Supplementary data

Analytical procedures

At several time-points during the studies, animals were fasted for 4 hours and blood was collected from the tail vein into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany). Blood glucose was determined using hand-held glucometers and glucose strips. Whole blood HbA1c was measured in the rats using the Bayer A1c Now meter and test cartridges and in the mice in fresh heparin blood using the kit of Diazyme. Plasma triglycerides and total cholesterol were determined using the Stanbio LiquiColor Triglyceride Test (Enzymatic) kit and the Wako Chemicals Cholesterol E Assay Kit (total), respectively. LDL and VLDL-cholesterol levels were estimated by agarose gel electrophoresis of plasma using a Helena Laboratories QuickGel Cholesterol system and staining of the gel for cholesterol. The gels were scanned and the amount of cholesterol contained within the various lipoprotein fractions was calculated from the plasma total cholesterol level. Plasma insulin was determined using the ultrasensitive mouse insulin ELISA from Mercodia. Homeostasis model assessment (HOMA) was used to calculate relative insulin resistance (IR). Four hours fasting plasma insulin and fasting blood glucose values were used to calculate IR, as follows: IR = [insulin (ng/ml) × glucose (mg/dL)]/405. Plasma adiponectin was measured using the ELISA of R&D Systems Inc. Plasma ALT was measured using a reflectance photometric analyser (Reflotron-Plus, Roche).

Histology

Liver samples (lobus sinister medialis hepatis and lobus dexter medialis hepatis) were collected (from non-fasted mice), fixed in formalin and paraffin embedded, and 3 µm sections were stained with haematoxylin and eosin (H&E) and Sirius Red. NASH was scored in H&E-stained cross sections using an adapted grading system of human NASH (1, 2). In short, the level of macrovesicular and microvesicular steatosis as well as hepatocellular hypertrophy was determined relative to the total liver area analysed and expressed as a percentage. Importantly, hepatocellular hypertrophy is not a substitute of ballooning (that in most rodent models is less prominent than in humans)

because, in contrast to ballooning, hypertrophy is not a sign of cellular injury, and merely refers to an abnormal enlargement of the cells without acknowledging the source of this enlargement. Inflammation was scored by counting the number of aggregates of inflammatory cells per field using a 100× magnification (view size of 3.1 mm^2) in five non-overlapping fields and expressed as the average number of aggregates per mm^2. Hepatic fibrosis was identified using Sirius Red stained slides and the level of collagen deposition was quantified using ImageJ software (version 1.48, NIH, Bethesda, MD, USA) to assess the area of liver tissue that was positively stained (expressed as the percentage of total tissue area). In addition, fibrosis was quantified by measuring the hydroxyproline (as a measure for collagen) and proline (as a measure for total protein) content of liver tissue using HPLC as previously described (3) and subsequent calculation of the ratio hydroxyproline : proline.

Hepatic lipid/lipidomics analysis

Liver samples of lobus sinister lateralis hepatis were collected, and the intrahepatic concentration of triglycerides, free cholesterol and cholesteryl esters was determined as described previously (4). Approximately 50 mg of tissue was homogenized in PBS, and samples of the homogenate were taken for measurement of protein content. Lipids were extracted and separated by high performance thin layer chromatography on silica gel plates. Lipid spots were stained with colour reagent (5 g MnCl2.4H2O, 32mL 95–97% H2SO4 added to 960mL of CH3OH:H2O 1:1 v/v) and quantified using Image Lab software (version 5.2.1, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).

For detailed lipidomics, liver metabolic profiles were semi-quantified as described previously (5). Two separate ultra-high performance liquid chromatography (UHPLC)-Time of Flight-MS based platforms analyzing methanol and chloroform/methanol extracts were used. Identified ion features in the methanol extract platform included fatty-acids, acyl carnitines, bile acids, monoacylglycerophospholipids, monoetherglycerophospholipids, free sphingoid bases, and oxidized fatty-acids. The chloroform / methanol extract platform provided coverage over glycerolipids, cholesterol esters, sphingolipids, diacylglycerophospholipids, and acyl-ether-glycerophospholipids.

Lipid nomenclature and classification follows the LIPID MAPS convention, www.lipidmaps.org. A specific metabolite extraction procedure was performed for each platform. Briefly, proteins were precipitated from 15 mg frozen liver samples by adding H_2O, and methanol and chloroform:methanol (2:1) containing the internal standards used for the platforms. The resulting mixture was homogenized and incubated at -20 ˚C. Then, 500 µl were collected for each platform and the supernatants, which were obtained after centrifugation, dried under vacuum, reconstituted and transferred to plates for UHPLC-MS analysis. Metabolite extraction procedures, chromatographic separation conditions and mass spectrometric detection conditions are also detailed in (6, 7). Metabolomics data were pre-processed using the TargetLynx application manager for MassLynx 4.1 (Waters Corp., Milford, MA). Intra-batch (multiple internal standard response correction) and inter-batch (variable specific inter-batch single point external calibration using repeat extracts of a commercial serum sample) normalization followed the procedure described in (6). Metabolomic data are represented as means ± SEM. Differences between groups were tested using Student's t-test. Significance was defined as $P<0.05$. All calculations were performed using statistical software package R v.3.1.1 (R Development Core Team, 2011; https://cran.r-project.org/).

Extraction Method and UPLC-MS conditions for assessment of hepatic reduced glutathione (GSH) and oxidized glutathione (GSSG).

The tissues were homogenized were homogenized in ice-cold methanol/water (25/75%vol/vol) and 10 µM SL-Methionine (IS)) with a tissue homogenizer (FastPrep24; MP Biomedicals, Santa Ana, CA, USA) in 1 x a 40''cycle at 6000 rpm. Subsequently, the homogenate was diluted 10 times in 25% methanol and shaken at 1400 rpm for 30 minutes at 4 °C. Then, to 75 µl of this diluted homogenate, 25µl of water and 150µl of acetonitrile was added. The resulting mix was shaken at 1400 rpm for 1 hour at 4 °C. After shaking, the samples were centrifuged at 14.000 rpm for 30 minutes at 4 °C. The supernatant was injected directly onto the ultra-high performance liquid chromatography–MS system.

For the samples of the calibration curve, 18 aliquots of approximately 50 mg mouse liver tissue were homogenized, pooled, split back into 18 aliquots, 100 times diluted and processed in order to create a similar matrix as present in the liver samples. For all analytes the 12-point calibration curve ranged from 100 µM to 0.025 µM. For the standard mixtures, separate 10 mM stocks of the standards were made. These were than pooled and further diluted in water in order to obtain the final concentrations as used for the calibration curve. The calibration curve was made by mixing 25 µL of pooled standard mixtures with 75 µL of 100x diluted matrix. To this mixture 150 µL of acetonitrile was added. The calibration sample where then treated in the same way as the samples. Quality control (QC) samples were prepared by pooling 30µL of each sample.

Samples were measured with a UPLC system (Acquity; Waters, Manchester, UK) coupled to a Time of Flight mass spectrometer (ToF MS, SYNAPT G2, Waters). A 2.1 x 100 mm, 1.7 µm BEH amide column (Waters), thermostated at 40 °C, was used to separate the analytes before entering the MS. Solvent A (aqueous phase) consisted of 99.5% water, 0.5% formic acid and 20 mM ammonium formate while solvent B (organic phase) consisted of 29.5% water, 70% acetonitrile, 0.5% formic acid and 1 mM ammonium formate. The following gradient was used: from 5% A to 50% A in 2.4 minutes in curved gradient (#8, as defined by Waters), from 50% A to 99.9% A in 0.2 minutes constant at 99.9% A for 1.2 minutes, back to 5% A in 0.2 minutes. The flow rate was 0.250 mL/min and the injection volume was 2 µL. All samples were injected randomly. After every 9 injections a QC sample was injected.

The MS was operated in positive electrospray ionization mode in full scan (50 Da to 1200 Da). The cone voltage was 25 V and capillary voltage was 250 V. Source temperature was set to 120 °C and capillary temperature to 450 °C. The flow of the cone and desolvation gas (both nitrogen) were set to 5 L/h and 600 L/h, respectively. A 2 ng/mL leucine-enkephalin solution in water/acetonitrile/formic acid (49.9/50/0.1 %v/v/v) was infused at 10 µL/min and used for a lock mass which was measured

each 36 seconds for 0.5 seconds. Spectral peaks were automatically corrected for deviations in the lock.

Extracted ion traces were obtained for GSH (m/z = 308.0916) and GSSG (m/z = 613.1598) in a 20 mDa window and subsequently smoothed (2 points, 2 iterations) and integrated with QuanLynx software (Waters). Concentrations in the samples were calculated with the power-fitted calibration curves. Reported are the adjusted tissue concentrations in nmol/mg tissue.

Transcriptome analysis

Nucleic acid extraction was performed as described previously in detail (8). Total RNA was extracted from individual liver samples using glass beads and RNA-Bee (Campro Scientific, Veenendaal, The Netherlands). RNA integrity was examined using the RNA 6000 Nano Lab-on-a-Chip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA expression was determined by RNA sequencing using the Illumina Nextseq 500 according Illumina's protocol by service provider GenomeScan B.V (Leiden, the Netherlands) using at least 15 million reads per sample, 75nt single-end reads. The genome reference and annotation file mus_Musculus.GRCm38p6 was used for analysis in FastA and GTF format. The reads were aligned to the reference sequence using the STAR 2.5 algorithm with default settings (https://github.com/alexdobin/STAR). Based on the mapped read locations and the gene annotation HTSeq-count version 0.6.1p1 was used to count how often a read was mapped on the transcript region. These counts serve as input for the statistical analysis using DEseq2 package (9). Selected differentially expressed genes (DEGs), corrected for multiple testing, were used as an input for pathway analysis (P-adjusted<0.05) through Ingenuity Pathway Analysis suite (www.ingenuity.com, accessed 2018).

Pathway analysis as well as the upstream regulator analysis tool of IPA was used to assess the activity of upstream regulators. Gene expression data were used to predict activation (e.g., of PPAR-α or the insulin receptor) or deactivation (e.g. STAT1) of upstream regulators. A negative *z*-score of

less than −2 indicates significantly reduced transcriptional activity based on the direction of gene expression changes of target genes. A positive *z*-score of greater than 2 indicates significant activation of the upstream regulator. For Table 2 a cut-off value of z-score <-3 or >3 was used to limit the table to an acceptable length.

References

1. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313-21.
2. Liang W, Menke AL, Driessen A, Koek GH, Lindeman JH, Stoop R, et al. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. PLoS One. 2014;9(12):e115922.
3. Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafeber FP, et al. A simplified measurement of degraded collagen in tissues: application in healthy, fibrillated and osteoarthritic cartilage. Matrix Biol. 1997;16(5):233-43.
4. Bijland S, Pieterman EJ, Maas AC, van der Hoorn JW, van Erk MJ, van Klinken JB, et al. Fenofibrate increases very low density lipoprotein triglyceride production despite reducing plasma triglyceride levels in APOE*3-Leiden.CETP mice. J Biol Chem. 2010;285(33):25168-75.
5. Barr J, Caballeria J, Martinez-Arranz I, Dominguez-Diez A, Alonso C, Muntane J, et al. Obesity-dependent metabolic signatures associated with nonalcoholic fatty liver disease progression. J Proteome Res. 2012;11(4):2521-32.
6. Martinez-Arranz I, Mayo R, Perez-Cormenzana M, Minchole I, Salazar L, Alonso C, et al. Enhancing metabolomics research through data mining. J Proteomics. 2015;127(Pt B):275-88.
7. Alonso C, Fernandez-Ramos D, Varela-Rey M, Martinez-Arranz I, Navasa N, Van Liempd SM, et al. Metabolomic Identification of Subtypes of Nonalcoholic Steatohepatitis. Gastroenterology. 2017;152(6):1449-61 e7.
8. Verschuren L, Wielinga PY, Kelder T, Radonjic M, Salic K, Kleemann R, et al. A systems biology approach to understand the pathophysiological mechanisms of cardiac pathological hypertrophy associated with rosiglitazone. BMC Med Genomics. 2014;7:35.
9. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.