

Aspartate- β -hydroxylase suppresses nonalcoholic fatty liver progression by regulating AMPK phosphorylation and SREBP-1c expression.

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B.S., Simmons University, 2016

Thesis
Submitted in partial fulfillment of the requirements for the degree of Master of Science
in the Department of Molecular Pharmacology, Physiology & Biotechnology at Brown
University

PROVIDENCE, RHODE ISLAND
MAY 2020

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Acknowledgements

I want to thank everyone in the Liver Research Center. I am grateful to all of those with whom I have had the pleasure to work with. I want to express my most profound appreciation to my committee, Chiung-Kuei Huang for his mentorship through each stage of my Master's training. Dr. Wands, for the opportunity to complete my thesis in his lab, and Ji Su Li for the additional mentorship. Each member of my committee has provided me extensive personal and professional guidance. I would also like to thank Dr. Xiaoqun Dong for her kind generosity of human ASPH transgenic mouse, and my fellow lab mate Kevin Cao for his help in this project. I'd also like to acknowledge the assistance of Rolf Carlson for always providing us with the necessary tools to execute experiments and Donna Palange for her help and support. My deep gratitude to my family and friends for their unconditional love and support. This accomplishment would not have been possible without them. Lastly, I would like to thank Brown and the Molecular Pharmacology, Physiology, and Biotechnology department for this invaluable experience.

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Background

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disorder, characterized as the accumulation of lipid, triglycerides, and free fatty acids (FFA) in hepatocytes, not caused by alcohol consumption [1]. It affects one-quarter of adults worldwide, 60% of diabetic patients, and 90% of obese patients [2]. It is the leading cause of chronic liver disease and liver transplant, causing a substantial economic burden of an annual estimate of \$292 billion in the United States [2, 4]. NAFLD's strong associations with obesity, metabolic syndrome and type 2 diabetes [1,3,4], makes it a growing epidemic and prevalent worldwide, affecting 30% of the United States population; 32% of the Middle East; 30% of South America; 27% of Asia; 24% of Europe; and 13% of Africa [3,4,7,8]. It is also the most common cause of chronic liver disease in pediatrics due to its increasing prevalence in children with severe obesity [1,6,5].

NAFLD encompasses simple steatosis and non-alcoholic steatohepatitis (NASH) [1,2,4]. Simple steatosis is the first stage of NAFLD; it is asymptomatic and characterized when $\geq 5\%$ of lipid accumulation occurs in the liver without causing hepatic injury [1,24]. Whereas NASH is advanced stages of steatosis, it occurs when hepatic lipid accumulation progresses, causing inflammation and hepatic injury. [1,2,4]. Severe cases of NASH lead to liver fibrosis, which is the scarring of liver tissue, cirrhosis, and, ultimately, hepatocellular carcinoma [1,2,4].

The pathophysiology of NAFLD is complex and multifactorial [1]. Hormonal factors play an essential role in the pathogenesis of NAFLD. Patients experience an increase in triglyceride (TG) levels from free fatty acid (FFA) uptake, which is derived

from hepatic de novo lipogenesis, dietary fat, and release of free fatty acids (FFA) from adipose tissues (Figure 1) [1,3,7,18]. NAFLD is also linked to Insulin resistance (IR) leading to hyperinsulinemia and increase in de novo synthesis of fatty acids by inducing the activity of sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) (Figure 1), ultimately leading to hepatic steatosis [18]. NAFLD patients also suffer from an imbalance in energy homeostasis [8], a decrease in glucagon-like peptide 1 (GLP-1), which is the gut-derived hormone that causes satiety, and increase in a gut-derived hormone that induces hunger (e.g., Ghrelin) [7, 8].

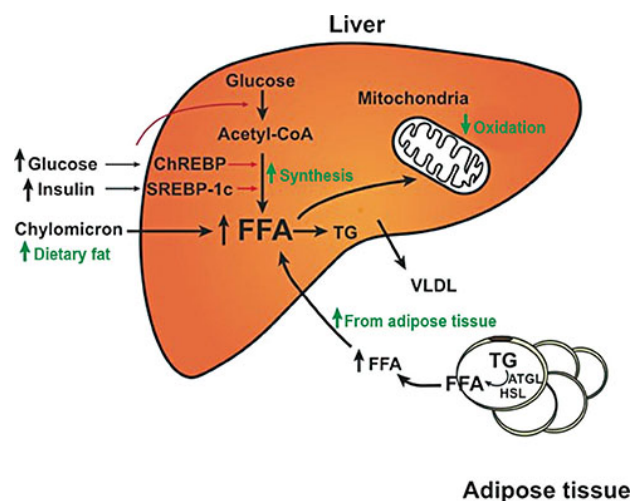


Figure 1: The mechanism of hepatic steatosis. Uptake of free fatty acids (FFA) is sourced from triglyceride (TG) lipolysis from adipose tissue, fatty acid synthesis, and dietary fat. Insulin resistance (IR) causes hyperinsulinemia resulting in de novo synthesis increase by inducing sterol regulatory element-binding protein-1c (SREBP-1c) activity. IR also leads to high levels of glucose that increases carbohydrate response element-binding protein (ChREBP) activity and FFA synthesis. FFA synthesis leads to the inhibition of Acetyl-CoA, reducing fatty acid oxidation in the mitochondria. Increased levels of FFA leads to triglyceride (TG) accumulation in the liver. VLDL, very low-density lipoprotein; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase [18]

Other factors in the pathogenesis of NAFLD are genetic and nutritional factors. Genome-wide association studies (GWAS) have shown polymorphisms of *PNPLA3*, *TM6SF2*, *FTO*, *LIPA*, *IFNλ4* *HFE*, and *HMOX-1* genes are associated with NAFLD pathogenesis [1,3,4,7]. These genes polymorphisms differ in their role in NAFLD severity and risk and are associated with different ethnic groups [7]. The nutritional factor is not

directly related to the pathogenesis of NAFLD. However, studies have shown that NAFLD patients have disturbed gut epithelial tight junctions that will allow bacteria to promote hepatic steatosis and inflammation by releasing inflammatory cytokines [7] (Figure 2). High fat (HF) and high-fat high-cholesterol (HFHC) diets in mice exhibit an increase in body weight, liver weight, liver to body weight ratio as compared to mice fed with normal chow diet (NC) (Figure 2A-2C). HF and HFHC diets lead to an increase in steatosis, inflammation, and NAFLD progression in liver tissue (Figure 2D-2F) [20].

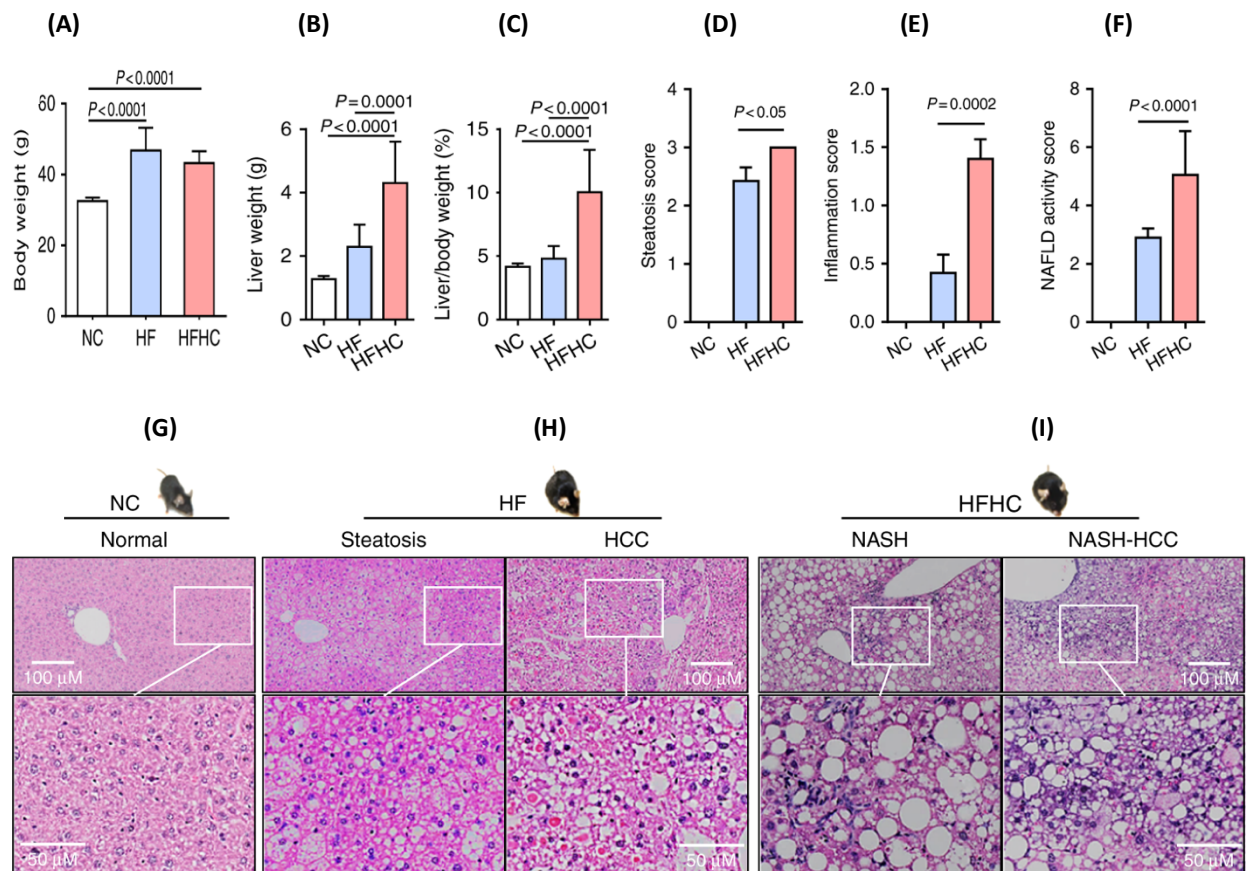


Figure 2: Nutritional factor affects steatosis, NASH, and HCC progression. Male C57BL/6J mice fed from 6 weeks of age to eight months, with normal chow (NC) high-fat (HF) and high-fat high cholesterol (HFHC) diets. Mice fed with HF and HFHC exhibit an increase in body weight (A), liver weight (B) and liver to body weight ratio (C), leading to high levels of steatosis (D), inflammation (E) and NAFLD activity (F). (G-I) Representative images using H&E staining histological images of the liver tissue from mice fed with NC, HF, and HFHC diets. Mice fed with HF diet exhibit hepatic steatosis, which progresses to hepatocellular

carcinoma (HCC) (H). Mice fed with HFHC diet exhibit inflammation resulting in NASH development and, ultimately, HCC (I) [20].

NAFLD can be clinically diagnosed using hepatic imaging such as ultrasonography, computed tomography, or magnetic resonance imaging of the liver [2,10]. Liver biopsy and histopathology are the most useful diagnostic tool to determine the severity of NAFLD [1,2,6,10]. There has been no effective drug for treating NAFLD; the most effective treatment is lifestyle intervention, increasing physical activity, and promoting a healthier diet. Most medications aim to reduce the symptoms of NAFLD and its associated diseases such as T2D, cardiovascular conditions, and obesity rather than reduce the progression of NAFLD. For example, insulin-sensitizing agents such as metformin are used to treat T2D [1,7]. Lipid-lowering agents and weight loss drugs are used to treat obesity, and surgery and liver transplantation is the treatment for the end-stage liver disease [1,7]. Previous clinical studies have used GLP-1 agonist, liraglutide, for NASH treatment, which was effective in 39% of patients who have taken it compared to 9% of individuals who received a placebo [7]. Since patients people poorly stick to these lifestyle interventions, there is a need for pharmaceutical development and effective treatment for NAFLD.

Aspartyl (asparaginy)- β -hydroxylase (AspH) is a type II transmembrane protein, it is part of α -keto glutarate-dependent dioxygenase family. It hydroxylates the Asp or Asn residues in the epidermal growth factor-like domains of proteins within the Notch pathway [11, 12, 13, 14, 20, 21, 22]. The upregulation of ASPH has been found in hepatocellular carcinoma and cholangiocarcinoma, making it a potential therapeutic target [12, 16, 20, 21, 22]. In this study, we investigate ASPH as a potential therapeutic

target to reduce the progression of NAFLD. Since NAFLD is affected by fatty acid synthesis [8,18], energy homeostasis [8] and type 2 diabetes, we aim to elucidate the mechanism of how ASPH reduces the progression of NAFLD in insulin-dependent mechanism of Protein Kinase B (Akt/PKB) signaling pathway, and muscle contraction mediated stimulation of AMP-activated protein kinase (AMPK) (Figure 3) [17, 19]. We further aim to investigate the effect of ASPH on the fatty acid synthesis and hepatic steatosis mechanism (Figure 1).

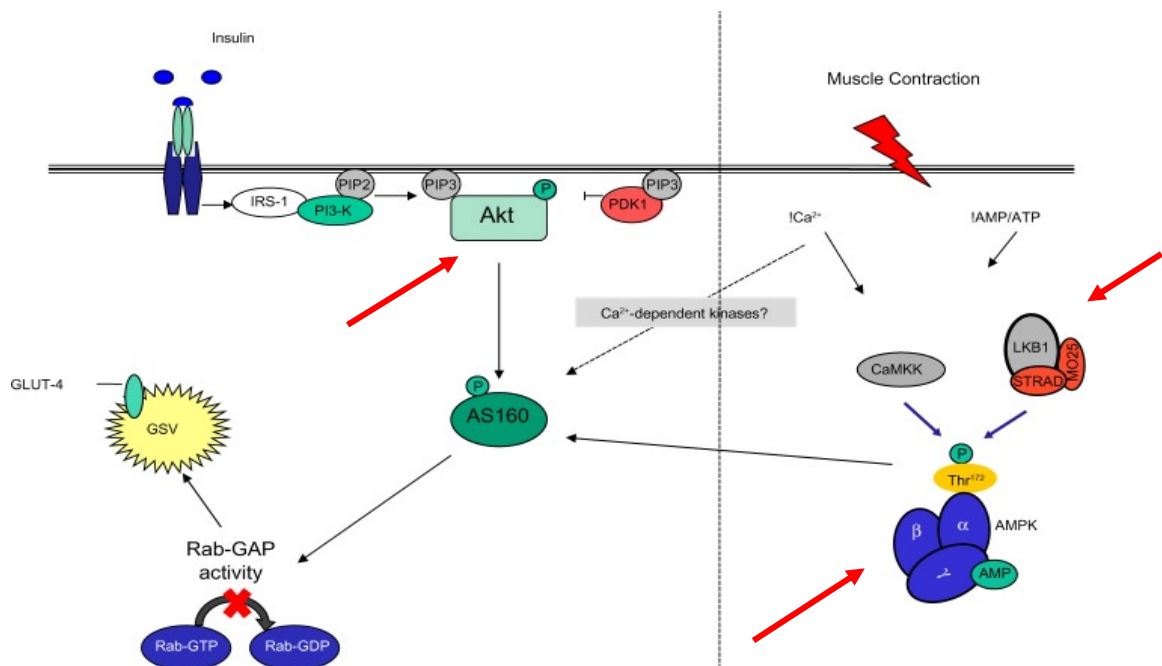


Figure 3: Insulin and muscle contraction signaling pathways in type 2 diabetes (T2D). Akt/PKB and AMPK play a significant role in glucose uptake and T2D. AMPK consists of three subunits; it is activated by phosphorylation of the α subunit at threonine 172 done by its upstream kinases, CaMKK, Ca^{2+} /calmodulin-dependent protein kinase, and LKB1 serine/threonine kinase 11 (Red arrow) [17]. Red arrows indicate the kinases and mechanisms investigated in this study to determine the ASPH mechanism to reduce NAFLD progression [17].

Materials and Methods

Transgenic mice (hASPH^{Tg})

Transgenic mice were generated by Applied StemCell Inc. (Milpitas, CA). Human ASPH gene was constructed into a pBT378 TARGATT and DNA microinjected into C57BL/6J mice, expressing the human ASPH transgene in every cell in the body. Genotyping was done using polymerase chain reaction (PCR) (Promega, M7805), dNTP mix (Promega, U1515) and results were visualized using 2% Agarose (BioRad, 1613100) and Ethidium Bromide (Invitrogen, 15585011) for gel electrophoresis.

In vivo study

Wild type (WT) and transgenic (hASPH^{Tg}) mice were fed with standard chow (NC) and high fat (HF) diets, in the form of soft pellets (Bio-Serv; F4031, F3282/S3282). High fat diet consists of 60% kcals from fat, whereas standard chow diet consists of 16.2% kcals from fat. The mice were kept in a clean room with access to clean food and sterilized water for 16 weeks, with body weight, food and water consumption monitored throughout that time. The mice were euthanized with CO₂ and cervical dislocation. Body, liver and fat were weighed, documented and collected. Blood samples were also collected and centrifuged at max speed for 30 minutes, serum was collected in new tubes and cell debris was discarded appropriately. Liver/body and fat/body ratio were calculated and p-value was determined using student's t-test. Small portions of each liver was collected in appropriate cassettes for Hematoxylin and Eosin (H&E) and Oil Red O (ORO) staining done by the Molecular Pathology Core Laboratory at Rhode

Island Hospital (Providence, RI). All protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital (Providence, RI).

Liver Tissue Protein Lysate

Proteins were collected from mouse liver tissues. The tissues were cut using sterilized blade and tweezer, and added to clean tubes with 5mm tungsten beads (Qiagen, 69997). 0.5mL of RIPA Lysis buffer (Alfa Aesar; J62524) containing 100X phosphate and phosphatase inhibitors (Thermo Fisher; 78428, 78430) were added to the tubes. The tissues were homogenized using Qiagen TissueLyser by shaking them at 20Hz for 3 minutes. The homogenate was left on ice for 20 minutes, then centrifuged at full speed (14,000 rpm) for 15 minutes at 4°C. Protein lysates were transferred to clean labeled tubes, cell debris were discarded appropriately and beads were collected and autoclaved for future use. Samples were stored at -80°C for long term use.

Protein Quantification

Protein concentration is determined using Bicinchoninic Acid (BCA) protein assay (Thermo Fisher; 23225) per manufacturer protocol, using 96-well plate and read on Spectramax M5 Plate Reader (Molecular Devices) at 562nm. Proteins were neutralized using the equation $V_1 = \frac{C_2 V_2}{C_1}$, to calculate the amount of protein (V_1) in μ l needed to reach the desired concentration (C_2) in μ g/mL, C_1 is the measured adjusted protein concentration from BCA in μ g/mL, and V_2 is the final volume of the diluted protein in μ l. The samples were diluted with the RIPA buffer using the equation $V_2 - V_1 =$ Volume of RIPA buffer (μ l).

Diluted samples were remeasured with BCA to get the precise concentration for western blot. Protein quantity for western blot was calculated using

$$y \text{ (}\mu\text{L)} = \frac{1x \text{ (}\mu\text{g)}}{\text{adj concnetration } (\frac{\mu\text{g}}{\text{mL}})} \times 1000 \text{ where } x \text{ is the desired concentration of protein,}$$

adj concentration is the adjusted measured absorbance of the sample using BCA in $\mu\text{g}/\text{mL}$, and y is the amount of protein lysate needed to get x , multiplied by 1000 to get the volume in μL . Protein lysates were added to protein sample buffer containing Bromophenol Blue, tris-HCl pH 6.8, glycerol, SDS and β –mercatopethanol , then denatured at 96°C for 5 minutes and rapidly cooled on ice for an additional 5 minutes.

Western Blot

SDS gels, along with the stacking gels, were prepared using the right amount of 30% acrylamide and TEMED to determine the percentage of gel depending on the size of the protein of interest. Protein lysates were added to the lanes in SDS PAGE gels with Precision Plus Protein Dual Color Standard (BioRad, 161-0374) in the first and last lane. Running buffer containing tris base, glycine and SDS was added to the gel tank, and the samples were stacked at 60V and separated at 80V.

Following SDS PAGE, the samples were transferred to a PVDF membrane, by making a transfer stack and immersing it in transfer buffer containing tris base, glycine and methanol. The samples were transferred for 2 hours at 100V while keeping the tank cold. After the transfer, membranes were blocked with 5% milk for a minimum of 1 hour then incubated with the primary antibody of interest overnight at 4°C, on the shaker (Table 1).

The following day, secondary antibodies in 2.5% milk were added for 1 hour and washed with TBST containing tris base, NaCl, and 0.1% tween-20 5 times for 10 minutes. Images were taken using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher; 34075) and FujiFilm Luminescent Image Analyzer LAS-1000.

Primary antibodies used in western blot are: In-house Polyclonal ASPH, α -tubulin (Sigma Aldrich, T5168), GAPDH (Santa Cruz, sc-32233), Akt (Cell Signaling, 2920S), pAkt (Cell Signaling, 4060S), AMPK (Cell Signaling, 5831S), pAMPK (Cell Signaling, 2535S), LKB1 (Cell Signaling, 3050S), pLKB1 (Cell Signaling, 3482S), CaMKII (Cell Signaling, 3362S), p-CaMKII (Cell Signaling, 3361S), and SREBP-1c (Santa Cruz, sc-13551). Secondary antibodies used are: Goat anti-rabbit IgG, peroxidase (Vector Laboratories Inc., PI-1000) and horse anti-mouse, peroxidase (Vector Laboratories Inc., PI-2000).

Lipid Profile

Lipid profile was generated to determine the enzymatic phenotype of WT and hASPH^{Tg} mice fed with standard chow (NC) and high fat (HFD) diets. Liver enzymatic activity functions, Alanine Aminotransferase (ALT); Aspartate Aminotransferase (AST); Alkaline phosphatase (ALP), were quantitatively determined using enzymatic kinetic assay from Aspartate aminotransferase (ALT) liquid reagents (23-666-087), Alanine transaminase (AST) liquid reagents (23-666-120) and Alkaline Phosphatase (ALP) reagent kit (23-666-074) from Pointe Scientific Inc, Canton Township, MI. Triglyceride levels were measured in serum and liver using the enzymatic kinetic assay from Pointe Scientific Triglyceride reagent set (23-666-410) and Triglyceride Standard (23-666-422).

Cholesterol levels were measured in serum and liver using Cholesterol Liquid Reagent for Diagnostic Set (SB-1010-430), and high-density lipoprotein (HDL) were measured in serum using Pointe Scientific HDL Cholesterol (PEG) reagents (23-666-301). Lastly we measured glucose serum levels using EKF Pointe Scientific Glucose (Hexokinase) liquid reagents (23-666-278).

The serum samples were collected from the blood samples in the animal studies. Liver protein lysates were prepared as previously mentioned, however; it was lysed with RIPA buffer without protease and phosphatase inhibitors to keep all enzymes active during the assays. Samples were quantified and normalized using BCA assay. Quantifications and analysis of the assays were completed per manufactures protocol. All assays were performed in duplicates.

Human Cell Lines

HEK-293T cell lines received from Dr. Jack Wands, and OUMS-29 cell lines received from Dr. Hironori Koga were used in this study. They were derived from human hepatocytes and embryonic kidney, respectively. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 2mM L-glutamine and 1% of Penicillin-Streptomycin solution in a humidified 37°C incubator with 5% CO₂.

Cell-based Assays

HEK-293T cells were transiently transfected using TransIT®-LT1 Reagent (Mirus Bio LLC, Madison, WI, USA). 15 µg of lentivirus plasmids (pLKO.1-shRNA-luciferase and pLKO.1-shRNA-ASPH from OriGene, Rockville, MD), 8.5 µg of psPAX2 (contains GAG,

POL) and 5 µg of pMD2.G (contains VSVg) were used to produce virus in a 10-cm dish. Medium containing the virus was collected 48 hours after transfection, filtered using a 0.45 µm filter, and added to the target cells used, OUMS-29 and HEK-293T cells. The infected cells were selected with puromycin at the concentration of 1 µg/ml. Puromycin concentration differs depending on cell type [36].

Cells were serum-starved overnight before challenging with fatty acids. Serum starvation is when culture medium, without fetal bovine serum, is added to the cells. Three fatty acids were used in our experiments for lipid accumulation in the cells: Palmitic acid, a saturated fatty acid [29] (Sigma Aldrich, 200-312-9), oleic acid, a monounsaturated omega-9 fatty acid [28], (Millipore Sigma, 112-80-1), and lastly fatty acid combination of Linoleic Acid-Oleic Acid-Albumin 100X (Sigma Aldrich, L9655).

ORO Histology staining for cell lines

OUMS293 cells were seeded to a density of 1.2×10^6 in 6-well plates. Cells were serum starved overnight, then treated with palmitic acid (PA) and oleic acid (OA) for 24 hours. Growth media was removed and cells were washed with 1X PBS 3 times. The cells were fixed with 37% formaldehyde and shaken for 1 hour. Oil red O staining solution was prepared by adding 0.5 g Oil Red O powder (Fisher Scientific Inc., AAA1298922) to 100% isopropanol. The working Oil Red O solution was prepared by adding 3 parts of the stock with 2 parts of fresh dH₂O, and stirred for 30 minutes. The working solution was added for 1 hour and shaken at room temperature to stain the lipid droplets in the cells. The solution was removed and cells were washed with H₂O three times every 10 minutes. Pictures were take using regular camera.

Luciferase Assay

HEK293T cells were seeded at a density of 1×10^5 cells per 12-well plate in DMEM containing 10% fetal bovine serum. Luciferase gene constructs containing a 550-bp mouse SREBP1c promoter fragment (SREBP1c-luc, addgene Cat# 60444) were transfected into the cells. HEK293T cells were also transiently transfected with empty vector control (EV) and ASPH overexpression (ASPH), serum starved the following day overnight, and treated with different concentrations of palmitic acid and oleic acid. Cells were lysed and luciferase reagent was added following the protocol in the Firefly Luciferase Assay Kit (Biotum, 30075-1, 30075-2). Cells were read on Packard TopCount NXT Microplate Scintillation and Luminescence Counter.

Results

Transgenic mice were successfully established with overexpressed human ASPH (hASPH) gene

The transgenic C57BL/6J mice were monitored to ensure successful modification of the human ASPH transgene (hASPH^{Tg}), using genotyping of mouse tail via polymerase chain reaction and western blot of the liver tissue. ASPH consists of 2265bp encoding a protein of 85-90 kDa [14]

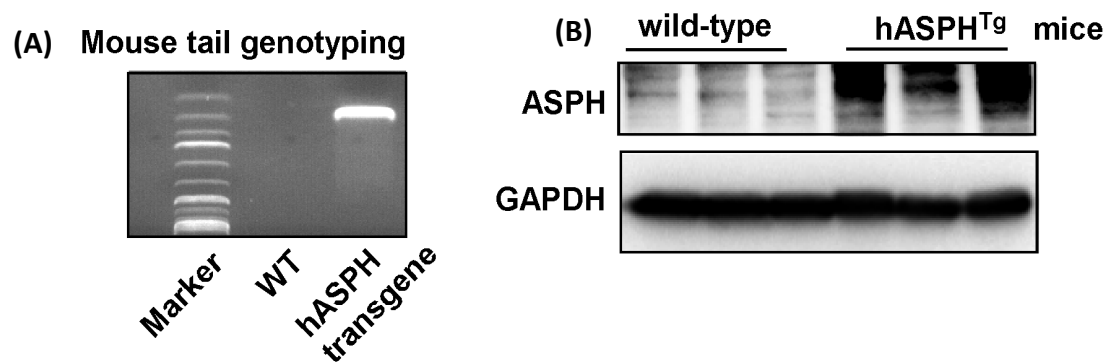


Figure 4: Successful genetic modification of hASPH^{Tg} transgenic mice. (A) The presence of the human ASPH (hASPH^{Tg}) transgene was monitored via mouse tail genotyping using polymerase chain reaction and (B) western blot against ASPH with GAPDH as control.

Food and water consumption of wild type (WT) and transgenic (hASPH^{Tg}) mice fed with standard normal chow (NC) and high fat diet (HFD).

To investigate the effect of ASPH on NAFLD, we first analyzed food and water consumption of WT and hASPH^{Tg} mice fed with NC (WT-NC, and hASPH^{Tg}-NC) and HFD (WT-HFD, and hASPH^{Tg}-HFD), respectively (Figure 5). WT and hASPH^{Tg} fed with NC demonstrate high food and water consumption as compared to WT and hASPH^{Tg} mice fed with HFD (Figure 5). In both food and water, hASPH^{Tg}-NC consumed less than WT-NC, yet there is no statistical significance. Both WT-HFD and hASPH^{Tg}-HFD consumed

less food and water compared to WT-NC, with statistical significance of $**p<0.01$, $*p<0.05$, and $***p<0.001$, respectively (Figure 5).

We further monitored body weights, which indicate there is no change between WT-NC and hASPH^{Tg}-NC, and also no change between WT-NC and hASPH^{Tg}-HFD. The body weight changes occur between WT-NC and WT-HFD, hASPH^{Tg}-NC and hASPH^{Tg}-HFD (Figure 5C, $*p<0.05$), due to the different composition of both diets.

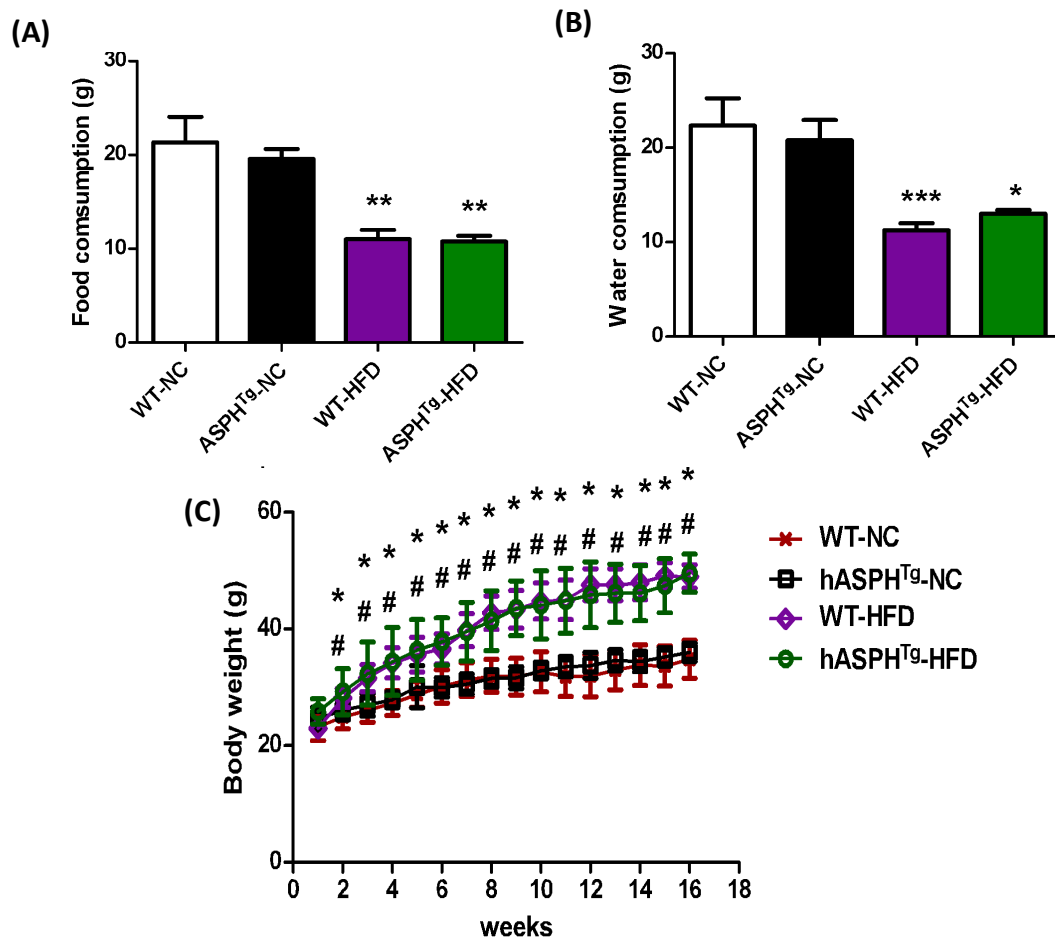


Figure 5: Comparison of food, water consumption and body weight between WT and hASPH^{Tg} fed with NC and HFD. (A) hASPH^{Tg}-NC demonstrates slightly lower food consumption compared to WT-NC. No change in food consumption between WT-HFD and hASPH^{Tg}-HFD, but they both consumed lower food than WT-NC with $**p<0.01$. (B) hASPH^{Tg}-NC demonstrates slightly lower water consumption compared to WT-NC. WT-HFD demonstrates lower water consumption than hASPH^{Tg}-HFD. WT-HFD and hASPH^{Tg}-HFD consumed lower water than WT-NC with $*p<0.05$ and $***p<0.001$, respectively. (C) No change in body weight between WT-NC and hASPH^{Tg}-NC, WT-HFD and hASPH^{Tg}-HFD. Change occurs between the different diets, $*p<0.05$ WT-NC vs WT-HFD; $*p<0.05$ hASPH^{Tg}-HFD vs hASPH^{Tg}-NC.

Overexpression of hASPH reduces liver weight in NAFLD development.

We speculated that ASPH suppresses NAFLD progression by reducing liver weight. Thus we examined the liver weights of WT-NC, hASPH^{Tg}-NC, WT-HFD and, hASPH^{Tg}-HFD using images of the liver and quantification of liver weight to body weight ratio (Figure 6). We found that there is no change in liver weight between WT-NC and hASPH^{Tg}-NC, in contrast, hASPH^{Tg}-HFD exhibits a significant decrease in liver weight as compared to WT-HFD (Figure 6A). By evaluating the statistical significance, hASPH^{Tg}-HFD have shown significant liver weight reduction compared to WT-HFD (Figure 6C, *** $p < 0.001$). WT-NC also demonstrates lower liver weight compared to WT-HFD (Figure 6C, * $p < 0.05$), indicating that the liver weight increase is due to high-fat diet consumption. We also examined ASPH effect on fat weight, and found that ASPH has no effect on fat weight (Figure 6B). These results indicate that ASPH decreases liver weight, with no effect on body fat in NAFLD development.

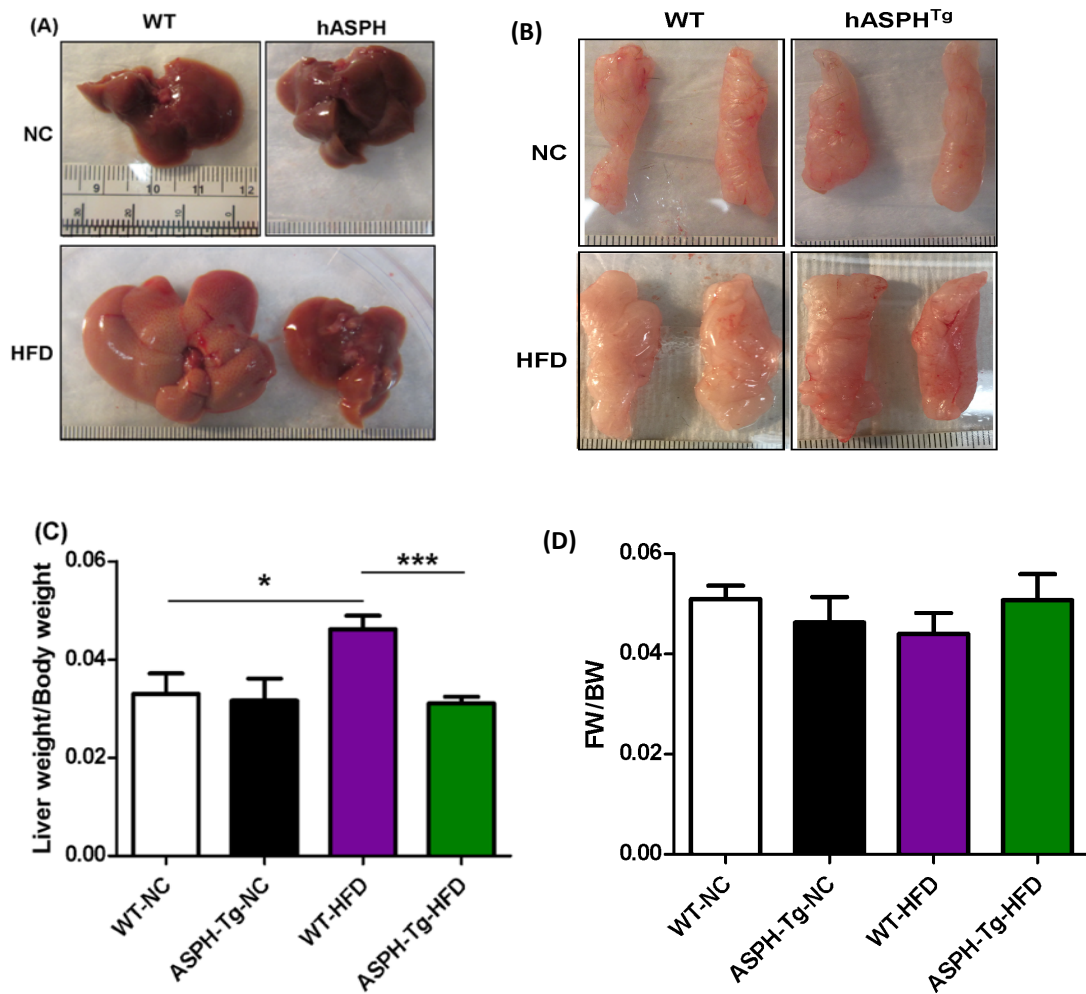


Figure 6: Overexpression of ASPH reduces liver weight in hASPH^{Tg}-HFD with no effect on fat. A) Images of livers derived from WT and hASPH^{Tg} mice fed with NC and HFD. (C) There is a significant liver weight decrease between WT and hASPH^{Tg} mice fed with HFD. No change between WT and hASPH^{Tg} mice fed with NC diet. * $p < 0.05$, WT-NC vs WT-HFD; *** $p < 0.001$, WT-HFD vs hASPH^{Tg} HFD. (B) Images show the fat between WT and hASPH^{Tg} mice fed with NC and HFD. (D) Quantification of fat weight over body weight (FW/BW) derived from (B). There is no change between in FW/BW between WT and hASPH^{Tg} mice fed with HFD nor WT and hASPH^{Tg} mice fed with NC diet, indicating that ASPH has no effect on body fat

ASPH suppresses hepatic lipid accumulation in NAFLD development

We further characterized the impact of ASPH on NAFLD development using Hematoxylin and Eosin (H&E) and Oil Red O (ORO) staining of liver tissue in WT and hASPH^{Tg} mice fed with NC and HFD. ASPH reduces NAFLD development by inhibiting hepatic lipid accumulation in hASPH^{Tg}-HFD (Figure 7), where hASPH^{Tg}-HFD liver showed a decrease in vacuoles (white lipid droplets), compared to WT-HFD (Figure 7A). In

contrast, WT-NC and hASPH^{Tg}- NC have shown similar vacuole structure. (Figure 7A).

ORO staining also confirms that ASPH suppresses hepatic lipid accumulation (Figure 7B), hASPH^{Tg}-HFD liver shows a decrease in vacuoles (red lipid droplets), as compared to WT-HFD (Figure 7B). WT and hASPH^{Tg} fed with NC show no change in vacuole structure (red lipid droplets). Both staining results show that ASPH reduces the progression of NAFLD by decreasing hepatic lipid accumulation.

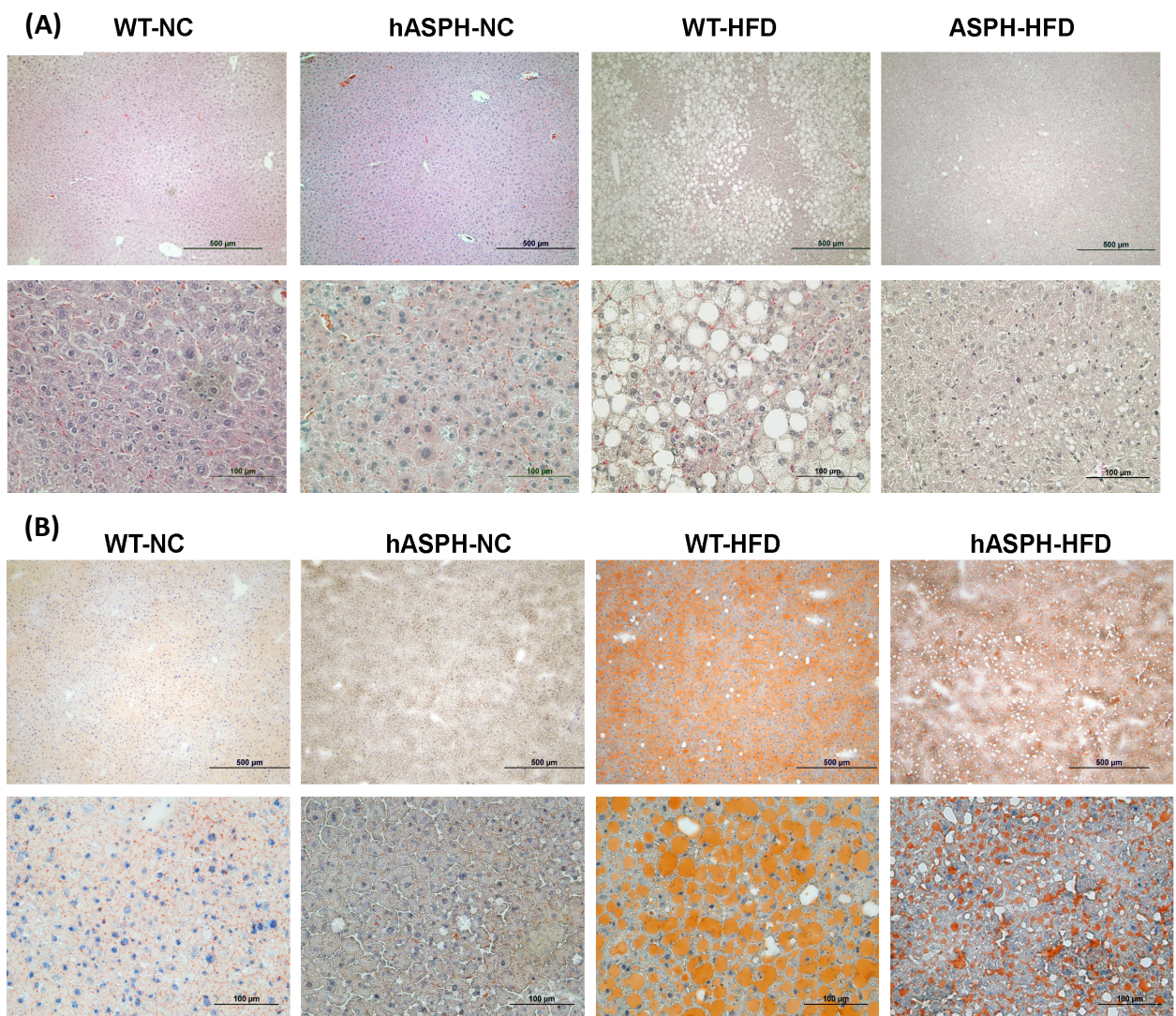


Figure 7: ASPH reduces hepatic lipid accumulation in hASPH^{Tg} mice fed with a HFD (A) H&E staining images of liver in WT and hASPH^{Tg} mice fed with NC and HFD show a decrease in vacuoles (lipid droplet) in the liver of hASPH^{Tg} with HFD as compared to WT mice with HFD. No change in vacuole structure in the liver between WT and hASPH^{Tg} mice with NC diet. First row (original magnification 40X) scale bar =

500 μ M, second row (original magnification 100X), scale bar =100 μ M. (B) Oil Red staining results of liver in WT and hASPH^{Tg} mice fed with NC and HFD show a decrease in lipid droplets (red color) in the liver of hASPH^{Tg} mice with HFD as compared to wild WT with HFD. No change in red color lipid droplets in the liver between WT and hASPH^{Tg} fed with NC. (original magnification 40X) scale bar = 500 μ M, second row (original magnification 100X), scale bar =100 μ M.

Overexpression of hASPH maintains the normal function of the liver enzymes in

NAFLD development

Liver enzyme screening is used for NAFLD diagnosis [24]. Increase and elevated levels in liver enzyme functions, Alanine Aminotransferase (ALT) Aspartate Aminotransferase (AST) and Alkaline phosphatase (ALP), have been shown to be associated features of NAFLD, T2D and metabolic syndrome [23]. In this study we show that ASPH can inhibit elevated levels of the liver enzymes by restoring its functions. We first examined ALT levels, which shows a significant decrease in hASPH^{Tg}-HFD and WT-NC compared to WT-HFD (Figure 8A, * $p < 0.05$).

We also examined AST levels and demonstrated that hASPH^{Tg}-HFD shows decreased levels in AST, as compared to WT-HFD (Figure 8B, ** $p < 0.01$). This indicates that ASPH decreases AST levels in hASPH^{Tg}-HFD. WT-NC is also significantly lower than WT-HFD (Figure 8B, ** $p < 0.01$), which indicates that high-fat diet consumption causes high levels of AST. Lastly, we examined ALP levels, which shows similar results as ALT and AST. hASPH^{Tg}-HFD shows decreased levels of ALP, as compared to WT-HFD (Figure 8C, * $p < 0.05$). ALP levels between WT fed with NC and HFD, is statistically significant (Figure 8C, * $p < 0.05$). The significant decrease in WT-NC as compared to WT-HFD in ALT, AST, ALP indicates that high-fat diet consumption causes elevated levels of these enzymes, which is an associated feature of NAFLD development. The results indicate

that ASPH inhibits NAFLD development, by restoring the functions of the liver enzymes ALT, AST, ALP.

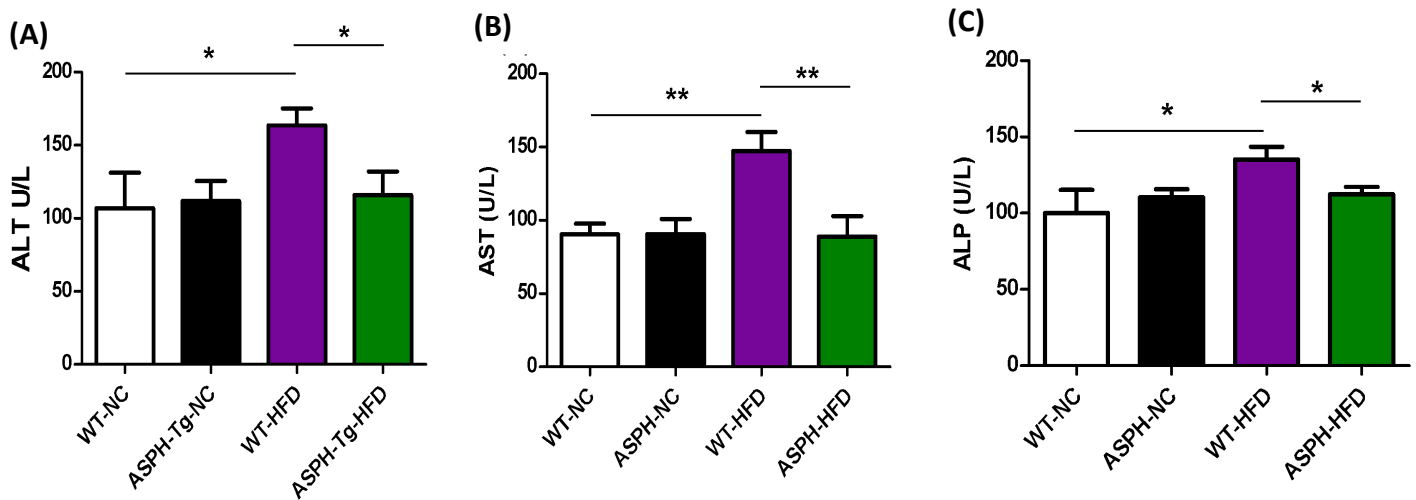


Figure 8: ASPH restores the function of ALT, AST, and ALP. Liver enzyme function tests, (A) ALT, (B) AST and (C) ALP, in hASPH^{Tg}-HFD are significantly lower compared to WT-HFD. These enzymes are also significantly lower in WT-NC than WT-HFD. * $p < 0.05$; ** $p < 0.01$. No significant change between WT-NC and hASPH^{Tg}-NC (A), (B), (C). Significant changes of liver enzyme levels between WT-NC and WT-HFD, indicate that elevated levels of these enzymes are important features in NAFLD diagnosis and development.

Overexpression of hASPH decreases cholesterol and triglyceride levels in hASPH^{Tg} mice fed with HFD.

NAFLD is linked to hyperinsulinemia and insulin resistance, leading to an increase in glucose levels [18]. Triglyceride (TRG) also plays a role in NAFLD development by releasing FFA in the liver (Figure 1) [1,2,7,18], thus we examined the effects of glucose and TRG to determine if ASPH reduces NAFLD development by decreasing their levels.

Glucose levels show a slight decrease in hASPH^{Tg}-NC as compared to WT-NC, (Figure 9A, * $p < 0.05$), yet we did not observe change between hASPH^{Tg}-HFD and WT-HFD. This indicates that ASPH does not affect glucose serum levels. Nevertheless, ASPH reduces TRG serum levels, which shows a decrease in hASPH^{Tg}-HFD compared to the WT of the same diet (Figure 9C, ** $p < 0.01$). Same effect with NC mice, hASPH^{Tg}-NC

shows a significant reduction compared to WT-NC in triglyceride serum levels (Figure 9C, *** $p < 0.001$). Whilst WT-NC has higher levels of triglyceride compared to WT-HFD (Figure 9C, * $p < 0.05$). These results indicate hASPH also reduces NAFLD development by decreasing elevated triglyceride levels.

Previous studies have also shown that steatosis in NAFLD affects HDL metabolism and cholesterol levels in patients [25], thus we analyzed ASPH effect on these enzymes in the blood. hASPH^{Tg}-HFD exhibits significantly lower cholesterol serum levels compared to WT-HFD (Figure 9B, *** $p < 0.001$). WT-NC also shows a significant reduction in cholesterol serum levels as compared to WT-HFD (Figure 9B, *** $p < 0.001$). Finally we found that ASPH decreases HDL serum levels shown in hASPH^{Tg}-HFD compared to WT-HFD (Figure 9D, * $p < 0.05$), however; there is no significant decrease in hASPH^{Tg}-NC compared to WT with the same diet (Figure 9D). Also no significant decrease in HDL in WT-NC compared to WT-HFD (Figure 9D). Together, these results show that ASPH reduces cholesterol and HDL serum levels, which ultimately slows down NAFLD progression.

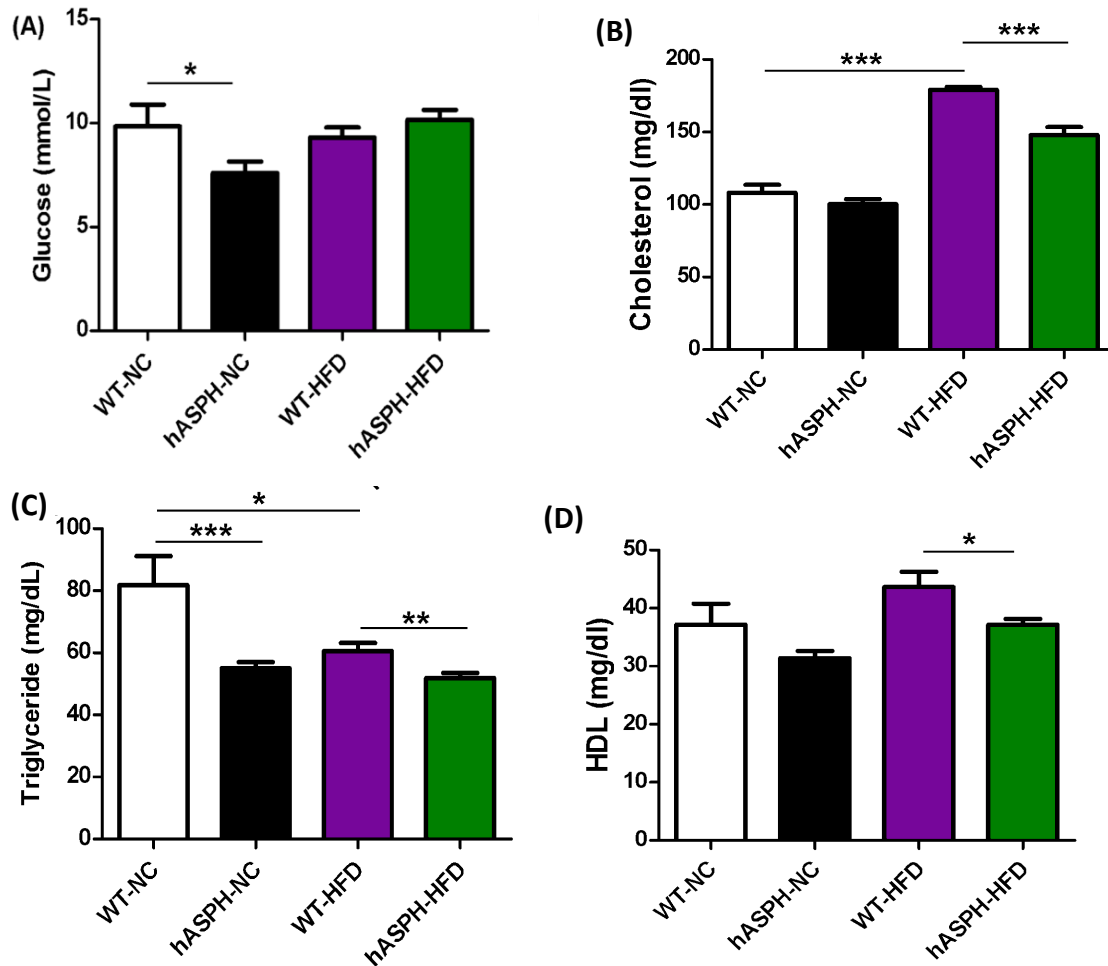


Figure 9: Serum levels of glucose, triglyceride, cholesterol and HDL are reduced in hASPH^{Tg}-HFD
 (A) Glucose test shows a slight decrease in hASPH^{Tg} mice with NC as compared to the WT mice, * $p < 0.05$. No change between WT-HFD and hASPH^{Tg}-HFD glucose levels. (B) Cholesterol serum levels show a decrease in hASPH^{Tg}-HFD compared to the WT mice with the same diet, and higher levels in WT-HFD compared to WT-NC, *** $p < 0.001$ (C) Triglyceride serum levels show a decrease in hASPH^{Tg}-HFD compared to the WT mice with the same diet, ** $p < 0.01$. Significant decrease in and hASPH^{Tg}-NC compared to WT-NC, *** $p < 0.00$, and WT-HFD compared to WT-NC, * $p < 0.05$. (D) HDL test shows a decrease in hASPH^{Tg}-HFD compared to WT-HFD, * $p < 0.05$. No change between WT-NC and hASPH^{Tg}-NC, and WT-NC and WT-HFD.

Overexpression of hASPH decreases cholesterol and triglyceride levels in the liver

samples derived from mice fed with a HFD

To further gain insight, we analyzed cholesterol and triglyceride levels in the liver of WT and hASPH^{Tg} mice fed with NC and HFD. Data is consistent in both cholesterol and triglyceride, hASPH^{Tg}-HFD have lower levels in both enzymes compared

to WT-HFD (Figure 10, * $p < 0.05$). hASPH^{Tg}-NC show increased levels in both enzymes compared to WT-NC (Figure 10, ** $p < 0.01$). There is a significant increase in WT-HFD compared to WT-NC (Figure 10, *** $p < 0.001$), which determines that high-fat diet consumption causes elevated levels of cholesterol and triglyceride compared to NC diet. These results indicate that ASPH decreases elevated levels of cholesterol and triglyceride in hASPH^{Tg}-HFD liver.

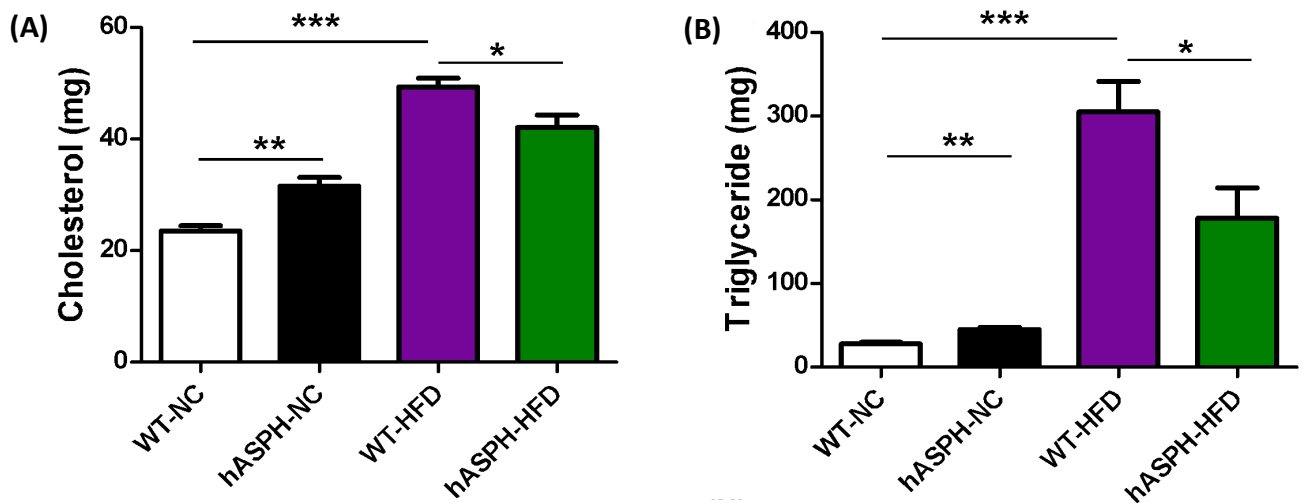


Figure 10: Cholesterol and triglyceride tests from liver samples. (A) Cholesterol and (B) triglyceride tests from liver samples show a decrease hASPH^{Tg}-HFD compared to the WT mice with the same diet, * $p < 0.05$. Both enzyme show lower levels in WT-NC compared to WT-HFD, *** $p < 0.001$, and lower levels in WT-NC compared to hASPH^{Tg}-NC, ** $p < 0.01$.

ASPH regulates phosphorylated AMP activated protein kinase (pAMPK) and sterol

regulatory element-binding protein 1 (SREBP-1c) in hASPH^{Tg} mice fed with HFD

To investigate the function of ASPH, we first examined its effect on Protein Kinase B (Akt/PKB) total and phosphorylated forms, which plays a role in the insulin transduction pathway and glucose uptake. Followed by ASPH effect on AMP-activated protein kinase (AMPK) total and phosphorylated forms, which plays a role in cellular

energy homeostasis, and lipid and glucose metabolism [17, 19]. It is also a therapeutic target in type 2 diabetes [31, 32, 33]. We further examined the effect of ASPH on sterol regulatory element-binding protein-1 (SREBP-1c), which is a transcription factor responsible for lipid homeostasis and fatty acid synthesis [19].

ASPH has shown to significantly enhance AMPK phosphorylation (pAMPK) and decrease SREBP-1c expression in hASPH^{Tg}-HFD (Figure 11A). In contrast, ASPH has no effect on Protein Kinase B (Akt) total and phosphorylated forms and AMP-activated protein kinase (AMPK) total form. The activity of AMPK is regulated by two upstream kinases CaMKK, Ca²⁺/calmodulin-dependent protein kinase, and LKB1 serine/threonine kinase 11 [17, 19] that activate AMPK by phosphorylating its α subunit at threonine 172 [17,19]. Thus ASPH has no effect on CAMKK and LKB1 (Figure 11A) indicating that ASPH has a direct effect on AMPK phosphorylation.

For further understanding, we examined the expression of AMPK and SREBP-1c forms in WT-NC, hASPH^{Tg}-NC, WT-HFD, and hASPH^{Tg}-HFD. We observed that ASPH enhances AMPK phosphorylation but decreases SREBP-1c expression. SREBP-1c has two forms affected by ASPH; the precursor SREBP-1c (P) form is located in the cellular membrane then cleaved in the nucleus to become a cleaved mature SREBP-1c (C) form (Figure 11B) [27]. The in-vivo results indicate that ASPH reduces NAFLD progression through pAMPK and SREBP-1c precursor and cleaved mature forms (Figure 11).

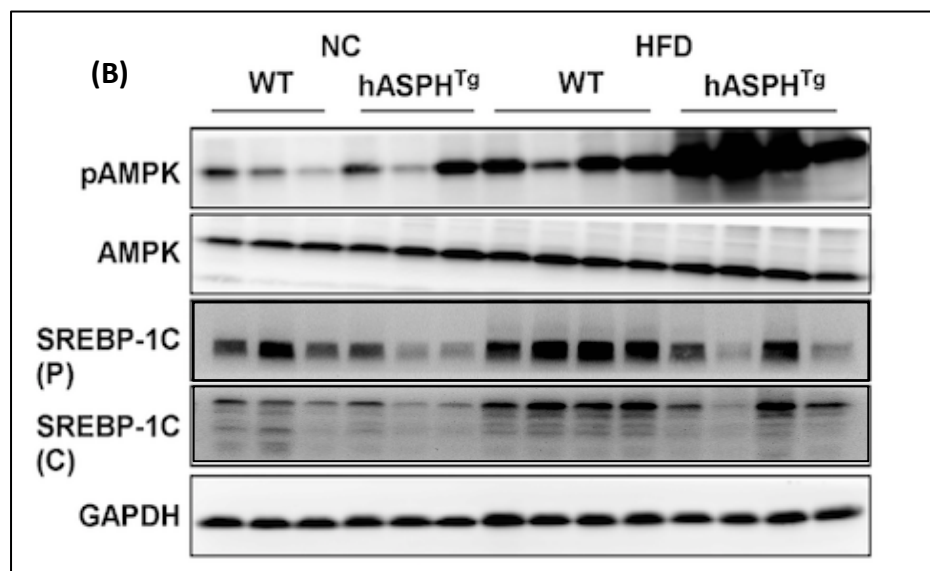
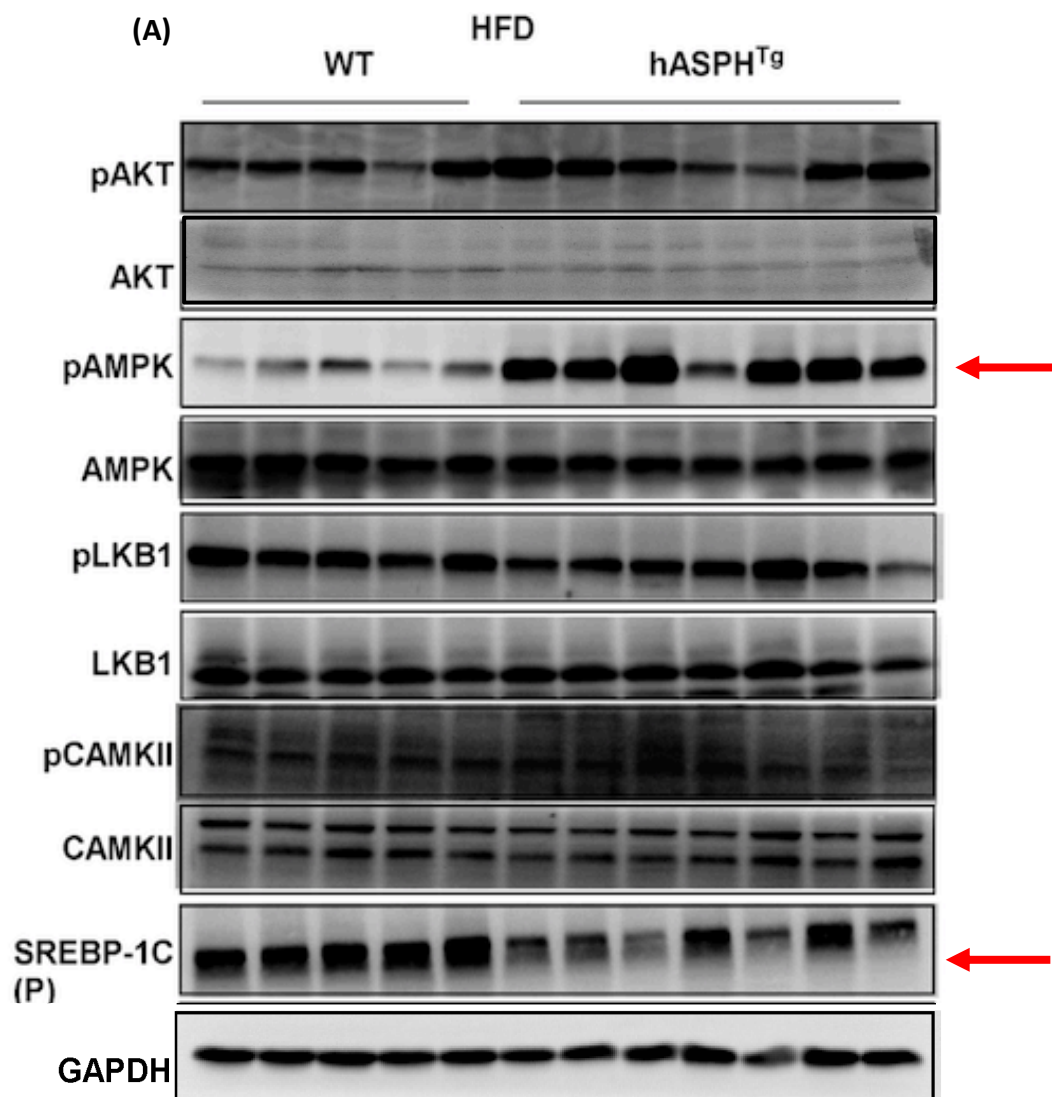


Figure 11: Western blot of WT and hASPH^{Tg} mice fed with NC and HFD. (A) hASPH^{Tg} mice fed with HFD affect phosphorylated AMPK and SREBP1c, compared to other kinases (Red arrow). ASPH does not affect both forms of Akt, LKB1, and CAMKII. It also has no effect on AMPK total form. GAPDH is used as control. (B) Western blot show hASPH in transgenic mice increases AMPK phosphorylation and suppresses total SREBP-1c. ASPH does not affect AMPK total form. GAPDH is used as control.

ASPH reduces the effect of SREBP-1c in OUMS-29 treated with fatty acids

To replicate the in-vivo findings, we first examined the effect of ASPH on SREBP-1c when treated with 0.2, 0.5 and 1 *mM* of oleic acid (OA) for 24 hours, a monounsaturated omega-9 fatty acid [29], in transient transfected OUMS-29 cells with empty vector control (EV) and ASPH overexpressed plasmid (ASPH). Cells were starved with DMEM without FBS overnight before challenging with OA (Figure 12A).

OUMS-29 (ASPH) cells have shown to reduce the expression of SREBP-1c (Figure 12A). OUMS-29 (ASPH) cells decrease the activity of SREBP-1c (C) cleaved mature form when the cells are challenged with 1.77 and 3.54 *mM* of oleic acid for 24 hours. However, OUMS-29 ASPH cells challenged with 5.31 *mM* of oleic acid have shown to enhance the effect of ASPH by decreasing the activity of both SREBP-1c forms; the cleaved mature form SREBP-1c (C), and precursor form SREBP-1c (P). In addition, OUMS-29 (EV) cells treated with 5.31 *mM* of oleic acid also induce a decrease in the activities of both SREBP-1c (C) and SREBP-1c (P). We conclude that 5.31 *mM* of oleic acid leads to saturation in OUMS-29 cells, which results in less fatty acid synthesis from SREBP-1c. OUMS-29 (ASPH) cells not treated with OA also induce a decrease in SREBP-1c (C) activity. This indicates that ASPH can decrease SREBP-1c (C) without OA, yet OA enhances the ASPH effect on both SREBP-1c (C) and SREBP-1c (P) (Figure 12A).

Next, we assessed whether a combination of fatty acids could enhance ASPH effect on SREBP-1c. We used a combination of linoleic acid, a polyunsaturated fatty acid,

and oleic acid (LA/OA). OUMS-29 cells with control empty vector (EV) and ASPH overexpression (ASPH) were serum starved overnight, then treated with 10, 50, 100 μ M of LA/OA for 24 hours (Figure 12B). In agreement with our previous observation, ASPH slightly decreases SREBP-1c (C) activity when treated with the different concentrations of LA/OA; thus there is no change in SREBP-1c (P). OUMS-29 (ASPH) cells not treated with LA/OA show no effect on SREBP-1c. The signal of LA/OA is weaker than OA alone, which explains why there is no substantial effect in LA/OA treatment. Yet, together, these results indicate that ASPH overexpression decreases SREBP-1c (C) expression in OUMS-29 cells, resulting in less fatty acid synthesis.

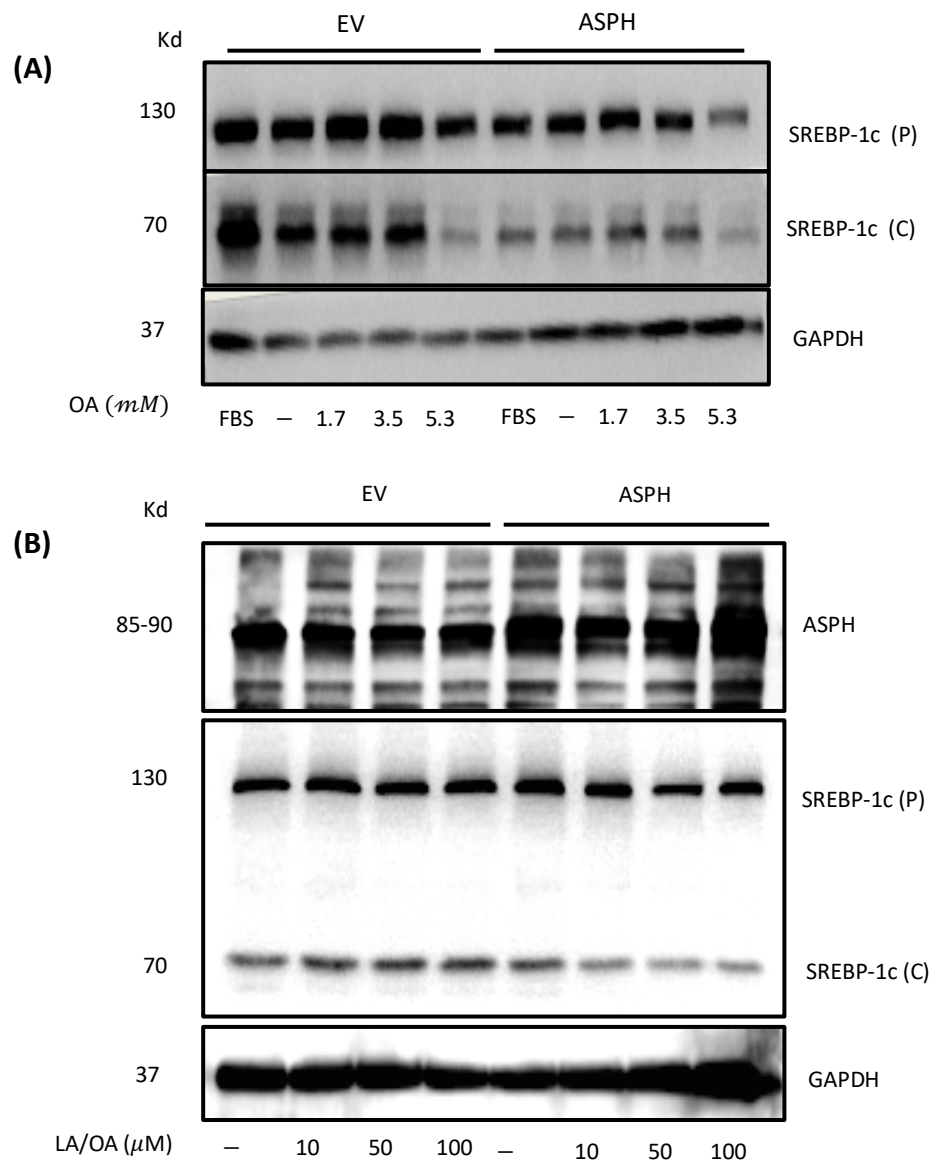


Figure 12: Overexpression of ASPH reduces SREBP-1c involved in fatty acid synthesis in OUMS-29 treated with oleic acid. (A) Empty vector control (EV) and ASPH overexpression (ASPH) OUMS-29 cells were serum starved from FBS, then treated with 1.77, 3.54 and 5.31 *mM* of OA for 24 hours. FBS is the control cell growing in regular DMEM without undergoing serum starvation. (–) is the control cell undergoing serum starvation overnight. 5.31 *mM* OA show decrease in both forms of SREBP-1c in both OUMS-29 EV and ASPH cells. Western blot of ASPH not shown. (B) Empty vector control and ASPH overexpression OUMS-29 cells were serum starved from FBS, then treated with 10, 50 and 100 (μ M) of LA/OA for 24 hours. ASPH OUMS29 cells have lower SREBP-1c (C) expression when treated with LA/OA, however; the signal is weaker compared to OA. Western blot of ASPH shows overexpression of ASPH compared to empty vector control cells. GAPDH is used as control.

Overexpression of ASPH suppresses lipid accumulation in OUMS-29 treated with fatty acids

We have recently shown that ASPH decreases hepatic lipid accumulation using H&E and ORO staining of mice liver tissue. To test these findings in-vitro, we treated OUMS-29 control and ASPH cells with different concentrations of oleic acid and palmitic acid. Due to LA/OA weak signaling (Figure 12), we speculated it would not produce the appropriate lipid accumulation in the cells.

Figure 13A shows OUMS-29 cell with empty vector control (EV) and ASPH overexpression (ASPH) treated with 200, 100, 50 μ M of palmitic acid (PA). OUMS-29 EV cells have more red staining than ASPH overexpressed cells, indicating there is more lipid and fat accumulated in the control cells. In addition, OUMS-29 cells treated with OA show similar results with less red staining in ASPH overexpressed cells compared to the EV cells (Figure 13B). Collectively these results support that ASPH reduces hepatic lipid accumulation in vitro.

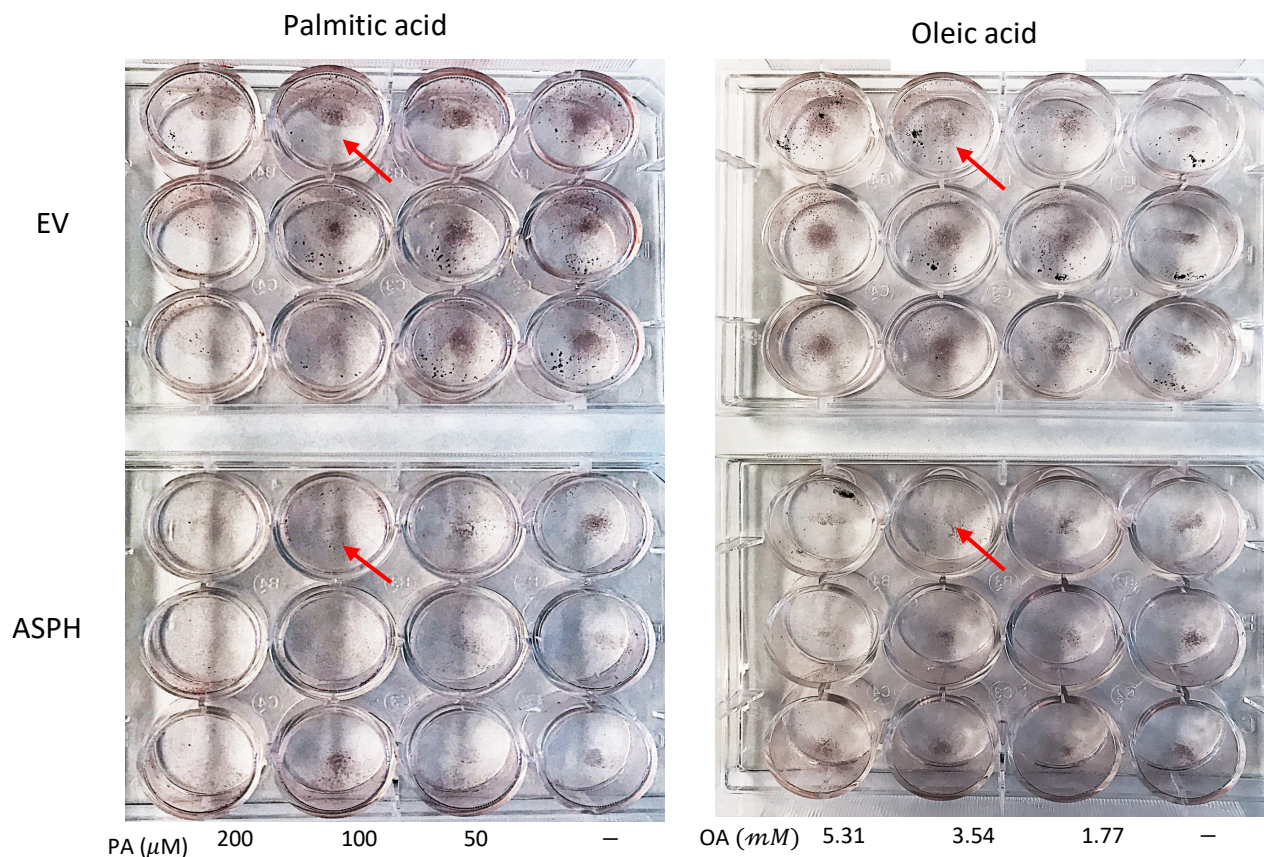


Figure 13: Red Oil O staining of OUMS-29 control and ASPH overdressed cells treated with oleic acid and palmitic acids. (A) OUMS-29 cell with control empty vector (top) and ASPH overexpression (bottom) treated with 200, 100, 50 μM of PA. (—) is the control without PA. Red arrows indicate the staining. (B) OUMS-29 cell with control empty vector (top) and ASPH overexpression (bottom) treated with 5.31, 3.54, 1.77 mM of OA. (—) is the control without OA. Red arrows indicate staining.

ASPH inhibits the transcriptional activity of SREBP-1c

Finally to assess the functional effect of ASPH on SREBP-1c, we measured its transcriptional assay in HEK-293T cells using luciferase gene construct with SREBP-1c promoter gene. HEK-293T cells were transiently transfected with empty vector control (EV) and ASPH overexpressed gene. The cells were serum starved overnight before challenging with PA and OA for 24 hours. The amount of light emitted from luciferase determines the rate of SREBP-1c transcriptional activity. Consistent with the previous

results, HEK-293T ASPH overexpressed cells has significantly lower SREBP-1c activity compared to the HEK-293T control cells (EV) (Figure). In addition, PA and OA have promoted lower SREBP-1c activity in the ASPH overexpressed cells (Figure 13). In short, these results indicate that ASPH suppresses SREBP-1c by inhibiting its transcriptional activity.

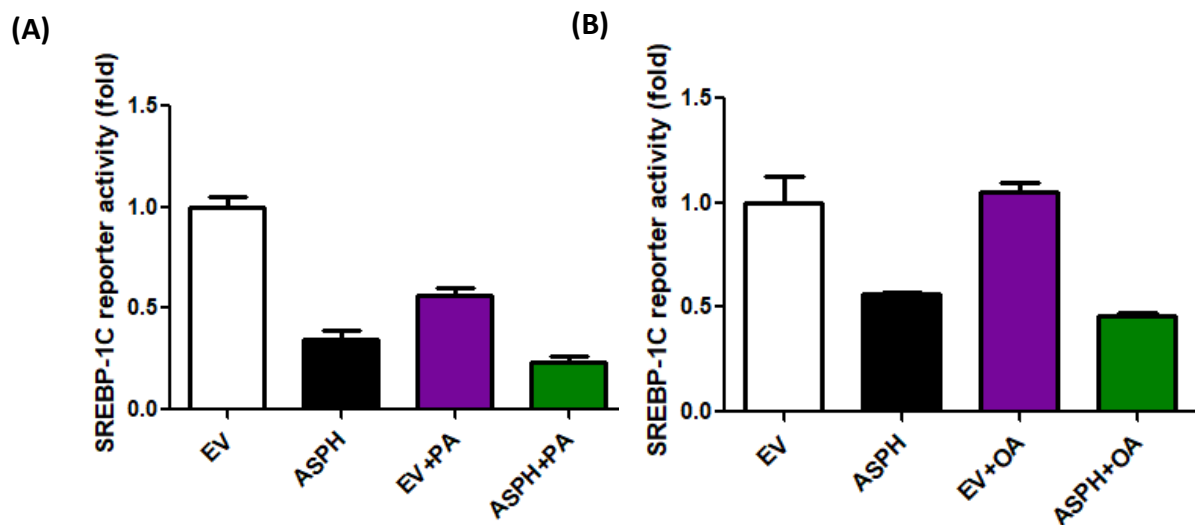


Figure 14: Luciferase assay determines SREBP-1c transcriptional activity in control and overexpressed ASPH HEK-293T cells with and without fatty acid treatment. SREBP-1c reporter activities were determined in HEK-293T cells transfected with EV and ASPH in the presence or absence of (A) palmitic acid (PA) as well as (B) oleic acid (OA). Cells were serum starved overnight before challenging with PA and OA.

Overexpression of ASPH did not affect AMPK and pAMPK expression

In vitro studies have shown that in hASPH^{Tg}-HFD promotes AMPK phosphorylation. To replicate the studies in vivo, we performed the same experiments by transfecting OUMS-29 cells with EV and ASPH, then serum starvation overnight before challenging with 500 μ M of Metformin. Previous studies have shown that Metformin is an antidiabetic drug that activates AMPK by promoting its phosphorylation [31, 32, 33]. The goal of this experiment is to determine if ASPH can function similarly to metformin by activating AMPK. However; we could not produce similar results as the

previous studies (Figure 14), OUMS-29 ASPH cells are overexpressed but they do not activate AMPK nor promote AMPK phosphorylation with the presence or absence of metformin. We conclude that the right conditions were not set for metformin in the cells, thus AMPK was not activated. ASPH has also no effect, which needs to be further investigated since our in vivo studies show AMPK phosphorylation by ASPH.

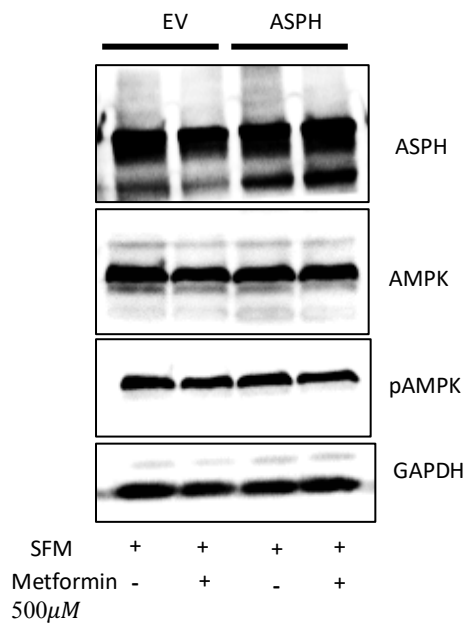


Figure 15: ASPH has no effect on pAMPK in-vitro

OUMS-29 cells with transfected with EV and ASPH. Western blot against ASPH shows successful transfection of the cells .No change in AMPK or pAMPK with the presence and absence of Metformin. GAPDH is used as control.

Discussion

In these studies, we have identified ASPH as an important therapeutic implication in NAFLD. We first developed a phenotype for C57BL/6J hASPH^{Tg}-HFD mice by inducing its ASPH expression and feeding it with either normal standard chow or high-fat diet. We then compared the models with wild type (WT) C57BL/6J mice fed with both diets. hASPH^{Tg}-HFD mice show a significant reduction in liver weight compared to the WT-HFD and controls. In contrast, ASPH did not affect fat weight.

Elevated levels of ALT, AST, and ALP are essential in NAFLD diagnosis. Our lipid profile shows a substantial decrease in hASPH^{Tg}-HFD enzymatic activity compared to WT-HFD and WT-NC in the liver enzymes. We further examined triglyceride, HDL, and cholesterol and glucose levels, and found that they were all significantly reduced in hASPH^{Tg}-HFD, except for glucose. This indicates that ASPH reduces elevated enzymatic activity in mice fed with HFD. The significant reduction of these enzymes in WT-NC compared to WT-HFD indicate that HFD causes the elevation of these enzymes that leads to NAFLD. Our last phenotype characterization determined that ASPH reduces hepatic lipid accumulation in the staining of lipid droplets in hASPH^{Tg}-HFD mice liver and OUMS-29 ASPH overexpressed cells.

The next question then arises, how does ASPH reduce NAFLD progression in hASPH^{Tg}-HFD mice? Through immunoblotting we have determined that ASPH overexpression in hASPH^{Tg}-HFD promotes AMPK phosphorylation and reduces SREBP-1c expression. We have also shown that ASPH overexpression did not induce AKT total and phosphorylated forms, which is an important kinase in the insulin transduction pathway.

In addition, it did not affect total form, which plays a role in cellular energy and glucose activation [17. 19. 31. 32. 33]. We further hypothesized that ASPH phosphorylates AMPK through AMPK upstream kinases LKB1 and CaMKK. Both kinases activate AMPK by phosphorylating its AMPK α subunit [19]. Results indicate that ASPH has no effect on the total and phosphorylated forms of LKB1 or CaMKK; this means that ASPH has a direct on AMPK phosphorylation.

Results indicate the mechanism of ASPH that contributes to reducing NAFLD progression. hASPH^{Tg}-HFD reduces SREBP-1c expression resulting in less fatty acid synthesis in the liver, which ultimately leads to slow progression in NAFLD. It also promotes the phosphorylation of AMPK, which leads to its activation and, thus, higher lipid and glucose metabolism.

We also found that ASPH overexpression transgenic mice fed with HFD (hASPH^{Tg}-HFD) regulates SREBP-1c expression in its precursor, and cleaved mature forms. SREBP-1c starts as an inactive precursor form located in the endoplasmic reticulum (ER). Low levels of cholesterol and lipid in the cell causes the SREBP-1c precursor form (P) to cleave and migrate to the nucleus where it undergoes proteolytic cleavage and becomes the active cleaved mature form (C) that is responsible for fatty acid synthesis [34]. Our in-vitro results show that OUMS-29 ASPH overexpressed cells decrease SREBP-1c (C); the results are similar when OUMS-29 ASPH cells are challenged with 1.77 and 3.54 *mM* OA for 24 hours. However, when both OUMS-29 EV control and ASPH overexpressed cells are challenged with 5.31 *mM* OA, we observed reduced activity in both SREBP-1c forms. This indicates that 5.31 *mM* OA causes fatty acid saturation in the liver, which

enhances the effect of ASPH by suppressing both SREBP-1c forms and restoring the activity of SREBP-1c [34]. It also indicates that saturation of OA in the OUMS-29 EV cells has the same effect of ASPH when the control cells are challenged with 5.31 *mM* OA.

Fatty acid combination of LA/OA also showed similar results to OA but was found to have a weaker signal than OA. This could be due to the insufficient lipid accumulation in the liver after 24 hours of challenging with LA/OA, thus LA/OA might need to be challenged for 48 hours or 72 hours for higher signal and sufficient lipid accumulation. Following the standard protocol for cell-based assays from previous studies, we serum-starved our cells to get optimum results [35]. We confirmed the previous studies by comparing the condition of serum starvation and normal cell growth in regular medium. We have found that serum starvation condition has more effect on SREBP-1c expression when treated with fatty acids (Data not shown). Lastly, by measuring SREBP-1c gene expression, we determined that ASPH decreases SREBP-1c expression by reducing its transcriptional activity and gene expression.

In the literature, it has been described that metformin, an antidiabetic drug, activates AMPK. Thus we aimed to replicate the results and hypothesized that ASPH will function similarly to metformin by phosphorylating AMPK. However, the results were inconsistent with the previous studies; this could be due to the different conditions we used. Further in-vitro studies are needed to assess the effect of ASPH on AMPK.

Conclusion

In conclusion, NAFLD is the accumulation of lipid in the liver with no relation to excessive drinking, there has been no effective pharmaceutical treatment other than life

style intervention. Our study has determined ASPH potential therapeutic target for NAFLD, which is an alpha-ketoglutarate dependent enzyme. Through in vivo studies we have found that ASPH reduces SREBP-1c expression and activates AMPK phosphorylation. We have provided solid evidence on how ASPH reduces hepatic lipid accumulation and decreases elevated enzymes that play a role in NAFLD.

Abbreviation

NAFLD: nonalcoholic fatty liver disease
NASH: nonalcoholic steatosis
GLP-1: glucagon-like peptide 1
IR: Insulin resistance
FFA: Fatty free acids
HCC: hepatocellular carcinoma
ASPH: Aspartate- β -hydroxylase
HFD: high-fat diet
NC: normal chow
T2D: Type 2 diabetes
AMPK: AMP-activated protein kinase
Akt/PKB: Protein kinase B
SREBP-1c: Sterol regulatory element-binding protein 1c

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