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Safety assessment of the dietary supplement OxyELITE™ Pro (New Formula) in inbred and outbred mouse strains

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Abstract

Herbal dietary supplements have gained wide acceptance as alternatives to conventional therapeutic agents despite concerns regarding their efficacy and safety. In 2013, a spate of severe liver injuries across the United States was linked to the dietary supplement OxyELITE Pro-New Formula (OEP-NF), a multi-ingredient product marketed for weight loss and exercise performance enhancement. The principal goal of this study was to assess the hepatotoxic potential of OEP-NF in outbred and inbred mouse models. In an acute toxicity study, significant mortality was observed after administering 10X and 3X mouse-equivalent doses (MED) of OEP-NF, respectively.

Increases in liver/body weight ratio, ALT and AST were observed in female B6C3F₁ mice after

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Appendix A. Supplementary data

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gavaging 2X and 1.5X MED of OEP-NF. Similar findings were observed in a 90-day feeding study. These alterations were paralleled by altered expression of gene- and microRNA-signatures of hepatotoxicity, including *Cd36*, *Nqo1*, *Aldoa*, *Txnrd1*, *Scd1* and *Ccng1*, as well as miR-192, miR-193a and miR-125b and were most pronounced in female B6C3F₁ mice. Body weight loss, observed at week 1, was followed by weight gain throughout the feeding studies. These findings bolster safety and efficacy concerns for OEP-NF, and argue strongly for implementation of pre-market toxicity studies within the dietary supplement industry.

Keywords

Dietary supplements; Drug-induced liver injury; Hepatotoxicity; Phytochemicals

1. Introduction

Despite a general insufficiency of proof regarding their efficacy, multi-ingredient herbal dietary supplements (HDS) have gained wide-spread acceptance in the United States. In fact, the estimated number of HDS has risen from ~4000 in 1993 to ~55,000 in 2012 (Bailey et al., 2011; de Boer and Sherker, 2017), and that number is even greater today. Unlike conventional medications, HDS are not required to undergo premarket approval testing for safety or efficacy; thus, the toxicity potential of such products is not realized until after their ingestion by the consuming public. This regulatory shortcoming is especially concerning with regard to unusual and heretofore untested combinations of exotic botanical extracts or purified phytochemicals.

The association of acute liver injury with HDS usage, while uncommon, is not without precedent. As reported by the Drug-Induced Liver Injury Network (DILIN), about 20% of all drug-induced liver injuries (DILI) in 2013 were attributable to HDS (Navarro et al., 2014), which gave rise to a separate category known as HDS-induced liver injury or HILI (de Boer and Sherker, 2017). In 2013, a spate of severe liver injuries across the United States was linked to use of a new formulation of the dietary supplement OxyElite Pro (OEP-NF), a multi-ingredient product marketed for weight loss and exercise performance enhancement (Chatham-Stephens et al., 2017; Foley et al., 2014; Heidemann et al., 2016; Johnston et al., 2016; Roytman et al., 2014). Shortly thereafter, the FDA removed OEP-NF from the U.S. market, in part, because one of its constituents, aegeline, did not meet the qualifications of a “new dietary ingredient” and the product was deemed adulterated (U.S. Food and Drug Administration, 2013a,b).

Until the development and marketing of OEP-NF, consumers had never been exposed to the unique combination of its principal ingredients: caffeine, aegeline, higenamine, yohimbine and coclaurine. A review of the medical literature suggests that the hepatotoxic potential of each individual component is either relatively low (i.e., caffeine, higenamine) (Bloomer et al., 2015; Lo and Chen, 1997) or unknown (i.e. yohimbine, aegeline, coclaurine), although one study demonstrated that the plant source of aegeline, *Aegle marmelos*, was hepatotoxic when fed to mice (Arseculeratne et al., 1985), and another found that aegeline is cytotoxic to the HepG2 cell line (Mohammed et al., 2016). In addition, yohimbine can exacerbate

lipopolysaccharide-induced liver injury (Chen et al., 2015), while chronic caffeine administration has been shown to intensify acute experimental hepatitis, possibly through its antagonism of adenosine 2A receptors (Ohta et al., 2007). Despite the equivocal findings related to OEP-NF's individual components, the hepatotoxic potential of its combined ingredients was never determined prior to its marketing. However, two short term, small scale clinical studies of a combination of caffeine, higenamine and yohimbine, sponsored by the makers of OEP-NF, demonstrated no serious adverse effects, apart from elevations in heart rate and systolic blood pressure (Bloomer et al., 2015; Lee et al., 2013). Unfortunately, the phytochemical content of this three-ingredient formula was not independently verified by the investigators. This oversight is a common shortcoming when performing dietary supplement research, as the product's label claim and its actual phytochemical content can be quite different, and without independent verification a study's findings can be called into question (Gurley, 2012; Gurley et al., 2017).

As mentioned previously, no pre-market safety assessment of the combined ingredients in the lot of OEP-NF linked to the outbreaks of acute liver injury was ever conducted; therefore, the goal of this study was to assess the hepatotoxic potential of OEP-NF in mice. For this purpose, we employed a multi-strain mouse model incorporating B6C3F₁ mice, an inbred strain used extensively in the preclinical assessments of hepatotoxicity and hepatocarcinogenesis, and an outbred CD-1 strain to address the idiosyncratic nature of OEP-NF toxicity. Both single-dose gavage studies to assess OEP-NF's acute capacity for hepatotoxicity, as well as feeding studies of 28 and 90 days duration to evaluate the product's sub-chronic hepatotoxic potential were performed (Avigan et al., 2016).

2. Materials and methods

2.1. Chemicals

Aegeline and higenamine were synthesized at the National Center for Natural Product Research (NCNPR) (University of Mississippi, University, MS) according to published methods (Albonico et al., 1967; Patra et al., 1981; Somanathan et al., 1983; Kamal et al., 2004; Pyo et al., 2008). Identity and purity was confirmed via published spectroscopic data. Caffeine and yohimbine were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and formic acid were of HPLC grade and these, along with dimethylsulfoxide, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water for the HPLC mobile phase was purified in a Milli-Q system (EMD Millipore, Temecula, CA, USA).

2.2. Phytochemical analysis

Phytochemical content of OEP-NF capsules was determined at the NCNPR via ultra-high performance liquid chromatography (UHPLC) with photodiode array detection. Briefly, the contents of OEP-NF capsules were extracted exhaustively in methanol. Five capsules were weighed, opened, and the contents emptied into a mortar and pestle then mixed and triturated. One hundred milligrams were then weighed and sonicated in 2.0 mL of methanol for 30 min followed by centrifugation for 30 min at 959×g. The supernatant was transferred to a 10-mL volumetric flask. The procedure was repeated four more times with 2.0 mL methanol and the respective supernatants were combined. The final volume was adjusted to

10 mL with methanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.45 µm polytetrafluoroethylene (PTFE) membrane filter. The first 1.0 mL was discarded and the remaining volume was collected in an LC sample vial. Dilutions were performed as required using methanol. If compounds were not detected in the 100-mg sample mass, the extraction was repeated using the average capsule content mass for the analysis and 2 µL aliquots were injected onto the UHPLC system. Phytochemical separations were performed on an Acquity UHPLC™ system (Waters Corp., Milford, MA, USA) equipped with the Waters Empower Software. Separation was achieved on an Acquity UHPLC™ BEH Shield RP18 (100 mm × 2.1 mm, i.d., 1.7 µm) column operated at 40 °C equipped with a 2 cm LC-18 guard column (Waters Corp., Milford, MA, USA) at a flow of 0.2 mL/min. A gradient separation was achieved with a mobile phase consisting of water (A) and acetonitrile (B), both containing 0.05% formic acid. The gradient elution was performed as follows: 97% A: 3% B isocratic for the first minute, followed by 75% B and over the next nine minutes, and up to 100% B for the last minute. Each run was followed by a 3-min wash with 100% acetonitrile and an equilibration period of 3.5 min. Higenamine was detected at 282 nm, while caffeine, yohimbine, and aegeline were detected at 272 nm. Individual stock solutions for each standard compound were prepared at a concentration of 1 mg/mL in methanol and calibration curves were prepared over a range of 1–200 µg/mL. The limits of detection of aegeline, caffeine, yohimbine and higenamine were 0.1 µg/mL, 0.1 µg/mL, 0.2 µg/mL and 0.25 µg/mL, respectively.

Additional OEP-NF extractions were performed at the University of Arkansas for Medical Sciences (UAMS) College of Pharmacy using both water and dimethylsulfoxide (DMSO) as extraction solvents. The contents of twelve OEP-NF capsules were transferred into six separate 20 mL round bottom, glass screw-cap tubes and extracted overnight with either 10 mL of water or 10 mL of DMSO using a rotational mixer (12 revolutions per min.). Tubes were centrifuged at 3300 rpm (Fisher Model 225 Centrifuge™ centrifuge, Fisher Scientific, Waltham, MA, USA) for one hour and supernatants were transferred to sterile 50 mL polypropylene tubes (Corning 430790, Corning, NY, USA). One mL aliquots of both water and DMSO extractions were sent to the NCNPR for phytochemical analysis. Combinations of the water/DMSO extractions were used for acute gavage administrations.

2.3. Animals

CD-1 mice of both sexes were purchased from Charles River Laboratories (Kingston, NY, USA). B6C3F₁ mice of both sexes were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were 7–8 weeks of age at the time of purchase. All animals were housed at the UAMS Division of the Laboratory Animal Medicine (DLAM) facility. Mice were given one week for acclimation before the initiation of experiments. Food and water were provided *ad libitum*. Each animal was individually tagged with an ear tag. Animal body weights were measured and recorded weekly. All procedures were approved by the UAMS Institutional Animal Care and Use Committee (protocol number: AUP #3701).

2.4. Diets

Custom-made diets were purchased from Envigo (former Teklad Diets) (Madison, WI, USA). The following diets were used: Diet #8640 (vehicle); OEP-NF – 1X (this diet was

formulated to deliver 110 mg/kg of total plant alkaloids per day per mouse); and OEP-NF – 3X (this diet was formulated to deliver 330 mg/kg of total plant alkaloids per day per mouse). These doses were based upon the product (lot# 421131) label recommendation (no more than 3 capsules daily) coupled with the total alkaloid (i.e., caffeine, aegeline, higenamine, yohimbine, coclaurine) content per capsule (209.5 mg) as determined by the NCNPR. Diet components were independently verified by Envigo before they were shipped to UAMS. Diets were stored in a cool dry place and a portion of the diet was provided to mice daily.

2.5. Animal gavage dosing

The maximum label-recommended human dose of OEP-NF is three capsules daily. Phytochemical analysis revealed that each capsule contained 209.45 mg of total plant alkaloids (i.e., caffeine, aegeline, higenamine, yohimbine, coclaurine). Based upon these findings, the maximum dose of total OEP-NF alkaloids for a 70-kg adult human is 8.98 mg/kg/day. Allometric scaling for mouse equivalent doses of total OEP-NF alkaloids were determined per the recommendation of Wojcikowski and Gobe (2014) which, in turn, is based upon the FDA Industry Guidance for Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Volunteers (U.S. Food and Drug Administration, 2005; Nair and Jacob, 2016). According to the scaling factor (12.3) commonly used for mice weighing between 11 and 34 mg, the mouse equivalent dose of total OEP-NF alkaloids was calculated to be 110 mg/kg/day. Gavage doses were assessed at the mouse equivalent dose (1X), as well as at three (3X) and ten times (10X) the mouse equivalent dose. This range of doses was well within the 100-fold uncertainty factor commonly recommended when designing HDS safety studies (Schilter et al., 2003).

Because aegeline is poorly extracted in water but readily recovered in DMSO, 1 mL each of the water and DMSO extractions were combined and then diluted with appropriate volumes of 50% DMSO/water such that a 300 μ L gavage volume would deliver either the 1X, 3X, or 10X mouse equivalent doses of total OEP-NF alkaloids. Gavage doses were prepared daily, 1–2 h prior to administration. Gavage solutions were vortex-mixed immediately prior to administration in each mouse. The sub-chronic feeding phase of the study incorporated only the 1X and 3X mouse equivalent doses into the mouse chow.

2.6. Animal experiments

Animal experiments were performed in three stages.

Stage 1. Assessment of acute OEP toxicity.—An aqueous extract of the dietary supplement formulation was initially planned to be gavaged at three concentrations: mouse equivalent dose (110 mg/kg), 3X mouse equivalent dose (330 mg/kg) and 10X mouse equivalent dose (1100 mg/kg). As stated above, these doses are based upon total OEP-NF alkaloids. Gavage was performed by trained DLAM personnel administering a total volume of 300 μ L. Due to the overt toxicity observed in male CD-1 mice 1 h after receiving the 10X dose, this dose was eliminated and a dosing range of 0.5–3X was adopted for further gavage studies. Male CD-1 mice receiving the 3X dose became lethargic after 3 h and, at the recommendation of the UAMS attending veterinarian, these animals were humanely

euthanized 4 h after gavage. For consistency, all other mice receiving the 3X dose were euthanized at 4 h. All other animals were humanely euthanized 24 h after gavage. Livers, kidneys and hearts were harvested for future molecular and histopathological examination.

Stages 2 and 3. Assessment of sub-acute and sub-chronic OEP-NF toxicity.—

To assess the sub-acute and sub-chronic effects of OEP-NF, animals were fed the supplemented diet at the 1X (110 mg/kg/day) and 3X (330 mg/kg/day) doses for 4 and 13 weeks, respectively.

2.7. Blood sampling

To measure the effects of OEP-NF on liver enzymes (e.g., ALT, AST) and biomarkers (e.g., miR-122) characteristic for drug-induced liver injury, blood was collected at the end of each stage of the experiments described above. Blood was collected from the mandibular plexus using a GOLDENROD animal lancet (MEDipoint, Inc., Mineola, NY, USA) by trained DLAM personnel into a 1.1 mL Z-gel microtube (Sarstedt, Newton USA). Tubes were kept on ice and centrifuged at 10,000 rpm for 20 min and the serum was immediately aliquoted and flash-frozen.

2.8. Histopathological assessment

Livers were excised and a 1 mm section was obtained from the left lateral lobe and another from the right medial lobe. The sections were fixed in 4% formalin for 24 h, then briefly rinsed in PBS and stored in 70% ethanol for 24 h. Livers were then processed at the UAMS Pathology Core Facility, stained with hematoxylin eosin and shipped to the Oklahoma Health Sciences Center (OHSC) for veterinary pathology assessment.

For histologic evaluation purposes, each liver was represented by two sections obtained from different locations within the liver. Each section was initially evaluated at magnifications of X40 and X100. The sections were then evaluated at X200 and X400 to better determine if significant changes were present and to check for the presence of mitotic figures and apoptotic bodies.

2.9. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement

Serum ALT and AST levels were detected using the activity colorimetric assays and quantitated by a standard curve. The assay kits were purchased from BioVision (Milpitas, CA, USA) (Cat. #K752–100 and K753–100 for ALT and AST, respectively). Serum samples from treatment and control groups were diluted in the assay buffer (1:2 or 2:5). Twenty microliter samples and standards were added to a 96-well plate. 100 μ L reaction mix composed of 86 μ L ALT (AST) Assay Buffer, 2 μ L reconstituted ALT (AST) Enzyme mix, 2 μ L OxiRed probe and 10 μ L reconstituted ALT (AST) substrate were added to each well, as well as the standards and positive controls. The plate was shaken for 3 min to mix the reagents. The absorbance (OD) was read at 570 nm (T1) and then again at T2 after the plate incubation at 37 °C for 30–45 min and protected from light. ALT (AST) activity in serum was calculated using a standard curve and formula below:

$$\text{ALT (AST) activity (mU/ml)} = B / (T2-T1) * V$$

where B is the glutamate amount calculated from the standard curve (in nmol); T1 is the time of the first reading (in min); T2 is the time of the second reading (in min); V is the original sample volume added into the reaction well (in mL).

2.10. miR-122 detection in serum

MicroRNAs (miRNAs) are a family of small non-coding RNAs that can affect xenobiotic metabolism and toxicity, and whose expression may be affected by certain drugs. They also play important roles in modulating inter-individual variability in drug metabolizing enzyme and transporter production (Koturbash et al., 2012, 2015). A miRNA important in hepatotoxicity is miR-122, the only liver-specific miRNA detectable in biological fluids following liver damage (Starkey Lewis et al., 2012). In this study, miR-122 levels were measured in serum by qRT-PCR. The standard was generated using synthetic miRNA 122–5p and added separately to RT reactions after serial dilution to generate a standard curve. The sample was diluted (1:10) in nuclease-free water treated with RNase inhibitor (0.4 U/ μ L). Reverse transcription reaction (RT), pre-amplification, and qRT-PCR were carried out for each sample or standard. (1) Two-step RT reaction was performed in a 15 μ L-RT reaction using the TaqMan miRNA Reverse Transcription Kit (Life Technologies, Foster City, CA). The first step RT reaction was carried out using the following cycling conditions: denature at 94 °C for 10 min; then anneal at 75 °C for 2 min; 60 °C, 50 °C, 40 °C, 30 °C for 3 min, respectively; and hold at 4 °C. The second step was run at 16 °C and 42 °C for 30 min, respectively, then at 85 °C for 5 min, and hold at 4 °C. (2) Pre-Amplification reaction: The RT product needs to be pre-amplified prior to the real-time PCR step to potentially enhance sensitivity. The pre-amplification step was carried out in a 25 μ L reaction comprised of 12.5 μ L Taqman PreAmp Master Mix (2X), 0.0375 μ L Taqman miRNA qPCR assay (20 X), 9.9625 μ L water and 2.5 μ L undiluted RT product. The Pre-Amp PCR reactions were carried out in the following cycling conditions: pre-denature at 95 °C for 10 min, then hold at 55 °C and 72 °C for 2 min, respectively; run 12 cycles at 95 °C for 15 Sec and 60 °C for 4 min, hold at 99.9 °C for 10 min, then hold at 4 °C. The pre-amplified PCR product was diluted in 35 μ L water with the ratio of 1:8. (3) qRT-PCR reaction: The generation of a standard curve was carried out in parallel with qRT-PCR of samples to calculate the amount of the mir-122 in the samples. qRT-PCR reaction was run in 20 μ L composed of 10 μ L TaqMan Universal PCR Master Mix (2 X), 4 μ L water, 1 μ L Taqman miRNA qPCR assay (20 X) and 5 μ L diluted Pre-Amp products on a ViiA7 qRT-PCR thermocycler at 95 °C for 10 min, 40 cycles at 95 °C for 15 Sec and 60 °C for 1 min and hold at 4 °C. The miRNA concentration was calculated based on the standard curve of the matching synthetic miRNA from the standard curve.

2.11. Gene expression array

Total RNA was extracted from flash frozen liver tissue using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Following purification, 1000 ng was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (ThermoFisher, Waltham, MA, USA). The cDNA was diluted to 5 ng/ μ L and 105 μ L was mixed with an equal volume of 2X TaqMan® Fast Advanced Master Mix. For real-time PCR, 100 μ L of the mix was applied to each of two channels on a TaqMan Low Density Hepatotoxicity Array (TLDA) (Supplementary Table 1) (ThermoFisher, Waltham, MA, USA). Four biological samples were loaded onto

each array with five samples analyzed per treatment group. Analysis was performed using the ExpressionSuite Software v1.1 (ThermoFisher, Waltham, MA, USA).

2.12. miRNA expression array

A panel of miRNAs whose expression was previously altered in response to hepatotoxicant exposure was utilized (Miousse et al., 2017). miRNAs were extracted from flash-frozen livers using the miRNeasy Kit (Qiagen, Germantown, MD, USA). RNA concentration was assessed by Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) and 1000 ng of RNA containing miRNAs was used for reverse transcription with the miScript reverse transcriptase and reverse transcription primers specific for nine miRNA targets and the internal reference U6). Transcript abundance was measured on a ViiA-7 instrument (Applied Biosystems, Foster City, CA, USA) and results calculated using the Ct method.

2.13. Statistical analyses

All statistical analyses were performed with the Graphpad Prism 6 software (Graphpad Software, San Diego, CA). Within gender and strain, treatment groups were compared with their respective untreated group using ANOVA followed by Dunnett's multiple comparison test. In cases where the data was not normally distributed, a Dunn's test was used instead.

3. Results

3.1. Analytical chemistry data

Phytochemical analysis of OEP-NF (lot #421131) capsules revealed the presence of caffeine (134.2 mg/cap.), aegeline (44.9 mg/cap.), higenamine (25.2 mg/cap), yohimbine (4.2 mg/cap), coclaurine (0.95 mg/cap.) and trace amounts of tannins (i.e., gallic acid and gallic acid derivatives). No characteristic marker phytochemicals were noted for extracts of either *Bauhinia purpurea* (e.g., isoquercetin, quercetin-3,4'-diglucoside, isorhamnetin-3,7-diglucoside, pelargonidin-3-triglucoside, quercetin 3-rutinoside-7-glucoside, kaempferol 3,4'-diglucoside) or *Hemerocalis fulva* (e.g., fulvanines A-E, kwansonine B-C). Besides yohimbine, no other characteristic marker phytochemicals for *Pausinystalia johimbe* extract (e.g., rauwolscine, β -yohimbine, corynanthine, dihydrositsirikine, pseudoyohimbine, yohimbic acid, alloyohimbine, hydroxyyohimbine) were detected. These findings indicate that neither of these botanical extracts were components of the formulation, despite label claims for each. In short, it appears that OEP-NF was a collection of synthetic alkaloids.

3.2. Acute effects of OEP-NF administration (4–24 h gavage study)

Gavaging CD-1 male mice with 10X MED resulted in 100% mortality within the first hour after OEP-NF administration. Two out of twelve CD-1 male mice gavaged with 3X MED were found dead by this time as well, and two others were moribund and were humanely euthanized along with the rest of the animals in this group at the request of the attending veterinarian. The observed overt toxicity led us to discontinue the 10X MED and the subsequent cohorts were gavaged within the range of 0.5–3X of the MED. All other mice gavaged with 3X MED formulation (i.e., CD-1 females) were humanely euthanized at 4 h so that biochemical findings would be consistent with CD-1 males. Mice receiving gavage doses less than 3X MED were euthanized at 24 h.

Despite the dosing reconsiderations noted above, two female and one male B6C3F₁ mice receiving the 2X MED expired within 24 h.

3.2.1. Liver/body weight ratio and histopathological evaluation—Significant increases in liver/body weight ratios at the 4 h time-point (+0.6%, $p < 0.01$) were noted for male CD-1 mice receiving the 3X MED. Gavaging both male and female B6C3F₁ mice was associated with statistically significant decreases in the liver body/weight ratios for all gavage doses of OEP-NF (range 0.2–0.8%, $p < 0.05$) (Fig. 1A). At 24 h, no significant histopathological changes were identified in the livers of mice in any of the gavage groups.

3.2.2. Clinical biochemistry—No statistically significant changes in ALT or AST levels were observed in CD-1 male and female mice gavaged with OEP-NF. In B6C3F₁ mice, gavaging with 1.5X and 2X MED resulted in statistically significant ($p < 0.05$ – 0.001) increases in the serum levels of both aminotransferases, the only exception being male mice receiving the 2X MED where the increase in ALT approached significance ($p = 0.059$) (Fig. 1B and C). Although, overall increases in ALT and AST were modest, clear dose-dependent trends were observed. Furthermore, compared to vehicle controls, some animals exhibited a 4–5 fold increase in serum aminotransferases. At the same time, no significant changes were observed in the levels of miR-122 in any of the experimental groups, although a greater than two-fold increase in miR-122 was observed in female B6C3F₁ mice gavaged with 2X MED ($p = 0.067$) (Fig. 1D).

3.3. Sub-acute effects of OEP-NF (4-week feeding study)

3.3.1. Effects of OEP-NF on body weight dynamics—Differences in the initial body weights of experimental animals were observed as a result of the outbred nature of CD-1 mice, as well as sex-, and strain-differences. Therefore, body weight dynamics were evaluated as a percent change from the initial weight of each individual animal.

Administration of OEP for 4 weeks affected body weight dynamics in all mice in a strain-, sex-, and dose-dependent manner. Specifically, CD-1 mice lost body weight over the first week, but then gained weight over the next 3 weeks, although at a slower pace than controls. (Supplementary Fig. 1). No treatment-related declines in body weight were observed in B6C3F₁ mice of either sex; however, similar to CD-1 mice, weight gain dynamics were slower when compared to controls.

3.3.2. Liver/body weight ratio and histopathological evaluation—No significant changes in liver/body weight ratios were noted in mice fed OEP-NF for 4 weeks (Fig. 2A). At the end of the 4-week feeding study a number of animals exhibited modest to moderate histopathological changes, mostly in the form of multiple mitotic foci (Fig. 2B).

3.3.3. Analysis of clinical biochemistry—Feeding OEP-NF for 4 weeks did not significantly affect serum ALT or AST (Fig. 2C and D). A statistically significant increase ($p < 0.05$) in the serum levels of miR-122 was observed in CD-1 males fed the 3X OEP-NF diet (Fig. 2E). Increases in serum miR-122 levels were also observed in male and female B6C3F₁ mice fed the 3X diet, although these differences did not reach statistical significance.

3.3.4. Hepatotoxicity gene expression array—To explore potential molecular alterations associated with dietary administration of OEP-NF, we performed expression analysis of a panel of genes previously linked to hepatotoxicity (Aleksunes et al., 2006; Chen et al., 2017; Heise et al., 2015; Koturbash et al., 2011; Minami et al., 2005). At four weeks, 17 genes were identified that were significantly (± 1.5 -fold change from control, $p < 0.05$) dysregulated in at least two treatment groups (Table 1). The most pronounced changes were observed in female B6C3F₁ mice in which 13 separate genes, including *Nqo1*, *Cd36*, and *Lpl* were differentially regulated.

3.3.5. Hepatotoxicity miRNA expression panel—As a result of the high inter-individual variability in miRNA expression, no statistically significant changes were observed in the expression of miRNAs related to hepatotoxicity after 4 weeks of OEP-NF exposure. However, dose-dependent trends in the expression of miR-122, miR-29a, and miR-101a were observed in the livers of female B6C3F₁ mice (Supplementary Fig. 2).

3.4. Analysis of OEP hepatotoxicity at a 13-week time-point

3.4.1. Body weight dynamics—Despite continuous dietary administration of OEP-NF, a purported weight-loss supplement, all animals in all treatment groups gained weight over the course of 13 weeks. The rate of weight gain was slowest in the CD-1 males on both the 1X and 3X diets with total body weight increases of 21% and 16%, respectively. For female mice on the 1X diet, the rate of weight gain was no different than that for controls, whereas female mice on 3X dose gained weight a slower rate than controls or females on the 1X dose (Fig. 3).

3.4.2. Liver/body weight ratio and histopathological evaluation

Administration of OEP-NF for 13 weeks resulted in significant ($p < 0.05$) increases in liver/body weight ratios for CD-1 male mice on both 1X and 3X doses, whereas female CD-1 and B6C3F₁ mice exhibited significant increases only on the high dose (Fig. 4A). There was a trend toward an increase in the liver/body weight ratio in B6C3F₁ male mice, however it lacked significance. The only notable histopathological findings was the appearance of multiple mitotic foci primarily in mice fed OEP-NF (Fig. 4B).

3.4.3. Analysis of clinical biochemistry—Statistically significant increases in both aminotransferases ($p < 0.01$, ALT; $p < 0.05$, AST) were noted in the serum of female B6C3F₁ mice (Fig. 4C and D). No significant changes in ALT or AST were observed in the serum of other animals. Statistically significant increases in serum miR-122 levels were observed in B6C3F₁ female mice fed low-dose OEP-NF (6.2-fold, $p < 0.05$). Increases in miR-122 were also observed in the serum of CD-1 female mice, as well as B6C3F₁ male and female mice fed high-dose OEP, but these trends did not reach statistical significance. (Fig. 4E).

3.4.4. Hepatotoxicity gene expression array—Administration of OEP-NF for 13 weeks also revealed that 12 genes associated with hepatotoxicity were significantly dysregulated in at least two groups, compared to control mice (Table 1). Interestingly, there

were 9 genes that were commonly affected at both 4- and 13-week time-points, including *Nqo1* and *Lpl*.

3.4.5. Hepatotoxicity miRNA expression panel—Analysis of microRNA expression revealed that the expression of various miRNAs was down-regulated at the 3X dose. Statistically significant reductions in miR-21 were noted for CD-1 females; whereas significant reductions were noted in miR-192, mir101a, B6C3F₁ mice (Supplementary Fig. 3).

3.5. Identification of individual responders to OEP-NF toxicity

During the data analysis, our attention was attracted to a number of outliers – mice that exhibited responses substantially different from the mean values of their respective groups. Given the idiosyncratic nature of OEP-NF hepatotoxicity in humans, we hypothesized that some mice exhibit individual susceptibility to diet-fed OEP-NF. Therefore, we created datasets that included the records of each individual “outlier” response and linked them to individually tagged mice. Only animals with endpoint values greater than 2 SEM from the control group mean were selected for inclusion into Table 2. In total, 39 animals displayed an “outlier” response for at least 1 endpoint. Among them, 2 animals were from the vehicle-fed mice, 15 received 1X MED, and 22 received 3X MED. Interestingly, a number of mice in the study (e.g., female CD-1 mouse #332: refer to Table 2) exhibited a spectrum of findings that included increased liver/body weight ratio, elevated serum levels of ALT, AST and miR-122, the appearance of hepatocellular mitotic foci, as well as altered gene expression profiles, all of which render these animals as genuine responders to OEP-NF. These findings also speak to the idiosyncratic nature of OEP-NF toxicity, in that select individuals, particularly those among the outbred CD-1 strain, appear more susceptible to the product than others.

3.6. Other non-liver-specific effects of OEP-NF

Feeding mice with OEP-NF was also associated with other, non-liver-specific effects. Among the most pronounced was a kidney/body weight ratio increase observed in CD-1 male mice at both 4- and 13-week time points (Supplementary Fig. 4). Other noteworthy observations were associated with affected behavior characterized by food-wasting, “asocial” activity, and unusual barbering patterns.

4. Discussion

It has been reported that about 50% of the adult population in the United States have used at least one HDS in the past month and these numbers continue to grow (Bailey et al., 2011). On the other hand, a significant increase in HDS-induced liver injury, from 7% to nearly 20% of all cases of liver injury has been observed in the last decade (Bailey et al., 2011; de Boer and Sherker, 2017). Of particular concern are dietary supplements marketed as weight-loss aids and performance enhancers, a number of which were suspected in causing liver injury and subsequently removed from the market (Bonkovsky, 2006; Church et al., 2015; Fong et al., 2010; Kanda et al., 2003; Stickel et al., 2009). The most recent suspect in this controversy involved OxyELITE Pro, a dietary supplement whose previous formulation was

removed from the market due to serious adverse health effects related to its combination of purportedly natural stimulants and sympathomimetics, including 1,3-dimethylamylamine (DMAA) (U.S. Food and Drug Administration, 2013a,b). As it turned out, DMAA was not of natural origin but rather a drug developed by Eli Lilly more than 70 years ago, which was removed from the market in the 1983 (Zhang et al., 2012). In its new formula, DMAA was replaced by aegeline, a phytochemical extracted from the leaves of the bael tree (*Aegle marmelos*). However, shortly after OEP-NF was introduced onto the market, a series of liver injuries was linked to its usage, with some cases requiring liver transplantation or resulting in death (Chatham-Stephens et al., 2017; Foley et al., 2014; Heidemann et al., 2016; Johnston et al., 2016; Roytman et al., 2014).

A paucity of scientific data exists regarding the safety of *A. marmelos*, and while extracts of its various plant parts may not appear hepatotoxic in some rodent models (Khan and Sultana, 2009; Singh et al., 2000; Veerappan et al., 2007), others have demonstrated significant liver injury (Arseculeratne et al., 1985). These equivocal findings for whole plant extracts are not exculpatory for synthetic aegeline, especially when combined with the other synthetic ingredients within OEP-NF. In fact, a recent *in vitro* screening found that purified aegeline was cytotoxic to HepG2 cells (Mohammed et al., 2016), and that when administered to rats aegeline was rapidly absorbed and concentrated in the liver (Manda et al., 2017).

While *A. marmelos* extracts have been used as traditional Asian medicines, an absence of historical records documenting its toxicity does not exclude the potential for aegeline to cause liver injury or other negative health effects when consumed in a cocktail of other drugs/phytochemicals not present in *A. marmelos*. From a historical perspective, pyrrolizidine alkaloids derived from *Senecio* spp. provide a useful example. The so-called “bush tea” brewed from *Senecio* was used in Jamaican medicine for centuries, but only in the 20th century was it linked to a commonly-observed hepatic sinusoidal obstruction syndrome and cirrhosis (Bras et al., 1954). When it became evident that the aegeline present in OEP-NF was not part of an *A. marmelos* extract, but chemically synthesized and imported to the U.S., questions arose regarding the toxicological contribution of potential contaminants either from synthetic intermediates, non-naturally occurring enantiomers, or both (Navarro et al., 2017).

While the pharmacological and toxicological profile of aegeline is virtually unknown, contributions from other components within OEP-NF cannot be dismissed. It is our contention that toxic manifestations of OEP-NF ingestion are attributable to the complete formulation and not a single phytochemical entity. Pharmacodynamic interactions among the various constituents are also likely contributing factors. For example, caffeine, a major constituent of OEP-NF, is recognized for its ability to augment the toxicity of other stimulants and sympathomimetics (Brown et al., 1991; Derlet et al., 1992; Haller et al., 2004; McNamara et al., 2006). Combinations of caffeine and ephedrine alkaloids can produce a host of cardiovascular and central nervous system pathologies that are more remarkable than those observed after administration of each individual component (Brown et al., 2012; Dunnick et al., 2007). Such findings reinforced the FDA’s decision to remove of *Ephedra*-containing dietary supplements from the U.S. market in 2004 (Anonymous, 2004). Today, many *Ephedra*-free formulations, like OEP-NF, which combine various caffeine

sources with other natural sympathomimetics, are also plagued by adverse health effects, especially when taken in conjunction with vigorous exercise (Gurley et al., 2015). In the case of OEP-NF, caffeine is known to intensify the hemodynamic response to the α_2 -antagonist yohimbine (Waluga et al., 1998), thus its augmentation of the sympathomimetic activities of higenamine and coclaurine is not unexpected (Lee et al., 2013). Supplement formulas containing caffeine and yohimbine are particularly concerning since *Yohimbe*-containing products have been associated with a significantly greater proportion of severe outcomes reported to the California Poison Control System (Kearney et al., 2010). Moreover, caffeine and yohimbine were also present in Hydroxycut, another weight loss product removed from the market in 2009 following reports of liver injury (Avigan et al., 2016; Fong et al., 2010). Finally, caffeine has been shown to exacerbate acute inflammatory liver injury (Ohta et al., 2007) while yohimbine's α_2 -antagonism aggravates lipopolysaccharide-induced liver injury (Chen et al., 2015).

Pharmacokinetic interactions among OEP-NF ingredients must also be taken into consideration. From that perspective, it is worth noting that aegeline, higenamine, yohimbine, and coclaurine are all CYP3A4 substrates and yohimbine, higenamine, and coclaurine are also CYP2D6 substrates, with the latter two also being CYP2D6 inhibitors (Le Corre et al., 2004; Liu and Santillo, 2016; Manda et al., 2016). Moreover, *CYP2D6* is highly polymorphic and individuals exhibiting a CYP2D6 "poor metabolizer" phenotype may be more susceptible to the effects of OEP-NF (Ingelman-Sundberg, 2005). Given that almost 50% of Asians exhibit a CYP2D6 poor metabolizer phenotype (Ingelman-Sundberg, 2005), it is interesting to note that most of those with severe liver injuries linked to OEP-NF use in Hawaii were of Asian descent (Johnston et al., 2016; Roytman et al., 2014). This observation also underscores the impact individual pharmacogenetic differences can have on dietary supplement safety and efficacy.

In this study, we investigated the hepatotoxic potential of OEP-NF in acute and sub-chronic protocols using both inbred and outbred mouse strains. Gavaging mice with 10X MED resulted in overt toxicity and 100% mortality within an hour, an effect most likely due to the high doses of caffeine and other OEP-NF ingredients. While the death of some of mice gavaged with 3X MED may have also been related to the combined cardiovascular effects of OEP-NF components, a hepatotoxic element cannot be ruled out, since an increase in liver/body weight ratio was observed in these mice. There were also significant increases in serum aminotransferases even at 1.5X MED, which contained individual quantities of caffeine, yohimbine, and higenamine well below the reported LD₅₀ values.

Perhaps of greater concern than the mortality associated with the 3X and 10X MED is the narrow safety margin implied by these findings. For a dietary supplement to produce adverse health effects at 10X the maximum daily label recommended dose, while concerning, is not unexpected; however, serious toxicity at doses just 3X greater than the maximum daily label recommended dose gives cause for great concern. That the OEP-NF warning language takes up almost half of the product label is a good indicator of the potential problems to be expected. Partial statements such as "Under no circumstances should initial serving size be exceeded or the warning on this bottle ignored;" "Do not exceed 3 capsules in any 24 h period;" or "Consult with your physician before using this product, especially if you are

using any prescription or over the counter medication or if you have any pre-existing medical condition including but not limited to high or low blood pressure, cardiac arrhythmia, stroke, heart, liver, kidney, or thyroid disease, seizure disorder, psychiatric disease, diabetes, difficulty urinating due to prostate enlargement or if you are taking a MOAI (monoamine oxidase inhibitor) or any other medication,” are tacit admissions to OEP-NF’s questionable safety.

In the feeding phase of the experiment, no marked histomorphological or biochemical changes were observed after 4 weeks. However, at the 13-week time-point, hepatotoxic effects of OEP-NF were evidenced by increases in liver/body weight ratios and in serum levels of ALT, AST and miR-122 of female B6C3F₁ mice. The magnitude of those changes, while not as dramatic as those observed following oral gavage, are indicative of mild liver injury after 13 weeks of OEP-NF exposure in the diet.

To further explore the molecular mechanisms underlying the OEP-NF-induced feeding effects, we performed an expression analysis of select genes and miRNAs commonly associated with hepatotoxicity. A number of critical genes were affected in response to OEP-NF ingestion both at 3X and 1X MED. Most notably, gene expression analysis revealed that OEP-NF ingestion similarly affected a number of genes that are also altered by potent liver toxicants like acetaminophen (acetyl-*para*-aminophenol; APAP), bromobenzene, carbon tetrachloride, dimethyl nitrosamine and thioacetamide (Minami et al., 2005). These genes included aldolase A, fructosebiphosphate (*Aldoa*); thioredoxin reductase 1 (*Txnrd1*); stearoyl-CoA desaturase-1 (*Scd1*); and cyclin G1 (*Ccng1*), just to name a few.

One of the genes most strongly affected by OEP-NF was *Cd36*. Expression of *Cd36* was increased 2-to-4-fold in the livers of CD-1 and B6C3F₁ mice at both 4 and 13-week time-points. *Cd36* is an adhesion molecule involved in hemostasis, thrombosis, inflammation, atherogenesis and lipid metabolism (Daviet and McGregor, 1997; Schneiderhan et al., 2001). As a recognized transcriptional target of *Ahr*, *Cd36* is required for *Ahr*-induced hepatosteatosis (Lee et al., 2010). Up-regulation of *Cd36* leads to increased fatty acid uptake from the bloodstream into the liver (Lee et al., 2010; Schneiderhan et al., 2001). This gene was previously reported to be up-regulated after 4 weeks of exposure to the hepatotoxic triazole fungicides - cyproconazole, epoxiconazole and prochloraz (Heise et al., 2015). Interestingly, the lipogenesis-associated gene *Cd36* has also been shown to play an important role in promoting caffeine’s ability to reduce hepatic lipid accumulation (Zheng et al., 2015). Yet, in the study by Zheng et al., the hepatoprotective effect of caffeine was mediated by down-regulation of *Cd36*, while caffeine-containing OEP-NF caused substantial up-regulation of *Cd36*. This paradoxical finding highlights another important message regarding multi-ingredient HDS: in the context of a complex mixture, phytochemicals may exhibit effects contradictory to those observed when administered alone. Interestingly, other plant alkaloids appear to induce liver steatosis via *Cd36*-mediated mechanisms. For instance, Choi and colleagues reported that berberine, an isoquinoline alkaloid with anti-microbial and anti-diarrheal activities, caused hepatic lipid accumulation by activating *Cd36* in the mouse model (Choi et al., 2017).

Another gene that was significantly up-regulated in response to OEP-NF in all mice independently of the strain or sex was the nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase-1 (*Nqo1*). This gene, usually poorly expressed in normal liver, is a potent scavenger of the superoxide anion, and serves as part of the antioxidant defense system; it also plays an important role in metabolism (Palming et al., 2007; Siegel and Ross, 2000; Strassburg et al., 2002). *NQO1* was shown to be induced in cases of APAP overdose, as well as in primary biliary cirrhosis (Aleksunes et al., 2006). Palming and colleagues also report that elevated levels of *NQO1* correlate with several clinical markers of liver dysfunction (Palming et al., 2007). In the same study, the authors also noted that *NQO1* was reduced during diet-induced weight loss a result opposite to that observed with OEP-NF. In a more relevant comparison, it was shown that usnic acid – a component in several previously banned weight-loss HDS linked to hepatotoxicity – caused up-regulation of *NQO1* in human HepG2 cells (Chen et al., 2017). Similarly, another known hepatotoxicant, Mequindox, caused up-regulation of *NQO1* in parallel with a modest increase in ALT and AST, an effect similar to that observed in our study (Liu et al., 2017). It is generally accepted that up-regulation of this cytoprotective enzyme is an adaptive stress response to limit further disease progression (Aleksunes et al., 2006).

Altogether, around two dozen genes were significantly dysregulated (at least 1.5-fold from control) in two or more treatment groups. Importantly, those genes whose expression was dysregulated are associated with multiple cellular or biochemical pathways, including, but not limited to: xenobiotic metabolism, cholestasis, oxidative stress response, fatty acid metabolism, hepatocarcinogenesis and necrosis. These effects were observed at the 4- and 13-week time-points, in both male and female CD-1 and B6C3F₁ strains; however, the most substantial effects were observed in B6C3F₁ female mice, congruent with other observed adverse effects, discussed above.

Feeding mice with OEP-NF also affected the expression of a number of miRNAs that have been associated with other liver toxicants. There was a clear dose-dependent response, in that feeding mice a 3X MED of OEP-NF caused substantial reductions in the expression of different miRNAs, where the most robust and significant effects were observed for miR-101a, miR-192, miR-193a and miR-125b. Taken together, the observed changes in gene and miRNA expression suggest that OEP-NF induces substantial molecular changes within the livers of experimental mice.

It can be argued that up-regulation of various oxidative stress genes can be advantageous by inducing the expression of redox enzymes within the liver. Many phytochemicals can indeed increase oxidative enzyme expression through their ability to activate the Keap1/Nrf2 pathway (Qin and Hou, 2016; Stefanson and Bakovic, 2014). Once activated, the transcription factor Nrf2 binds to the antioxidant response element in the hepatocyte nucleus to promote expression of a plethora of antioxidant enzymes. In fact, Nrf2 activators have been investigated as potential therapeutic agents, although the development of some has been stopped due to liver injury concerns (Zoja et al., 2013). Despite their purported benefits, excessive exposure or prolonged use of natural Nrf2 activators may become pro-oxidant or give rise to a state of reductive stress, both of which can be detrimental to the hepatocyte (Brewer et al., 2013; Dodson et al., 2015). Only recently has this redox paradox

garnered attention as a pathological consequence of Nrf2 activation, and may explain certain types of herbal hepatotoxicity (Lei et al., 2016). For example, oleanolic acid is a potent phytochemical Nrf2 activator that acts as a hepatoprotectant at low concentrations but becomes hepatotoxic at higher doses (Liu et al., 2013). Other phytochemicals or botanicals that display hormesis with regard to antioxidant properties at low doses and pro-oxidant effects at higher doses include quercetin (Vargas and Burd, 2010), rhubarb (Wang et al., 2011), epigallocatechin gallate (EGCG) (Calabrese et al., 2012) and various other polyphenolics (Skibola and Smith, 2000). Because the phytochemical content of HDS is often much greater than that found in the plant itself, pro-oxidant or reductive stress effects may be a more common problem with these products than heretofore anticipated.

This comprehensive and integrative pre-clinical assessment reports two major outcomes of administration of OEP-NF in the mouse model. First, the dietary supplement OEP-NF is clearly liver-reactive, exhibiting a number of molecular and biochemical responses e from a modest increase in liver/body weight ratios and elevations in serum aminotransferase levels to marked dysregulation of genes involved in numerous detoxification and pathological pathways. These effects were cumulative and were most strongly pronounced after 13 weeks of ingestion. These findings suggest that administration of OEP-NF for periods longer than 13 weeks increase the risk for progressive liver injury.

The second noteworthy observation is identification of individual mice that exhibited a spectrum of affected endpoints despite a lack of statistical significance in the group mean for those parameters. This finding speaks towards the potential idiosyncratic nature of OEP-NF-induced liver toxicity observed in humans.

Finally, it must be emphasized that while individual compounds of a given HDS formulation may be safe/non-toxic, atypical combinations of ingredients, especially those with little to no data regarding their pharmacology, toxicology, or drug interaction potential (i.e., aegeline), may produce serious adverse health effects. The potent mixture of plant alkaloids present in the OEP-NF formulation gave rise to a spectrum of adverse cardiovascular, neurological, and hepatotoxic effects in inbred and outbred mouse strains. While these findings were not unexpected at higher gavage doses, those emanating from allometrically scaled doses mimicking a 1–3X human equivalent are disconcerting and indicate that this and similar products pose significant health risks. These risks are likely amplified in individuals whose pharmacogenetic profile may lead to altered pharmacokinetics and pharmacodynamics of the phytochemical cocktail. Our findings underscore the need for dietary supplement companies to conduct integrative pre-clinical toxicological studies of multi-ingredient HDS, especially those marketed for weight-loss and/or exercise performance enhancement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ALT	alanine aminotransferase
APAP	acetaminophen
AST	aspartate aminotransferase
DILI	drug-induced liver injury
DILIN	Drug-Induced Liver Injury Network
DMAA	1,3-dimethylamylamine
DMSO	Dimethyl sulfoxide
HDS	herbal and dietary supplements
HILI	Herbal-induced liver injury
MED	mouse equivalent dose
miRNA	microRNA
OEP-NF	OxyELITE Pro New Formula
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
TLDA	TaqMan Low Density Arrays
UHPLC	ultra-high performance liquid chromatography

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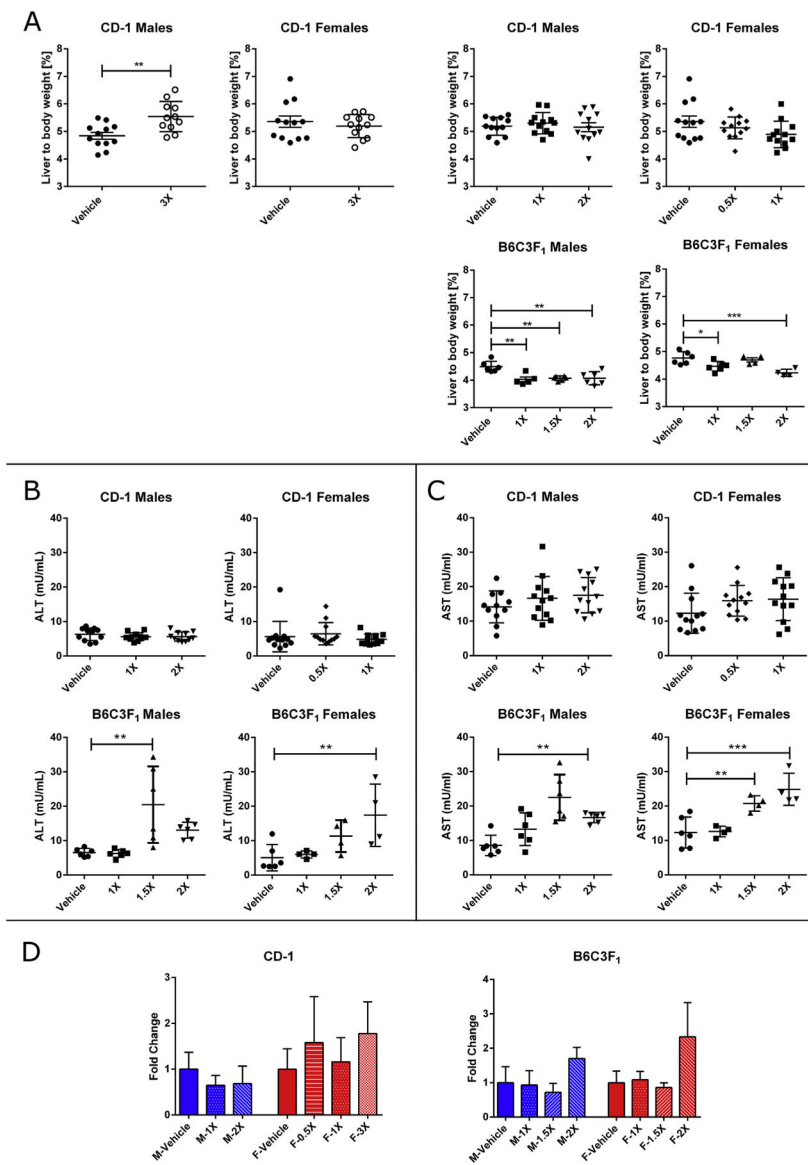


Fig. 1. Effects of OEP-NF gavaging in male and female CD-1 and B6C3F1 mice. A) Liver to body weight ratio in mice after gavaging with 3X MED (4 h time-points) and 0.5X-2X MED (24 h time-point); B) Levels of ALT, and C) Levels of AST in mice 24 h after gavaging; D) Levels of miR-122 in the serum of mice 24 h after gavage, data expressed as fold-change from respective control values. Mean \pm SD. Asterisks “*” denotes significant ($p < 0.05$), “**” ($p < 0.01$), and “***” ($p < 0.001$) difference from control.

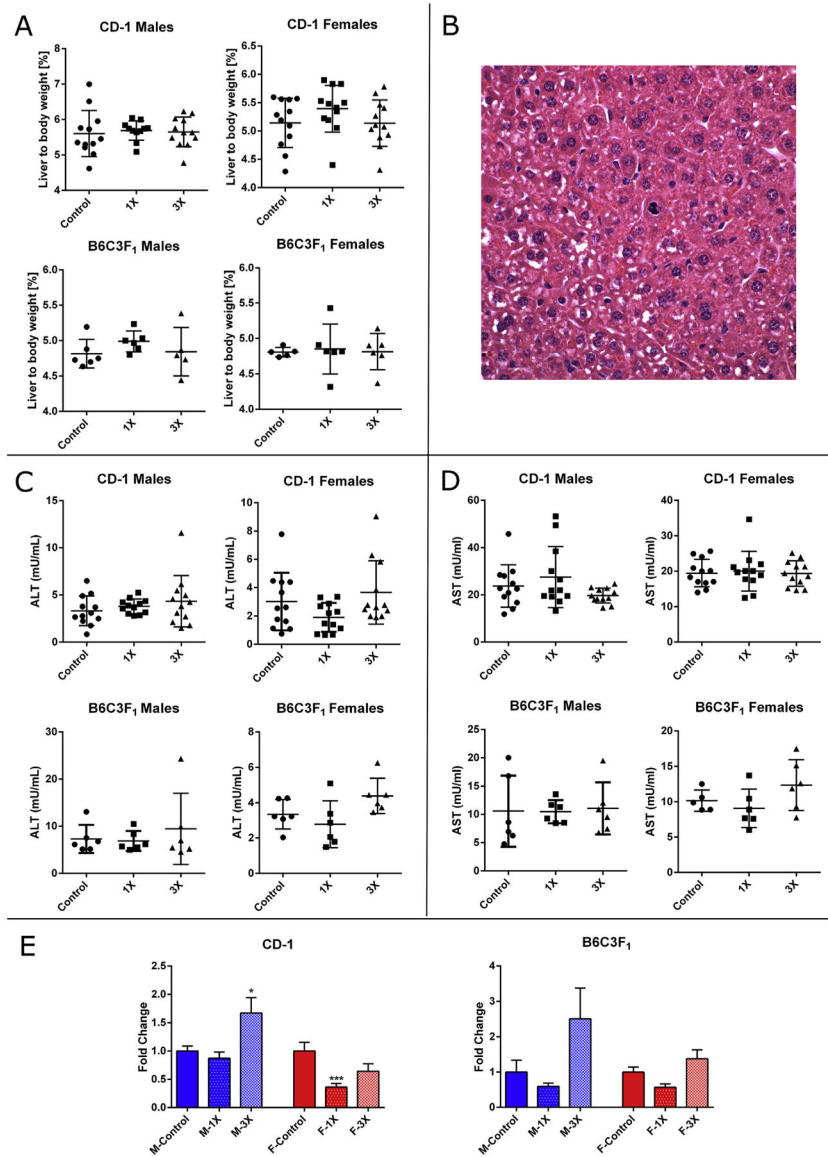


Fig. 2. Effects of feeding mice with OEP-NF for 4 weeks. A) Liver to body weight ratio; B) Mitotic figures in the liver of a mouse (ID# 291) fed 1X OEP-NF (magnification 40×); C) Levels of ALT, and D) Levels of AST in the serum of mice; E) Levels of miR-122 in the serum of mice, data expressed as fold-change from respective control values. Mean ± SD. Asterisks “*” denotes significant ($p < 0.05$) difference from control.

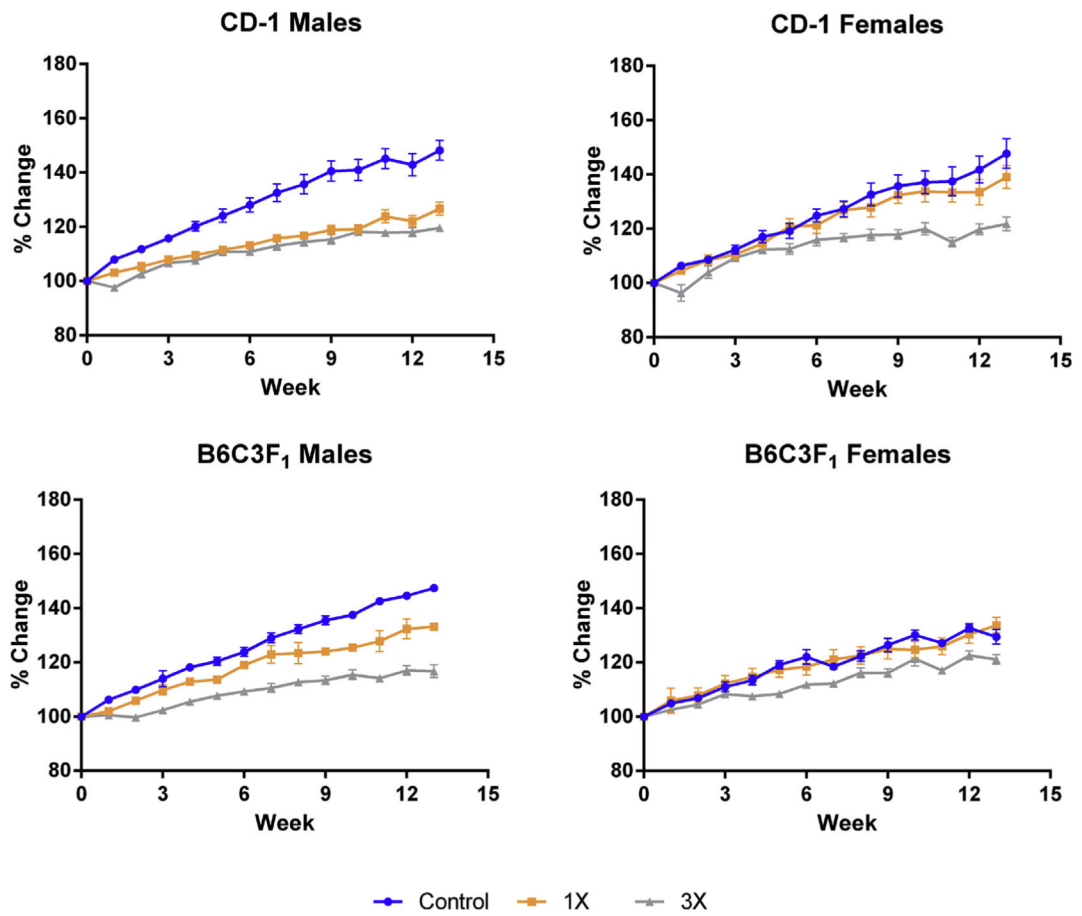


Fig. 3. Body weight dynamics in mice fed OEP for 13 weeks. Data presented as a percent-change from control values at Week 0.

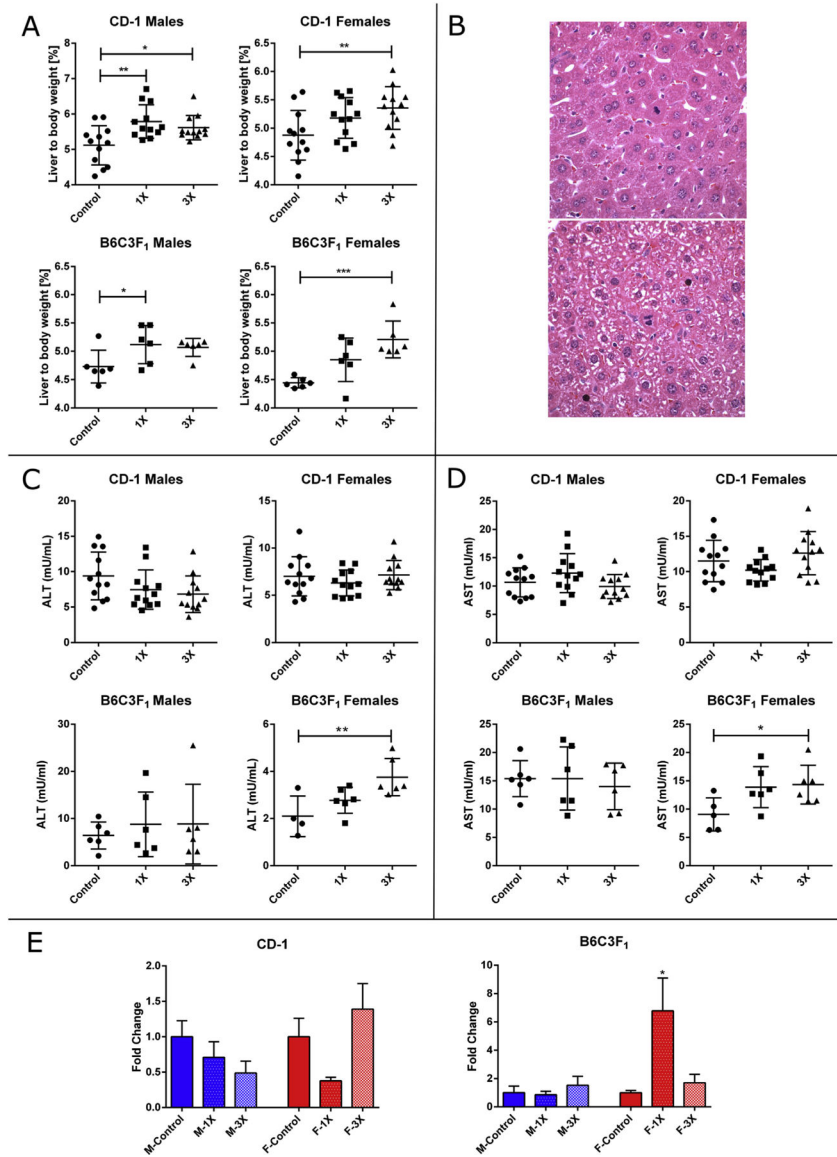


Fig. 4. Effects of feeding mice with OEP-NF for 13 weeks. A) Liver to body weight ratio; B) Mitotic figures in the livers of mice (ID# 259 and 332) fed OEP-NF (magnification 40×); C) Levels of ALT, and D) Levels of AST in the serum of mice; E) Levels of miR-122 in the serum of mice, data expressed as fold-change from respective control values. Mean ± SD. Asterisks “*” denotes significant ($p < 0.05$), “**” ($p < 0.01$), and “***” ($p < 0.001$) difference from control.

Table 1

List of dysregulated genes as a result of feeding mice with OEP for 4 or 13 weeks.

	B6C3F ₁															
	4 weeks				CD-1				Males				Females			
	Control	1X	3X	IX	Control	1X	3X	IX	Control	1X	3X	IX	Control	1X	3X	IX
<i>Abcb4</i>	1.00 (±0.16)	1.12 (±0.12)	1.05 (0.09)	1.39 (±0.14)	1.00 (±0.05)	1.05 (0.09)	1.45 (0.19)	1.18 (±0.07)	1.00 (±0.07)	1.45 (0.19)	1.18 (±0.07)	1.53* (±0.17)	1.00 (±0.11)	1.52 (±0.04)	1.58* (±0.20)	
<i>Abcc2</i>	1.00 (±0.14)	1.04 (±0.12)	0.93 (0.12)	1.56 (±0.28)	1.00 (±0.04)	0.93 (0.12)	1.65 (0.27)	0.97 (±0.06)	1.00 (±0.08)	1.65 (0.27)	0.97 (±0.06)	1.38** (±0.08)	1.00 (±0.10)	1.27 (±0.09)	1.38* (±0.07)	
<i>Abcc3</i>	1.00 (±0.13)	0.84 (±0.06)	1.34 (0.13)	1.30 (±0.10)	1.00 (±0.02)	1.34 (0.13)	1.46 (0.29)	1.02 (±0.18)	1.00 (±0.18)	1.46 (0.29)	1.02 (±0.18)	2.38# (±0.42)	1.00 (±0.15)	1.31 (±0.17)	1.84** (±0.13)	
<i>Aldoa</i>	1.00 (±0.17)	0.98 (±0.08)	1.06 (0.07)	1.36 (±0.18)	1.00 (±0.09)	1.06 (0.07)	1.43 (0.02)	1.47* (±0.15)	1.00 (±0.03)	1.43 (0.02)	1.47* (±0.15)	1.66** (±0.14)	1.00 (±0.08)	1.58 (±0.22)	1.55 (±0.24)	
<i>Avpr1a</i>	1.00 (±0.06)	1.40* (±0.10)	1.26 (0.13)	0.94 (±0.12)	1.00 (±0.06)	1.26 (0.13)	0.75 (0.15)	1.37 (±0.08)	1.00 (±0.06)	0.75 (0.15)	1.37 (±0.08)	1.59# (±0.21)	1.00 (±0.06)	1.54 (±0.18)	1.45 (±0.21)	
<i>Bhmt</i>	1.00 (±0.13)	1.36 (±0.13)	1.11 (0.39)	1.12 (±0.14)	1.00 (±0.22)	1.11 (0.39)	0.98 (0.10)	1.08 (±0.11)	1.00 (±0.06)	0.98 (0.10)	1.08 (±0.11)	1.14 (±0.15)	1.00 (±0.08)	1.64 (±0.06)	1.65 (±0.27)	
<i>Ccgn1</i>	1.00 (±0.17)	1.23 (±0.34)	0.84 (0.08)	1.34 (±0.18)	1.00 (±0.15)	0.84 (0.08)	1.13 (0.05)	1.30 (±0.08)	1.00 (±0.03)	1.13 (0.05)	1.30 (±0.08)	1.32* (±0.12)	1.00 (±0.04)	1.70* (±0.20)	1.76* (±0.21)	
<i>Cd36</i>	1.00 (±0.19)	0.50 (±0.09)	1.06 (0.23)	1.50 (±0.30)	1.00 (±0.36)	1.06 (0.23)	2.98** (0.29)	0.86 (±0.08)	1.00 (±0.33)	2.98** (0.29)	0.86 (±0.08)	2.27 (±0.68)	1.00 (±0.14)	1.18 (±0.08)	3.70# (±0.67)	
<i>Cdc14b</i>	1.00 (±0.16)	1.41 (±0.15)	1.40 (0.14)	1.32 (±0.22)	1.00 (±0.07)	1.40 (0.14)	1.14 (0.14)	1.02 (±0.10)	1.00 (±0.05)	1.14 (0.14)	1.02 (±0.10)	1.72** (±0.14)	1.00 (±0.14)	1.73* (±0.13)	1.87** (±0.19)	
<i>Cxcl12</i>	1.00 (±0.06)	0.80 (±0.08)	0.67# (0.02)	0.87 (±0.09)	1.00 (±0.04)	0.67# (0.02)	0.93 (0.06)	0.86 (±0.04)	1.00 (±0.12)	0.93 (0.06)	0.86 (±0.04)	1.08 (±0.06)	1.00 (±0.09)	1.40* (±0.09)	1.08 (±0.10)	
<i>Cyp11a2</i>	1.00 (±0.24)	0.85 (±0.14)	0.94 (0.07)	1.36 (±0.22)	1.00 (±0.10)	0.94 (0.07)	1.98* (0.25)	1.24 (±0.12)	1.00 (±0.01)	1.98* (0.25)	1.24 (±0.12)	1.73## (±0.11)	1.00 (±0.07)	1.61* (±0.16)	2.24*** (±0.16)	
<i>Fasn</i>	1.00 (±0.16)	0.63 (±0.09)	0.94 (0.12)	0.65 (±0.38)	1.00 (±0.37)	0.94 (0.12)	0.61 (0.07)	0.71 (±0.08)	1.00 (±0.10)	0.61 (0.07)	0.71 (±0.08)	1.28 (±0.07)	1.00 (±0.25)	0.55 (±0.10)	0.73 (±0.06)	
<i>Galc</i>	1.00 (±0.12)	1.48 (±0.21)	1.79* (0.28)	1.41* (±0.12)	1.00 (±0.03)	1.79* (0.28)	1.52* (0.14)	1.06 (±0.11)	1.00 (±0.04)	1.52* (0.14)	1.06 (±0.11)	1.83* (±0.37)	1.00 (±0.10)	1.40 (±0.22)	1.63 (±0.24)	
<i>Gsr</i>	1.00 (±0.21)	0.78 (±0.14)	1.09 (0.14)	1.00 (±0.18)	1.00 (±0.07)	1.09 (0.14)	1.43 (0.12)	1.00 (±0.11)	1.00 (±0.15)	1.43 (0.12)	1.00 (±0.11)	1.23 (±0.15)	1.00 (±0.04)	1.54 (±0.36)	1.48 (±0.17)	
<i>Lpl</i>	1.00 (±0.17)	1.15 (±0.32)	1.34 (0.29)	1.03 (±0.27)	1.00 (±0.08)	1.34 (0.29)	2.05 (0.57)	1.23 (±0.19)	1.00 (±0.15)	2.05 (0.57)	1.23 (±0.19)	1.50 (±0.09)	1.00 (±0.09)	1.67* (±0.24)	2.06** (±0.08)	
<i>Lss</i>	1.00 (±0.11)	0.55** (±0.06)	1.01 (0.05)	0.91 (±0.32)	1.00 (±0.15)	1.01 (0.05)	1.07 (0.13)	0.90 (±0.06)	1.00 (±0.11)	1.07 (0.13)	0.90 (±0.06)	1.58* (±0.18)	1.00 (±0.08)	1.11 (±0.04)	1.21 (±0.17)	
<i>Mlxipl</i>	1.00 (±0.15)	1.05 (±0.03)	1.70# (0.25)	1.06 (±0.30)	1.00 (±0.13)	1.70# (0.25)	0.87 (0.09)	1.07 (±0.10)	1.00 (±0.04)	0.87 (0.09)	1.07 (±0.10)	1.50*** (±0.09)	1.00 (±0.07)	1.81# (±0.26)	2.00# (±0.49)	

	13 weeks				CD-1							
	Males		Females		Males		Females					
	Control	1X	3X	1X	Control	1X	3X	1X				
<i>Nqo1</i>	1.00 (±0.17)	1.22 (±0.28)	1.76 (0.53)	1.00 (±0.18)	0.93 (±0.19)	1.85* (0.27)	1.00 (±0.07)	1.09 (±0.05)	2.55## (±0.41)	1.00 (±0.17)	1.79 (±0.10)	2.40** (±0.33)
<i>Plazg12a</i>	1.00 (±0.12)	0.87 (±0.07)	0.99 (0.22)	1.00 (±0.13)	1.41 (±0.09)	1.51 (0.22)	1.00 (±0.09)	1.35 (±0.16)	1.54* (±0.11)	1.00 (±0.07)	1.87*** (±0.06)	1.51** (±0.13)
<i>pygl</i>	1.00 (±0.14)	1.20 (±0.14)	1.04 (0.15)	1.00 (±0.16)	0.93 (±0.23)	0.86 (0.05)	1.00 (±0.06)	1.36 (±0.12)	1.69 (±0.25)	1.00 (±0.10)	1.10 (±0.16)	1.16 (±0.17)
<i>Scd1</i>	1.00 (±0.33)	0.67 (±0.17)	1.55 (0.45)	1.00 (±0.69)	0.68 (±0.36)	0.83 (0.23)	1.00 (±0.16)	0.67 (±0.14)	0.99 (±0.15)	1.00 (±0.13)	0.77 (±0.24)	0.85 (±0.07)
<i>Thrsp</i>	1.00 (±0.25)	0.85 (±0.21)	0.81 (0.18)	1.00 (±0.36)	0.22 (±0.07)	0.89 (0.33)	1.00 (±0.13)	0.62 (±0.10)	0.88 (±0.07)	1.00 (±0.12)	0.83 (±0.36)	0.50 (±0.10)
<i>Tmem2</i>	1.00 (±0.07)	0.89 (±0.06)	1.01 (0.07)	1.00 (±0.06)	1.14 (±0.16)	1.20 (0.10)	1.00 (±0.08)	0.92 (±0.04)	1.23 (±0.10)	1.00 (±0.06)	1.38* (±0.06)	1.52** (±0.11)
<i>Txnrd1</i>	1.00 (±0.21)	0.89 (±0.09)	1.02 (0.12)	1.00 (±0.03)	1.01 (±0.13)	1.40 (0.30)	1.00 (±0.08)	1.12 (±0.09)	1.63 (±0.19)	1.00 (±0.15)	1.36 (±0.19)	1.67 (±0.27)
B6C3F₁												
	Males		Females		Males		Females					
	Control	1X	3X	Control	1X	3X	Control	1X	3X	Control	1X	3X
<i>Abcb4</i>	1.00 (±0.13)	0.96 (±0.06)	1.29 (0.12)	1.00 (±0.09)	1.31 (±0.22)	1.33 (0.17)	1.00 (±0.06)	0.89 (±0.12)	1.26 (±0.09)	1.00 (±0.12)	1.11 (±0.16)	1.07 (±0.12)
<i>Abcc2</i>	1.00 (±0.08)	1.37 (±0.20)	1.37 (0.14)	1.00 (±0.04)	1.17 (±0.13)	1.30 (0.10)	1.00 (±0.10)	0.81 (±0.09)	1.12 (±0.12)	1.00 (±0.06)	1.17 (±0.12)	1.05 (±0.05)
<i>Abcc3</i>	1.00 (±0.27)	0.61 (±0.02)	1.66 (0.07)	1.00 (±0.09)	1.57 (±0.25)	2.07 (0.27)	1.00 (±0.09)	0.78 (±0.05)	1.22 (±0.10)	1.00 (±0.11)	1.05 (±0.11)	1.70 (±0.21)
<i>Aldoa</i>	1.00 (±0.18)	1.18 (±0.11)	1.39 (0.14)	1.00 (±0.06)	1.21* (±0.04)	1.54*** (0.05)	1.00 (±0.04)	1.33 (±0.08)	1.57** (±0.13)	1.00 (±0.12)	1.12 (±0.15)	1.17 (±0.12)
<i>Avpr1a</i>	1.00 (±0.20)	1.54 (±0.25)	1.28 (0.15)	1.00 (±0.07)	1.09 (±0.13)	0.98 (0.15)	1.00 (±0.10)	1.06 (±0.11)	1.14 (±0.07)	1.00 (±0.11)	0.82 (±0.10)	1.30 (±0.12)
<i>Bhmt</i>	1.00 (±0.08)	0.87 (±0.02)	0.38# (0.04)	1.00 (±0.05)	0.82 (±0.11)	0.55* (0.10)	1.00 (±0.06)	1.00 (±0.15)	0.81 (±0.09)	1.00 (±0.15)	0.80 (±0.13)	0.79 (±0.15)
<i>Ccgn1</i>	1.00 (±0.15)	1.78 (±0.59)	1.00 (0.08)	1.00 (±0.10)	0.95 (±0.09)	1.08 (0.07)	1.00 (±0.12)	0.92 (±0.01)	0.96 (±0.07)	1.00 (±0.06)	1.19 (±0.15)	1.04 (±0.04)
<i>Cd36</i>	1.00 (±0.16)	0.65 (±0.14)	2.56 (0.96)	1.00 (±0.22)	1.07 (±0.55)	2.52* (0.48)	1.00 (±0.21)	0.57 (±0.14)	0.74 (±0.15)	1.00 (±0.11)	1.23 (±0.16)	3.66* (±0.50)
<i>Cdc14b</i>	1.00 (±0.17)	0.68 (±0.12)	1.14 (0.04)	1.00 (±0.05)	0.93 (±0.05)	0.74 (0.06)	1.00 (±0.13)	0.62 (±0.15)	1.10 (±0.20)	1.00 (±0.10)	0.99 (±0.12)	1.17 (±0.10)
<i>Cxcl12</i>	1.00 (±0.13)	0.99 (±0.13)	1.05 (0.06)	1.00 (±0.11)	1.09 (±0.09)	1.28 (0.12)	1.00 (±0.10)	0.82 (±0.07)	1.10 (±0.09)	1.00 (±0.10)	0.90 (±0.09)	0.82 (±0.06)
<i>Cyp1a2</i>	1.00 (±0.14)	1.15 (±0.12)	1.34 (0.07)	1.00 (±0.22)	1.16 (±0.17)	1.68 (0.09)	1.00 (±0.12)	0.97 (±0.16)	1.52 (±0.16)	1.00 (±0.10)	1.42 (±0.24)	1.31 (±0.12)

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	4 weeks				B6C3F ₁							
	Males		Females		Males		Females					
	Control	1X	3X	Control	1X	3X	Control	1X	3X			
<i>Fasn</i>	1.00 (±0.06)	0.49** (±0.12)	0.40*** (0.04)	1.00 (±0.18)	0.64 (±0.05)	0.81 (0.11)	1.00 (±0.16)	0.36# (±0.03)	0.37# (±0.04)	1.00 (±0.08)	0.75 (±0.02)	0.85 (±0.20)
<i>Gata</i>	1.00 (±0.12)	0.86 (±0.13)	1.75 (0.14)	1.00 (±0.11)	0.96 (±0.09)	1.30 (0.22)	1.00 (±0.17)	0.93 (±0.11)	1.02 (±0.03)	1.00 (±0.13)	1.04 (±0.13)	1.47 (±0.14)
<i>Gsr</i>	1.00 (±0.09)	0.93 (±0.09)	1.17 (0.05)	1.00 (±0.11)	0.81 (±0.05)	1.26 (0.10)	1.00 (±0.04)	1.06 (±0.08)	1.28* (±0.07)	1.00 (±0.07)	1.14 (±0.08)	1.41** (±0.07)
<i>Lpl</i>	1.00 (±0.16)	1.67 (±0.33)	1.99* (0.17)	1.00 (±0.15)	1.56 (±0.42)	1.00 (0.16)	1.00 (±0.11)	1.37 (±0.16)	2.82## (±0.72)	1.00 (±0.10)	1.26 (±0.23)	2.12** (±0.23)
<i>Lss</i>	1.00 (±0.12)	0.94 (±0.18)	0.92 (0.06)	1.00 (±0.12)	0.85 (±0.04)	1.37 (0.13)	1.00 (±0.12)	0.70 (±0.04)	0.66 (±0.03)	1.00 (±0.09)	0.65 (±0.15)	0.70 (±0.12)
<i>Mxip1</i>	1.00 (±0.09)	0.92 (±0.12)	1.03 (0.11)	1.00 (±0.14)	0.87 (±0.10)	0.73 (0.06)	1.00 (±0.13)	0.66 (±0.10)	0.96 (±0.05)	1.00 (±0.05)	1.27 (±0.25)	1.13 (±0.11)
CD-1												
Males												
	Control	1X	3X	Control	1X	3X	Control	1X	3X	Control	1X	3X
<i>Nqo1</i>	1.00 (±0.20)	0.96 (±0.13)	2.68*** (0.22)	1.00 (±0.11)	0.99 (±0.21)	1.86* (0.24)	1.00 (±0.22)	0.66 (±0.06)	1.59* (±0.05)	1.00 (±0.07)	1.57** (±0.12)	2.28*** (±0.13)
<i>Pla2g12a</i>	1.00 (±0.21)	1.57 (±0.31)	1.81 (0.17)	1.00 (±0.13)	1.37 (±0.06)	1.60 (0.22)	1.00 (±0.12)	1.02 (±0.11)	1.41 (±0.17)	1.00 (±0.15)	1.15 (±0.19)	0.88 (±0.08)
<i>Pygl</i>	1.00 (±0.09)	0.92 (±0.18)	0.70 (0.07)	1.00 (±0.10)	0.95 (±0.16)	0.79 (0.08)	1.00 (±0.08)	0.84 (±0.13)	0.68 (±0.02)	1.00 (±0.14)	0.96 (±0.05)	0.91 (±0.04)
<i>Scd1</i>	1.00 (±0.10)	0.33** (±0.08)	0.44** (0.11)	1.00 (±0.08)	0.44* (±0.14)	0.52* (0.14)	1.00 (±0.09)	0.46** (±0.12)	0.26*** (±0.05)	1.00 (±0.30)	0.83 (±0.15)	1.08 (±0.28)
<i>Thrsp</i>	1.00 (±0.21)	0.31* (±0.09)	0.17** (0.04)	1.00 (±0.23)	0.51 (±0.10)	0.39 (0.15)	1.00 (±0.27)	0.28 (±0.05)	0.14** (±0.01)	1.00 (±0.26)	0.78 (±0.18)	0.36 (±0.10)
<i>Tmem2</i>	1.00 (±0.08)	0.97 (±0.20)	0.93 (0.04)	1.00 (±0.15)	0.92 (±0.06)	1.12 (0.17)	1.00 (±0.08)	0.81 (±0.04)	0.97 (±0.10)	1.00 (±0.11)	1.00 (±0.06)	1.26 (±0.17)
<i>Txnd1</i>	1.00 (±0.14)	1.40* (±0.10)	1.79** (0.13)	1.00 (±0.05)	1.11 (±0.11)	1.68*** (0.06)	1.00 (±0.11)	1.35 (±0.11)	1.26 (±0.13)	1.00 (±0.08)	1.20 (±0.18)	1.17 (±0.09)

Values are mean (±SEM). Significant values are presented in bold with asterisks (*) for parametric analysis and number signs (#) for non-parametric analysis (*p 0.05, **p 0.01, ***p 0.001).

Table 2

List of animals that exhibited outlier responses as a result of feeding mice with OEP for 4 or 13 weeks.

Animal #	Strain	Sex	Time-point	Diet	L/BW	ALT	AST	miR-122	Pathology
200	CD-1	M	4 wks	Vehicle			↑		
212	CD-1	M	4 wks	1X			↑		
223	CD-1	M	4 wks	1X			↑		
233	CD-1	M	4 wks	3X		↑		↑	
283	CD-1	F	4 wks	Vehicle		↑			
285	CD-1	F	4 wks	1X			↑		
307	CD-1	F	4 wks	3X		↑		↑	
358	B6C3F ₁	M	4 wks	3X		↑		↑	
359	B6C3F ₁	M	4 wks	3X	↑				
387	B6C3F ₁	F	4 wks	1X	↑				
388	B6C3F ₁	F	4 wks	1X		↑			
392	B6C3F ₁	F	4 wks	3X		↑			
393	B6C3F ₁	F	4 wks	3X	↑				
395	B6C3F ₁	F	4 wks	3X				↑	
248	CD-1	M	13 wks	1X	↑				
258	CD-1	M	13 wks	1X	↑			↑	
259	CD-1	M	13 wks	1X	↑				Mild mitotic changes
268	CD-1	M	13 wks	3X	↑				
332	CD-1	F	13 wks	3X	↑			↑	Mild mitotic changes
336	CD-1	F	13 wks	3X	↑			↑	
338	CD-1	F	13 wks	3X	↑				
340	CD-1	F	13 wks	3X	↑			↑	
341	CD-1	F	13 wks	3X	↑			↑	
371	B6C3F ₁	M	13 wks	1X	↑			↑	
372	B6C3F ₁	M	13 wks	1X	↑			↑	
375	B6C3F ₁	M	13 wks	3X	↑			↑	Mild mitotic changes
376	B6C3F ₁	M	13 wks	3X	↑			↑	
415	B6C3F ₁	M	13 wks	3X	↑			↑	Mild mitotic changes

Animal #	Strain	Sex	Time-point	Diet	L/BW	ALT	AST	miR-122	Pathology
402	B6C3F ₁	F	13 wks	1X	↑		↑		
403	B6C3F ₁	F	13 wks	1X	↑			↑	
404	B6C3F ₁	F	13 wks	1X	↑				
406	B6C3F ₁	F	13 wks	1X	↑		↑	↑	
407	B6C3F ₁	F	13 wks	1X	↑				
408	B6C3F ₁	F	13 wks	3X	↑	↑	↑		
409	B6C3F ₁	F	13 wks	3X	↑				
410	B6C3F ₁	F	13 wks	3X	↑				
411	B6C3F ₁	F	13 wks	3X	↑				
412	B6C3F ₁	F	13 wks	3X	↑	↑		↑	
413	B6C3F ₁	F	13 wks	3X	↑				

M – male mice, F – female mice; 4 wks – animals that received vehicle or OEP-NF-containing diet for 4 weeks; 13 weeks - animals that received vehicle or OEP-NF-containing diet for 4 weeks; 1X – animals received 1x MED OEP-NF with the chow; 3X - animals received 3x MED OEP-NF with the chow; L/BW – liver to body weight ratio; ALT – alanine aminotransferase, AST – aspartate amino transferase. Only animals with endpoint values greater than 2 SEM from the control group mean were selected for inclusion into the table. Mice highlighted in grey exhibited a “responder” phenotype for 2 or more evaluated parameters.