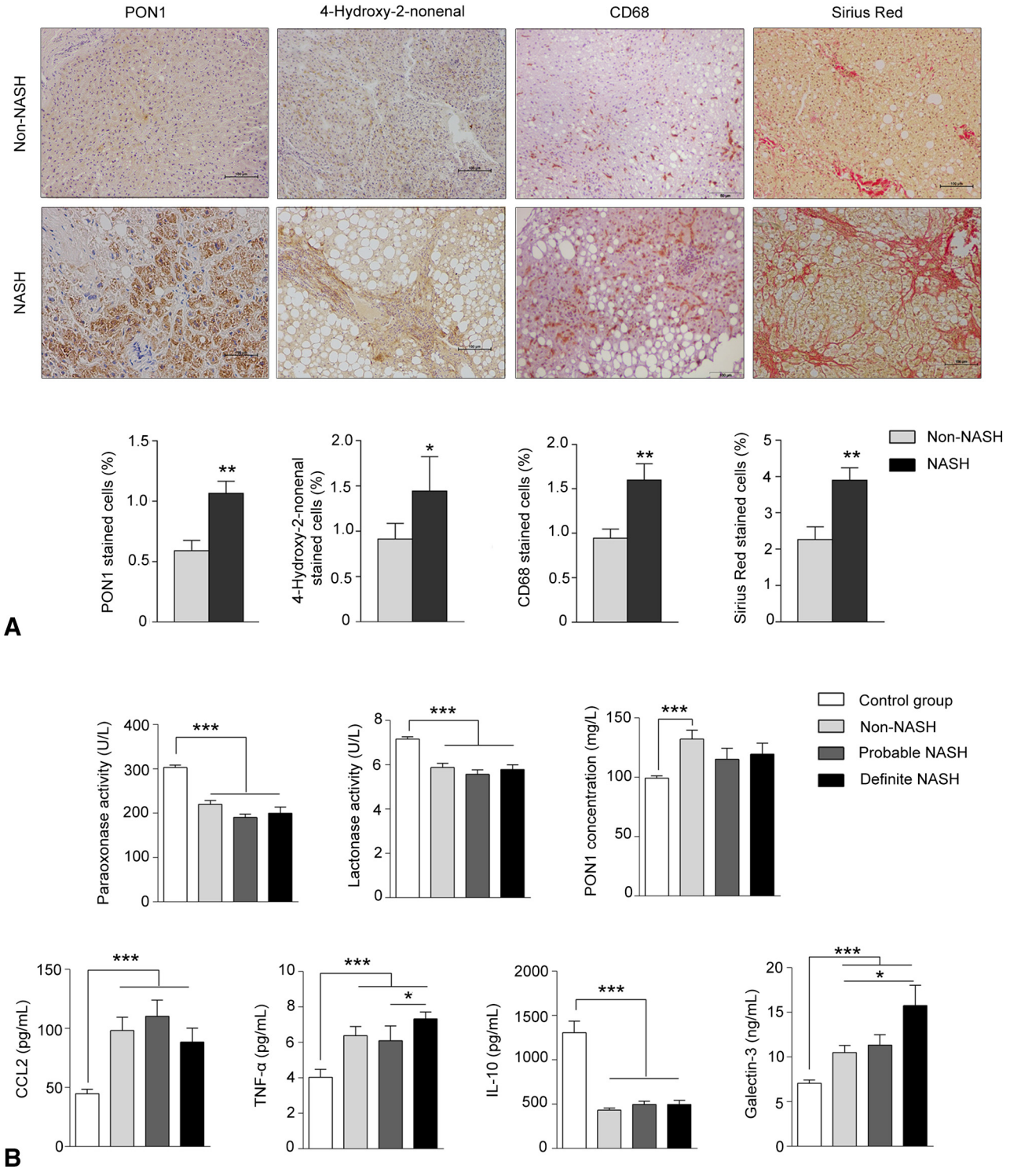

Fig. 2. Hepatic oxidation and inflammation discriminate patients with definite NASH from those without. (A) Definite NASH patients had higher hepatic paraoxonase-1 (PON1), 4-hydroxy-2-nonenal, and cluster of differentiation 68 (CD68) expressions and Sirius Red staining compared to non-NASH individuals (bars indicate 100× magnification). (B) Circulating levels of paraoxonase and lactonase activities, and paraoxonase-1 (PON1), chemokine (C-C motif) ligand 2 (CCL2), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10) and galectin-3 concentrations. Results are shown as means±SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by the Mann-Whitney *U* test.

IL-10. Variations in the expression of CD163 did not reach statistical significance. We also assessed the effect of LSG in relation to the hepatic expression of STAT-3 and phosphorylated STAT-3. Both had 4-fold increase in expression post-surgery, which would indicate increased production and activation following NAFLD remission. The extent of hepatic glycated PON-1 (the 45 kD band), which is less effective in providing protection against oxidative response, was not significantly reduced. However, the unmodified, more active enzyme (the 40 kD band) that had been practically absent pre-surgery, was prominent post-surgery. Finally, we observed a significant decrease in the expression of α-smooth muscle actin (α-SMA) and sonic hedgehog (Shh) protein, indicating regression of liver fibrosis-activating pathways (Fig. 4).

Significant variations were observed in circulating paraoxonase activity and galectin-3 levels post-surgery. Circulating PON-1 and CCL2

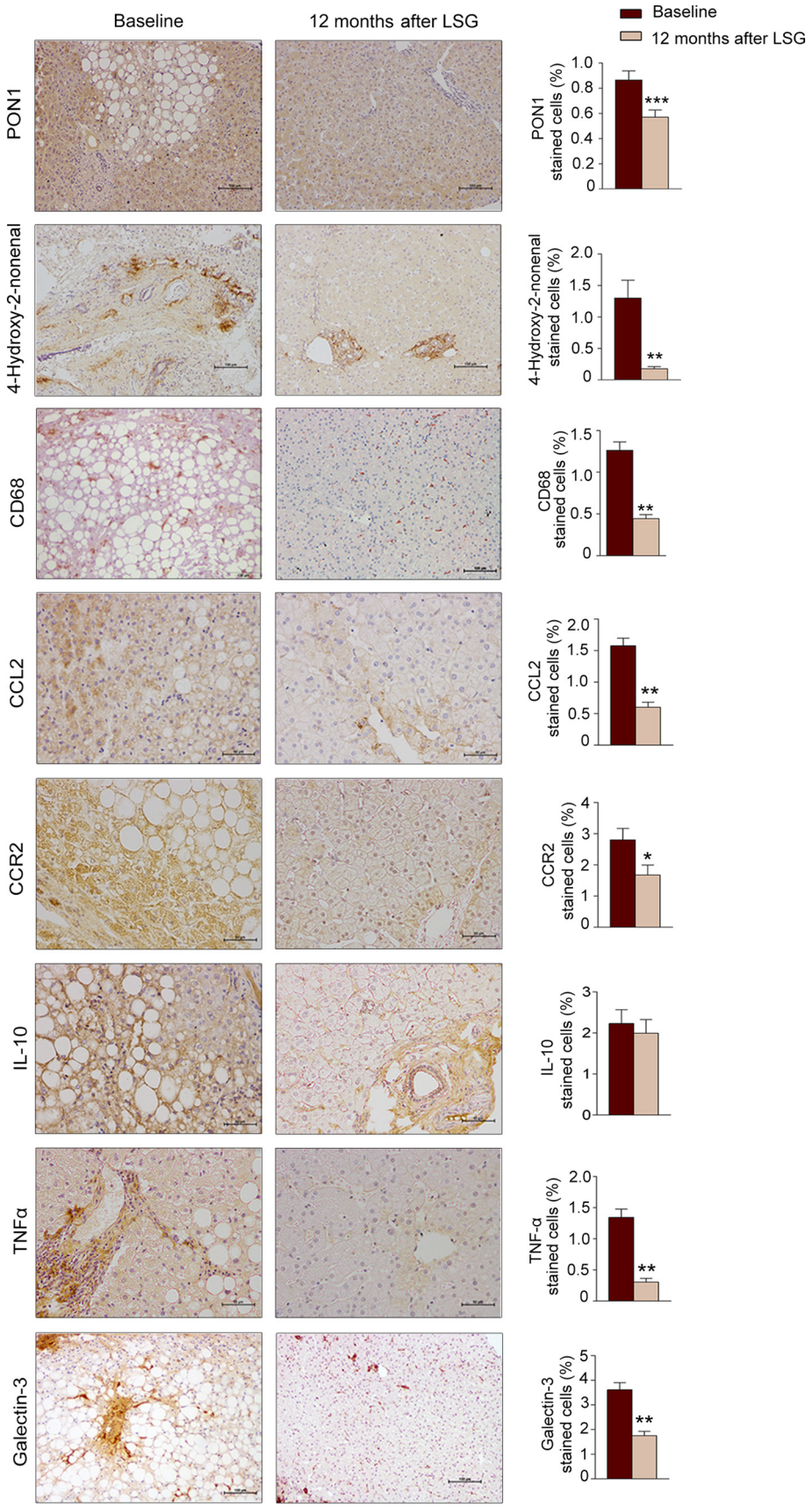

Fig. 3. Effect of laparoscopic sleeve gastrectomy in oxidation and low-grade systemic inflammatory balance. Differences in the hepatic immunochemical staining of paraoxonase-1 (PON1), 4-hydroxy-2-nonenal, cluster of differentiation 68 (CD68), chemokine (C-C motif) ligand 2 (CCL2), C-C motif chemokine receptor 2 (CCR2), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10) and galectin-3 in patients pre- and 12 months post-surgery (bars indicate 100× magnification). Results are shown as means±SEM. *$p < 0.01$, **$p < 0.001$ by the Mann-Whitney *U* test.

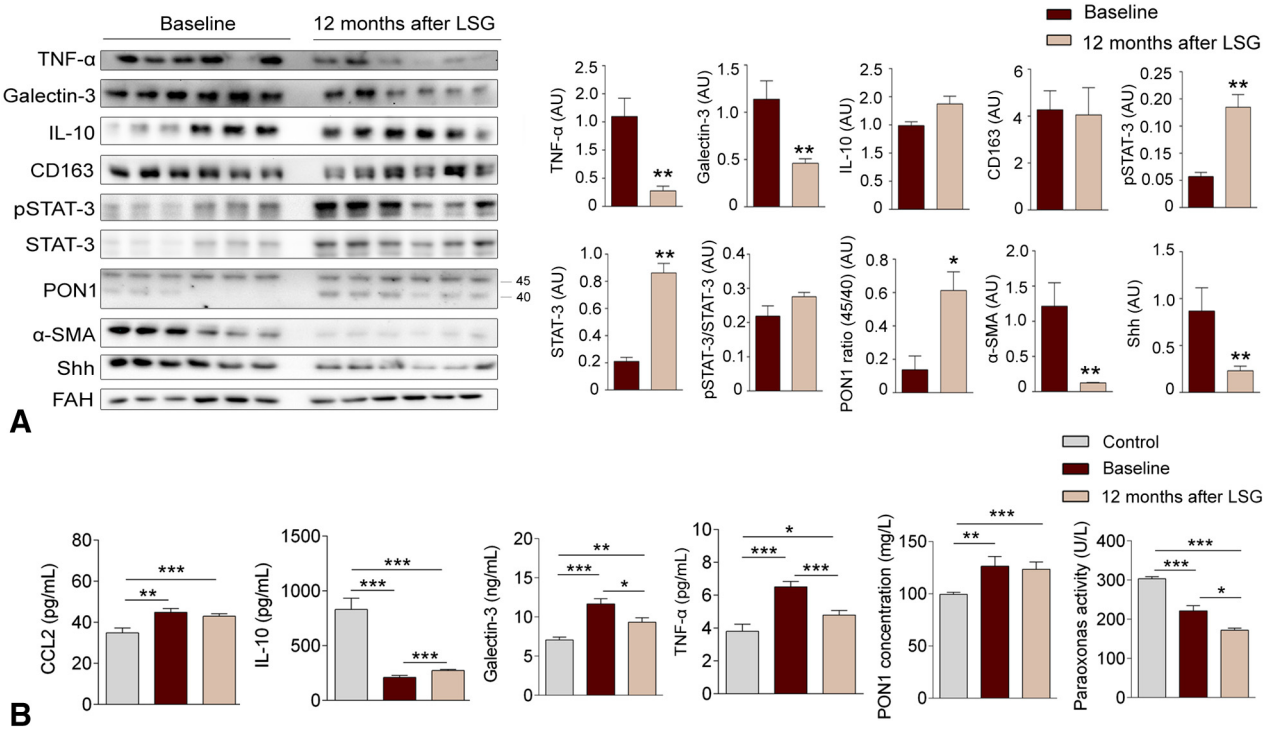

Fig. 4. Laparoscopic sleeve gastrectomy (LSG) improves the hepatic levels of oxidative stress and inflammation markers. (A) Western Blot analysis of tumor necrosis factor-α (TNF-α), galectin-3, interleukin-10 (IL-10), cluster of differentiation 163 (CD163), phosphorylated signal transducer and activator of transcription-3 (pSTAT3), signal transducer and activator of transcription-3 (STAT3), paraoxonase-1 (PON1), α-smooth muscle actin (α-SMA), and sonic hedgehog protein (Shh). Pooled liver extracts were used for cross validation (left) and mean values of variations in the expression of selected markers are shown on the right. The graph of paraoxonase-1 shows the ratio between the 40 kD and the 45 kD isoforms. (B) Circulating levels of chemokine (C-C motif) ligand 2 (CCL2), interleukin-10 (IL-10), galectin-3, tumor necrosis factor-α (TNF-α), and paraoxonase-1 (PON1) concentration and activity, in obese patients before and after surgery, and in the control group. Results are shown as means±SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by the Mann-Whitney *U* test.

concentrations remained high in patients with biopsy-proven NAFLD remission. Mean plasma TNF-α concentrations were normalized, and circulating IL-10 levels were even higher following remission (Supplementary Fig. 3).

4. Discussion

Bariatric surgery is a safe and effective procedure for weight loss in persons with severe obesity refractory to lifestyle modifications [18]. However, the clinical take-up of this procedure remains low even in patients meeting all criteria for eligibility. Here we provide evidence that all comorbidities, including NAFLD, significantly improved within one year post-surgery, following weight loss and metabolic improvement. Our findings of the impact of LSG on NAFLD regression are consistent with previous studies [1]. Indeed, it has already been reported that bariatric surgery reduces the incidence of NASH and fibrosis [19–22] and of the circulating levels of aminotransferases [23,24] in obese patients, in addition to decreasing insulin resistance and BMI [20].

In the present study, indices of oxidation, inflammation and fibrosis were clearly altered in patients with definite NASH compared to those without NASH. Moreover, oxidation, inflammation and fibrosis in the liver substantially improved post-surgery. The measurement of molecules that have been shown to be good indicators of these phenomena confirmed the improvement. In particular, we had previously observed the close relationship between PON1 and CCL2 in the regulation of hepatic oxidative stress and inflammation [25,26]. In mice, *pon1* gene deficiency promotes fatty liver disease and *ccl2* gene deficiency abrogates it [25]. In humans, polyphenols attenuate liver damage by modulating gene expression pathways that regulate the roles of PON1 and CCL2 in oxidative stress and the inflammatory response [27]. Both processes are important in macrophage polarization, with potential impact on promoting the resolution of liver disease [28]. Increasingly, galectin-3 has been recognized as a modulator of oxidative stress, inflammation, fibrosis and angiogenesis [29]. The decrease in liver galectin-3 expression and the simultaneous decrease in the liver expression of α-SMA post-surgery appears to modify the hedgehog-signaling pathway; indicating that transition from the quiescent stellate cells to myofibroblastic stellate cells may be reversible [30]. In the current study we observed that LSG resulted in a significant increase in hepatic STAT-3, a cytoplasmic protein that, when phosphorylated, induces transcription of genes promoting cellular protective and proliferative effects [31]. Several authors have recently investigated the relationship between inflammation markers and NASH. CD44-deficient mice with steatohepatitis presented low hepatic CCL2 expression with partial correction of liver inflammation and injury and, in obese patients, hepatic CD44 was upregulated in subjects with NASH and correlated with the NAS score and hepatic CCL2 levels [32]. Similar correlations in patients were observed between TNF-α, CCL3, and NAFLD severity [33]. In addition, toll-like receptor-9, a protein that plays a key role in the regulation of immunity, it has been reported to be a pro-inflammatory trigger in NASH [34].

Preliminary results in some of our patients that have been followed for 5 years indicate that their metabolic status has not worsened and this makes us feel optimistic about the evolution of their levels of oxidative stress and inflammation parameters. However, these data must be confirmed in the future with an adequate number of cases and the relevant laboratory analyses.

Our obese patients were mostly women and this is a relevant topic, since recent studies showed that sex hormones affect the incidence and severity of NAFLD and NASH [35–38]. Estrogen deficiency worsens NASH in mice models with fatty liver and the prevalence of NAFLD is higher in postmenopausal compared with premenopausal women [38]. Menopause seems to interplay with an increase in abdominal adipose mass and inflammation, which are both closely related to the severity and progressive forms of NAFLD [36]. Experimental studies in mice showed that sex hormones affect the severity of NASH, and regulate pro-inflammatory cytokine levels [37].

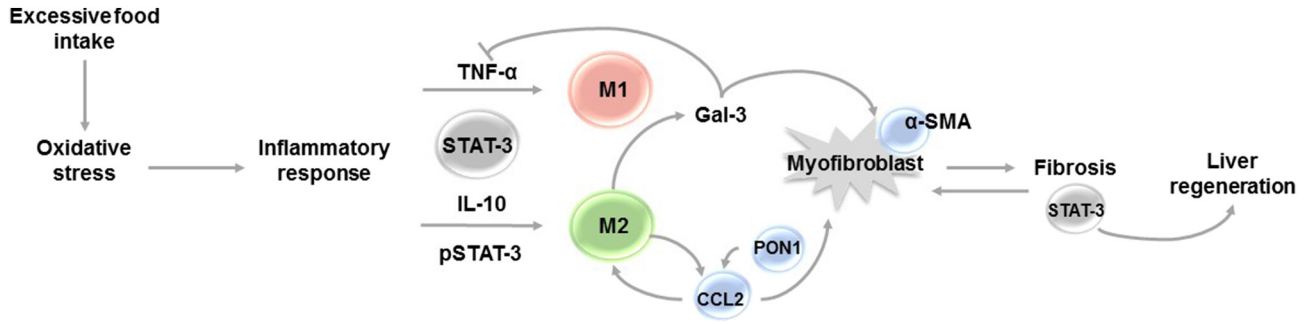

Fig. 5. Hypothetical scheme for the sequence of events during the development of non-alcoholic fatty liver disease, and the multi-factor effects of laparoscopic sleeve gastrectomy. Excessive energy intake promotes pro-inflammatory M1 macrophage polarization, resulting in an increase in cytotoxicity, inflammation and fibrosis. Gastrectomy, by reducing food intake, counteracts these effects and induces a "polarization switch" of macrophages to the anti-inflammatory M2. CCL2: chemokine (C-C motif) ligand 2; Gal-3: Galectin-3; IL-10: interleukin-10; PON1: paraoxonase-1; pSTAT3: phospho signal transducer and activator of transcription 3; α-SMA: α-smooth muscle actin; STAT3: signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor-α.

Limitations of this study are inherent in the design; in particular, a relatively short-term follow-up, enrolment of referral patients at a single hospital, and being a per protocol study, without randomized control subjects. Further, criteria for entry into the study were strict and carefully characterized; aspects that are not feasible in routine clinical practice. It would have been very informative to have a control group of morbidly obese patients, with paired liver biopsies, not having being subjected to bariatric surgery and followed-up for similar time with those subjected to surgery. However, we could not investigate a group with these characteristics for obvious ethical reasons. As such, surveillance bias cannot be ruled out. Future research should investigate long-term outcomes post-surgery. However, the sparse data available indicate a clear association with sustained weight loss, reduced comorbidities, and higher effectiveness compared to intensive lifestyle interventions [39]. Moreover, our study was restricted to a limited set of biomarkers associated with oxidative stress and inflammation. We do not rule out the possibility that other factors such as changes in lipogenesis, endoplasmic reticulum stress, insulin resistance or fibrogenesis could be related to the remission of hepatic alterations [40,41].

Our results suggest a sequential involvement of multiple cellular responses, and support the concept of applying a combination of different therapies to achieve non-invasive regulation of several molecular networks (Fig. 5). Assaying a single, expensive and potentially toxic new compound does not seem a desirable strategy, considering the multifactorial nature of NAFLD development. Positive modulation of liver function requires considerable weight loss and profound changes in lifestyle. Some well-tried and safe drugs may help improve insulin sensitivity but are fairly ineffective without dietary restraint [42]. Our histology evidence confirmed that reducing oxidative stress and suppressing activation of liver inflammatory cells are valuable therapeutic targets. Dietary antioxidants, insulin sensitizers, and lipid-lowering agents can, when used in combination, boost intracellular protection against lipoperoxides, suppress key inflammatory signaling systems, and induce reparative stress signaling [43,44]; all of which warrant further randomized controlled trials with a multi-targeting approach to determine dosage, duration of treatment, and modes of action when used in combinations.

In conclusion, our study suggests that LSG improves the histology and liver function of patients with morbid obesity. The mechanism involves the reduction of oxidative stress and inflammatory processes. These data encourage the use of this procedure as a therapeutic option to improve, or resolve, obesity-associated liver disease.

Declaration of Competing Interest

No potential conflicts of interest declared.

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Appendix A. Supplementary data

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