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RESEARCH ARTICLE

# Methyl donor deficient diets cause distinct alterations in lipid metabolism but are poorly representative of human NAFLD [version 1; referees: 1 approved]

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## Abstract

**Background:** Non-alcoholic fatty liver disease (NAFLD) is a global health issue. Dietary methyl donor restriction is used to induce a NAFLD/non-alcoholic steatohepatitis (NASH) phenotype in rodents, however the extent to which this model reflects human NAFLD remains incompletely understood. To address this, we undertook hepatic transcriptional profiling of methyl donor restricted rodents and compared these to published human NAFLD datasets.

**Methods:** Adult C57BL/6J mice were maintained on control, choline deficient (CDD) or methionine/choline deficient (MCDD) diets for four weeks; the effects on methyl donor and lipid biology were investigated by bioinformatic analysis of hepatic gene expression profiles followed by a cross-species comparison with human expression data of all stages of NAFLD.

**Results:** Compared to controls, expression of the very low density lipoprotein (VLDL) packaging carboxylesterases (*Ces1d*, *Ces1f*, *Ces3b*) and the NAFLD risk allele *Pnpla3* were suppressed in MCDD; with *Pnpla3* and the liver predominant *Ces* isoform, *Ces3b*, also suppressed in CDD. With respect to 1-carbon metabolism, down-regulation of *Chka*, *Chkb*, *Pctyl1a*, *Gnmt* and *Ahcy* with concurrent upregulation of *Mat2a* suggests a drive to maintain S-adenosylmethionine levels. There was minimal similarity between global gene expression patterns in either dietary intervention and any stage of human NAFLD, however some common transcriptomic changes in inflammatory, fibrotic and proliferative mediators were identified in MCDD, NASH and HCC.

**Conclusions:** This study suggests suppression of VLDL assembly machinery may contribute to hepatic lipid accumulation in these models, but that CDD and MCDD rodent diets are minimally representative of human NAFLD at the transcriptional level.

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## Introduction

Non-alcoholic fatty liver disease (NAFLD) is the predominant cause of chronic liver disease in the developed world, with an estimated prevalence of between 20–68%<sup>1</sup>. The accumulation of hepatic fat in the form of triglycerides and other lipid species in NAFLD has two major clinical consequences. Firstly, a subgroup of patients with hepatic steatosis will progress to an inflammatory hepatitis, hepatic cirrhosis and in some cases hepatocellular carcinoma (HCC)<sup>2</sup>. Secondly, almost all patients with NAFLD also exhibit hepatic insulin resistance, which can be associated with impaired glucose uptake, increased gluconeogenesis and type 2 diabetes, possibly as a direct consequence of the increased hepatic lipid load<sup>1,3,4</sup>. Together, these conditions are responsible for significant morbidity and mortality and represent a substantial burden for health resources<sup>5</sup>.

The molecular mechanisms underpinning NAFLD pathology are incompletely understood, and as such there is a need for accurately representative rodent models in which to investigate this common disease and to trial novel therapeutics. Given the association of NAFLD with human obesity, the use of high fat diet feeding in rodents remains a popular model in which to investigate mechanisms. However whilst high fat feeding generates a NAFLD-like picture, the disadvantages with this model include the protracted time required to induce even mild non-alcoholic steatohepatitis (NASH), the lack of malignant transformation to HCC even with prolonged exposure, and the variation in the histological and transcriptional changes due to the behavioural characteristics of mice in social groups<sup>6–8</sup>. Thus, a number of alternative models have been employed. In rodents, dietary restriction of the methyl donors methionine and/or choline rapidly and reliably induces a spectrum of liver injury histologically similar to human NAFLD, within weeks of instigation<sup>9,10</sup>. Although the precise biological mechanisms responsible for the predictable phenotypic changes are poorly understood, the histological similarity to human steatosis (choline deficient diets; CDD) and NASH (methionine and choline deficient diets; MCDD) means that these models have been used in mechanistic and therapeutic studies for a number of years<sup>11–13</sup>. Since impaired metabolism of the key methyl donor S-adenosylmethionine (SAME) is a well documented feature of chronic liver disease regardless of aetiology<sup>14–16</sup>, there may be common molecular mechanisms which may present an opportunity for therapeutic intervention. Although a number of transcriptional changes have been reported during the progression of human NAFLD, no detailed transcriptional comparisons have been performed to identify similarities or differences between human disease and the CDD and MCDD models of NAFLD, despite their widespread use. In this study, we set out firstly to dissect potential mechanisms underpinning the development of liver pathology in CDD and MCDD models by mapping pathways of lipid and one-carbon metabolism, and secondly to evaluate their potential usefulness as models of human disease. To address these aims, we have examined in detail the transcriptional profiles in liver from mice maintained on CDD and MCDD, and compared these with published human NAFLD transcriptome data series.

## Materials and methods

### Animals

All experiments were carried out under a UK Home Office Licence (PPL 70/7874), and with local ethical committee approval and adhering to the ‘Animal Research: Reporting In Vivo Experiments’ (ARRIVE) guidelines. Adult C57BL/6J mice (purchased from Charles River, Tranent, UK) were maintained under controlled conditions in social groups of 5 animals per cage; n=10/group. A 12-hour light cycle (07.00h to 19.00h) and twelve hour dark cycle was implemented throughout. The temperature was maintained at 22°C +/- 2°C. Mice were maintained on control, CDD or MCDD diets (Dyets, Bethlem, PA) for 4 weeks. All efforts were made to ameliorate any suffering of animals, in particular, since MCDD diets can result in weight loss, mice were weighed and their health status checked daily by experienced technicians for the 4 weeks they remained on the diets. All mice remained well, with no concerns about health other than weight loss in the MCDD group. After 4 weeks mice were killed by Schedule 1 (CO<sub>2</sub> inhalation), and tissues were collected and used for histology or snap-frozen and stored at -80C. The diet composition can be found in [Supplementary Table 1](#).

### Histology staining, triglyceride and SAME quantification

Livers were removed and sections were fixed in methacarn solution (methanol:chloroform:glacial acetic acid; ratio 6:3:1) and mounted in paraffin blocks prior to staining with haematoxylin and eosin or picosirius red. Hepatic triglyceride concentration was determined by spectrophotometric analysis (BioVision, Milpitas, USA) as previously described<sup>17</sup>. Image analysis for fibrosis content was performed in ImageJ (<http://imagej.nih.gov/ij/>). SAME was quantified by matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) using the 12T SolariX MALDI-FTICR-MS (Bruker Daltonics, MA, US), as previously described<sup>18</sup>.

### Reverse transcription and qPCR

RNA was extracted from snap frozen liver tissue using the RNeasy kit (Qiagen, Manchester, UK). 800ng of RNA was DNase treated using Promega RQ1 DNAase (Promega, Southampton, UK) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley, UK). Quantitative real time PCR was performed using Roche Universal Probe Library assays or TaqMan qPCR assays (Life Technologies, Paisley, UK) (please see [Supplementary Table 2](#) for the primer sequences), using the Roche Lightcycler 480 and associated Lightcycler 480 software: release 1.5.1.62 (Roche, West Sussex, UK). Gene expression is displayed relative to mean of three housekeeping genes (Gapdh, Ppia, Ldha).

### Transcript analysis

RNA labelling was performed on 500ng RNA using the Illumina Total Prep RNA amplification kit (Life Technologies, Paisley, UK) and subsequently hybridised to Illumina Mouse-ref6 expression bead arrays as per the manufacturer’s instructions, at the Edinburgh Clinical Research Facility, Western General Hospital, Edinburgh,

UK. Intensity data were generated using a HiScan array scanner (Illumina, San Diego, USA) and analysed using iScan Illumina software. Data analysis and generation of plots were performed in RStudio (<http://www.rstudio.com>), with R version 3.1.2. Data import, quality control, normalisation and between array adjustment was performed using the Lumi package, and differential expression was determined using the Limma package (Bioconductor.org). Unsupervised clustering was performed using Euclidean distance. Where multiple probes mapped to the same gene, the median result was used. Data have been uploaded to EBI-Array Express, accession number E-MTAB-3943.

### Pathway analysis

Gene Ontology and pathway enrichment was performed using the GOstats package (Bioconductor.org). Investigation of fat handling was performed by interrogating relevant pathways of lipid metabolism and insulin signalling from the Kyoto encyclopedia for genes and genomes (KEGG) module database (<http://www.genome.jp/kegg/module.html>). KEGG module sets 'M00003 gluconeogenesis', 'M00086 beta-Oxidation, acyl-CoA synthesis', 'M00083 Fatty acid biosynthesis, elongation' and 'mmu\_M00089 Triacylglycerol biosynthesis', 'mmu00071 Fatty acid degradation', and KEGG pathways 'Fat Digestion and Absorption' and 'Insulin Signalling Pathway' and 'Glycerolipid Metabolism' were used. In addition, the family of carboxylesterase (Ces) genes were analysed due to their recently discovered role in triglyceride hydrolysis<sup>19–21</sup>. Finally, to dissect the link between lipid metabolism and one carbon metabolism, relevant mediators were analysed and mapped to known biochemical pathways.

### Cross species comparison

A comparison of transcriptional data from both CDD and MCDD was made with published human expression sets of normal liver, simple steatosis and NASH (GSE48452, E-MEXP-3291), and HCC (GSE63898)<sup>22–24</sup>. Datasets were retrieved from the ArrayExpress archive (<http://www.ebi.ac.uk/arrayexpress/>). Predicted gene orthologues were determined using *homologene* via the Hugo Gene Nomenclature Committee server (<http://www.genenames.org>). For all human NASH, HCC and all mouse datasets, a transcriptional threshold of 1.5 with an adjusted p value of <0.05 was applied. For human steatosis gene sets alone, the fold change transcriptional threshold was reduced to 1.2 to allow comparison with the relatively mild transcriptional derangement observed. All genes found to be dysregulated at each stage of human NAFLD were then examined in the CDD and MCDD datasets to determine if expression was altered, and if so, what was the direction of change. Genes dysregulated in human and mouse models were then depicted in scatter plot analyses with linear regression used to compare datasets.

### Statistical analysis

Animal model and qPCR statistical analysis was performed using Prism GraphPad software (GraphPad Software Inc.). Data were routinely analysed for outliers, normalisation and sphericity where required. Non-parametric data were either log transformed or a non-parametric test used as indicated.

## Results

### Phenotype

Mice on CDD gained significantly less weight than animals on a control diet, whereas MCDD fed mice lost weight from the outset, consistent with previous observations<sup>17,25</sup> (Figure 1A). At the end of the experiment, hepatic triglyceride content was significantly higher in both CDD and MCDD groups compared to controls, but there was no significant difference between the two interventions (Figure 1B). MCDD liver weights were lower in MCDD fed mice but not CDD (Figure 1C). Histological analysis revealed severe hepatic steatosis in both groups (Figure 1D), with a significant increase in hepatic fibrosis in the MCDD group (Figure 1E).

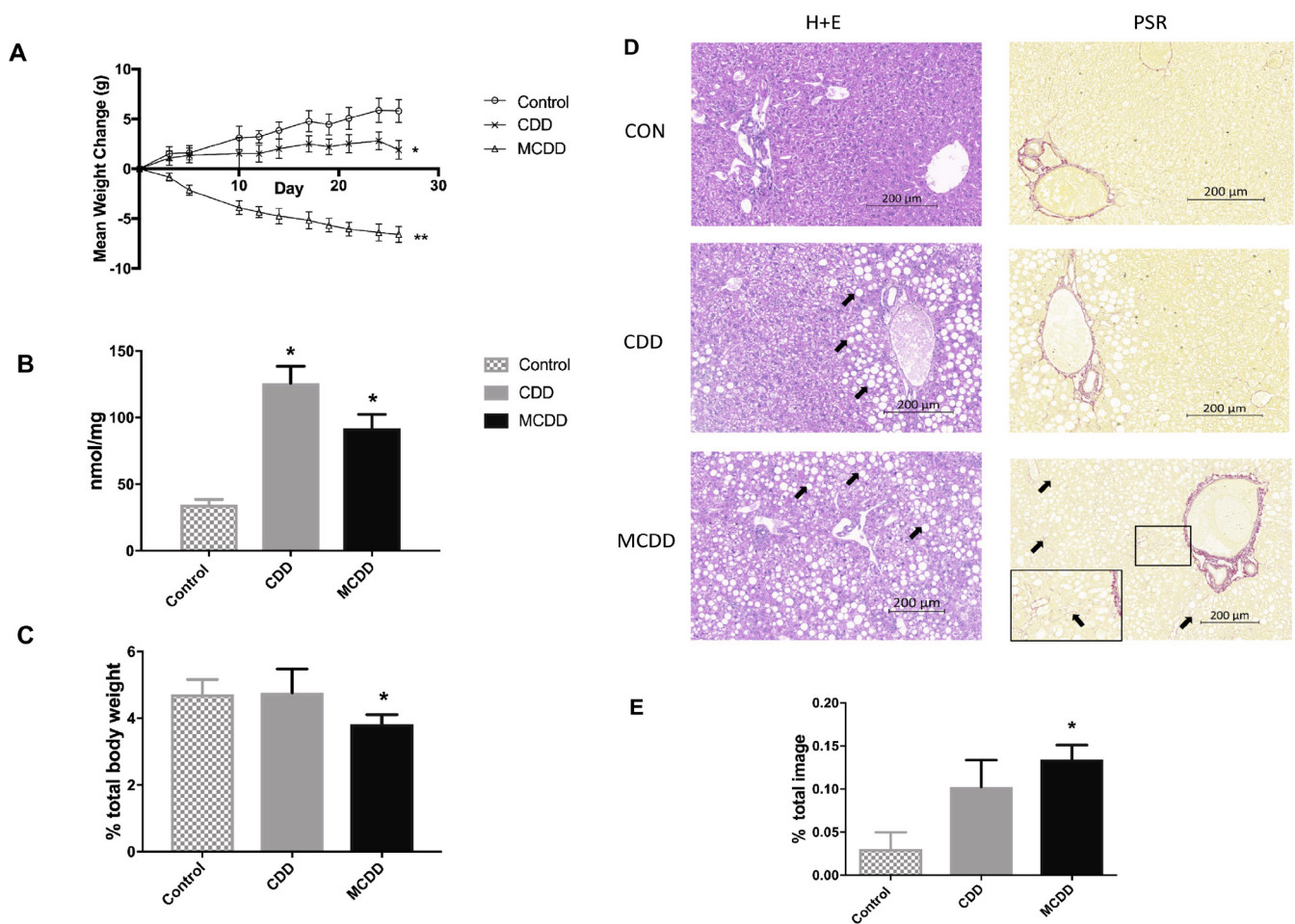
### Gene expression changes

Next we carried out analysis of the transcriptome in control, CDD or MCDD mouse livers, each n=4. This approach allowed us to interrogate ~18,000 transcripts per mouse liver and analysis of total datasets revealed a number of transcriptional differences between animals. Unsupervised clustering of the 500 most variable transcripts between all animals was sufficient to cluster into the different dietary interventions (Figure 2A). Both interventions induced a >1.5-fold differential expression in multiple transcripts when corrected for multiple testing (adjusted P value <0.05, Benjamini-Hochberg test) (Figure 2B–D). The top 100 up- and down-regulated genes in each group are shown in Supplementary Table 3 and Supplementary Table 4. Of the 234 genes differentially expressed in CDD, 194 (82.4%) were also differentially expressed in MCDD. The additional restriction of methionine over and above choline induced the differential expression of a further 1032 transcripts (Figure 2B).

Transcripts showing at least a 2-fold change were segregated into up-regulated and down-regulated gene lists and examined for over-representation within all detected transcripts on the array platform. GO-terms for lipid, sterol, fatty acid and organic acid biosynthesis were markedly over-represented in the list of suppressed genes. Over-expressed pathways in CDD mice included 'immune system process' and 'inflammatory response'. Up-regulated pathways in mice on the MCDD diet included 'positive regulation of mitotic cell cycle' and 'negative regulation of cell cycle arrest' (Supplementary Figure 1).

The most up-regulated genes in CDD mice included immune mediators (*GpnmB*, *Ly6d*); fibrosis mediators (*Mmp12*, *Mmp13*); and the detoxification enzymes *Gsta1* and *Gsta2* and the microsomal enzyme *Cyp4a14*. The most suppressed genes in CDD included lipid synthesis genes (*Sqle*, *Elovl3*, *Elovl6*, *Aacs*, *Acly*, *Acss2*, *Acacb*), the endopeptidase inhibitor *Serpina4-ps1* and the multifunctional triglyceride metabolism enzyme *Pnpla3*. Whilst these genes were similarly differentially expressed in MCDD mice, volcano plots revealed considerably more severe transcriptional derangement in MCDD compared with CDD, both in the number and fold change of differentially expressed genes (Figures 2C and 2D). Additional genes up-regulated in MCDD included the mitotic proteins *Cdc20*, *Nupr1*, the metalloproteinase *Adam32*, the inflammatory mediator *Slpi* and the aldo-ketoreductase *Akrb7*.





**Figure 1. Body weight and hepatic lipid content.** **A**) In comparison with control animals, mice on CDD gain less weight whereas those on MCDD lose weight. **B**) Hepatic liver triglyceride content was increased on both CDD and MCDD diets. **C**) Liver weight was reduced in MCDD fed mice but not CDD. **D**) Liver histology from control, CDD and MCDD mice. H+E = Hematoxylin and Eosin staining showing marked macrovesicular steatosis in both CDD and MCDD mice (white arrows). PSR = Picosirius Red stain staining for new collagen formation showing increased periportal and interstitial fibrosis in MCDD animals (black arrows). In MCDD liver stained with PSR, insert at higher magnification shows increased fibrosis more clearly. **E**) Quantified fibrosis content (PSR positive staining as a percentage of entire image).  $n = 10$  per group for all figures. \*  $P < 0.05$  \*\*  $P < 0.01$  (one way ANOVA with Tukey post hoc test versus control animals). Error bars =  $\pm$  SEM.

qPCR validation of array findings was performed for known mediators of lipid uptake (*Lpl*), putative contributory genes to hepatic fat accumulation in NAFLD (*Scd1*, *Aacs*, *Fasn*, *Mlxipl*, *Acs11*) and hepatic fibrogenesis (*Mmp12*), and *Pdk1* which is an important link between insulin signaling and HCC. In addition, we analysed gene expression of four members of the *Ces* family due to their functional role in triglyceride hydrolysis. Gene expression by qPCR was consistent with array findings for all genes (Figures 3A and B).

#### Lipid pathway analysis

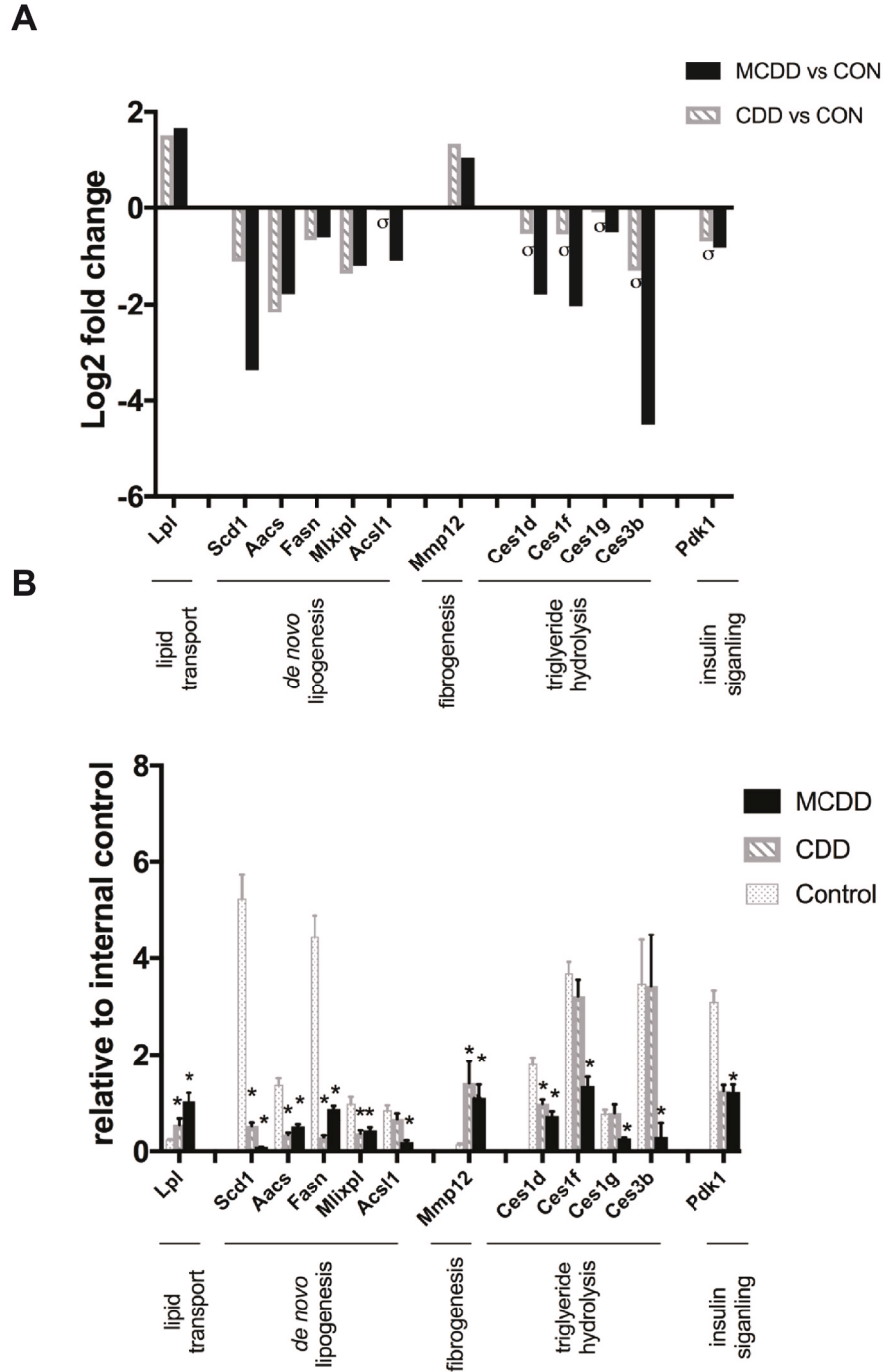
We then proceeded to examine pathways of lipid uptake, synthesis and disposal. Differentially expressed genes in relevant KEGG pathways in either group are depicted in Figure 4. Expression changes were generally greater in MCDD than CDD although they occurred in the same direction. Previous studies have suggested an

increase in lipid uptake with MCDD, with upregulation of some of the FATP/solute carrier family 27 genes in association with increased sequestration of isotope labelled fatty acids<sup>17,26</sup>, however we noted only the down-regulation of the fatty acid translocase *Scla27a5* (FATP5) with no change in the other FATP isoforms. We did identify marked upregulation of lipoprotein lipase, a key mediator in triglyceride hydrolysis from lipoproteins. In keeping with the gene ontology analysis, the global picture suggests suppressed hepatic lipid synthesis. Perturbed genes in pathways of fatty acid biosynthesis initiation, Acyl-CoA synthesis, fatty acid elongation and cholesterol synthesis were almost universally down-regulated.

We then examined dominant pathways of hepatic fatty acid fate (triglyceride synthesis,  $\beta$ -oxidation, oxidation and peroxisomal oxidation). These were found to be relatively unaffected,



**Figure 2. Differential gene expression.** **A)** Transcript profiles of each dietary intervention were sufficiently consistent to cluster by Euclidean distance. **B)** Venn diagram demonstrating high degree of overlap in dysregulated transcripts ( $\geq 2$  fold change) in each group and the direction of transcriptional change. **C** and **D)** Volcano plots demonstrating transcriptional changes in CDD (**C**) and MCDD (**D**) mice. Dotted lines and blue colour represent adjusted P values  $< 0.05$  and two fold differential expression change.

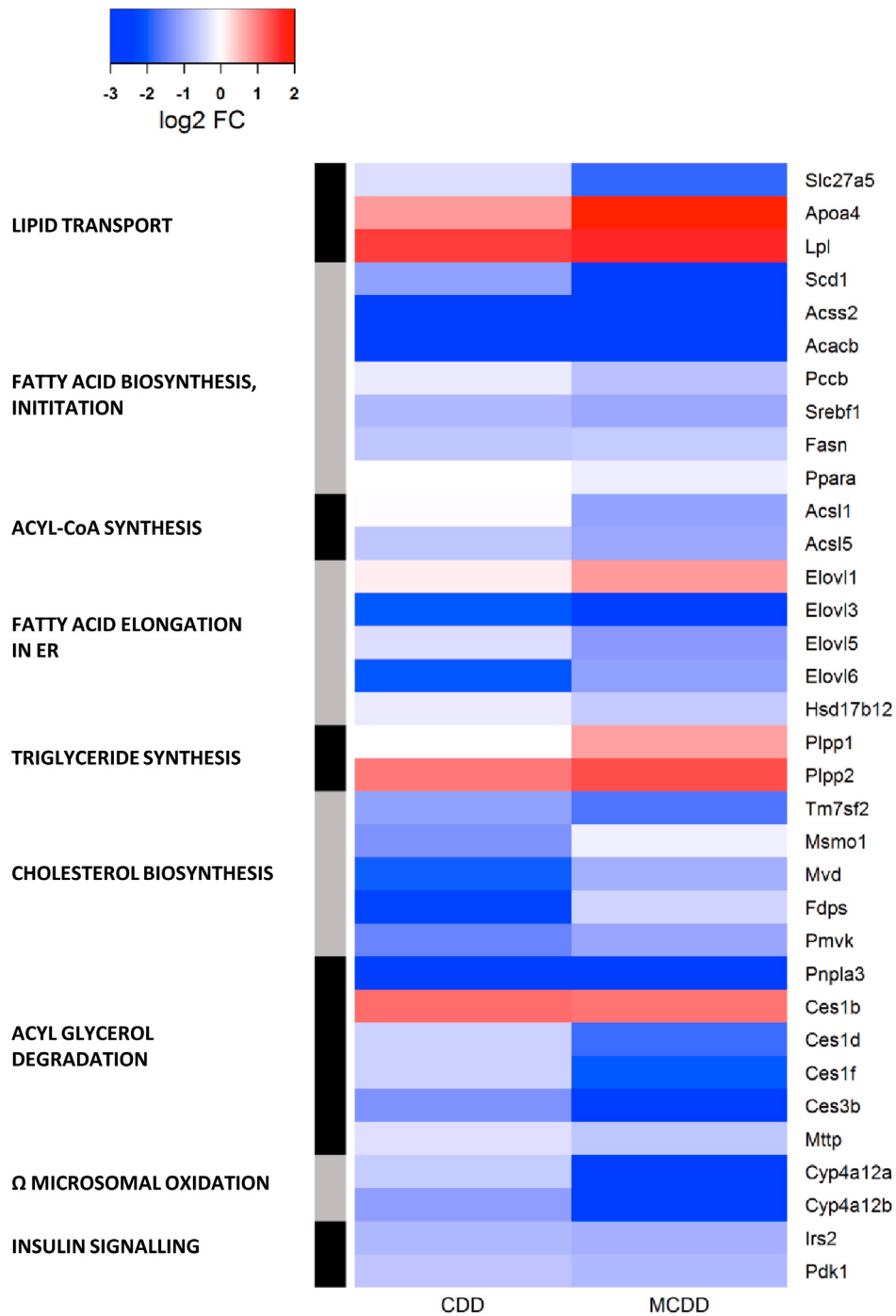


**Figure 3. CDD and MCDD induce changes in the hepatic expression of genes important in lipid metabolism and storage.** (A) Microarray analysis and (B) qPCR validation of selected genes important in lipid transport, *de novo* lipogenesis, fibrogenesis, triglyceride hydrolysis and insulin signalling. (A) Adjusted P value < 0.05 (FDR) for all samples except those marked  $\sigma$ . (B) \* = P < 0.05 versus control, one-way ANOVA with Bonferroni post hoc analysis. Error bars = +/- SEM.

apart from an upregulation of the phosphatidic acid phosphatases *Pap2a* and *Pap2c* (which convert phosphatidic acids to diacylglycerol) and the peroxisomal fatty acid elongation enzyme *Elovl1* in MCDD. The Cyp4a family of enzymes broadly catalyse the microsomal ( $\omega$ ) oxidation of saturated and unsaturated fatty

acids and have reported to be upregulated by dietary and drug-induced hepatic inflammation<sup>27-29</sup>. Interestingly, isoforms of Cyp4a enzymes demonstrated marked bi-directional differential expression with Cyp4a12a and Cyp4a12b strongly suppressed in CDD and MCDD whilst Cyp4a14 was upregulated by 4-fold with both





**Figure 4.** Heatmap depiction of expression changes in KEGG pathways of lipid transport, lipid synthesis and degradation and insulin signalling. Up- and down-regulated genes in each dietary intervention versus control animals are demonstrated by colour key. Pathways of lipid and cholesterol synthesis are globally suppressed.

diets. Other microsomal oxidation Cyp450 isoforms (Cyp4a10, Cyp4a32, Cyp4a29, Cyp4a30b) were unchanged. Cyp2e, which has previously been reported to be upregulated in MCDD was also unchanged<sup>29</sup>. In keeping with the theory of impaired very-low-density lipoprotein (VLDL) secretion in NAFLD<sup>17,30</sup>,

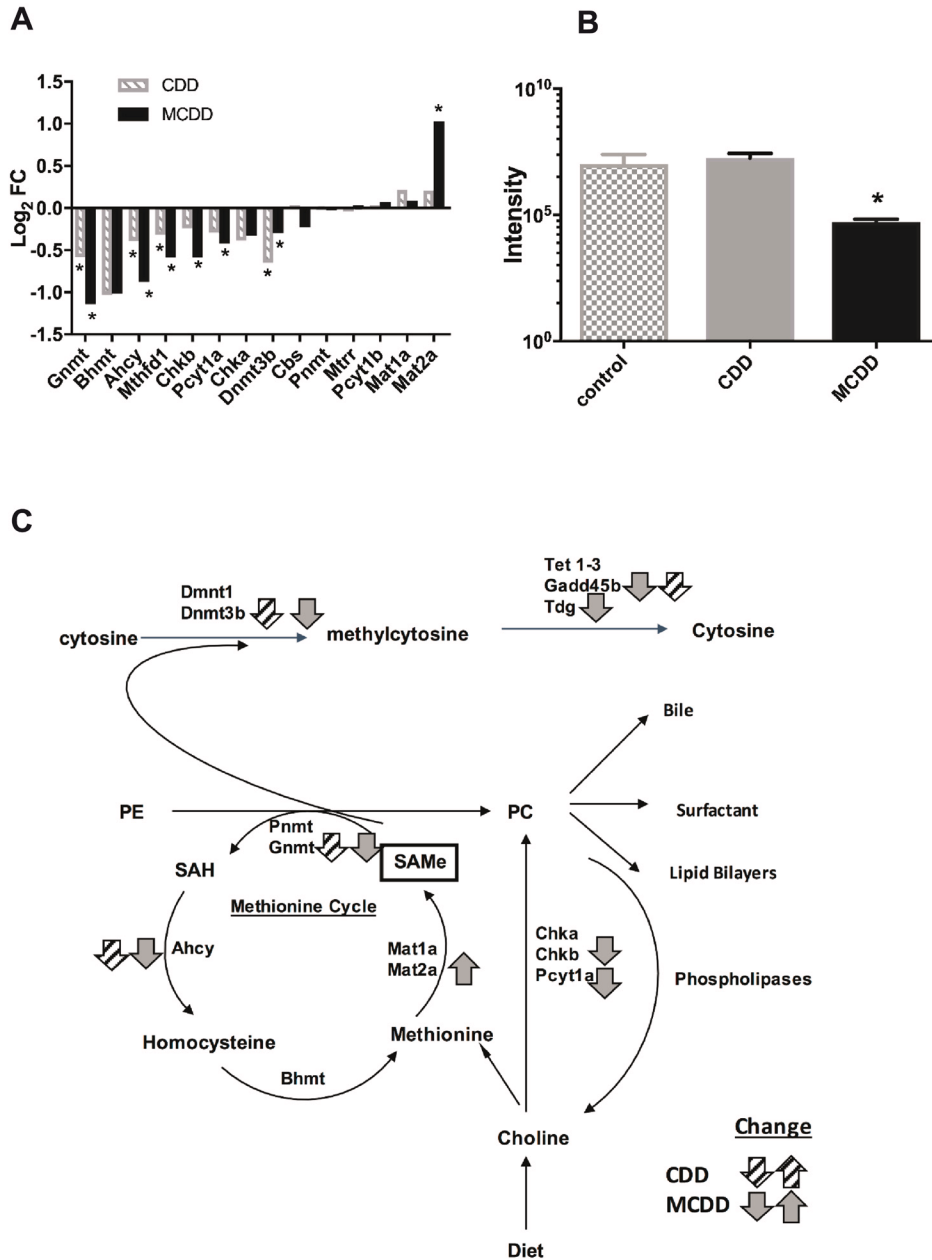
endoplasmic reticulum (ER)-associated mediators of triglyceride hydrolysis and VLDL assembly (*Pnpla3*, *Mttp* and the carboxylesterase enzymes: *Ces1d*, *Ces1f*, *Ces3b*) were markedly suppressed in MCDD, with *Pnpla3* also suppressed in CDD. Interestingly, expression of the *Ces1b* isoform was clearly up-regulated in both

groups, in contrast to the other members of this class. ApoA4, a lipid binding protein involved in the expansion and secretion of VLDL particles was significantly induced. Finally, two key intermediaries in hepatic insulin signal transduction (*Irs2* and *Pdk1*) were also down-regulated.

### One-carbon metabolism

Given the importance of choline and methionine as methyl donors, we then proceeded to examine the expression of genes important in

one-carbon metabolism. There were striking changes in the expression of genes associated with choline, methionine and phosphatidylcholine (PC) metabolism in both interventions, broadly in the same direction (Figure 5A). Two pathways demonstrated significant down-regulation of key enzymes in MCDD: including genes important in the synthesis of PC from choline (*Chkb*, *Pcyt1a*) and in the conversion of SAdMe to homocysteine (*Gnmt*, *Ahcy*). Furthermore, the expression of enzymes that contribute to the clearance of methionine, SAdMe and S-adenosylhomocysteine



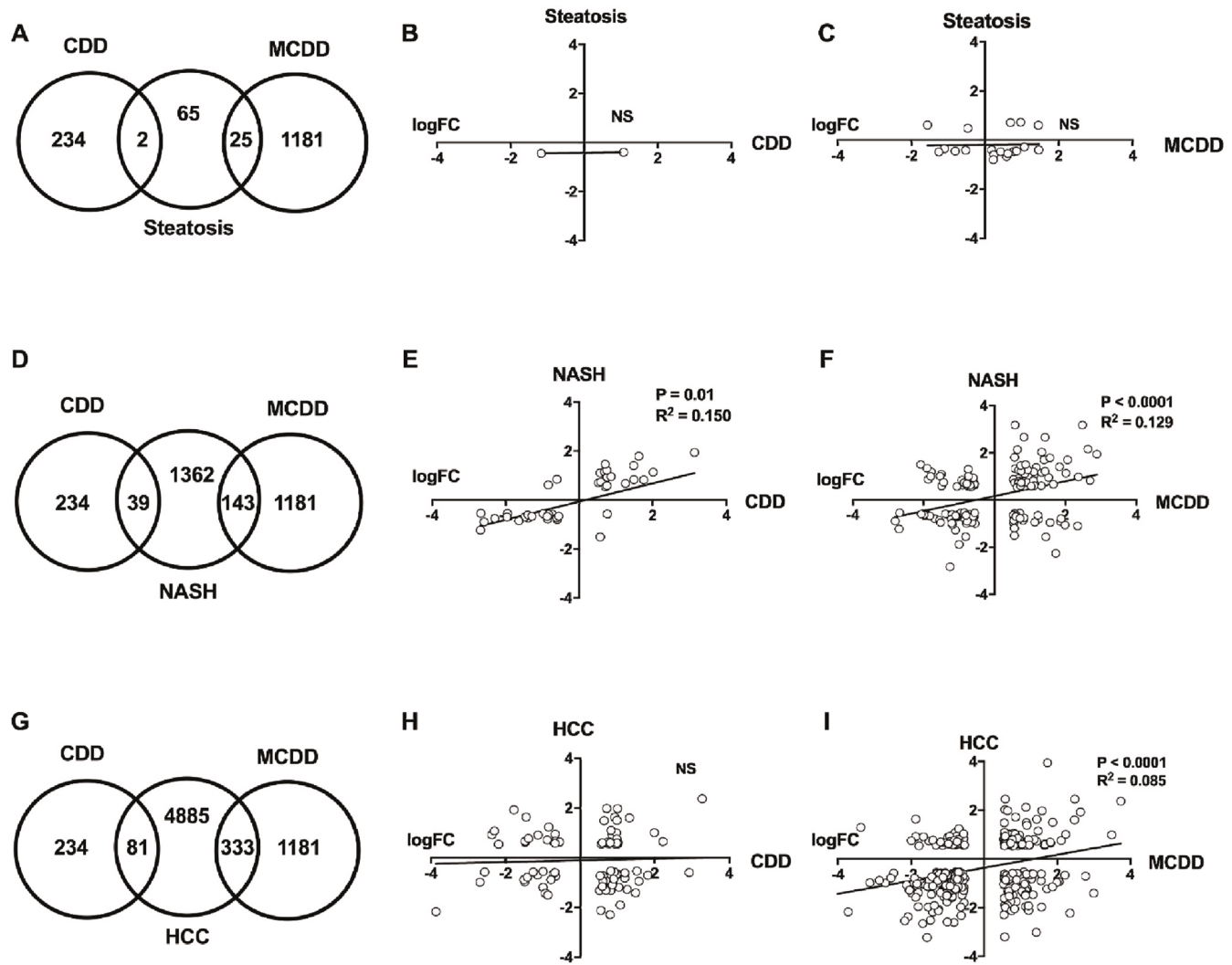
**Figure 5. CDD and MCDD induce changes in the hepatic expression of genes important in one-carbon metabolism.** (A) Transcriptional changes in one-carbon metabolism enzymes induced by CDD and MCDD diets (\* = adjusted P value < 0.05, Benjamini – Hochberg analysis). (B) SAdMe levels as measured by MALDI analysis. (\* = P < 0.01 one-way ANOVA with Bonferroni post hoc analysis). (C) Metabolic interactions between methionine cycle, phosphatidylcholine synthesis and DNA methylation. Grey (CDD) and black (MCDD) arrows depict expression changes in each pathway. PE=phosphatidylethanolamine, PC=phosphatidylcholine, SAH=S-adenosylhomocysteine, SAdMe = S-adenosylmethionine. Adapted from Li and Vance 2008.

(*Mthfd1*, *Gnmt*, *Achy*, *Dnmt3b*) were suppressed in both groups. In MCDD mice, there was also marked upregulation of expression of *Mat2a*, an enzyme necessary for the synthesis of SAME from methionine. In the light of these results, we measured SAME concentrations in each group using MALDI-MSI. This demonstrated a significant reduction in SAME in MCDD mice with no change in CDD (Figure 5B). A summary of pathways showing changes in enzyme expression is shown in Figure 5C.

#### Comparison with human NAFLD datasets

We then proceeded to compare our transcriptional findings with large published expression sets of three stages of NAFLD: simple steatosis, NASH and HCC<sup>22-24</sup> (Figure 6). While a number of microarray studies have been performed in NAFLD<sup>31-33</sup> we selected datasets from Ahrens *et al* and Lake *et al* (accession numbers:

GSE48452 and E-MEXP-3291), due to the detailed patient and histological descriptors (including Kleiner NAFLD activity score) confirming NAFLD stage<sup>22,23</sup>. In addition, these datasets include all three NAFLD stages and control samples in the same data series, reducing assay variation, and are directly available from the ArrayExpress repository. This allowed direct comparison of obese subjects with simple steatosis (n=22, NAS score <3), and patients with NASH (n = 24, NAS score 3-5) with well characterised controls (n = 37). Details of subject numbers and arrays used are in Supplementary Table 5. There are no current datasets available from exclusively NAFLD-induced HCC. We therefore used a large dataset of mixed Hepatitis C and alcohol-induced HCC samples (n=228), which are directly compared with cirrhotic liver samples (n=168) (accession number: GSE63898). In this way, we aimed to identify the transcriptional changes associated with HCC malignant



**Figure 6. Cross-species comparison of CDD and MCDD transcriptional changes with human stages of NAFLD.** Venn diagrams showing common and distinct dysregulated gene orthologues between CDD and MCDD livers and human hepatic steatosis (A), human NASH (D) and human HCC (G). Scatter plots demonstrate Log<sub>2</sub> fold change in common dysregulated orthologues between CDD and MCDD mouse livers and corresponding orthologues in human steatosis (B+C), human NASH (E+F) and human HCC (H+I). Trend line, P value and R<sup>2</sup> value calculated by linear regression analysis.

transformation and compared these with our findings in murine methyl donor deficiency.

The CDD and MCDD transcriptomes demonstrated very limited similarity to all stages of human NAFLD. Only 2 (3%) of genes identified as dysregulated in human steatosis were also dysregulated in CDD livers (Figure 6A and B). 26 (40%) of genes identified as dysregulated in human steatosis were also altered in MCDD mice, however changes in expression of the most commonly dysregulated genes were not in the same direction and there was no significant correlation on linear regression analysis ( $P = 0.9$ ) (Figure 6C). There was a greater but still comparatively small overlap in dysregulated gene sets from human NASH studies and CDD and MCDD mice (39 (2.8%) and 143 (10.4%) respectively (Figure 6D). Those transcripts that were dysregulated in NASH and CDD or MCDD mice did demonstrate a weak but significant correlation in terms of directional change ( $P < 0.01$ ,  $R^2 0.150$  for CDD and  $P < 0.001$ ,  $R^2 0.129$  for MCDD) (Figure 6E and F). Upregulated transcripts common to both CDD mice and NASH were almost exclusively involved in inflammatory (*Lgals3*, *Cd52*, *Clec7a*) and malignant processes (*Tm4sf4*, *S100A11*, *Gpnm3*). These genes were also upregulated in MCDD and NASH, in which there were additional changes in fibrosis regulators (*Lum*, *Osbp13*, *Col6a3*, *Tgfb1*, *Tmsb10*, *Tpm1*) and oncogenes (*Golm1* and *Emp1*). Down-regulated genes common to CDD, MCDD and NASH were overrepresented in the GO terms “GO:0006629 Lipid metabolic process” and included the master lipid regulator *Mlxip* and the lipid synthesis enzymes *Acat2*, *Agpat2*, *Lss*, *Acacb* and *Mvd*.

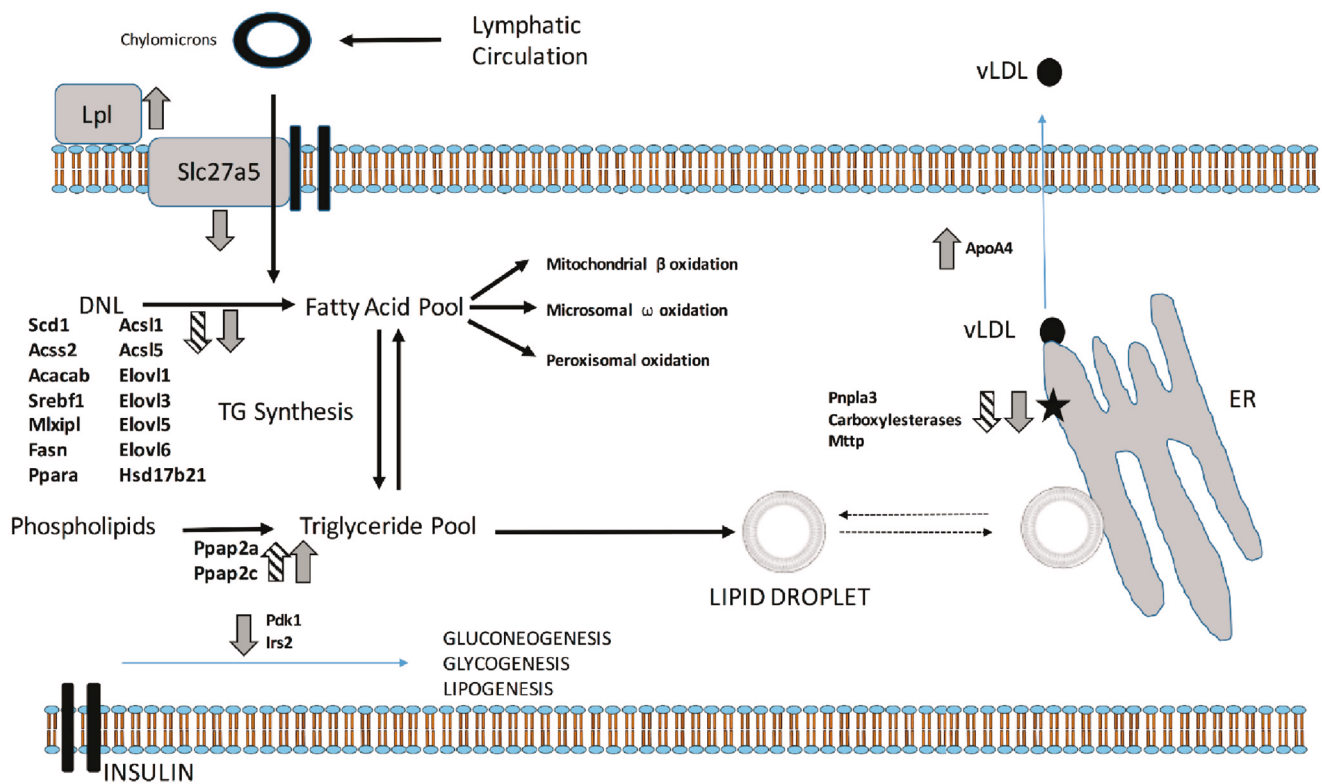
When comparing the transcriptome of each dietary intervention to HCC, there was again only minimal overlap in perturbed transcripts (81 (1.6%) in CDD and 333 (7.6%) in MCDD) (Figure 6G). There was no correlation in terms of directional change between CDD and HCC (Figure 6H). Overlapping transcripts between MCDD mice and HCC did show a weak and highly significant agreement in direction of transcriptional change ( $R^2 0.085$ ,  $P < 0.0001$ , Figure 6I). Genes which were dysregulated in both MCDD and CDD datasets and in HCC were overrepresented in GO terms ‘GO:0000278 mitotic cell cycle’, ‘GO:0007599 Haemostasis’ and ‘GO:0070373 negative regulation of ERK1 and ERK2 cascade’ and include putative HCC oncogenes *Cdc20*, *Osgin1* and *Cdk1*.

## Discussion

It is widely assumed that the steatosis induced by CDD and MCDD results from impaired export of VLDLs, which are required for triglyceride clearance from hepatocytes, perhaps because deficiency of choline and methionine results in an inability to synthesise the major lipid bilayer component phosphatidylcholine (PC) required for VLDL synthesis<sup>30,34</sup>. Our study supports the concept that both decreased PC synthesis and impaired VLDL secretion may play a role in the hepatic pathology in these models and suggest a potential role for the carboxylesterase (Ces) enzymes in mediating the reduction in VLDL secretion.

The importance of reduced hepatic lipid clearance in MCDD is supported by studies demonstrating i) reduced clearance of radiolabelled hepatic fatty acids, ii) a decrease in serum VLDL concentrations and iii) reduced serum triglyceride accumulation in the context of the peripheral lipase inhibitor tyloxalol<sup>17,26</sup>. Additionally, increased hepatic sequestration of radiolabelled fatty acids and increased incorporation of <sup>14</sup>C into hepatic triglycerides suggest that increased lipid uptake and/or increased *de novo* lipogenesis may also occur with MCDD<sup>17,26,34</sup>. These effects have not been reported in rodents exposed to CDD alone<sup>17,35</sup>; indeed *ex vivo* studies using primary hepatocytes isolated from rats maintained on CDD has shown that the presence of methionine is sufficient to maintain normal levels of PC synthesis and VLDL export into culture media<sup>34</sup> and similar experiments in mouse primary hepatocytes demonstrated only a minor reduction in triglyceride export and no change in apolipoprotein secretion in choline deficient media<sup>9</sup>. These findings may be due to the presence of an accessory pathway for PC synthesis which is only present in liver, where in the absence of choline, PC can be directly synthesised from phosphatidylethanolamine (PE) by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) using methionine as a methyl donor. Indeed ~30% of PC is synthesised in this way in rodent liver<sup>36</sup>.

In our study, detailed analysis of the expression of genes in *de novo* lipogenesis pathways in CDD and MCDD strongly suggest an appropriate compensatory response to the high hepatic triglyceride content, with a clear suppression of key mediators of fatty acid synthesis and elongation and cholesterol synthesis (Figure 7). This supports the concept that impaired lipid clearance rather than impaired *de novo* lipogenesis is responsible for the hepatic fat accumulation that occurs with both diets. Consistent with this, the expression of the Ces enzymes (*Ces1d*, *Ces1f*, *Ces3b*) was markedly suppressed in MCDD and the expression of the liver predominant Ces isoform, *Ces3b*, was also suppressed in CDD. These enzymes are important regulators of VLDL lipid packaging and assembly in the hepatic endoplasmic reticulum (ER), and as such a reduction in expression would be expected to result in reduced hepatic lipid clearance<sup>20</sup>. Mice lacking liver specific *Ces3* (also known as triacylglycerol hydrolase) have a reduction in circulating VLDL triglycerides and cholesterol levels on a standard chow diet with altered hepatic lipid droplet morphology<sup>20,37</sup>. Furthermore, *Ces1* overexpression in mice reduces hepatic triglyceride content and plasma glucose levels whereas liver specific knock-down results in increased hepatic triglyceride<sup>19</sup>. Whilst it is unclear why the expression of these genes is suppressed in the presence of an increased hepatic lipid load (notably in MCDD), we suggest that these models may present an opportunity for investigating the mechanism of action of these important hepatic lipid clearance enzymes and the screening of therapeutics that exploit these molecular targets. The expression of Patatin-like phospholipase domain containing 3 (*Pnpla3*) was also suppressed in both CDD and MCDD models. The human PNPLA3<sup>1148M</sup> variant is strongly associated with human NAFLD<sup>38</sup>; humans homozygous for the PNPLA3<sup>1148M</sup> allele are reported to have ~73% more hepatic triglyceride when



**Figure 7. Transcriptional dysregulation mapped to pathways of lipid transport and metabolism in hepatocytes.** Arrows demonstrate direction of transcriptional change in CDD (hatched) and MCDD (grey) diets. *Pnpla3* and *Ces* suppression suggest impaired packaging of lipid into VLDL particles on the surface of the ER (black star), which may represent a novel site of lipid accumulation.

compared with matched heterozygote controls, and both *in vivo* and *in vitro* studies suggest that this is due to impaired triglyceride hydrolysis and VLDL export<sup>39–42</sup>. Thus, *Pnpla3* suppression may also contribute to triglyceride accumulation in methyl donor deficiency.

Dissection of the interacting pathways involved in choline, methionine and PC metabolism in these models provides further insights into the mechanisms by which the murine liver responds to dietary choline and methionine deficiency. Whilst choline has a major role as a substrate for PC synthesis<sup>36</sup>, the essential amino acid methionine is also necessary for the methylation of a large variety of substrates including DNA, proteins and lipids and for the synthesis of polyamines, and it is also crucial for normal hepatocyte function<sup>43</sup>. Both substrates are important for the maintenance of hepatic SAME levels, which are normally tightly regulated to maintain normal hepatic function<sup>15</sup>, and the direction of transcriptional changes with choline and methionine deficiency strongly suggest a drive to maintain hepatic SAME concentrations. The down-regulation of *Chkb* and *Pcyta* which are involved in the synthesis of PC from choline, coupled with the upregulation of methionine adenosyl-transferase (*Mat2a*), which synthesises SAME from methionine, suggest a forward drive to maintain SAME levels. The concurrent down-regulation of *Gnmt* and *Ahcy* (which metabolise SAME and

SAH respectively) may act as a further cellular buffer to maintain SAME concentrations<sup>44</sup>. Nevertheless, despite these changes, we found reduced levels of hepatic SAME in MCDD mice in agreement with other studies<sup>45</sup>, suggesting an inability to maintain SAME levels with severe deficiency of both substrates. Thus, the cumulative effect of the observed transcriptional changes in MCDD mice is directed at maintaining SAME concentrations at the expense of PC synthesis, with the potential to result in decreased VLDL synthesis. Further evidence in support of the importance of SAME deficiency in the pathogenesis of liver disease in MCDD mice is supported by the fact that the deleterious effects of MCDD diets can be rescued by the administration of SAME<sup>46</sup>.

Whilst there are some clear biological similarities between the hepatic pathology induced by methyl donor deficiency in rodents and human NAFLD/NASH<sup>35,47–49,50</sup>, there are also a number of major differences. In humans, NAFLD is closely associated with obesity and insulin resistance, whereas in rodents, CDD results in profound hepatic steatosis without insulin resistance<sup>17,51</sup> and MCDD causes an inflammatory steatohepatitis with fibrogenesis and significant weight loss with an *increase* in peripheral insulin sensitivity<sup>30,52</sup>. Our transcriptomic analysis also suggests that CDD and MCDD produce a hepatic phenotype which is markedly dissimilar to human NAFLD in terms of lipid handling. Whereas human



NAFLD is associated with an upregulation of genes important in *de novo* lipogenesis (FASN, MLPXL, ACACA, SREB-1c)<sup>53,54</sup>, this is either not seen, or indeed the reverse is observed in mice maintained on CDD/MCDD diets. Furthermore, although some findings in human NASH support the concept that NAFLD may result at least in part from an inability to synthesise PC<sup>55,56</sup>, none of the genes dysregulated in the one carbon metabolism pathways of interest in CDD/MCDD were also altered in the human simple steatosis or NASH datasets. Although SAMe depletion is a feature of human NAFLD and correlates with severity of disease in NAFLD biopsies, and oral SAMe preparations are currently under review as a treatment for chronic liver disease<sup>14,16</sup>, mediators of SAMe metabolism were not altered in the human steatosis or NASH datasets. Some enzymes important in SAMe metabolism were dysregulated in the human HCC data (upregulated AHCY (LogFC 0.58); down-regulated BHMT (logFC -1.35), GNMT (LogFC -1.05) and MAT1a (logFC -0.97)), however these did not reflect the changes seen in the mouse model apart from a similar change in the expression of GNMT.

In conclusion, our data suggest a novel alternative mechanism for methyl donor deficient liver injury involving impaired VLDL particle assembly due to suppression of key triglyceride hydrolysis proteins. Although these CDD and MCDD models are widely used for the study of NAFLD, their translational impact in studies of NAFLD/NASH is likely to be limited by fundamental differences in the global transcriptional profiles between these models and human disease states. Our data do suggest that MCDD may be a useful

model for studying the development of HCC secondary to the premalignant inflammatory steatohepatitis NASH. We suggest that there remains an urgent need for novel, more representative models of the full spectrum of NAFLD pathology.

#### Data availability

Data are available on Open Science Framework at DOI [10.17605/OSF.IO/CY5PH](https://doi.org/10.17605/OSF.IO/CY5PH)

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#### Competing interests

No competing interests were disclosed.

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#### Supplementary material

**Supplementary Figure 1: Gene Ontology analysis of CDD and MCDD mouse liver.** Overrepresented pathways of up (black) and down (hatched) dysregulated genes in CDD (A+B) and MCDD (C+D) mouse liver microarray analysis.

[Click here to access the data.](#)

**Supplementary Table 1: Dietary constituents for CDD and MCDD diets.** Mice were maintained on control, Choline deficient (CDD) or methionine and choline deficient (MCDD) diets (Dyets, Bethlem, PA) for 4 weeks.

[Click here to access the data.](#)

**Supplementary Table 2: Primer sequences for qPCR validation.** Candidate genes identified as differentially expressed on the microarray were validated using qPCR.

[Click here to access the data.](#)

**Supplementary Table 3: Top 100 differentially expressed genes in CDD mice versus control animals.**

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**Supplementary Table 4: Top 100 differentially expressed genes in MCDD mice versus control animals.**

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**Supplementary Table 5: Details of subject numbers and arrays used from human studies.**

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We read with interest the paper submitted by Lyall and colleagues. Non-Alcoholic Fatty Liver Disease (NAFLD) is a truly global health issue with significant associated morbidity and mortality, the only currently available therapeutic intervention is weight loss and exercise. The development of new therapeutics is hampered by the lack of animal models that have been validated against human disease for both NAFLD and the associated phenotype; NASH (Non-Alcoholic SteatoHepatitis) and attempts to validate models are most welcome.

The paper described the expected steatosis and fibrosis within both the CDD and MCDD cohorts, which superficially resembles NASH, but as in human disease histo-pathological similarity does not imply common aetiology. The authors rightfully comment on the inherent physiological and phenotypic confounders within the models compared with human NAFLD subjects. NAFLD is principally, albeit not exclusively, characterised by peripheral insulin resistance, obesity and hepatic accumulation of triglycerides. Within the MCDD rodents there was significant weight loss and insulin sensitivity, while the CDD mice whilst developing hepatic steatosis, did not demonstrate insulin resistance.

Within the gene analysis groups there were small numbers interrogated, likely related to the cost of analysis being prohibitive. Within this cohort however; there was marked upregulation of “immune system process” and “inflammatory process” with significant concordance between both groups.

Interestingly, dominant pathways of hepatic fatty acid fate were relatively unperturbed; although there is invariably some discordance between murine models and humans, particularly related to peroxisome metabolism.

Whilst the study attempted to use closely modelled comparators; there were difficult variables with which the authors had to contend. This relates in particular to the HCC cohort; which comprised of composite HCC-related to HCV and ALD, which while similar have understated intrinsic differences. The CDD and MCDD transcriptomes grossly demonstrated very little relation to human NAFLD. Of the genes that bore commonality, the expression profiles were often oppositional and of no statistical significance. The observation of changes in PNPLA3 expression, which is the gene most strongly associated with Human disease, should not be taken as evidence of similarity between the models and human disease, the activity of PNPLA3 is not specific to NAFLD but important in regulating lipid handling and appears in other liver diseases to have an impact on adaptive responses to oxidative stress

It is difficult to extrapolate the findings in relation to s-adenosylmethionine (SAdMe). Recent work has suggest amelioration of steatotic features within certain murine models but it is interesting that there was no metabolic variability in SAdMe mediators. Certainly the work presents a novel mechanism for methyl donor deficient liver injury in a murine model affecting impaired VLDL particulate assembly as a consequence of suppressed triglyceride hydrolysis proteins. It does not however; bear significant transcriptional resemblance to human NAFLD or HCC.

This study does definitively demonstrate that whilst there are common histological features between murine

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MCDD, CDD and human NAFLD; there are obviously inherent differences in how these pertain to transcriptomic expression profiles. This would of course suggest that there are conceivably alternative pathways which produce identical histological outcomes. Finally, the paper seeks to raise important concerns about the limitations of both CDD and MCDD models in replicating human NAFLD and efforts should continue to focus on developing more accurate models for future studies to utilise.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

*Competing Interests:* No competing interests were disclosed.

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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