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The ALIOS diet in male and female rodents recapitulates the clinical and transcriptomic features of NAFLD and NASH

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Abbreviated title: The ALIOS diet recapitulates NASH in mice

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Abbreviations: ALT: alanine aminotransferase. ALP: alkaline phosphatase. ALIOS: American lifestyle induced obesity syndrome diet. AST: aspartate aminotransferase. HCC:

hepatocellular carcinoma. HDL: high-density-lipoprotein. HFD: high fat diet. LDL: low-density protein. NAFLD: non-alcoholic fatty liver disease. NASH: non-alcoholic steatohepatitis. NC: normal chow. TAG: triacyclglycerols.

Abstract: 226 words

Background & Aims: The pathogenesis of non-alcoholic fatty liver disease (NAFLD) and the progression to non-alcoholic steatohepatitis (NASH) and increased risk of hepatocellular carcinoma (HCC), remain poorly understood. Additionally, there is increasing recognition of the extra-hepatic manifestations associated with NAFLD and NASH. We demonstrate that intervention with the American lifestyle induced obesity syndrome diet (ALIOS) in male and female mice recapitulates many of the clinical and transcriptomic features of human NAFLD and NASH. **Methods**: Male and female C57BL/6N mice were fed either normal chow (NC) or ALIOS from 11 to 52-weeks and underwent comprehensive metabolic analysis throughout the duration of the study. **Results:** From 26-weeks, ALIOS-fed mice developed features of hepatic steatosis, inflammation and fibrosis. ALIOS-fed mice also had an increased incidence of hepatic tumours at 52-weeks compared to those fed NC. Hepatic transcriptomic analysis revealed alterations in multiple genes associated with inflammation and tissue repair in ALIOS-fed mice. Ingenuity Pathway Analysis confirmed dysregulation of metabolic pathways as well as those associated with liver disease and cancer. In parallel the development of a robust hepatic phenotype, ALIOS-fed mice displayed many of the extra-hepatic manifestations of NAFLD including hyperlipidaemia, increased fat mass, sarcopaenia and insulin resistance. **Conclusions**: The ALIOS diet in mice recapitulates many of the clinical features of NAFLD and therefore represents a robust and reproducible model for investigating the pathogenesis of NAFLD and its progression.

New and Noteworthy: NAFLD affects 30% of the general population and can progress to NASH and potentially hepatocellular carcinoma. Pre-clinical models rely on mouse models which often display hepatic characteristics of NAFLD, but rarely progress to NASH and seldom depict the multi-system effects of the disease. We have conducted comprehensive

metabolic analysis of both male and female mice consuming a western diet of trans-fats and sugar, focussing on both their hepatic phenotype, but also the extra-hepatic manifestations.

Key words: Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, diet, transcriptome.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and is the most common form of liver disease in the western world (2, 25). NAFLD currently affects around 30% of the normal population and rises to 80% in patients with obesity and type 2 diabetes (24, 25). It is a spectrum disease, ranging from simple steatosis through to the necro-inflammatory disease, non-alcoholic steatohepatitis (NASH). Development of NASH subsequently increases the risk of fibrosis and cirrhosis and eventually, hepatocellular carcinoma (HCC) (51, 52). Recent evidence supports the concept that NAFLD is a multi-systemic condition impacting upon a variety of organs and systems (5, 48) and is associated with multiple extra-hepatic clinical features, including insulin resistance, hyperlipidaemia and sarcopenia.

Pre-clinical mouse models of NAFLD and NASH typically rely on genetic manipulation, hepatic cytotoxic injury or formulated dietary extremes (11, 16, 29). Although some of these models exhibit the histological features of NAFLD, they rarely progress to NASH or HCC and therefore do not reflect the mechanisms of the human disease. Commonly used genetic models of obesity, the leptin-deficient (*ob/ob*) mouse and leptin receptor-deficient (*db/db*) mouse, have excess hepatic fat deposition, but do not develop NASH or HCC (1, 11, 36), most likely because leptin is involved in regulating inflammation and fibrosis. In addition, models using high fat diet (HFD; 60% fat) cause simple steatosis but do not progress to NASH or develop hepatic injury (11, 34). Fructose has been shown to be a driver of hepatic *de novo* lipogenesis (43), however, fructose only dietary interventions often fail to induce dyslipidemia, hepatic steatosis and inflammation (28, 41). Previous studies have utilised "fast food" diets (7, 22), which contain 40% fat (12% from saturated fat) with the addition of fructose in drinking water, though these studies progress to characteristic NASH, they seldom highlight the extra-hepatic features of the disease and sexual dimorphism has not

been explored. Alternative models use hepatic toxins to drive liver injury. These commonly include carbon tetrachloride with which animals develop hepatic histological features of NASH and fibrosis from as early as 8 weeks, but often do not present with other clinical features of NASH such as weight gain and insulin resistance (47). Diethynitrosamine is able to induce HCC but does so without the progression from NAFLD and NASH (19). Furthermore, the use of genetic manipulation, in combination with toxins and HFD in generating models of NAFLD poses questions as to the relevance and similarity to human NAFLD and NASH.

The American lifestyle-induced obesity syndrome (ALIOS) mouse model is a dietary intervention based on the nutritional content of commonly consumed fast foods of the western world (46). Mice are fed high fat chow (45%), including trans fats, with high fructose corn syrup added to the drinking water; animals become obese, insulin resistant and develop hepatic steatosis with a necro-inflammatory response (10, 46). When aged to 12 months, mice fed ALIOS also develop NASH driven HCCs (10). To date, studies using ALIOS have only treated male mice, therefore the effects of ALIOS on female metabolism is entirely unexplored. In addition, with the increasing evidence suggesting the importance of the extra-hepatic impact of NAFLD, models that can accurately replicate a clinical condition are likely to be more highly informative, both with respect to natural history, but also the potential impact of intervention. In the published literature, studies have largely focused on the hepatic phenotype associated with the ALIOS diet, and its multi-system impact has not been evaluated in detail.

We have therefore conducted a comprehensive metabolic analysis of both male and female mice consuming either an ALIOS diet or standard chow from 11 to 52 weeks of age, focussing on both their hepatic phenotype, but also the extra-hepatic manifestations.

Methods

Mouse husbandry

Male and female C57BL/6NTac mice were kept and studied in accordance with UK Home Office legislation and local ethical guidelines issued by the Medical Research Council (Responsibility in the Use of Animals for Medical Research, July 1993; home office license 30/3146). All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (SI 4 2012/3039). Mice were kept under controlled light (light 7am–7pm, dark 7pm–7am), temperature (21±2°C) and humidity (55±10%) conditions. They had free access to water (9–13 ppm chlorine) and were fed *ad libitum* on a commercial diet (SDS Rat and Mouse No. 3 Breeding diet, RM3) until 10 weeks of age when they were then transferred to a control (NC; SDS Rat and Mouse No. 3 Breeding diet, RM3, Essex, UK) or ALIOS diet (D06031302, Research Diets, USA and TD06415 with hydrogenated vegetable fats, Envigo, USA, [45% fat of which 30% is trans-fat] with 55% fructose:45% glucose in drinking water) for 26- or 52-weeks before culling by cervical dislocation and tissue analysis.

Experimental design

Cohorts of male and female mice were bred for longitudinal metabolic phenotyping tests (26w cohort: NC males n=12, ALIOS males n=15, NC females n=15, ALIOS females n=15. 52w cohort: (NC males n=17, ALIOS males n=12, NC females n=15, ALIOS females n=15). Mice were housed in single sex groups across multiple litters and were not randomised into groups. Animal IDs and diets were recorded on the cages and were not blinded to the operator carrying out the animal procedure although subsequent tests only include animal

ID information. Sample size estimates were based on previous experience of mouse models in which relevant traits were measured (9, 10, 38).

Body weight and composition

Body weight was measured weekly in the morning using average weights (g) calculated by Adventure Pro balances (Ohaus, US). Fat and lean mass was assessed by Dual-energy X-ray absorptiometry (DEXA) at 17, 25, 39 and 51 weeks of age.

Calorimetry

Calorimetry data was collected in a PhenoMaster system (TSE Systems, Germany) at 14, 25, 36 and 48 weeks of age. Mice were kept under controlled light (light 7am–7pm, dark 7pm–7am), temperature (21±2°C) and humidity (55±10%) conditions. They had free access to water (9–13 ppm chlorine) and were fed *ad libitum.* O_2 consumption (VO_2), CO_2 production (VCO_2) and respiratory exchange ratio (RER (VCO_2/VO_2), an estimate of fuel usage) were calculated and recorded electronically over 12 hours for each mouse. Total locomotor activity (measured by x, y, and z axis infrared beam breaks) and diet consumption were also recorded electronically for each mouse. Data were collected at 3 to 4 time points each hour.

Intraperitoneal glucose tolerance test (ipGTT)

Glucose tolerance was assessed at 15, 25, 37 and 49 weeks of age. Mice were fasted overnight for 16 hours then injected intraperitoneally with 20% glucose solution (2g glucose/kg body weight; Sigma, Dorset, UK). Blood was sampled from the tail vein and glucose concentration measured at t=0, 15, 30, 60, and 120 minutes (Alphatrak, Abbott, UK). Insulin concentrations at t=0, 60 and 120 minutes were determined by ELISA (Crystal Chem, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as [fasting glucose (mmol/L) x fasting insulin (μU/ml)/22.5] (13).

Intraperitoneal insulin tolerance test (ipITT)

Insulin tolerance was assessed at 25, 37 and 49 weeks. Mice were fasted for 4-5 hours then injected intraperitoneally with insulin at a concentration of 0.75 or 1.25 IU/kg for females and males, respectively (Hypurin Bovine Insulin). Blood was sampled from the tail vein and glucose concentration measured at t=0, 15, 30, 45 and 90 minutes (Alphatrak, Abbott, UK).

Blood biochemistry

At termination, mice were anaesthetised with isoflurane and blood collected *via* retro-orbital bleed. Samples were kept on ice then centrifuged for 10 minutes at 8,000 x g at room temp. Alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, high-density-lipoprotein (HDL), low-density-lipoprotein (LDL), triglycerides, free fatty acids and bilirubin were determined from plasma on an AU680 Clinical Chemistry Analyser (Beckman Coulter, High Wycombe, UK). Creatinine was analysed using a colorimetric kit appropriate for mouse serum (Cayman Chemicals, Cambridge, UK).

Tissue biochemistry

Hepatic triacylglycerol content was measured on an AU480 Clinical Chemistry Analyser (Beckman Coulter, High Wycombe, UK), from a homogenate of frozen liver tissue (100mg in500µl of PBS/0.1% Triton-X).

Tissue Histology

Liver tissue was fixed in 4% (vol/vol) buffered formaldehyde and samples were subsequently paraffin-embedded and 5-µm sections prepared on a microtome. Sections were stained with

H&E and viewed at 200x magnification. Inflammation score was determined by counting the number of inflammatory foci in 5 fields of view over 3 sections from each mouse liver (20, 23) and the average scored as follows: no foci = 0, <2 per field of view = 1, 2-4 per field of view = 2, >4 per field of view = 3. A foci was determined as a cluster of 5 or more inflammatory cells. Percentage of hepatic fibrosis was determined by staining 3 sections from each mouse liver with Sirius Red and quantifying percentage of positive staining over 6 fields of view by ImageJ (NIH, USA; http://rsb.info.nih.gov/ij).

Immunohistochemistry

Immunohistochemistry was performed on wax embedded liver sections (5µm). Briefly, sections were dewaxed and rehydrated before incubation with antibodies against glutamine synthetase (5µg/ml; Millipore, Watford, UK) and Sox9 (1µg/ml; Millipore) after heat inducted antigen retrieval. Bound primary antibody was detected using a peroxidase-conjugated secondary antibody (Dako) with visualisation using 3,3- diaminobenzidine (SigmaFast, Sigma, Dorset, UK). For negative control samples, non-immune goat's serum replaced primary antibodies.

Protein extraction and immunoblotting

Total protein was extracted from whole liver tissue using RIPA buffer (150mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0; Sigma, Dorset, UK), with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Loughborough, UK). Protein concentrations were measured using a BCA protein quantification kit according to the manufacturer's protocol (Thermo scientific). Primary Col1A1 (Cell Signalling Technology, Leiden, The Netherlands) and secondary antibodies from Dako (Agilent Technologies, Santa Clara, USA) were used at a dilution of 1/1000 and 1/2000 respectively. Bands were visualised with ECL (Pierce Thermo Fisher Scientific) and

ChemiDocXS imager (Biorad, Watford, UK). Bands were quantified by densitometry and normalised to total protein using ImageJ (NIH, Bethesda, MD, http://rsb.info.nih.gov/ij).

RNA sequencing

Total liver RNA was extracted using Tri-Reagent (Sigma, Dorset, UK). Concentration was determined spectrophotometrically at OD260 on a Nanodrop spectrophotometer (Thermo Scientific, Hemel Hempstead, UK) and quality checked on a 2100 Bioanazlyer system (Agilent Technologies, Stockport, UK). Only samples with RNA integrity numbers >7 were used for analysis. cDNA was generated from total RNA using first oligodT and subsequently random priming using the TruSeq Stranded mRNA HT Sample Prep Kit for Illumina (according to manufacturer's instructions). The prepared libraries were QC'ed and multiplexed, followed by pair-end sequencing (75bp) over one lane of a NextSeq 75SR flow cell (Illumina, Cambridge, UK) to a total depth of 130 million read pairs on the Illumina NextSeq 500 platform (Illumina, Cambridge, UK). Reads were mapped with Stampy (31) on default settings with GRCm38/mm10 as genome reference and bam files merged using bamtools. Gene level read counts for all protein-coding RNA transcripts present in refGene mm10 were quantified in a strand-specific manner using FeatureCounts from the Subread package v1.6.0. Differential expression analysis was performed using DESeq2 (30). Differentially expressed genes (DEGs) were reported for $q \leq 0.05$ and fold change of 2 or $q \leq 0.1$. Statistical significance was determined by unpaired parametric t-test and differentially regulated genes were defined by a false discovery rate (Benjamini-Hochberg method) adjusted p-value<1%. The online bioinformatics tools Metascape (metascape.org) and Enrichr (http://amp.pharm.mssm.edu/Enrichr/) were used for enrichment analysis of the DE genes. Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc., USA) was used to examine biological functions and disease and functional relationships between gene networks.

Bioinformatic Data Analysis

RNA-seq data was downloaded from NCBI (GSE126848). Gene counts (GSE126848_Gene_counts_raw.txt) and sample identification were determined from the series matrix (GSE126848_series_matrix.txt). The clinical characteristics of patients are described in Supp. Table 1 (https://doi.org/10.6084/m9.figshare.12666860). Gene symbols were converted from Ensembl ID using (org.Hs.eg.db v3.11.4). Differential gene expression was determined using EdgeR (3.11). As the human NASH dataset contained 12 male samples and 4 female samples, RNA-seq libraries from female samples were removed. All remaining samples were included (Normal weight, obese, NAFLD and NASH), low counts were removed (CPM>0.25 in 2 libraries), and differential expression for NASH vs Normal weigh samples were determined, this gave 3152 down regulated and 3491 up regulated genes(using EdgeR glmLRT). Differentially expressed genes from male mice (ALIOS vs normal chow, 2153 down regulated and 2865 up regulated) were used to convert to human symbols. Mouse Gene symbols (5018) were converted using the package BiomartR (v2.440). 4701 genes were matched between human and mouse. NASH regulated genes were then compared to ALIOS regulated genes. A list of 2052 genes were identified as overlapping, with significance determined using Fisher's Exact Test (a Venn diagram was produced, using the ggVennDiagram R package). The overlapping genes were used for pathway analysis and plotted on heatmaps (using ggplot2). The top 30 significant genes (sorted by false discovery rate) in the NASH dataset were extracted. LogCPM counts were used for each heatmap, each dataset (mouse and human) was scaled separately before plotting.

Statistics

Data are presented as mean ± standard error. Data analysis was performed using Graphpad Prism software (Graphpad Software Inc, La Jolla, USA) and considered statistically significant

at $p<0.05$. Normality was assessed using the Shapiro-Wilk test. Two-tailed, unpaired t-tests were used to compare differences in the mean between diets within each sex. Mann-Whitney tests were used with datasets of non-parametric distributions. For data collected across time, repeated measure two-way analysis of variance (ANOVA) was used.

Results

Male and female mice fed ALIOS have progressive weight gain and increased fat mass

Weight gain was greater in both male and female ALIOS fed mice and body weight continued to rise throughout the study, from the onset of ALIOS at 11-weeks until 52-weeks (Fig 1*A,B*). From 15-weeks onwards, DXA analysis confirmed that ALIOS fed mice of both sexes had an increase in fat mass compared to normal chow controls (Fig. 1*C,D*). Interestingly, at 15- and 37-weeks female mice also displayed a decrease in lean mass. There was no change in lean mass in the male mice throughout the duration of the study.

At 26-weeks, male ALIOS fed mice had increased fat pad weights across all depots compared to NC controls. However, by 52-weeks, gonadal fat was the only enlarged depot (Fig.1*E*). ALIOS fed female mice had increased fat depots at both 26- and 52-weeks compared to NC controls (Fig.1*F*). Consistent with increased lipid storage, ALIOS fed male and female mice had increased serum levels of total, HDL and LDL cholesterol from 25-weeks onwards (Table 1). Circulating levels of triacyclglycerols (TAG) and free fatty acids (FFA) were significantly decreased in ALIOS fed mice compared to NC controls, at 52-weeks and from 25-weeks in males and females, respectively (Table 1). In addition, data from metabolic cage experiments revealed that male mice were more sedentary than females and that ALIOS fed mice had reduced total activity compared to NC controls (Supp. Tables 2 and 3). As expected, respiratory exchange ratios were reduced in male and female ALIOS fed mice, indicating increased fat catabolism for energy production (Supp. Tables 2 and 3).

At 26-weeks, kidney size was reduced in both male and female mice and quadriceps weight decreased in female mice only (Supp. Fig.1). By 52-weeks, male ALIOS fed mice had decreased heart, quadricep and testes mass compared to those fed NC, alongside an increase in spleen mass (Supp. Fig.1*A*). Serum creatinine levels were elevated in male ALIOS fed mice compared to NC controls at 52-weeks (Table 2). Female ALIOS fed mice mirrored the changes in quadricep and spleen mass seen in the males and also had a reduction in kidney mass (Supp. Fig.1*B*) and increase in serum creatinine (Table 2).

ALIOS fed mice have normal glucose tolerance but are insulin resistant.

There was no difference in glucose tolerance in ALIOS fed male mice throughout the duration of the study (Fig. 2*A*). In females, glucose tolerance was impaired at 25- and 37-weeks in ALIOS mice, although by 49-weeks, there was no difference in comparison with NC fed animals (Fig.2*B)*. However, the ALIOS diet increased serum insulin levels in response to the glucose bolus, in both male and female mice, compared to NC controls consistent with the development of insulin resistance (Fig. 2*E,F* & Supp. Fig. 2).

Further evidence of insulin resistance was obtained from insulin tolerance testing; in both male and female ALIOS fed mice from 37-weeks onwards, there was an impaired glycaemic response to intraperitoneal insulin injection (Fig.3A,B & Supp. Fig. 3). There was no change in insulin sensitivity at 25-weeks in ALIOS fed mice of either sex. The HOMA-IR was increased in both male and female ALIOS-fed mice from 15-weeks onwards (Fig. 3C,D).

ALIOS drives hepatic steatosis and inflammation

Liver to body weight mass was increased in both male and female ALIOS fed mice compared to NC at 26- and 52-weeks (Fig.4*A,B*). Additionally, hepatic triglyceride was elevated in ALIOS fed mice of both sexes at 26-weeks and in male mice at 52-weeks (Fig.4*C,D*). Histological

examination of all ALIOS livers identified steatosis, with evidence macro- and micro-vesicular lipid droplets and ballooning of hepatocytes (Fig.4*E,F*). ALIOS fed male and female mice had altered expression of genes associated with lipid metabolism (*Srebf1, Elovl3, Lpl*; Fig.4*G,H*) insulin signalling (*Irs1*, *G6pc*, *Glut4, Pck1*; Fig.4*I,J*).

At 26-weeks, male ALIOS fed mice displayed no change in histologically determined inflammation score, however this was increased by 52-weeks (Fig.5*A*). In contrast, female mice fed ALIOS had an increased inflammation score from 26-weeks which persisted to 52-weeks (Fig.5*B*). ALIOS fed male and female mice had increased hepatic expression of genes involved in inflammation, including macrophage infiltration, (*Tnf*, *Ccl2*, *Cd68, f4/80*; Fig 5*C,D*) compared to NC fed mice at 52-weeks.

Male ALIOS fed mice have increased incidence of fibrosis and HCCs

ALIOS fed mice of both sexes, had elevated serum levels of ALT and AST from 26-weeks onwards, and continued to rise throughout the duration of the study suggesting hepatocyte damage and the presence of fibrosis (Table 2); the AST/ALT ratio was decreased in ALIOS fed mice compared to controls, which is suggestive of NAFLD. Serum levels of bilirubin, however, were unchanged in ALIOS fed mice compared to NC controls (Table 2). Hepatic fibrosis percentage was increased in male ALIOS fed mice compared to NC controls at 26- and 52-weeks (Fig.6*A*), while in female ALIOS fed mice percentage fibrosis was only increased at 52-weeks (Fig.6*B*). Both ALIOS fed male and female mice had increased hepatic expression of genes associated with cell adhesion (*Col1a1*, *Col1a2*, *Dpt*, *Lum*; Fig.6*C,D*) as well as increased protein levels of collagen type 1 (Col1a1; Fig.6*E,F*).

Advanced fibrotic disease increases the risk of HCC; male mice fed ALIOS had an increased frequency of macroscopic liver growths (NC: 5.8%, ALIOS: 25%, $P<0.05$). There was no

389 evidence of liver lesions in female ALIOS fed mice. The lesions were associated with
390 compressed adjacent non-tumour tissue, though there appeared to be no invasion of blood
391 vessels or surrounding liver tissue (Fig.6*I*). Histological assessment revealed that the lesions
392 were composed of atypical hepatocytes with increased nuclear to cytoplasmic ratios, as well
393 as some multinucleated cells (Fig.6*J*). As there was no obvious evidence of invasion to
394 confirm malignancy, characterisation was performed using HCC markers commonly used in
395 mice and humans. Sox9 is a marker of hepatic stem cell activation previously implicated in
396 tumour pathogenesis (18, 37) and labelling was positive in the nucleus of the atypical
397 hepatocytes of only 1/3 lesions (Fig.6*K*). Glutamine synthetase (GS) has previously been
398 used to determine well differentiated HCCs from pre-malignant tumours in human liver (10,
399 37, 49). Diffuse GS was also only present in 2/3 ALIOS lesions (Fig.6*L*).
400
401 *ALIOS diet alters the hepatic transcriptome in male and female mice*
402 Clustering of NC and ALIOS of the top 100 DEGs revealed two distinct groups between NC
403 and ALIOS with almost no overlapping (Fig.7*A,B*). In male mice, 5018 genes were
404 differentially expressed between NC and ALIOS, 2153 were down-regulated and 2865 were
405 up-regulated (Table 3). Within the top 10 up-regulated genes, three were associated with
406 the major histocompatibility complex (MHC). Gene ontology analysis highlighted that the
407 most up-regulated genes were associated with reorganisation of cellular structures and
408 collagen binding as well as inflammatory and immune response (Fig.7*C*). The down-
409 regulated genes were mostly clustered to biological pathways involved in metabolism and
410 protein processing (Fig.7*D*). In line with these findings, the top diseases and functions
411 captured in IPA included cancer, organismal injury and endocrine disorders (Table 4). The
412 top toxicology lists by IPA included liver necrosis and hepatic fibrosis, as well as NRF2-
413 mediated Oxidative Stress Response.

In livers of ALIOS fed female mice, a total of 4222 genes showed differential expression; 2350 genes were up- and 1871 were down-regulated (Table 3). The most up-regulated genes were associated exclusively with inflammatory processes (Fig.7*E*), while down-regulated genes, as in male mice, clustered to metabolic processes (Fig.7*F*). Similarly, the top diseases and functions identified by IPA included cancer, injury and endocrine disorders (Table 4). The top toxicology lists as determined by IPA included hepatic fibrosis, liver necrosis and steatosis.

To identify DEGs associated with NASH in humans, published RNAseq data from liver biopsies of patients with NASH was re-analysed alongside biopsies from patients with normal bodyweight. A total of 4558 human DEGs were identified. Comparative analysis revealed that 2052 (22.5%) genes were shared by the human NASH patients and male ALIOS fed mice (Fig.8*A*). Out of the 2052 common genes, 1018 were up-regulated and 1034 were down-regulated. The clustering of the top 30 overlapped genes revealed similarities between livers of ALIOS fed mice and human NASH liver biopsies (Fig.8*B*). Genes associated with lipid metabolism (Fig.8*C*; LPL), insulin signalling (G6PC, PCK1), inflammation (TNFA1P3, CCL2, CXCR4) and cell adhesion (DPT, LUM, COL1A1) were among those that which were altered in both human NASH and ALIOS fed male mice. Additionally, gene ontology analysis revealed that the top 100 shared genes were enriched in pathways associated with immune response, metabolic processes and cell migration (Fig.8*D*), confirming that most of the genes and pathways conserved between the ALIOS mouse model and human NASH are associated with inflammation and fibrosis.

Discussion

NAFLD is rapidly becoming the most common cause for liver transplantation. However, there are aspects of its pathology that remain poorly understood, and informative pre-

clinical models that accurately reflect the clinical condition, particularly its natural history and progression, can provide valuable mechanistic insight. In this study, we have shown that male and female mice fed the ALIOS diet for 52-weeks develop a classical and reproducible hepatic phenotype (including elevated liver chemistry, histological features of NASH and the development of HCC). However, we also have shown that they develop many of the extra-hepatic features associated with NAFLD including, increased fat mass, sedentary behaviour, abnormal circulating lipid profiles, insulin resistance, and sarcopenia. Kidney weights were reduced and serum creatinine levels were elevated in ALIOS fed mice. Recent reports have highlighted the association between NAFLD and renal dysfunction in humans (32, 44). Furthermore, we have highlighted the differential gene expressions in ALIOS fed mice compared to NC controls that may prove highly informative in enhancing our understanding of the pathogenesis of NAFLD.

Male and female ALIOS fed mice had increased bodyweight from as early as 11-weeks, driven by increased fat mass. Of note, bodyweights in female ALIOS-fed mice continue to increase at 52-weeks and do not plateau, contrasting with male ALIOS-fed mice. It's interesting to speculate that this sexually dimorphic trajectory might extend to the liver in that the females might also develop HCCs as seen in the males, if left for longer than one year. Unfortunately we did not extend our observations beyond one year in either sex. Detailed body composition analysis has not been undertaken in this model previously and we have been able to show increased fat depot mass, with additional evidence for reduced skeletal muscle (quadriceps mass). This finding mirrors the sarcopenia that is associated with NAFLD (8); Koo *et al*. (21), reported that sarcopenia was present in 17.9% and 35% of patients with NAFLD and NASH, respectively and was associated with significant fibrosis and insulin resistance (21). Previous studies have demonstrated abnormal glucose handling and insulin resistance in mice fed the ALIOS diet (10, 46) and we have now shown that this

persists throughout the duration of the intervention (at least to 52-weeks).Total (including both LDL and HDL) cholesterol became elevated soon after the commencement of the diet, although circulating triglyceride levels were lower in both male and female mice on the ALIOS diet at 52 weeks. It is possible that this may reflect impaired hepatic lipid export therefore contributing to increased hepatic triglyceride accumulation.

The liver phenotype that we have observed is similar to that which has been described previously (10, 46) and we have now extended the detailed phenotyping to 52-weeks duration. At 26-weeks steatosis is predominately periportal, but, by 52-weeks, macro- and micro-vesicle steatosis has extended to the centrilobular region. The development of micro-vesicular steatosis may be linked to increased disease progression; in humans micro vesicular steatosis from liver biopsies correlated positively with increased NASH diagnosis and advanced fibrosis (45). By 52-weeks, there was clear evidence of hepatic fibrosis in both male and female mice. In male mice, the ALIOS diet was associated with an increased incidence of liver tumours as we have shown previously (10). However, female mice appeared to be completely protected from this. Male predisposition to HCC is well described (15, 17, 35) and in this regard, the ALIOS model appears to replicate clinical findings. The atypical hepatocytes seen in the liver lesions from ALIOS fed male mice are a recognised feature of human hepatic tumours. Unfortunately, there is no established panel of murine HCC markers that are comparable to human (40); the use of IHC markers was variable across different lesions from different mice. Glutamine synthetase is a target gene of β-catenin, and its overexpression is associated with mutations of β-catenin and/or activation of its pathway. In mice, hepatocellular tumours express differing levels of GS depending on the type of mutation within the neoplasm (40).

Unbiased transcriptomic analysis provides a powerful tool with which to interrogate the processes that drive NAFLD to the more advanced disease stages. Our analysis demonstrated increased mRNA expression of pro-inflammatory cytokines (*Tnf, Ccl2, Ccl3*) in male and female ALIOS fed mice, as well as markers of macrophage (*Cd68, Cd40, F4/80*) and Kupffer cell infiltration. Endorsing these observations, a direct comparison of the transcriptome from ALIOS fed male mice with publically available RNAseq data from biopsies of patients with NASH highlights a significant overlap of genes associated with NAFLD and NASH. Moylan *et al*., (33) reported a 64-gene profile of up-regulated genes in severe NAFLD in humans, which included genes associated with inflammation, cell adhesion and liver progenitor cells. These pathways are believed to be crucial in the progression from steatosis to NASH as well as to the development of HCC (11, 12), further validating the ALIOS diet as a good model of the full NAFL spectrum of disease. In addition, the top pathways highlighted from IPA mirror those seen in patients with severe NAFLD (33), including cancer.

Of the most up-regulated genes in ALIOS-fed mice, two are common between males and females and both genes have key roles in driving the inflammatory phenotype. *Clec7a* encodes membrane receptors that play a role in the innate immune response. Activation of *Clec7a* leads to production of the transcription factor *NF-κβ* (14), which induces synthesis of inflammatory cytokines such as TNF, IL-6 or IL-2 (6), suggesting that *Clec7a* is key contributor to the inflammatory profile of the liver in NAFLD. *Mmp12* is predominately expressed by macrophages and in human adipose tissue, *Mmp12* expression correlates positively with macrophage infiltration, inflammation and insulin resistance (26). *Mmp12* expression also correlates positively with arterial stiffening in mice (27), suggesting that the hepatic macrophage filtration in ALIOS-fed mice further drives progression of the disease. Among the most strongly down-regulated genes in ALIOS fed mice was *Ces2A*, encoding a hepatic serine hydrolase. In humans and mice, obesity decreases the activity of *Ces2* which leads to

hepatic dyslipidaemia (39). Indeed, normal expression of *Ces2* contributes to suppression of hepatic inflammation, improving adiposity and glucose tolerance (39); downregulation in the ALIOS-fed mice may therefore be a key driver of NAFLD progression. In males, three genes associated with MHC Class II were also up regulated. Previous reports have indicated disease susceptibility is strongly influenced by the MHC-II pathway; increased expression of MHC-II related genes is associated with increased hepatic fibrosis in response to toxic insults and hepatocyte damage (4, 42), suggesting the progression of NASH may be due to antigen presentation through MHC particularly in males.

Previous studies using "fast food" diets have also highlighted the characteristics of NASH (7, 22). However, these diets do not contain trans-fats which Tetri *et al*., suggested is the main driver of hepatic injury to promote fibrotic disease and its potential progression to HCCs. The use of trans-fats has recently been phased out from the food industry, due to their impact on metabolic disease. The ALIOS diet aimed to generate a rodent model which replicated the clinical characteristics of human NAFLD and NASH, and therefore trans-fats were used to drive an adverse liver phenotype rather than recapitulate current human diets.

Previous "fast-food" diets studies have focused primarily on the hepatic phenotype and have also detailed mitochondrial dysfunction in NASH. The ALIOS study did not include a fructose only cohort as the aim was to fully characterise a diet which induces steatosis, inflammation and fibrosis. The role of fructose in hepatic lipid accumulation has been well characterised, however recent studies using fructose only interventions have previously failed to induce hepatic steatosis and inflammation (28, 41). The combination of adverse diets and different genetic backgrounds have also made substantial contributions to pre-clinical NASH models (3, 11, 50). The ALIOS model has investigated a dietary driver of NAFLD and NASH and its plausible this may behave differently on different genetic backgrounds, but this is beyond the scope of this study. Additionally, previous pre-clinical NASH mouse

models have primarily neglected to analyse females and their response to these altered diets. The current ALIOS study has extended the comprehensive metabolic analysis to one year, detailed the extra-hepatic phenotype of the syndrome, as well as full transcriptomic analysis, in both male and female mice.

In conclusion, we have provided the most comprehensive, longitudinal assessment of the ALIOS diet, both with regards to its hepatic phenotype, but also its extra-hepatic manifestations. The ALIOS diet closely recapitulates many of the features of clinical NAFLD and our transcriptomic analysis has revealed many common pathways that are shared between clinical samples and the ALIOS intervention. The ALIOS model therefore represents a robust and reproducible tool to further understand the complex nature of NAFLD and its progression to the most advanced stages including NASH and HCC.

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Fig.1. The ALIOS diet increased bodyweight and fat mass in male and female mice from 11- to 52-weeks. (A) Body weight curves for male (NC n=29, ALIOS n=27) and (B) female NC (n=30) and ALIOS (n=30) fed mice and DXA determined lean and fat mass in (C) male (NC n=17, ALIOS n=12) and (D) female (NC n=15, ALIOS n=15) mice from 15- to 52-weeks. Changes in adipose depot mass in male (E) and (F) female NC and ALIOS fed mice at 26- and 52-weeks. GF=gonadal fat, Mes=mesenteric fat, PR=perirenal fat, BAT=brown adipose tissue. Data expressed as mean ± SEM. 26-weeks: NC males n=12, ALIOS males n=15, NC females n=15, ALIOS females n=15. 52-weeks: NC males n=17, ALIOS males n=12, NC females n=15,

ALIOS females n=15. *P<0.05, **P<0.01, ***P<0.001 significantly different from mice fed NC at each time point.

Fig.2. ALIOS fed mice have normal glucose tolerance. ipGTTs in NC and ALIOS fed male (A) and female (B) mice at 15-, 25-, 37- and 49-weeks. ipGTT represented as AUCs in (C) male and (D) female NC and ALIOS fed mice at 15-, 25-, 37- and 49-weeks. AUCs of serum insulin collected at t=0, 60 and 120 minutes during the ipGTT in (E) male and (F) female mice. ipGTT: i.p. glucose tolerance test, AUC; area under the curve. Data expressed as mean ± SEM. n=7 in each group at each time point, *P<0.05, **P<0.01.

Fig.3. Male and female ALIOS fed mice are insulin resistant. ipITT represented as AUCs in (A) male and (B) female NC and ALIOS fed mice at 25-, 37- and 50-weeks. ipITT: i.p. insulin tolerance test. HOMA-IR of (C) male and (D) female ALIOS and NC fed mice at 15-, 25-, 37- and 49-weeks. Data expressed as mean ± SEM. n=7 in each group at each time point, *P<0.05, **P<0.01, ***P<0.001.

Fig.4. ALIOS drives hepatic steatosis. Liver mass of (A) male and (B) female and hepatic TAG content in (C) male and (D) female NC and ALIOS fed mice at 26- and 52-weeks (26-weeks: NC males n=12, ALIOS males n=15, NC females n=15, ALIOS females n=15. 52-weeks: NC males n=17, ALIOS males n=12, NC females n=15, ALIOS females n=15). Representative H&E images from male mice at 52-weeks fed NC (E) and ALIOS (F) depicting macro- and micro-vesicular lipid droplets. Data expressed as mean ± SEM. Scale bar is 25µm. ALIOS fed male and female mice had altered expression of genes associated with lipid metabolism (G: males, H: females) insulin signalling (I: males, J: females). n=8 in each group. Genes are

784 expressed as fold changes in log_{10} compared to NC. *P<0.05, **P<0.01, ***P<0.001,
785 ****P<0.0001.
786
787 **Fig.5.** Mice fed ALIOS have increased hepatic inflammation. Histologically determined
788 inflammation score of (A) male and (B) female NC and ALIOS fed mice at 26- (NC males
789 n=12, ALIOS males n=15, NC females n=15, ALIOS females n=15) and 52-weeks (NC males
790 n=17, ALIOS males n=12, NC females n=15, ALIOS females n=15). Data expressed as mean ±
791 SEM. Both male (C) and female (D) mice had increased hepatic expression of genes involved
792 in inflammation, including macrophage infiltration compared to NC fed mice at 52-weeks.
793 n=8 in each group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001
794
795 **Fig.6.** ALIOS drives hepatic fibrosis in aged mice. Percentage fibrosis determined by Sirius red
796 staining in male (A) and female (B) NC and ALIOS fed mice at 26- (NC males n=12, ALIOS
797 males n=15, NC females n=15, ALIOS females n=15) and 52-weeks (NC males n=17, ALIOS
798 males n=12, NC females n=15, ALIOS females n=15) and hepatic expression of genes
799 associated with cell adhesion in male (C) and female (D) ALIOS fed mice (n=8 in each group,
800 genes are expressed as fold changes in log_{10} compared to NC). (E) Western blotting of Col1a1
801 in male and female (F) NC and ALIOS fed mice at 52-weeks (n=10 in each group). Data
802 expressed as mean ± SEM, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Representative
803 images depicting Sirius red staining in male NC (G) and ALIOS (H) mice at 52-weeks. (I) H&E
804 staining of a potential HCC from a male ALIOS fed mouse at 52-weeks highlighting (J) the
805 compressed border of cells (*) and multinucleic cells (arrow). An example of positive Sox9
806 (K) and glutamine synthetase (L) labelling in a HCC from an ALIOS fed male mouse at 52-
807 weeks.
808

Fig.7. Hierarchical clustering analysis of the top 100 DEGs from male (A) and (B) female NC and ALIOS fed mice at 52-weeks. Data presented in a heat-map format in which NC and ALIOS separated into columns and genes in rows. Red corresponds to genes which are upregulated in ALIOS compared to NC and blue corresponds to those that are downregulated. Enriched gene pathways of ALIOS fed mice. (C) Upregulated and (D) downregulated gene ontology pathways in male mice; (E) upregulated and (F) down regulated pathways in females. Pathways ranked by p-values.

Fig.8. (A) ALIOS fed male mice and human patients with NASH share 22.5% of DEGs. (B) Hierarchical clustering analysis of the top 30 DEGs from human NASH and ALIOS fed male mice. (C) Overlapping DEGs are associated with metabolism, inflammation and cell adhesion (genes are expressed as fold changes in $\log_{10}$ compared to NC) and (D) gene ontology of the top 100 overlapping DEGs are associated with alterations in metabolic pathways, immune response and cell adhesion. Pathways ranked by p-values.

Table 1. Mean ± SEM circulating serum levels of lipids

	16w		25w		37w		52w	
Males (mmol/l)	**NC**	**ALIOS**	**NC**	**ALIOS**	**NC**	**ALIOS**	**NC**	**ALIOS**
Total cholesterol	2.6 ± 0.1	3.9 ± 0.2***	2.1 ± 0.1	4.1 ± 0.2****	2.4 ± 0.1	4.6 ± 0.3****	2.5 ± 0.2	5.8 ± 0.4****
HDL	1.8 ± 0.1	2.7 ± 0.1****	1.5 ± 0.1	2.9 ± 0.1****	1.7 ± 0.1	3.1 ± 0.2****	1.7 ± 0.1	3.7 ± 0.4****
LDL	0.59 ± 0.02	0.9 ± 0.1***	0.43 ± 0.03	0.9 ± 0.1****	0.6 ± 0.1	1.3 ± 0.2****	0.5 ± 0.1	1.8 ± 0.2****
TAG	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.94 ± 0.04	0.67 ± 0.04****
Free fatty acids	1.7 ± 0.1	1.5 ± 0.1	0.9 ± 0.1	0.81 ± 0.05	1.9 ± 0.1	1.8 ± 0.1	0.8 ± 0.1	0.67 ± 0.04*
Females (mmol/l)								
Total cholesterol	2.0 ± 0.1	2.4 ± 0.2*	2.0 ± 0.1	2.8 ± 0.2***	1.7 ± 0.1	2.9 ± 0.2***	1.7 ± 0.1	3.2 ± 0.4***
HDL	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	2.0 ± 0.1***	1.2 ± 0.1	2.1 ± 0.1***	1.2 ± 0.1	2.1 ± 0.2***
LDL	0.48 ± 0.03	0.57 ± 0.04	0.45 ± 0.02	0.61 ± 0.04***	0.43 ± 0.01	0.71 ± 0.08**	0.36 ± 0.02	0.74 ± 0.13***
TAG	0.8 ± 0.1	1.0 ± 0.1	0.65 ± 0.03	0.55 ± 0.02*	0.97 ± 0.02	0.71 ± 0.04***	0.81 ± 0.03	0.55 ± 0.03**
Free fatty acids	1.7 ± 0.1	1.7 ± 0.1	0.81 ± 0.04	0.64 ± 0.04**	1.95 ± 0.15	1.37 ± 0.07***	0.97 ± 0.06	0.69 ± 0.04**

* Significantly different from NC at same age P<0.05, ** P<0.01, *** P<0.001, *****P<0.0001. n=12-15 in each group.

Table 2. Mean ± SEM circulating serum levels of renal and liver biochemistry

	16w		25w		37w		52w	
Males	**NC**	**ALIOS**	**NC**	**ALIOS**	**NC**	**ALIOS**	**NC**	**ALIOS**
Creatinine (mg/dl)	N/A		0.69 ± 0.06	0.81 ± 0.11	N/A		0.67 ± 0.08	0.96 ± 0.08*
ALP (U/l)	76.6 ± 3.3	72.6 ± 4.0	64.9 ± 2.0	75.2 ± 7.6	70.1 ± 2.9	88.7 ± 12.1	66.2 ± 3.1	118.5 ± 11.0****
ALT (U/l)	42.1 ± 1.9	83.7 ± 23.9	33.0 ± 2.6	99.9 ± 16.4**	34.4 ± 2.9	238.0 ± 82.4**	38.0 ± 4.0	291.2 ± 47.4****
AST (U/l)	93.7 ± 6.3	126.3 ± 22.1	69.5 ± 8.1	135.9 ± 17.0**	72.4 ± 7.9	250.7 ± 80.5*	72.4 ± 4.6	346.3 ± 50.8****
AST/ALT ratio	2.2 ± 0.1	1.7 ± 0.1*	2.2 ± 0.3	1.6 ± 0.2	2.2 ± 0.3	1.1 ± 0.1**	2.0 ± 0.1	1.2 ± 0.1****
Total bilirubin (µmol/l)	3.7 ± 0.5	2.9 ± 0.4	1.9 ± 0.1	1.8 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Females								
Creatinine (mg/dl)	N/A		0.62 ± 0.07	0.65 ± 0.08	N/A		0.64 ± 0.07	0.91 ± 0.08
ALP (U/l)	138.9 ± 11.3	120.9 ± 8.1	105.1 ± 3.6	108.4 ± 4.4	122.7 ± 15.1	142.3 ± 9.8	130.5 ± 7.5	138.5 ± 13.7
ALT (U/l)	43.9 ± 7.2	46.7 ± 6.8	28.5 ± 1.4	136.3 ± 32.8***	33.0 ± 1.7	226.3 ± 54.4***	37.7 ± 4.1	310.4 ± 41.5***
AST (U/l)	114.9 ± 20.5	125.9 ± 11.0	68.3 ± 3.6	251.7 ± 47.0***	83.7 ± 6.4	338.5 ± 55.4***	101.3 ± 8.6	501.3 ± 45.3***
AST/ALT ratio	2.6 ± 0.1	2.8 ± 0.2	2.4 ± 0.1	2.2 ± 0.2	2.5 ± 0.1	1.7 ± 0.1***	2.8 ± 0.2	1.7 ± 0.1***
Total bilirubin (µmol/l)	3.2 ± 0.6	4.4 ± 0.4	1.7 ± 0.1	1.9 ± 0.1	2.3 ± 0.2	2.1 ± 0.2	1.7 ± 0.1	2.0 ± 0.1*

* Significantly different from NC at same age P<0.05, **P<0.01, *** P<0.001,**** P<0.0001. N/A Serum not analysed at this time point. n= 12-15 in each group. Creatinine n=10 in each group.

Table 3. The 10 most up- and down-regulated genes in NC vs ALIOS liver tissue at 52-weeks

Gene	Description	Log Fold	FDR
Males			
Clec7a	C-type lectin domain family 7	2.3128	$7.98x10^{-33}$
Mmp12	matrix metallopeptidase 12	4.1723	$7.04x10^{-31}$
H2-Ab1	histocompatibility 2, class II antigen A, beta 1	1.9017	$4.10x10^{-29}$
Cx3cr1	chemokine (C-X3-C motif) receptor 1	2.8669	$1.24x10^{-28}$
H2-Aa	histocompatibility 2, class II antigen A, alpha	2.1164	$2.10x10^{-28}$
Tmem86a	transmembrane protein 86A	1.7757	$4.03x10^{-28}$
Col1a1	collagen, type I, alpha 1	3.5204	$1.72x10^{-26}$
Ephb2	Eph receptor B2	4.7443	$2.27x10^{-25}$
Cd63	CD63 antigen	3.0092	$3.45x10^{-25}$
H2-Eb1	histocompatibility 2, class II antigen E beta	1.9343	$1.11x10^{-24}$
Ces2a	carboxylesterase 2A	-1.9190	$7.04x10^{-31}$
Ces1b	carboxylesterase 1B	-1.3651	$6.89x10^{-23}$
Tnfaip8l1	tumour necrosis factor, alpha-induced protein 8-like 1	-1.0119	$1.99x10^{-17}$
Scarb2	scavenger receptor class B, member 2	-0.7034	$3.34x10^{-14}$
Retsat	retinol saturase (all trans retinol 13,14 reductase)	-1.0527	$6.13x10^{-14}$
Marf1	meiosis regulator and mRNA stability 1	-0.7292	$2.89x10^{-13}$
Tuba4a	tubulin, alpha 4A	-0.9631	$3.80x10^{-13}$
Hectd1	HECT domain E3 ubiquitin protein ligase 1	-0.6121	$9.50x10^{-13}$
Angptl4	angiopoietin-like 4	-1.0041	$1.27x10^{-12}$
Pxmp4	peroxisomal membrane protein 4	-0.9919	$1.46x10^{-12}$
Females			
Wfdc2	WAP four-disulfide core domain 2	2.6371	$1.97x10^{-40}$
Uap1l1	UDP-N-acteylglucosamine pyrophosphorylase 1-like 1	2.6742	$6.04x10^{-39}$
Ifi27l2b	interferon, alpha-inducible protein 27 like 2B	2.8447	$2.95x10^{-37}$
Ly6d	lymphocyte antigen 6 complex, locus D	3.6763	$7.04x10^{-29}$
Clec7a	C-type lectin domain family 7, member a	2.4672	$4.90x10^{-28}$
Mmp12	matrix metallopeptidase 12	5.0590	$5.69x10^{-28}$
Ms4a6d	membrane-spanning 4-domains, subfamily A, member 6D	2.0780	$7.87x10^{-28}$
Hcar2	hydroxycarboxylic acid receptor 2	2.5398	$1.12x10^{-27}$
Lpl	lipoprotein lipase	2.5220	$1.16x10^{-27}$
Osbpl3	oxysterol binding protein-like 3	3.5456	$2.10x10^{-27}$
Abhd6	abhydrolase domain containing 6	-1.1633	$6.92x10^{-27}$
Gm3787	Predicted gene 3787*	-2.6100	$1.97x10^{-22}$
Ces2a	carboxylesterase 2A	-1.4585	$6.56x10^{-19}$
Mttp	microsomal triglyceride transfer protein	-0.8436	$2.59x10^{-17}$
Cyp2c23	cytochrome P450, family 2, subfamily c, polypeptide 23	-1.6328	$1.10x10^{-16}$
Avpr1a	arginine vasopressin receptor 1A	-1.5448	$2.88x10^{-16}$
Fam234b	family with sequence similarity 234, member B	-1.2050	$9.72x10^{-16}$
Sult5a1	sulfotransferase family 5A, member 1	-2.6802	$1.79x10^{-15}$
Cyp4a10	cytochrome P450, family 4, subfamily a, polypeptide 10	-1.2606	$2.61x10^{-14}$
Sult3a2	sulfotransferase family 3A, member 2	-3.7032	$9.57x10^{-14}$

*function of predicted genes unknown in current annotation

Table 4. IPA of differentiated genes in male and female ALIOS fed mice at 52-weeks

Biological Function	P-value	Genes (n)
Males		
Diseases and Disorders		
Cancer	$1.04x10^{-14} – 1.69x10^{-88}$	4093
Organismal Injury and Abnormalities	$1.04x10^{-14} – 1.69x10^{-88}$	4206
Endocrine System Disorders	$1.03x10^{-27} – 2.92x10^{-70}$	3316
Gastrointestinal Disease	$6.12x10^{-15} – 9.14x^{-57}$	3682
Inflammatory Response	$9.02x10^{-15} – 7.51x10^{-54}$	1364
Molecular and Cellular Functions		
Cell Death and Survival	$7.61x10^{-15} – 2.49x10^{-72}$	1622
Cellular Movement	$7.21x10^{-15} – 6.10x10^{-68}$	1216
Cellular Compromise	$5.16x10^{-19} – 7.51x10^{-54}$	354
Cell-To-Cell Signalling and Interaction	$1.07x10^{-14} – 6.98x10^{-41}$	953
Lipid Metabolism	$1.10x10^{-14} – 1.40x10^{-39}$	797
Top Toxicology list		**Overlap (ratio)**
NRF2-mediated Oxidative Stress Response	$2.83x10^{-15}$	42.1% (101/240)
Liver Necrosis/Cell Death	$6.98x10^{-15}$	38.5% (124/322)
Renal Necrosis/Cell Death	$1.40x10^{-14}$	33.3% (191/573)
Hepatic Fibrosis	$1.41x10^{-13}$	51.4% (57/111)
Xenobiotic Metabolism Signalling	$1.19x10^{--11}$	35.2% (123/349)
Females		
Diseases and Disorders		
Cancer	$8.17x10^{-15} – 2.35x10^{-78}$	3434
Organismal Injury and Abnormalities	$1.86x10^{-14} – 2.35x10^{-78}$	3528
Endocrine System Disorders	$1.94x10^{-36} – 3.14x10^{-64}$	2815
Inflammatory Response	$1.29x10^{-14} – 7.07x10^{-56}$	1187
Gastrointestinal Disease	$1.09x10^{-14} – 3.97x10^{-51}$	3122
Molecular and Cellular Functions		
Cell Death and Survival	$1.35x10^{-14} – 7.31x10^{-58}$	1375
Cellular Movement	$1.32x10^{-14} – 2.23x10^{-55}$	999
Cellular Compromise	$1.35x10^{-14} – 4.09x10^{-55}$	374
Cellular Function and Maintenance	$2.32x10^{-15} – 1.81x10^{-50}$	1237
Cell-To-Cell Signalling and Interaction	$1.75x10^{-14} – 1.79x10^{-41}$	707
Top Toxicology list		**Overlap (ratio)**
Hepatic Fibrosis	$2.00x10^{-16}$	50.5% (56/111)
Renal Necrosis/Cell Death	$5.35x10^{-13}$	28.4% (163/573)
Liver Necrosis/Cell Death	$1.11x10^{-12}$	32.6% (105/322)
Increases Liver Steatosis	$3.83x10^{-12}$	45.0% (49/109)
LXR/RXR Activation	$5.63x10^{--11}$	41.5% (51/123)

Data represent the number of genes up- or down-regulated in ALIOS fed mice at 52w. Biological functions, molecular functions and top toxicology lists were assigned using findings extracted from literature and stored in IPA. P-values were determined by IPA software using Fisher's Exact Test and determine the probability that the pathway or function assigned is explained by chance alone. The percent overlap and ratio were calculated from the number of observed genes compared to the number of known genes for that category in the Ingenuity Knowledge Base.