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## Vertical Sleeve Gastrectomy Attenuates the Progression of Non-Alcoholic Steatohepatitis in Mice on a High-Fat High-Cholesterol Diet

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### Abstract

**Objective**—To determine whether vertical sleeve gastrectomy (VSG) attenuates fibrosis in mice on a high-fat high-cholesterol (HFHC) diet.

**Background**—Bariatric surgery mitigates non-alcoholic steatohepatitis in 85–90% of obese patients. While animal models demonstrate similar results on a high-fat diet, none have observed the effects of bariatric surgery on a combined HFHC diet.

**Methods**—Mice on a HFHC diet were used to confirm the development of hepatic fibrosis at 8 ( $n = 15$ ) and 24 ( $n = 15$ ) weeks. A separate cohort of mice on a HFHC diet for 12 weeks was subjected to either VSG ( $n = 18$ ) or sham ( $n = 12$ ) operations and remained on a HFHC diet for an additional 20 weeks. Changes in weight, dyslipidemia, and the development of steatosis and fibrosis were documented. Serum was obtained for bile acid analysis by liquid chromatography

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The animal studies were approved by the Institutional Animal Care and Use Committee at UCLA.

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Statement** All applicable institutional and/or national guidelines for the care and use of animals were followed.

**Informed Consent** Does not apply.

**Disclosure** The content is solely the responsibility of all the authors and does not necessarily represent the official views of the Children's Discovery Institute.

and mass spectrometry, while hepatic gene expression by RT-PCR was performed to evaluate intrahepatic lipid metabolism.

**Results**—Hepatic steatosis and fibrosis developed after 8 weeks on the HFHC diet. After VSG, mice demonstrated a sustained decrease in weight with a significant decrease in fibrosis compared to sham mice. Serum total cholesterol, HDL, and LDL were significantly reduced following surgery, while serum bile acids were significantly elevated. Intra-hepatic cholesterol excretion was not upregulated based on hepatic gene expression of CYP7A1 and ABCG5/8.

**Conclusions**—VSG attenuates the development of hepatic fibrosis in diet-induced obese mice, presumably through enhancement of cholesterol elimination at the intestinal level.

### Keywords

Vertical sleeve gastrectomy; VSG; NASH; Non-alcoholic steatohepatitis; Fibrosis

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### Introduction

Non-alcoholic fatty liver disease (NAFLD) describes varying degrees of hepatic steatosis, from simple steatosis to hepatic inflammation, injury, and fibrosis, also known as non-alcoholic steatohepatitis (NASH) [1]. Since the 1980s, NAFLD has increased 2.7-fold [2, 3], making it the most common chronic liver disease in adults and children [4, 5]. Without medical therapy or treatment, the disease is capable of progressing to cirrhosis and end-stage liver disease, ultimately requiring liver transplantation [6].

Current treatments for NASH are limited to weight loss strategies through diet modification and physical activity, which remain difficult for many patients. It is estimated that a decrease in weight by at least 7% is required to improve or resolve NASH, but long-term weight loss is only achieved by 3–6% of these patients [7, 8]. Although used to induce sustained weight loss in morbidly obese patients, bariatric surgery is currently not indicated for patients with NASH [9]. Preliminary data, however, demonstrates that the resolution of NASH occurs in 85–90% of patients who undergo weight loss surgeries [10, 11]. While this phenomenon was initially attributed to caloric restriction, animal models now reveal that the mechanism transcends weight loss with much more complexity than previously thought. Recent studies have cited alterations in gut microbiota [12] and serum bile acids [13, 14] as a consequence of bariatric surgery, but the implication of these observations in the resolution of NASH remains unclear.

A major barrier to understanding the pathogenesis of NASH hinges upon poor animal models that depict NAFLD progression in humans. High-fat diets alone have been used to generate hepatic steatosis, but fibrosis fails to develop without the addition of cholesterol [15, 16]. In order to mimic human intake, high-fat high-cholesterol (HFHC) diets have recently been employed [17, 18]. Prior studies have successfully induced hepatic steatosis and fibrosis with the use of high-fat diets with 1–2% cholesterol, but the utility of high-cholesterol diets beyond 2% has not been determined. Therefore, we sought to clarify whether an increase in cholesterol supplementation would worsen and expedite fibrosis in mice. Though the addition of 10% cholesterol did not increase or hasten fibrosis, we report

that it maintains the ability to induce NASH in mice. Next, to address the effect of bariatric surgery in this particular model of NASH, mice were subjected to vertical sleeve gastrectomy (VSG) while remaining on a HFHC diet. We report that sleeve gastrectomy attenuates the progression of NASH in mice and proceed to characterize the alterations in intra-hepatic lipid metabolism after VSG. To our knowledge, this is the first study to document the effects of VSG on fibrosis in mice on a HFHC diet.

## Materials and Methods

### Animals and Diet

The animal studies were approved by the Institutional Animal Care and Use Committee at UCLA. Five-week-old male C57BL6 mice were obtained from Jackson Laboratory and housed at  $22 \pm 2$  °C on a standard 12-h light/dark cycle. Since prior studies have shown that fibrosis does not occur without both high fat and cholesterol intake [15], all mice were subjected to a high-fat diet with varying amounts of cholesterol. Five-week-old mice were allowed ad libitum access to a control diet versus a high-fat diet of 45 kcal% fat and 20 kcal % fructose with 2% ( $n = 5$ ) or 10% ( $n = 5$ ) cholesterol for 8 and 24 weeks (Fig. 1a). Another cohort of 5-week-old mice was fed a high-fat diet of 45 kcal% fat and 20 kcal% fructose with 10% cholesterol for 12 weeks before undergoing vertical sleeve gastrectomy ( $n = 18$ ) or sham operations ( $n = 12$ ). After surgery, these mice remained on a high-fat diet with 10% cholesterol for an additional 20 weeks (Fig. 1b). Mice that received VSG were housed in different cages from those that underwent a sham operation. Weight and food intake were documented weekly.

### Surgery

VSG surgery was performed using isoflurane anesthesia. Seventy to eighty percent of the lateral stomach was excised along with a surgical clip, leaving a tubular gastric remnant in continuity with the esophagus superiorly and the pylorus and duodenum inferiorly. Care was taken to remove the whole fundus of the stomach. The sham procedure involved analogous isolation of the stomach followed by manually applying pressure with blunt forceps along a vertical line between the esophageal sphincter and the pylorus. After surgery, mice consumed a liquid diet (Osmolite OneCal) for 3 days post-operatively and were reintroduced to solid food (high-fat high-cholesterol diet) on post-operative day 4. Both VSG and sham mice received amoxicillin for 10 days after surgery.

### Serum and Hepatic Biochemical Assays

At the time of sacrifice, blood was obtained and serum was isolated for the analysis of hepatic transaminases, lipid profiling, and insulin levels. All mice endured a 6 h fast prior to blood collection. The levels of serum transaminases, lipids, and insulin were measured using the diagnostic laboratory at the IDEXX bioresearch company. HOMA-IR indices were calculated as a measure of insulin resistance, using the following formula: fasting insulin (mIU/L)  $\times$  fasting glucose (mmol/L)/22.5. GTT was obtained as previously described [19] at 24 weeks on the HFHC diet and at 12 weeks post-operatively.

## Histology

Murine livers were obtained for hematoxylin-eosin and Sirius red staining. Histologic analyses of NASH included evaluation of steatosis and hepatic fibrosis. To quantify fibrosis, multiple quadrants from each slide were photographed at  $\times 10$  magnification. The percentage of fibrosis from each section was quantified using imageJ software. The sections were subsequently averaged to achieve a final fibrosis percentage for each sample.

## Quantitative Real-Time PCR

Hepatic tissue was obtained upon euthanasia and snap frozen in liquid nitrogen. RNA was extracted using the RNeasy plus mini kit (Qiagen) and quantitative real-time PCR was performed using the q-script one-step RT-qPCR kit (Quantabio). Gene expression results were normalized using GAPDH.

## Serum Bile Acid Analysis

Serum was isolated to compare bile acid content prior to and after VSG and sham operations. Liquid chromatography and mass spectrometry were utilized to isolate and quantify the following bile acids in serum: cholic acid, a-muricholic acid, b-muricholic acid, tauromuricholic acid, and tauro-cholic acid. Methods were performed as previously described [20].

## Statistical Analysis

All data is presented as a mean  $\pm$  standard error in age-matched mice, receiving various treatments. Two mice that underwent VSG were excluded due to incomplete data, while 3 mice that received sham operations were excluded due to co-housing with VSG mice. A total of 7 mice from the VSG cohort and 8 mice from the sham group were ultimately included in the analysis for all reported indices (Fig. 1b). Statistical significance was assessed by the student's *t* test and  $p < 0.05$  was considered significant.

## Results

### The High-Fat High-Cholesterol Diet Induces NASH

Male C57BL6 mice were placed on a high-fat diet (45 kcal % fat) supplemented with either 2% or 10% cholesterol for 8 and 24 weeks. Compared to WT mice on standard chow, all mice on the HFHC diet demonstrated a significant increase in body weight, AST and ALT levels, liver to body ratios, hepatic steatosis, and hepatic fibrosis regardless of cholesterol content at 8 and 24 weeks (Fig. 2a–f). The addition of 10% cholesterol did not lead to increased hepatic steatosis and fibrosis compared to 2% cholesterol at 8 weeks and 24 weeks, indicating that cholesterol content beyond 2% does not expedite or worsen the development of NASH in mice. Plasma cholesterol, HDL, and LDL were significantly increased in both the HF + 2% and HF + 10% cholesterol groups versus control. Insulin resistance was achieved at 24 weeks based on glucose tolerance testing in mice on the HF + 10% cholesterol diet, though glucose, insulin, and HOMA-IR indices were no different compared to control (Table 1).

## VSG Reverses the Progression of Fibrosis in NASH

After 12 weeks on a HFHC diet, mice were randomized to receive VSG or sham surgery. Mice that underwent VSG experienced a mortality rate of 50% (9/18), while the mortality rate in sham mice was only 8% (1/12). Despite remaining on a HFHC diet, VSG mice demonstrated a sustained decrease in percentage total weight compared to sham post-operatively (Fig. 3a, b). Sham mice had an overall percentage total weight gain of 17%, while VSG mice had an average percentage total weight loss of 7.1% ( $p = 0.005$ ). Food intake was no different between sham and VSG mice at all time points (Fig. 3c). In fact, VSG mice demonstrated an increase in oral intake compared to sham and naïve mice; however, this did not reach statistical significance.

Serum AST ( $p = 0.041$ ), ALT ( $p = 0.019$ ), and liver to body ratios ( $p = 0.015$ ) were significantly decreased in mice that received VSG (Fig. 3d–f). Histologically, hepatic steatosis was reduced and the percentage of fibrosis was significantly decreased ( $p = 0.00005$ ) after VSG, but not after sham operation (Fig. 3g, h). Fibrotic markers, TIMP1 and Col1a1 were decreased in VSG mice, but this was not statistically significant compared to sham mice (Fig. 3i, j). Surprisingly, VSG mice demonstrated a significant decrease in serum cholesterol ( $p = 0.00009$ ), HDL ( $p = 0.00003$ ), and LDL ( $p = 0.028$ ) despite remaining on a HFHC diet. Sham mice, on the other hand, continued to reflect an elevation in serum cholesterol, HDL, and LDL levels (Fig. 4a–c). Serum triglycerides in both groups were no different compared to control (Fig. 4d). GTT at 12 weeks post-operatively revealed significantly elevated glucose levels in sham mice, while VSG mice demonstrated a normal response to glucose loading (Fig. 4e). Glucose levels, insulin levels, and HOMA-IR indices were no different between groups (Fig. 4f–h).

To determine the effect of VSG on hepatic cholesterol excretion, we interrogated the three pathways for cholesterol clearance from the liver: the conversion of cholesterol to bile acids by CYP7a1, the direct excretion into the biliary tract via ABCG5/8, and the loading of cholesterol into nascent HDL by ABCA1. We found mRNA expression of upstream mediators, LXR and FXR, to be decreased in the livers of both sham and VSG mice compared to control mice on standard chow. CYP7a1 and ABCG5/8 were also downregulated in both groups; however, intra-hepatic ABCA1 was significantly upregulated in VSG mice ( $p = 0.0026$ ) (Fig. 5). Intrahepatic enzymes involved in the fatty acid synthesis, SREBP1c, FAS, and scd1 were upregulated in both VSG and sham mice (Fig. 5). Intra-hepatic cytokine profiles were also assessed for mRNA expression. Surprisingly, TNF $\alpha$ , IL1b, IL6, CCL2, and TGFb levels were comparable in VSG and sham groups (Fig. 5). CXCL10 was the only cytokine that was significantly downregulated after VSG compared to sham ( $p = 0.029$ ).

Prior studies in VSG mice on a high-fat diet have shown that VSG leads to a change in serum bile acids. In our experimental system, serum was obtained prior to VSG and sham operations, as well as 8 weeks post-surgery. Tauro-muricholic acid ( $p = 0.020$ ), tauro-cholic acid ( $p = 0.021$ ), and cholic acid ( $p = 0.020$ ) were all significantly elevated after VSG compared to levels before VSG (Fig. 6).

## Discussion

We first evaluated the development of NASH in inbred mice on a high-fat diet with varying amounts of cholesterol. We hypothesized that a higher amount of cholesterol would accelerate the rate of fibrosis and/or lead to more severe fibrosis. Our results, however, indicate that mice in the 10% cholesterol group did not develop hepatic fibrosis at an earlier time point than the 2% group. Furthermore, the 10% cholesterol cohort did not exhibit more severe fibrosis by 24 weeks. Nonetheless, our diet successfully achieved hepatic steatosis and fibrosis, as well as insulin resistance and hypercholesterolemia, which is consistent with other murine studies [17, 18] that used diets with similar ratios of fat, cholesterol, and fructose. It is interesting to note that insulin resistance was demonstrated by GTT, but not by HOMA-IR. This may be due to an elevation in glucose and insulin levels that were observed in older mice on standard chow. For this reason, GTT may provide a better representation of glucose intolerance in aging mice.

After remaining on a HF 10% cholesterol diet for 12 weeks, mice were subjected to either VSG or sham operations and remained on a HF 10% cholesterol diet for an additional 20 weeks. VSG mice experienced a much higher mortality rate than sham mice, which was not surprising based on similar mortality rates reported by others [21, 22]. Most of our mice died within the first 2 weeks of VSG secondary to dehydration versus contained leaks. Of the surviving mice, no evidence for subclinical leaks were observed in VSG mice upon sacrifice. We do not believe that the mortality rates influenced our study results since we excluded dying mice and provided an adequate sample of healthy mice for analysis.

While prior studies have observed the outcome of VSG mice on a high-fat diet, none have determined how the addition of cholesterol impacts the overall effect of VSG. To address this discrepancy and provide a more accurate representation of human intake, we employed a high-fat 10% cholesterol diet. Our results corroborate other human and animal studies that demonstrate sustained weight loss with a significant reduction in serum transaminases and hepatic steatosis after VSG [23, 24]. Furthermore, we proceed to show that VSG halts the progression of fibrosis and significantly reduces serum cholesterol, HDL, and LDL, despite analogous cholesterol intake to sham. This suggests that VSG enhances cholesterol clearance via hepatic cholesterol excretion or through trans-intestinal cholesterol excretion [25, 26]—the only known pathways by which mice eliminate cholesterol.

Intra-hepatic cholesterol clearance occurs via bile acid synthesis, biliary excretion, and HDL secretion [27]. CYP7a1 and ABCG5/8 were downregulated in sham and VSG mice suggesting that cholesterol clearance from the liver was not enhanced through conversion to bile acids or via biliary excretion after VSG. More notably, the ABCA1 transporter was significantly upregulated in VSG mice compared to sham, indicating that HDL secretion by the liver was increased following VSG. However, it is unclear whether the increase in HDL secretion was a direct result of VSG or a compensatory response to low serum HDL levels. Nonetheless, since intrahepatic biliary excretion of cholesterol was not increased after VSG and serum HDL and cholesterol were significantly decreased, the mechanism for the elimination of cholesterol after VSG is likely due to an increase in trans-intestinal cholesterol excretion (TICE). Evidence for this phenomenon has been demonstrated in bile



duct ligation studies, where cholesterol excretion into the gut persists in the absence of biliary secretion [28, 29]. The transporters involved in TICE include intestinal ABCG5/8, which is mediated by intestinal LXR. Coincidentally, selective activation of intestinal LXR has been shown to reduce hepatic triglycerides and cholesterol accumulation in prior studies [30]. Based on this account, we suspect that the upregulation of intestinal LXR may play a role in cholesterol excretion after VSG; however, further studies will need to be performed.

Another potential mechanism to explain the reduction in hepatic steatosis after VSG includes alterations in gut micro-biota [22] that lead to subsequent changes in bile acid species. Prior studies have shown that bariatric surgery in humans and mice alter the intestinal microbiota with a reduction of *Firmicutes* and an increase in *Bacteroidetes* [31–33]. Furthermore, the beneficial effects of bariatric surgery were shown to be transferrable by fecal transplant into non-operated mice [34]. To prove that intestinal dysbiosis alters serum bile acids, another study demonstrated that antibiotic-treated mice on a high-fat diet exhibited an increase in muricholic acid (MCA) with subsequent improvement in hepatic steatosis [35]. This finding is of interest because muricholic acid is a known intestinal FXR antagonist and selective inhibition of intestinal FXR has been shown to attenuate hepatic steatosis in mice on a high-fat diet [36]. Although we did not evaluate for changes in fecal microbiota post-surgery, we infer from previous studies that VSG modulates gut microbiota. We proceed to show that VSG leads to a significant increase in serum tauro-muricholic acid post-operatively, which has been observed in prior animal studies [37, 38]. Additional experiments are needed to illustrate that the change in fecal micro-biota after VSG leads to a direct increase in MCA that inhibits intestinal FXR with subsequent improvement in hepatic steatosis. To date, no studies have linked these features together in VSG. Moreover, how intestinal FXR inhibition leads to decreased hepatic steatosis is unclear and warrants in-depth investigation.

In conclusion, our results confirm that VSG improves hepatic steatosis and fibrosis in a murine model of NASH. While preliminary human studies have demonstrated this effect, no animal models have documented the attenuation of fibrosis after VSG, which was observed in our experimental series. What is most striking in our study is the stark reduction of serum cholesterol after VSG despite HFHC supplementation, suggesting that VSG plays a role in enhancing cholesterol elimination. It is unclear whether the improvement in hepatic steatosis and fibrosis was a direct result of VSG via altered cholesterol metabolism or due to weight loss, since the study was not performed on pair-fed, weight-matched mice. It is interesting to note, however, that there was no significant difference in weight between sham and VSG mice at 7 weeks post-operatively (Fig. 3b), yet bile acids at 8 weeks after surgery were significantly increased in VSG mice. This implies that a change in bile acids occurred without a significant decrease in weight, though future experiments in pair-fed, weight-matched mice will need to confirm this observation. Despite this limitation, our results demonstrate reversal of hepatic steatosis, improvement in dyslipidemia, and increased serum bile acids after VSG, which is in agreement with published data where weight loss was accounted for [14, 24].



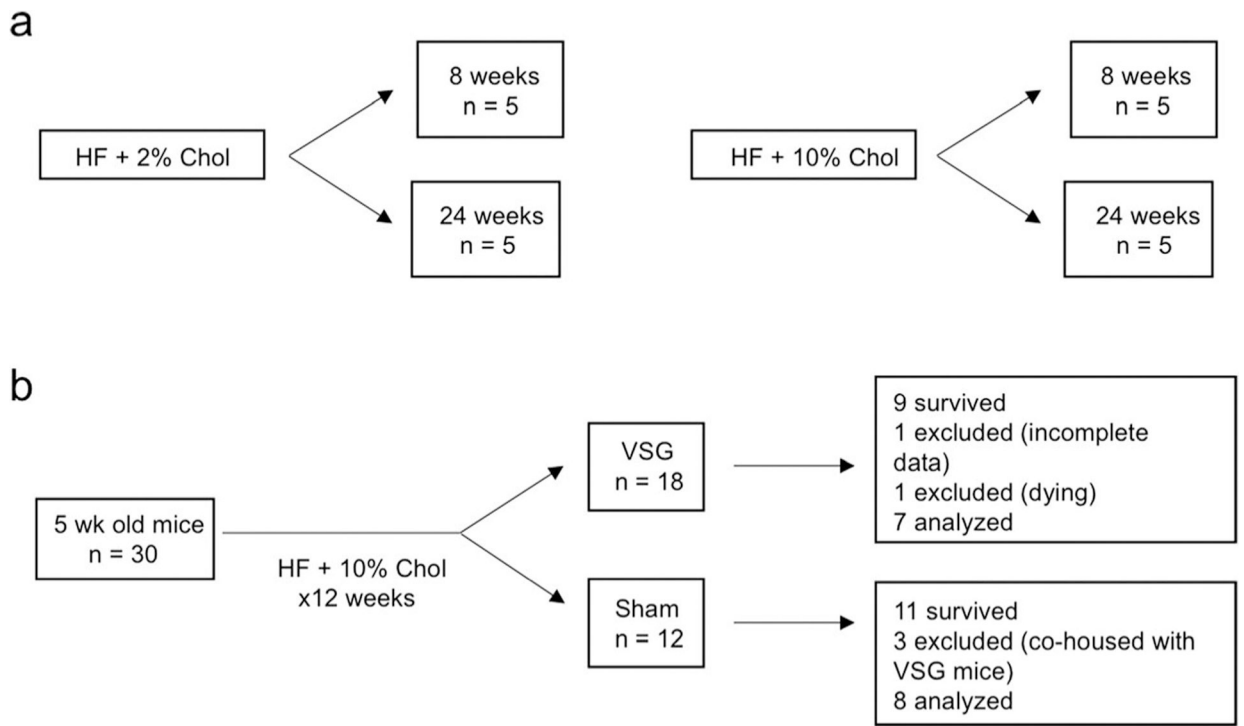
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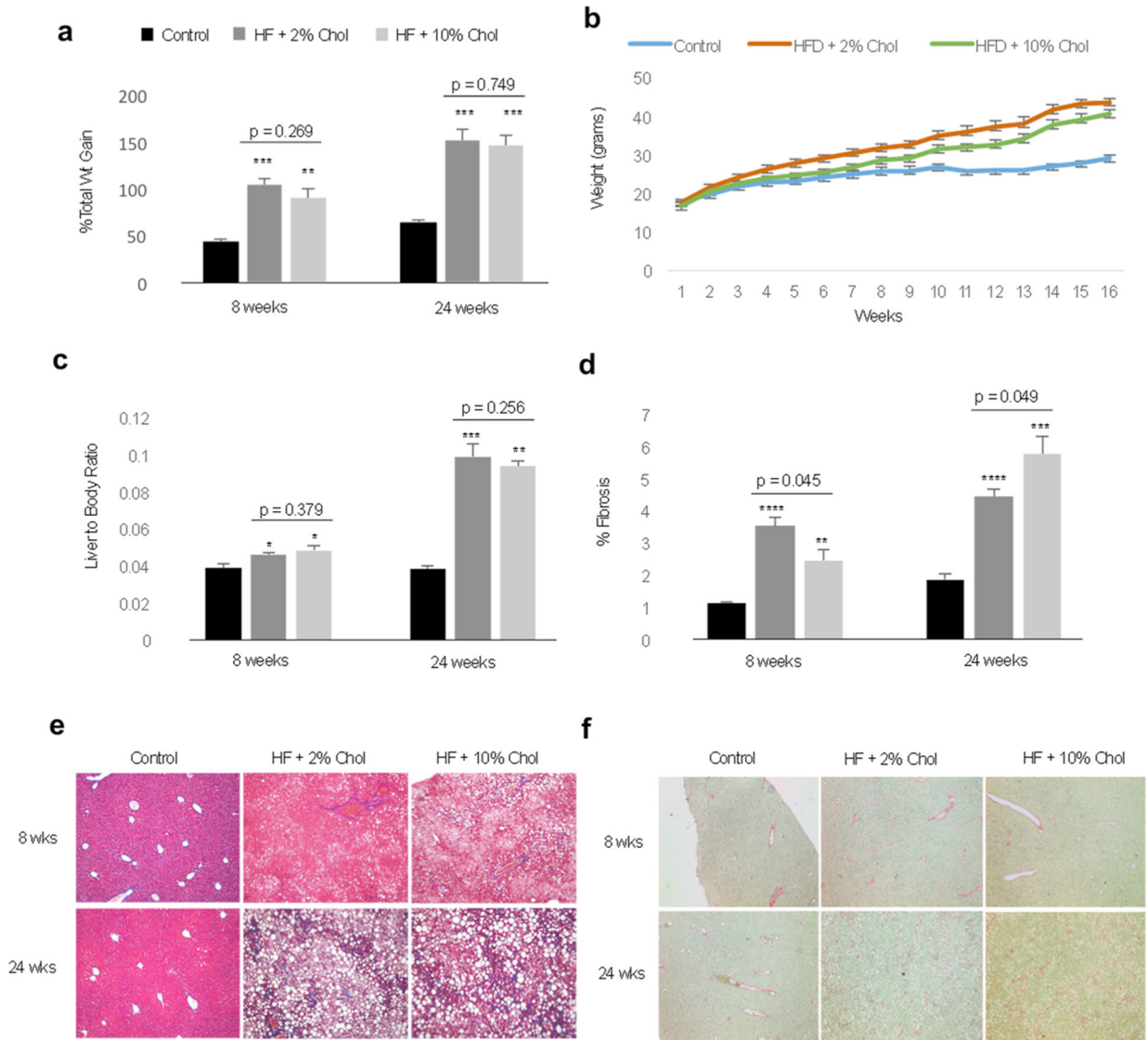
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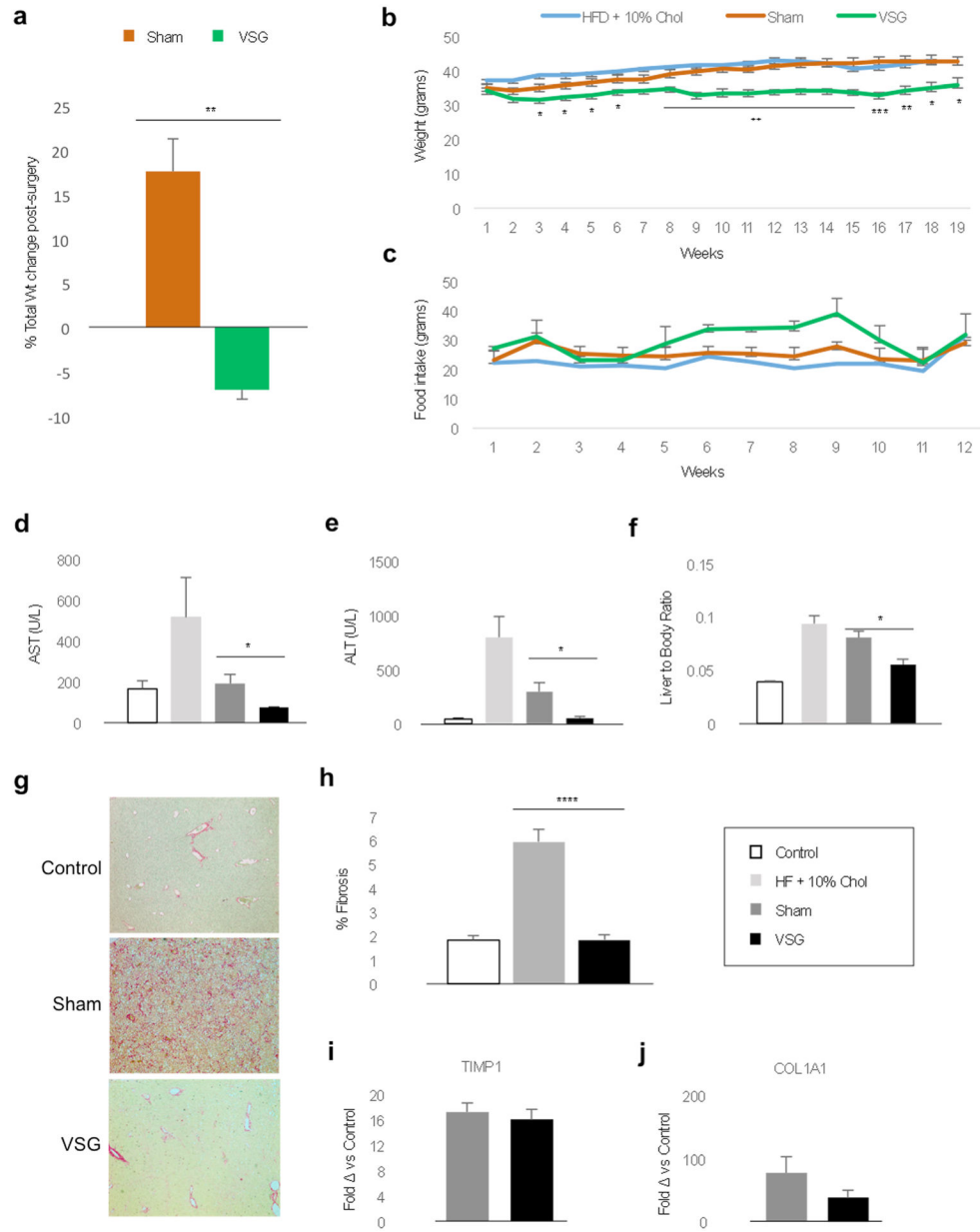
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**Fig. 1.**  
Schematic of experimental design

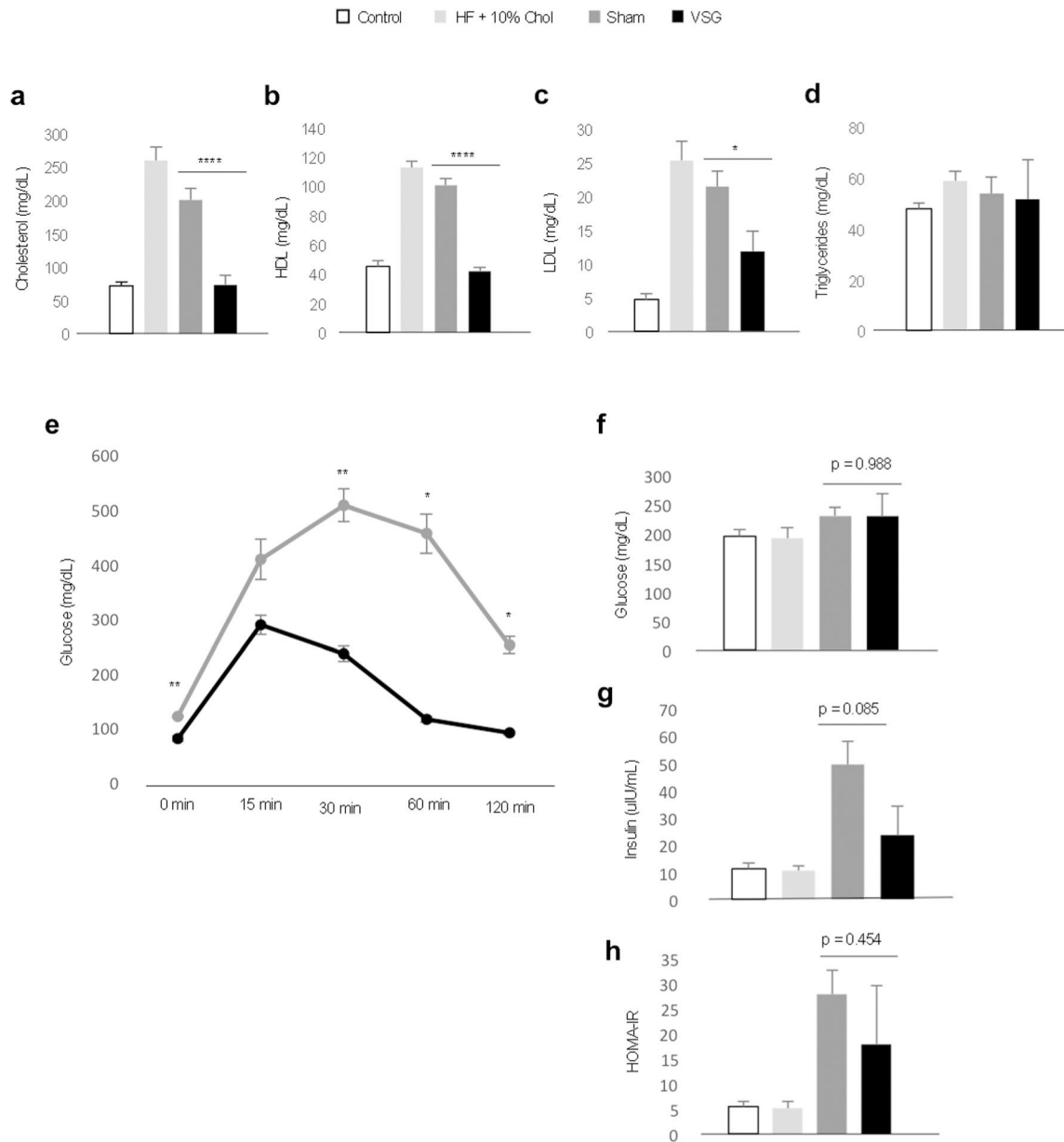


**Fig. 2.** The features of NASH in HF-fed mice with 2% or 10% cholesterol supplement vs. controls (at 8 weeks and 24 weeks of diet). **a, b** Weight gain; **c** liver/body weight ratio; **d, f** % fibrosis, as determined by Sirius red staining; **e** steatosis on hematoxylin and eosin staining. The HF + 10% cholesterol diet did not worsen any of these features as compared to the HF + 2% cholesterol diet. There were 5 animals per group. \**p* 0.05, \*\**p* 0.01, \*\*\**p* 0.001, \*\*\*\**p* 0.0001



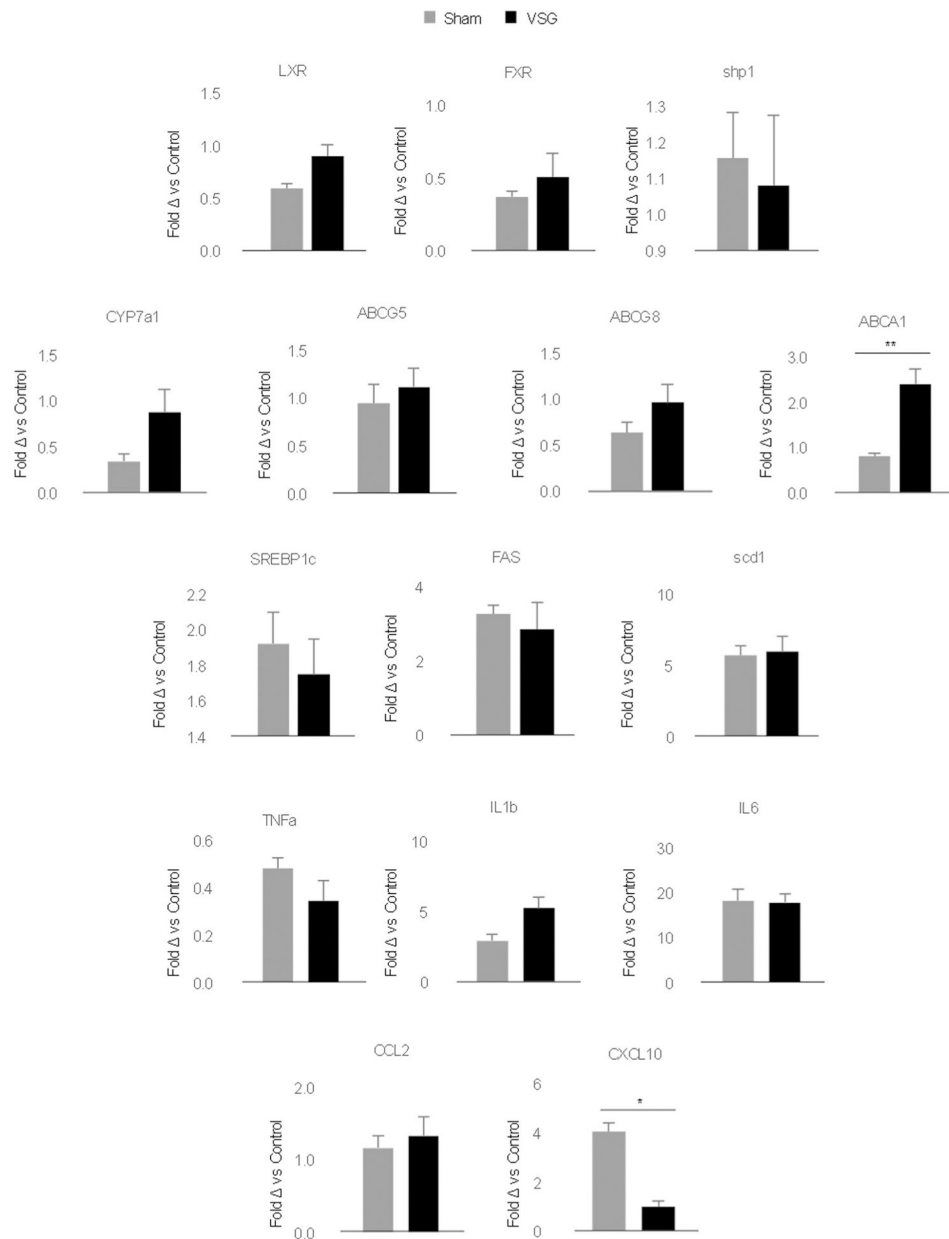
**Fig. 3.** The effects of VSG in mice on HF + 10% cholesterol diet at 20 weeks after the surgery. **a, b** Weight loss; **c** food intake; **d, e** hepatocellular liver function, as determined by serum AST and ALT levels; **f** liver/body weight ratio; **g, h** % fibrosis, as determined by Sirius red staining; **i, j** TIMP1 and Col1a1 fibrotic markers, as determined by qRT-PCR. VSG reversed the progression of NASH in HFD + 10% cholesterol-fed mice. 7 mice were included in the VSG group and 8 mice in the sham group.

\**p* 0.05, \*\**p* 0.01, \*\*\**p* 0.001, \*\*\*\**p* 0.0001

**Fig. 4.**

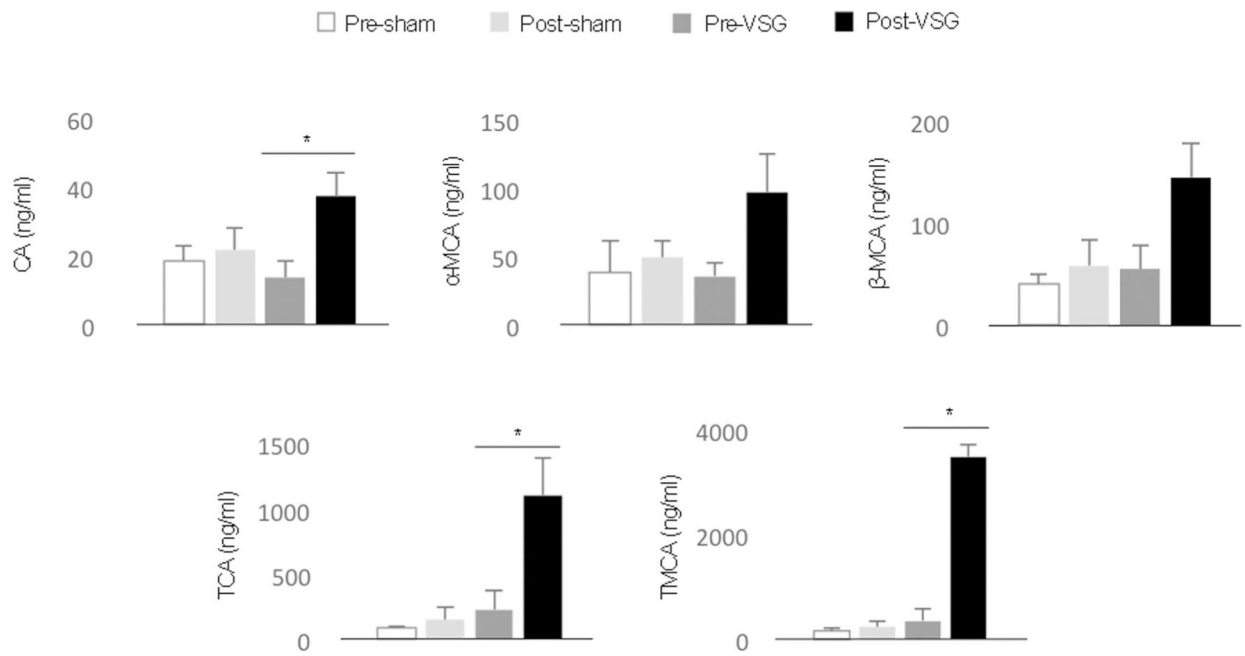
The effects of VSG at 20 weeks post-surgery. **a-d** Serum cholesterol, HDL, LDL, and triglyceride levels; **e** GTT (obtained at 12 weeks post-operatively); **f-h** serum glucose, insulin, and HOMA-IR indices. VSG mice demonstrated a significant decrease in serum cholesterol, HDL, and LDL despite remaining on a HFHC diet. 7 mice were included in the VSG group and 8 mice in the sham group.

\**p* 0.05, \*\**p* 0.01, \*\*\**p* 0.001, \*\*\*\**p* 0.0001



**Fig. 5.** The effects of VSG on hepatic cholesterol excretion and pro-inflammatory cytokine profile. Upregulation of qRT-PCR assisted expression of ABCA1 transporter ( $p = 0.0026$ ) and depressed CXCL10 chemokine levels ( $p = 0.029$ ) in VSG mice despite remaining on a HFHC diet. 7 mice were included in the VSG group and 8 mice in the sham group





**Fig. 6.** The effects of VSG on serum bile acids. Liquid chromatography/mass spectrometry-assisted levels of tauro-muricholic acid (TMCA,  $p=0.020$ ), tauro-cholic acid (TCA,  $p=0.021$ ), and cholic acid (CA,  $p=0.020$ ) were all significantly elevated at 8 weeks after VSG compared to levels before surgery. 7 mice were included in the VSG group and 8 mice in the sham group

**Table 1**

Metabolic parameters in control vs HF + 2% Chol vs HF + 10% Chol at 24 weeks.

Serum levels	Control	HF + 2% Chol ( <i>p</i> value vs control)	HF + 10% Chol ( <i>p</i> value vs control)	<i>p</i> value 2% vs 10%
AST (U/L)	165.8 ± 76.1	743 ± 631.6 (0.02) *	605.2 ± 342.4 (0.04) *	0.553
ALT (U/L)	48.8 ± 5.6	511.4 ± 214.9 (0.0009) **	951.2 ± 536.2 (0.02) *	0.127
Triglycerides (mg/dL)	48 ± 4.2	59.8 ± 11.1 (0.076)	55.6 ± 9.4 (0.16)	0.535
Total cholesterol (mg/dL)	72.3 ± 11.2	307 ± 69 (0.001) **	251.8 ± 48.7 (0.0008) **	0.182
HDL (mg/dL)	45.3 ± 7.6	113 ± 14.1 (6.9 × 10 <sup>-5</sup> ) *****	111.2 ± 6.8 (7.9 × 10 <sup>-6</sup> ) *****	0.804
LDL (mg/dL)	4.8 ± 1.7	29.4 ± 7.8 (0.002) **	23.6 ± 6.6 (0.002) **	0.238
Fasting glucose (mg/dL)	195.5 ± 22.8	168.8 ± 22.7 (0.126)	186.8 ± 57.5 (0.768)	0.533
Fasting insulin (microU/mL)	11.3 ± 3.9	18.3 ± 5.4 (0.06)	10.6 ± 3.7 (0.785)	0.031 *
HOMA-IR	5.5 ± 2.0	7.6 ± 2.9 (0.22)	5.0 ± 3.1 (0.814)	0.202
GTT (mg/dL) at 0 min	149 ± 26.7		181 ± 51.6 (0.313)	
GTT (mg/dL) at 15 min	342.3 ± 30.0		47.3 ± 69.5 (0.012) *	
GTT (mg/dL) at 30 min	294.3 ± 31.8		573.5 ± 21.9 (6.87 × 10 <sup>-6</sup> )	
GTT (mg/dL) at 60 min	224 ± 27.9		642 ± 239.6 (0.013) *	
GTT (mg/dL) at 120 min	168.8 ± 13.0		426 ± 48.7 (5.15 × 10 <sup>-5</sup> ) *****	

\* *p* 0.05,

\*\* *p* 0.01,

\*\*\* *p* 0.001,

\*\*\*\*\* *p* 0.0001