

Supplementary Materials

Moderate chronic ethanol consumption exerts beneficial effects on nonalcoholic fatty liver in mice fed a high-fat diet. Possible role of higher formation of triglycerides enriched in monounsaturated fatty acids

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Diets		SD	HFD
Macronutrients			
Crude Proteins	%	16.4	20.0
Fat	%	4.0	36.0
Carbohydrate	%	48.5	36.7
Energy Density	kcal/g (kJ/g)	3.0 (12.6)	5.5 (23.1)
Calories from protein	%	22	14.6
Calories from fat	%	12	58.8
Calories from carbohydrates	%	66	26.7
Minerals			
Calcium	%	1.0	0.7
Phosphorus	%	0.7	0.4
Sodium	%	0.2	0.4
Potassium	%	0.6	0.6
Chloride	%	0.4	0.2
Magnesium	%	0.2	0.1
Zinc	mg/kg	70	55.9
Manganese	mg/kg	100	14.9
Copper	mg/kg	15	4.6
Iron	mg/kg	200	64.6
Vitamins			
Vitamin A	IU/g	15.0	5.2
Vitamin D3	IU/g	1.5	1.6
Vitamin E	IU/kg	110	97.5
Vitamin K3	mg/kg	50	8
Vitamin B1	mg/kg	17	7.8
Fatty Acids			
C16:0 Palmitic	%	0.5	11.0
C18:0 Stearic	%	0.1	3.7
C18:1 ω 9 Oleic	%	0.7	6.4
C18:2 ω 6 Linoleic	%	2.0	1.7
C18:3 ω 3 Linolenic	%	0.1	0.3
Total Saturated	%	0.6	23.3
Total Monounsaturated	%	0.7	7.1
Total Polyunsaturated	%	2.1	2.3

Supplementary table 1: Nutrient composition of the standard diet (SD) and high-fat diet (HFD) supplied by Teklab and Safe respectively.

Supplementary methods 1: Protocol of ethanol exposure and blood withdrawal.

After one month on standard diet (SD) or high-fat diet (HFD), one HFD group was exposed to ethanol (70% v/v, VWR International, Fontenay-sous-Bois, France) via the drinking water for 3 months, while the other group of HFD mice drank water. Water provided to all mice in this study was filtered and autoclaved tap water. Since our study was specifically designed to determine the effects of a moderate ethanol consumption on NAFLD, we did not include a group of mice receiving standard chow and ethanol. Daily ethanol consumption in HFD mice was 5 g/kg of body weight (bw) for the first week and then kept around 10 g/kg bw until the end of the experiment (i.e. for 11 weeks). To respect this dose of exposure, we adjusted the percentage of alcohol in the drinking water twice a week (Supplementary figure 1a), taking into account the mean body weight of the mice (Figure 1a) and the average of their liquid intake (Supplementary figure 1b). Indeed, consumption of liquid was evaluated twice a week, while body weight was measured mostly every week or every two weeks thrice during the study (i.e. when mice were fasted overnight for blood withdrawal at week 4, 8 and 12). With this protocol of alcohol administration, the mean percentage of calories from alcohol during the 11-week period of ca. 10 g/kg ethanol consumption was $15.9 \pm 0.5\%$, whereas the mean percentage of ethanol in the drinking water during the same period was $20.3 \pm 0.8\%$. Finally, food consumption was assessed once a week.

At the end of each month, blood samples were drawn in the morning after an overnight fast. During this fasting period, mice were allowed to drink water containing ethanol or not. Blood withdrawal for biochemistry analyses was carried out from the submandibular vein using a Goldenrod animal lancet from MEDIpont (Mineola, NY). One mouse fed the SD diet did not survive after the blood withdrawal carried out at the end of the first month. At the end of the fourth month, all mice (n=29) were sacrificed by cervical dislocation and liver was quickly removed. While a majority of the liver fragments were immediately frozen in liquid nitrogen, some of them were rapidly processed for appropriate histological staining. Collected liver fragments frozen in liquid nitrogen were subsequently stored at -80°C until use. All experiments were performed according to national guidelines for the use of animals in biomedical research and approved by the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche (agreement n°904-201506221708455).

Supplementary methods 2: Fibrosis quantification by Second-Harmonic Generation (SHG) microscopy.

Multiphoton SHG and auto-fluorescent images were acquired on an inverted microscope (Leica DMI 6000 CS) adapted to a confocal scanning head (Leica TCS SP5, Leica Microsystems, Mannheim, Germany) and equipped with a MAITAI femtosecond laser (Spectra Physics, Santa Clara, CA, USA). A 10X (NA=0.4) Leica oil objective and Leica condenser S23 (NA=0,53) were used respectively to apply fixed 12 mW of 820 nm wavelength laser excitation at the sample and for photons trans-detection. For forward SHG signal trans-detection, a BG39 bandpass and 410 nm IR (10 nm FWHM) filters were placed before the photomultiplier tube (PMT) detector whose gain was fixed at a constant value of 950 V. For autofluorescence signal epi-detection, 500-650 nm bandpass filter was placed before the PMT fixed at constant 888 V gain. All images were acquired from 4 μ m thick slices of liver samples colored with Red Sirius. From a motorized microscope stage, sequentially scanned SHG/autofluorescent tile images were then reassembled by automat tile merging to reconstitute the entire image of each liver slice prior analysis and quantification by the open source ImageJ software. Edges of slices and main blood vessels were then deleted from the reconstituted image and background pixels were set to NaN (Not-a-Number) values with a fixed common threshold set for all samples. SHG signal was next quantified from areas having larger than 5 pixels. The results are expressed as percentage of fibrosis area representing the percentage of SHG pixels above threshold relative to the total number of corresponding pixels in the duplex auto-fluorescence image.

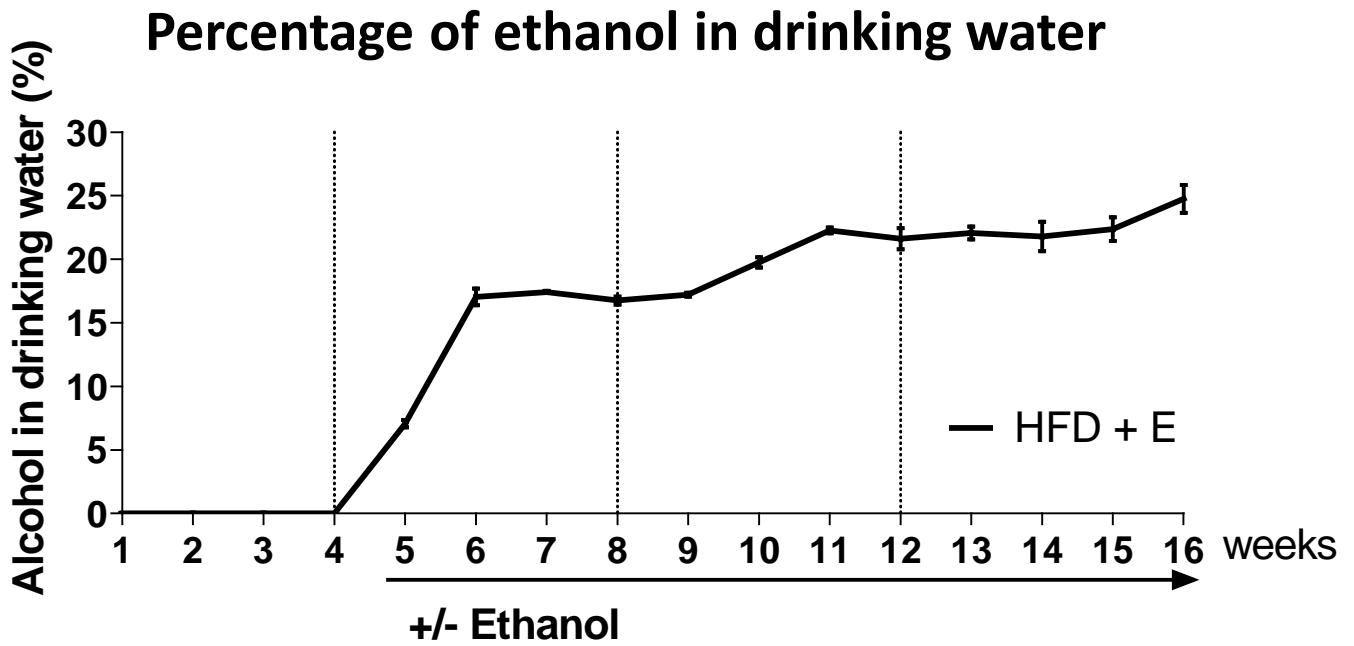
Supplementary methods 3: Transcriptomic analysis and gene set enrichment analysis (GSEA).

Total RNA were amplified and labeled with Cy3 fluorescent dye using Agilent one color low-input QuickAmp labeling kit. Cy3-labeled cRNA were fragmented and hybridized to the microarrays using the Agilent gene expression hybridization kit. Microarrays were then washed and scanned immediately with the Agilent DNA Microarray C Scanner (G2565). The scanned images were analyzed with Feature Extraction Software 10.7.3.1 (Agilent Technologies) using default parameters. After a standard quality control, all 10 microarrays (i.e. 5 replicates per condition) from the genomic dataset were qualified and further analyzed using GeneSpring software (Agilent Technologies). Microarray data were submitted to the gene expression omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo; GSE116417). Unsupervised gene set enrichment analysis (GSEA), performed using the Java tool developed at the Broad Institute (Cambridge, MA, USA), was commonly used in order to select the most significant Gene Ontology (GO) datasets. A supervised GSEA was used in two instances with the datasets pertaining to “GO Proteasome complex” and “GO Response to oxidative stress”. Significance was represented by the Normalized Enrichment Score (NES), the nominal p value (NOM p-val) and the False Discovery Rate q value (FDR q-val).

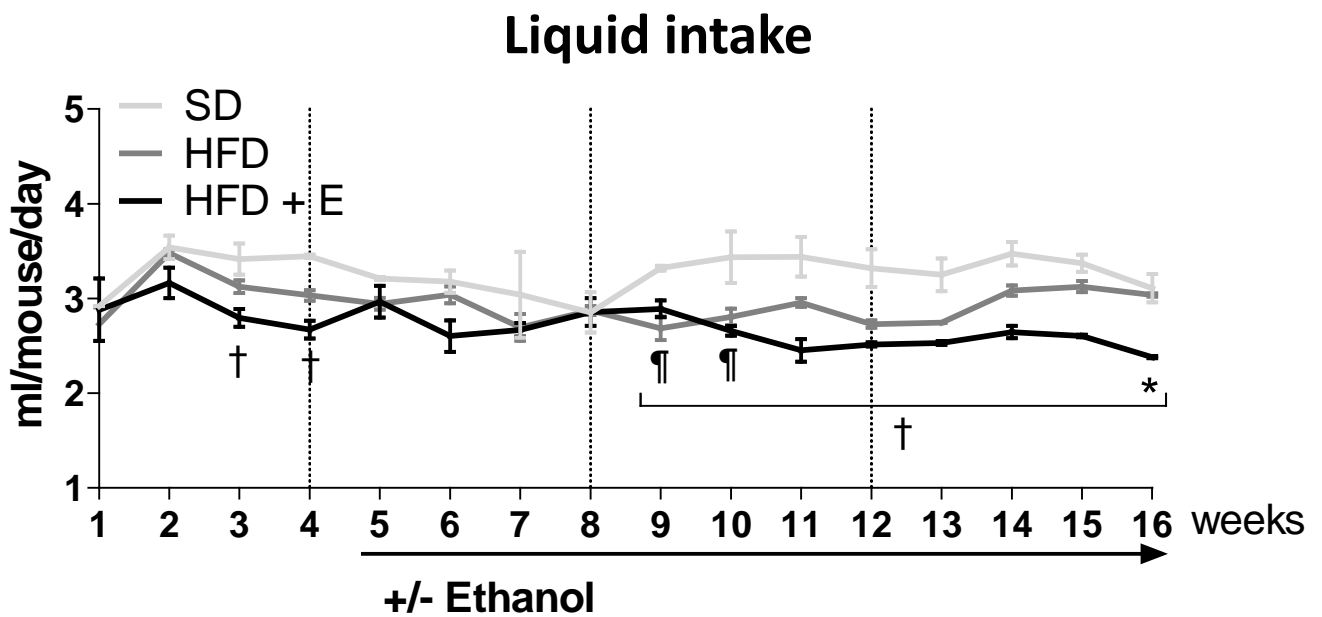
Supplementary methods 4: Hepatic lipid extraction and fatty acid analysis.

Hepatic lipids were extracted from 200 mg of liver with 20 volumes of dimethoxymethane:methanol (4:1, v/v) and converted to Fatty Acid Methyl Esters (FAMES) [30]. Heptadecanoic acid (C17:0) was added as an internal standard. Alternatively, lipid species (triglycerides, phospholipids, cholesterol esters and non-esterified fatty acids) were separated by thin-layer chromatography (TLC), as previously described [31]. Gas chromatography-mass spectrometry (GC-MS) analysis was performed using an Agilent Technologies 7890A GC system (Agilent, Les Ulis, France), with a bonded silica capillary column (BPX 70, 60 m × 0.25 mm; SGE, Melbourne, Australia), as previously described [32]. Mass spectra were recorded with an Agilent Technologies 5975C inert MSD with triple axis detector. Identification of the FAMES was based upon retention times (Rt) obtained for methyl ester of authentic standards. All identified fatty acids with a signal/noise above 10 were considered in the analysis. Results were expressed as mg of total fatty acids/g of liver and as percentage of total identified fatty acids.

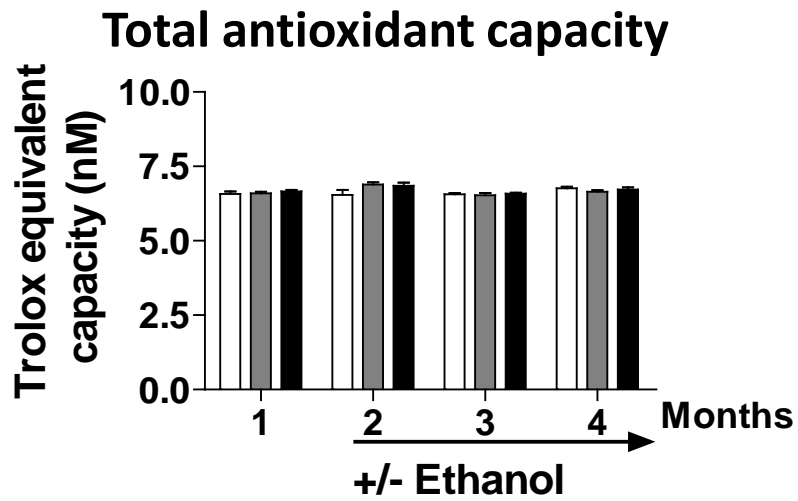
a.



b.



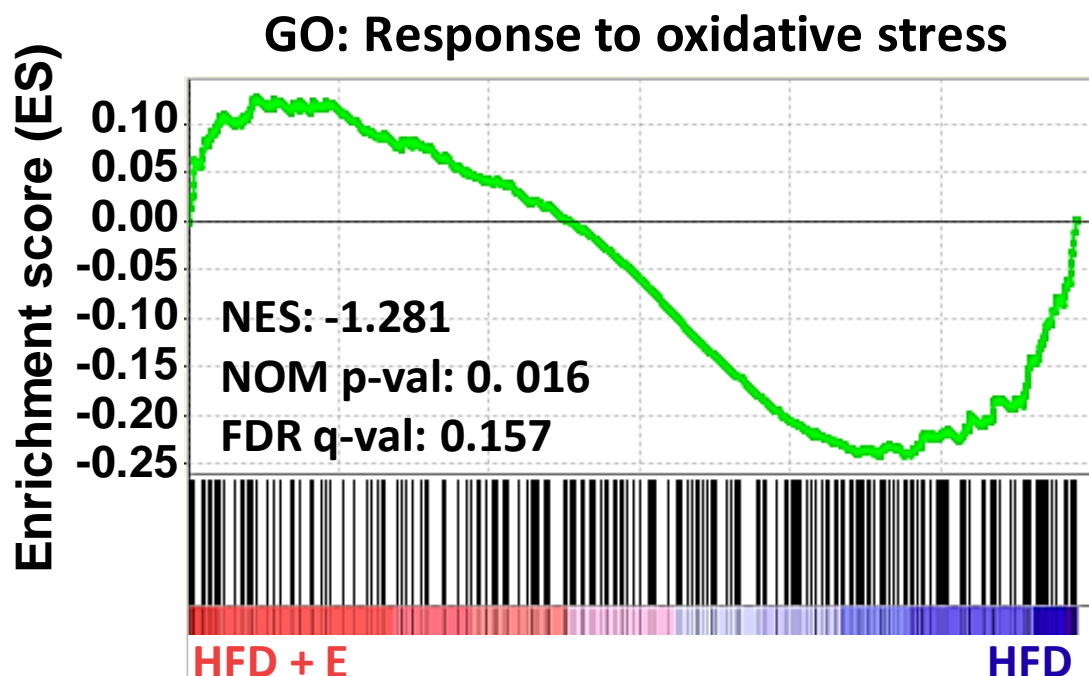
Supplementary figure 1: (a) Percentage of ethanol in drinking water for the HFD+E group (corresponding to ca. 10 g/kg/day). (b) Liquid intake in mice fed the standard diet (SD), mice fed the high-fat diet (HFD) and ethanol-treated HFD mice (HFD+E). Values are mean \pm SEM for 9-10 mice per group. *Significantly different between HFD+E and HFD mice, †Significantly different between HFD+E and SD mice, ¶Significantly different between HFD and SD mice, $p < 0.05$ with a two-way ANOVA test.



Mouse groups: SD HFD HFD + E

Supplementary figure 2: Serum total antioxidant capacity serially measured during the 4-month experiment in mice fed the standard diet (SD), mice fed the high-fat diet (HFD) and ethanol-treated HFD mice (HFD+E). Values are means \pm SEM of 9-10 mice per group.

a.



b.

Gene		Fold change
symbol	name	HFD + E vs HFD
<i>Gpx3</i>	glutathione peroxidase 3	0.68 ± 0.11
<i>Gpx7</i>	glutathione peroxidase 7	0.73 ± 0.15
<i>Gpx8</i>	glutathione peroxidase 8	0.71* ± 0.07
<i>Hmox1</i>	heme oxygenase 1	0.84 ± 0.05
<i>Ogg1</i>	8-oxoguanine DNA glycosylase	0.87* ± 0.02
<i>Sod3</i>	superoxide dismutase 3	0.76 ± 0.05

Supplementary figure 3: mRNA expression of hepatic genes involved in the response to oxidative stress in high-fat diet (HFD) and ethanol-treated HFD mice (HFD+E). Genome-wide expression profiling performed in naïve (n=5) and ethanol-treated (n=5) HFD mice (GSE116417) was used for subsequent gene set enrichment analysis (GSEA). (a) GSEA revealing in naïve HFD mice a significant enrichment of the gene ontology (GO) gene set corresponding to “Response to oxidative stress”, in comparison with ethanol-treated HFD mice. (b) The hepatic mRNA expression of different representative genes involved in the response to oxidative stress extracted from the transcriptomic analysis (GSE116417), is shown below the GSEA plots. *Significantly different from naïve HFD mice, $p < 0.05$ with a Mann-Whitney test.