Adverse Maternal Environment and Postweaning Western Diet Alter Hepatic CD36 Expression and Methylation Concurrently with Nonalcoholic Fatty Liver Disease in Mouse Offspring

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ABSTRACT

Background: The role of an adverse maternal environment (AME) in conjunction with a postweaning Western diet (WD) in the development of nonalcoholic fatty liver disease (NAFLD) in adult offspring has not been explored. Likewise, the molecular mechanisms associated with AME-induced NAFLD have not been studied. The fatty acid translocase or cluster of differentiation 36 (CD36) has been implicated to play a causal role in the pathogenesis of WD-induced steatosis. However, it is unknown if CD36 plays a role in AME-induced NAFLD.

Objective: This study was designed to evaluate the isolated and additive impact of AME and postweaning WD on the expression and DNA methylation of hepatic Cd36 in association with the development of NAFLD in a novel mouse model.

Methods: AME constituted maternal WD and maternal stress, whereas the control (Con) group had neither. Female C57BL/6J mice were fed a WD (40% fat energy, 29.1% sucrose energy, and 0.15% cholesterol [wt/wt]) 5 wk prior to pregnancy and throughout lactation. Non invasive variable stressors (random frequent cage changing, limited bedding, novel object, etc.) were applied to WD dams during the last third of pregnancy to produce an AME. Con dams consumed the control diet (CD) (10% fat energy, no sucrose or cholesterol) and were not exposed to stress. Male offspring were weaned onto either CD or WD, creating 4 experimental groups: Con-CD, Con-WD, AME-CD, and AME-WD, and evaluated for metabolic and molecular parameters at 120 d of age.

Results: AME and postweaning WD independently and additively increased the development of hepatic steatosis in adult male offspring. AME and WD independently and additively upregulated hepatic CD36 protein and mRNA expression and hypomethylated promoters 2 and 3 of the Cd36 gene.

Conclusions: Using a mouse AME model together with postweaning WD, this study demonstrates a role for CD36 in AME-induced NAFLD in offspring and reveals 2 regions of environmentally induced epigenetic heterogeneity within Cd36. J Nutr 2021;151:3102–3112.

Keywords: NAFLD, CD36, perinatal, methylation, DNA, maternal environment

Introduction

The incidence of NAFLD is steadily increasing and occurs in ~30% of Americans (1–3). Consumption of a high-fat diet (HFD) or Western diet (WD) contributes to the development of NAFLD. Interestingly, the incidence of adult NAFLD also increases following exposure to an adverse maternal environment (AME) (4–9). It is unclear if the development of NAFLD following an AME occurs via a similar mechanism to diet-related NAFLD.

The development of NAFLD following the consumption of HFD or WD occurs in part through the hepatic uptake of fatty acids in excess of metabolic requirements. Excess fatty acids are then stored inside hepatocytes and result in steatosis (10). Increasing numbers of studies have shown the role of the fatty acid translocase or cluster of differentiation 36 (CD36) in
driving the onset of hepatic steatosis (4, 11, 12). CD36 plays a key role in increasing fatty acid uptake in hepatocytes and other cell types in the liver (10, 13, 14).

CD36 is a member of the class B scavenger receptor family and mediates long-chain fatty acid uptake in many tissues (15). CD36 also regulates lipid homeostasis in many cell types through multiple mechanisms, including regulation of fatty acid oxidation (16), VLDL secretion (17), and lipophagy (18). CD36 expression is low in hepatocytes but increases with lipid-rich diets and in NAFLD in both human and mouse models (19–23). A liver-specific Cd36 knockout attenuates steatosis in mouse models of NAFLD (4, 12). Overexpression of hepatic CD36 resulted in a marked increase in hepatic fatty acid uptake and storage in both primary cultured hepatocytes and mouse livers (11). These indicate that CD36 expression has significant effects on liver lipid content and plays a causal role in the pathogenesis of NAFLD.

Multiple groups have studied the impact of maternal HFD or WD on the development of NAFLD in offspring in animal models (24–30). The offspring from these models developed NAFLD when fed a postweaning HFD or WD (5–9, 31). Despite this appreciation for the maternal and dietary impact on NAFLD, none of these studies ascertained if CD36 plays a role in AME-induced NAFLD.

To identify a mechanism through which relevant environmental stimuli alter CD36 expression, we have generated a novel AME model by combining long-term maternal WD and maternal stress. This combination of diet and stress was thoughtfully developed to model 2 of the most common environmental pressures during pregnancy as well as important factors for the development of NAFLD. Importantly, these factors are also modifiable and translatable from animals to humans.

Epigenetics, such as DNA methylation, provide insights into the mechanisms of early life predisposition to adult disease risk (32, 33). Understanding these epigenetic changes is important not only to understanding the mechanisms at play, but may also provide a molecular marker of potential disease. We hypothesized that, like HFD, AME induction of NAFLD will increase CD36 expression and that the addition of a postweaning WD will further increase CD36 expression and NAFLD. Additionally, AME-driven increase in CD36 will be accompanied by decreased methylation at key regulatory regions of Cd36.

Methods

Animals

All animal procedures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. This mouse model of AME has been previously published by our group (34). Briefly, Female C57BL/6j mice were maintained with standard light cycles at a constant temperature of 23°C with food and water available ad libitum. The mice were randomly allocated to either an experimental WD or a control diet (CD) (Table 1 and Supplemental Table 1). The WD (D12079B, Research Diets, New Brunswick, NJ) has been previously published by other groups (35). It contained increased fat (40% compared with 10% in kcal), sucrose (29.1% compared with 0% in kcal), and cholesterol [0.15% compared with 0% (wt/wt)] compared with the CD (D14020502, Research Diets). The diets were provided for 5 wk before the females were mated with males receiving Laboratory Rodent Diet (SLOD, LabDiet), throughout pregnancy, and during lactation.

Dams fed the WD experienced a “stressed” environment during the last third of pregnancy. The combination of chronic WD and gestational stress is designated as AME (34). Briefly, the stressed environment consisted of daily random environmental changes as well as a static change in the maternal environment consisting of one-third of the standard amount of bedding from embryonic day (E)13 to E19. The daily random environmental changes included altered light cycles on 3 nonconsecutive d, 3 repeat cage changes throughout the day on E15, and the short-term introduction of a novel object in the cage for a day. Control (Con) dams consumed CD and were not exposed to stress (34). Food intake in dams was recorded from E6 through E19. At the end of pregnancy, 6 pregnant female mice from both Con and AME groups were anesthetized with isoflurane and killed by decapitation after 5 h of fasting. Trunk blood and serum were collected for the measurement of corticosterone. On postnatal day 5, litters were randomly culled to 6, 3 of each sex. Male offspring from both the Con and AME dams were weaned onto either the CD or WD, generating 4 experimental groups: Con-CD, Con-WD, AME-CD, and AME-WD (34). At the age of 120 d (n = 6 litters of each of the 4 groups, respectively) mice were anesthetized with isoflurane and killed by decapitation after 5 h of fasting. Trunk blood and serum were collected. Fasting glucose was measured using a OneTouch Verio IQ meter (LifeScan Inc.). Retroperitoneal fat pads and livers were quickly removed, and the wet weights were recorded. A piece of the right medial lobe of the liver was fixed in 10% formalin, the remaining liver was flash frozen in liquid nitrogen and stored at −80°C.

Serum concentrations of corticosterone, insulin, and alanine transaminase

Dam serum concentrations of corticosterone were measured by using a corticosterone enzyme immunoassay kit (Cat# K014-H1, Arbor assays) per the manufacturer’s instructions.

Serum insulin was measured via the ELISA method using a Rat/Mouse Insulin ELISA kit (EMD Millipore) according to manufacturer’s recommendations. HOMA-IR is widely used clinically for the diagnosis of human insulin resistance (IR). It has been validated in rats (36–38). It has been used as a diagnostic criterion for IR in mice studies, although it has not been validated in mice (39–42). HOMA-IR was calculated by using fasting insulin and fasting glucose. In the following equation: HOMA-IR = fasting plasma insulin (in milliunits per liter) × fasting glucose (in millimoles per liter)/22.5 (43).

Serum ALT was measured by using EnzyChrom Alan Transaminase Assay Kit (BioAssay Systems) per the manufacturer’s recommendations.

Glucose tolerance test (GTT)

The glucose tolerance test (GTT) was performed at week 14 of life after 6 h of fasting. Fasting glucose (time 0) was measured followed by intraperitoneal injection of glucose 60 mg in 20% (wt/vol) solution. The fixed dose of glucose was calculated as 2 g/kg of mean body weight of the Con-CD mice, based on the recommendations of the NIH Mouse Metabolic Phenotyping Center Consortium (44, 45). Glucose concentrations at 15, 30, 60, 90, and 120 min after glucose injection were measured using a OneTouch Verio IQ meter. Serum insulin was measured using ELISA at 60 min after glucose injection, as aforementioned.
Hepatic histology
Livers were fixed in 10% formalin and paraffin embedded. Slices (4 μm) were stained by hematoxylin and cosin and Masson’s trichrome. The NAFLD activity score (NAS) was proposed by the NIDDK-sponsored NASH Clinical Research Network and has been widely used for clinical trials in patients since its validation (46). NAS was evaluated by a pathologist blinded to the experimental groups by using the Kleiner scoring system (46). The score is defined as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2). A minimum of 5% steatosis (steatosis score of 1) was used for the operational minimal definition of histological NAFLD. NAS scores of 1–2 were largely considered mild NAFLD and of 3–4 as moderate NAFLD. An NAS score of ≥5 is interpreted as nonalcoholic steatohepatitis (NASH). A score of <2 was diagnosed as “not NAFLD” (46). The score was based on value differences between the target and Hprt control using the comparative Ct method.

Membrane protein extraction and immunoblotting
Liver membrane protein was extracted using a Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific) and quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific).

Immunohistochemistry
Immunohistochemistry (IHC) analysis and staining were performed on formalin-fixed paraffin-embedded (FFPE) sections of mouse livers using the Bond RX autostainer platform (Leica Biosystems Inc.) in the Children’s Research Institute Histology Core in Medical College of Wisconsin. Deparaffinized sections underwent heat-induced epitope retrieval (Cat. No. S1699, pH 6.0, Agilent Dako) at 100°C for 20 min. The primary antibody was anti-CD36 rabbit monoclonal antibody (Cat. No. ab133625, abcam) at 1:100 dilution. Bound antibody was detected with biotinylated donkey anti-rabbit IgG (711-066-152, Jackson ImmunoResearch Laboratories, Inc.), streptavidin-HRP (P039701-2, Agilent Dako), and a DAB kit (K346811-2, Agilent Dako).

**TABLE 1** Compositions of experimental diets

| Ingredients          | CD   | kcal | WD   | kcal |
|----------------------|------|------|------|------|
| Casein               | 195  | 780  | 195  | 780  |
| DL-methionine        | 3    | 12   | 3    | 12   |
| Corn starch          | 695  | 2780 | 50   | 200  |
| Maltodextrin 10      | 150  | 600  | 100  | 400  |
| Sucrose              | 0    | 0    | 341  | 1364 |
| Milk fat, anhydrous  | 35.5 | 320  | 200  | 1800 |
| Corn oil             | 17   | 153  | 10   | 90   |
| Cellulose, BW200     | 50   | 0    | 50   | 0    |
| Mineral mix S10001   | 35   | 0    | 35   | 0    |
| Calcium carbonate    | 4    | 0    | 4    | 0    |
| Vitamin mix V10001   | 10   | 40   | 10   | 40   |
| Choline bitartrate   | 2    | 0    | 2    | 0    |
| Cholesterol          | 0    | 0    | 1.5  | 0    |
| Total                | 1196.6| 4685 | 1001.5| 4686 |
| Protein, %           | 17   | 17   | 20   | 17   |
| Carbohydrate, %      | 71   | 73   | 50   | 43   |
| Fat, %               | 4    | 10   | 21   | 40   |
| kcal/g               | 3.9  | 4.7  |      |      |

CD, control diet (D14020502, Research Diets); WD, Western diet (D12079B, Research Diets).
A negative control was prepared for all tissue samples by omitting the primary antibody. Macrophages, which strongly express CD36, provided an internal positive control (48).

**Bisulfite pyrosequencing**

Liver tissue was ground in liquid nitrogen and used for genomic DNA isolation with the DNeasy Blood & Tissue Kit (Qiagen) including RNAse treatment. DNA quantity and purity were estimated spectrophotometrically. Briefly, spectrophotometry was done on a Biotek Cytation Hybrid Multi-Mode Reader with the Take 3 micro-volume plate (Biotek) following the manufacturer’s instructions. The ratio of absorbance at 260 and 280 nm (A260/280) > 1.8 is considered “good purity.”

Bisulfite treatment of genomic DNA was performed using an EpiTect Bisulfite Kit (Qiagen) as instructed in the manual. For each PCR, bisulfite-treated DNA equivalent to 20 ng of the DNA prior to bisulfite treatment was used.

Primers for PCR and sequencing were designed by using PyroMark Assay Design 2.0 software (Qiagen). Four amplions around the 3 promoters were studied (Supplemental Table 3). The amplicons of Promoters 2 and 3 are in regulatory regions indicated by chromatin features of enhancer, promoter, promoter flank, and DNase I hypersensitive site (DHS) provided by Ensembl Regulatory Build (Ensembl.org) (49). Two CpG sites immediately upstream of the transcription start site (TSS) were chosen to be studied because the promoter 1 region does not have obvious DHS regions based on Ensembl.org. Three primer sets were also designed around regions in the intron 1 around −31 kb, −27.3 kb, and −13.4 kb upstream of exon 2 TSS because of the presence of regulatory chromatin features based on Ensembl.org (Supplemental Table 3). PCR conditions were 95°C for 10 min, followed by 50 cycles of 94°C for 30 s, primer-specific annealing temperature for 30 s, and 72°C for 30 s.

To avoid PCR artifacts which generate bias on the methylation status of CpG sites (50), annealing temperature was optimized using mouse methylated genomic DNA standards (EpigenDx, Inc.) as the DNA template. An annealing temperature that generated the percentage of CpG methylation closest to the known percentage of methylation in the methylation standard DNA was used for final experiments. Pyrosequencing was performed using Q48 Aвуropр (Qiagen) in conjunction with PyroMark Q48 Advanced CpG Reagents (Qiagen) and PyroMark Q48 Magnetic Beads (Qiagen). Results were analyzed using Pyromark Q48 Aвуropр software (Qiagen).

**Laser capture microdissection followed by bisulfite pyrosequencing**

Laser capture microdissection was performed using a PALM MicroBeam system (Zeiss Microscopy) with PALM Robo 3.2 software on a 10μm thickness of anti-CD36 IHC sections from the Con-CD group. Both CD36-positive (CD36+) and -negative (CD36−) hepatocytes were dissected. An EpiTect Fast FFPE Bisulfite Kit (Qiagen) was used for deparaffinization, proteinase digestion, reverse crosslinking, and bisulfite conversion as instructed by the manufacturer’s manual. PCR and pyrosequencing were performed as aforementioned.

**Statistics**

All analyses were performed using GraphPad Prism 8 software (GraphPad Software). The diagnosis of no, mild, or moderate NAFLD (based on the NAS score as described in the histology Method section) was analyzed using the χ2 test. To determine the additive effect of AME and postweaning WD on the development of steatosis, a suitable test with 2 categorical variables—the degree of insulin [single insult (Con-CD and AME-WD combined) compared with double insults (AME-WD)] and the severity of steatosis—was created (51). Other data were analyzed for normality or lognormality first by using a Shapiro-Willik normality test and Kolmogorov-Smirnov normality test with Dalli-Wilkinson-Lillie for the P value. All data except the serum ALT and food intake were normally distributed. Serum ALT was therefore analyzed by using a Kruskal-Wallis test followed by Dunn’s multiple comparisons test to adjust the P value. Food mass and calorie intakes were analyzed using a Mann-Whitney test. The DNA methylation in the CD36+ and CD36− hepatocytes was analyzed by using unpaired multiple t tests, followed by false discovery rate (FDR) testing by using the 2-stage step-up method of Benjamini et al. with desired FDR q < 0.05 (52). All other data from the 4 groups were analyzed by using 2-way ANOVA to assess main and interaction effects, with diet and maternal environment as independent variables. When a significant main or interaction effect was detected, Bonferroni post hoc testing was used to identify the means that differ. When P-interaction was significant, a synergetic effect was reported. When significant P values of both AME-WD compared with AME-CD and AME-WD compared with Con-WD were found, but P-interaction was not significant, an additive effect was reported (51, 53).

All data except the NAFLD diagnosis data were means ± SDs. The level of significance was set at P < 0.05 for all statistical tests.

**Results**

**AME dams had elevated serum corticosterone concentrations**

AME dams had significantly higher serum concentrations of corticosterone compared with Con dams at the end of pregnancy (Table 2). Serum corticosterone concentrations did not differ between the AME and Con offspring at birth, at weaning (data not shown), or at day 120 (34).

Consistently, the dams’ food intake also indicated the impact of stress on AME dams. Stress significantly decreased food mass intake in the AME dams compared with Con dams during E13–E19 when stress was implemented (Table 2).

**AME-WD significantly elevated liver weight in offspring**

As our group has recently reported, all mice weaned onto the WD were significantly heavier than those weaned onto the CD, regardless of the maternal environments. This body weight difference was consistent with the significantly greater energy intake in the 2 WD groups compared with the 2 CD groups (34).

The intake of diet mass was not different between the 4 groups (data not shown).

At day 120, both AME and postweaning WD significantly increased the ratio of retroperitoneal fat weight to body weight (P < 0.0001 and P = 0.0009; P-interaction ≥ 0.05) (Table 3). More importantly, postweaning WD augmented the AME effect on the increase of the ratio of liver to body weight (P-interaction = 0.0048) (Table 3).

**AME-WD offspring developed IR**

Neither postweaning WD nor AME affected fasting glucose (Table 3). However, WD and AME synergistically elevated both fasting insulin and HOMA-IR (P-interaction < 0.0001 and = 0.0013, respectively) (Table 3), indicating that postweaning WD exacerbated the predisposition of AME upon the development of IR in offspring.

These results were further supported by GTT. Only WD had a significant effect on the increase of blood glucose AUC during the GTT (P = 0.0005) (Table 3). In contrast, both WD and AME had significant effects on the increase of insulin concentrations 60 min after glucose injection (P = 0.0025 and 0.0011, respectively) (Table 3). A trend of significant interaction between WD and AME was observed for both the glucose AUC (P-interaction = 0.0676) and 60-min insulin concentrations (P-interaction = 0.051), indicating that AME induced the offspring a greater predisposition to slower glucose clearance.

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Taken together, these data indicate that AME-WD offspring developed IR at day 120 of age.

**AME-WD offspring developed hepatic steatosis**
In male offspring, postweaning WD alone (Con-WD) and AME alone (AME-CD) developed similar levels of mild steatosis (Figure 1A and B). These interesting results suggest that an adverse maternal environment has a long-term effect of steatosis, which is similar to the effect of an adult WD (Con-WD). More importantly, AME and postweaning WD had an additive effect on the development of steatosis, e.g., the AME-WD group had moderate but significantly more severe steatosis than the 2 single-insult groups combined (AME-CD and Con-WD) ($P < 0.0001$) (Figure 1A and B). There was no fibrosis in any specimens, confirmed by Trichrome staining (result not shown). Out of all the liver sections of the 4 groups evaluated, only 1 liver in the AME-WD group had lobular inflammation of the lowest level, which contributed to an NAS score of 1 to the sample. Additionally, AME-WD had elevated serum ALT of the lowest level, which contributed to an NAS score of 1 to the sample. Additionally, AME-WD had elevated serum ALT concentrations compared with the corresponding control group (AME-CD) ($P = 0.0021$) (Figure 1C). This result indicates that postweaning WD exacerbated the effect of the maternal insults on hepatic steatosis.

**AME and WD additively elevated hepatic Cd36 mRNA expression**
Among the genes important for fatty acid transport, oxidation, and synthesis, Cd36 mRNA expression showed a distinctive expression pattern between the 4 experimental groups (Figure 2 and Supplemental Table 4). Both WD and AME had significant effects on the increase of Cd36 mRNA expression ($P = 0.0005$ and 0.0002, respectively). They additionally further increased Cd36 mRNA expression compared with WD alone ($P = 0.0022$) and the AME alone group ($P = 0.0043$) ($P$-interaction $= 0.122$) (Figure 2A and Supplemental Table 4).

We have also quantified the mRNA for genes related to hepatic inflammation, including Mcp1, Il1b, and Tgfβ1. None of them revealed significant differences between groups (Supplemental Table 4), which is consistent with the histological findings.

**AME and WD additively elevated hepatic Cd36 protein expression**
Mirroring the pattern of hepatic Cd36 mRNA expression, postweaning WD and AME additively increased hepatic Cd36 protein expression ($P$-interaction $= 0.1731$) (Figure 3A).

IHC staining for Cd36 revealed a distinct lobular distribution pattern of Cd36$^+$ hepatocytes in sections from the Con-CD, Con-WD, and AME-CD groups. The Cd36$^+$ hepatocytes were found to be in locations surrounding the central veins, whereas periportal hepatocytes were negative or only lightly positive for Cd36. In contrast, in the AME-WD sections, the pattern of hepatocellular positivity for Cd36 expanded to encroach upon perportal regions (Figure 3B).

**AME-WD elevated hepatic Cd36 mRNA variants expression**
Multiple mRNA variants of Cd36 have been recorded in Ensembl.org (54), with transcription started from promoter 1, 2, or 3 (Supplemental Figure 1). All the mRNA variants are translated into the same protein product.

Mirroring the expression pattern of Cd36 total mRNA, both WD and AME had significant effects on the increase of expression of the P2 and P3 transcripts ($P$ values $< 0.05$)

### Table 2: Serum corticosterone concentrations and food intake in Con and AME dams

| Dam group | Con          | AME          | $P$ value |
|-----------|--------------|--------------|-----------|
| Serum corticosterone, ng/ml | 32.7 ± 19.4 | 80.4 ± 50.6 | 0.0462    |
| Food intake, g/d | E6–E12 4.08 ± 0.452 | 4.93 ± 1.51 | 0.315 |
| E13–E19 4.76 ± 0.539 | 3.71 ± 0.440 | 0.0009 |
| Energy intake, kcal/d | E6–E12 15.9 ± 1.76 | 23.2 ± 7.10 | 0.0003 |
| E13–E19 18.6 ± 2.10 | 17.4 ± 2.07 | 0.397 |

**Table 3:** Measurement of fat, liver, and insulin resistance parameters in day 120 male offspring of Con and AME dams fed postweaning CD or WD

| Maternal group | Con | AME | $P$ value |
|----------------|-----|-----|-----------|
| Postweaning diet | CD | WD | CD | WD | Diet | Maternal environment | $D \times M$ |
| Retinoneoneal fat weight, g/100 g BW | 0.006 ± 0.001 | 0.011 ± 0.002 | 0.007 ± 0.002 | 0.013 ± 0.002 | $<0.0001$ | 0.0009 | 0.815 |
| Liver weight, g/100 g BW | 0.036 ± 0.002 | 0.038 ± 0.002 | 0.035 ± 0.002 | 0.049 ± 0.008 | $<0.0001$ | 0.0003 | 0.0078 | 0.0048 |
| Fasting glucose, mmol/L | 9.59 ± 1.38 | 10.2 ± 1.70 | 10.3 ± 1.01 | 11.2 ± 1.98 | 0.198 | 0.266 | 0.88 |
| Fasting insulin, ng/mL | 3.41 ± 1.80 | 2.70 ± 1.24 | 2.89 ± 0.92 | 2.09 ± 1.19 | 0.0004 | 0.0008 | $<0.0001$ | 0.0004 | 0.0008 | 0.0008 |
| HOMA-IR | 29.6 ± 14.1 | 71.5 ± 15.2 | 33.8 ± 18.2 | 125 ± 52 | $<0.0001$ | 0.0108 | 0.0271 |
| AUC glucose in GTT, mol/L-120 min | 1.65 ± 0.142 | 2.74 ± 0.483 | 2.28 ± 0.423 | 2.70 ± 0.412 | 0.0005 | 0.107 | 0.0676 |
| AUC insulin 60 min post glucose injection, ng/mL | 3.34 ± 0.389 | 3.62 ± 1.32 | 3.94 ± 0.881 | 5.95 ± 1.33 | 0.0025 | 0.011 | 0.051 |

Data are means ± SDs. $n = 8$. AME, adverse maternal environment; Con, maternal control group; E, embryonic day. 

1Different from corresponding postweaning CD group, $P < 0.05$.
2When the diet × maternal environment interaction was significant, only values with differing superscripts are significant, $P < 0.05$. Values that share a superscript are not significant.

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Liver histology (A), diagnosis of hepatic steatosis (B), and serum ALT (C) in d120 mice from the Con and AME maternal groups and on the postweaning diet of CD or WD. Representative images were H&E-stained liver sections from each group and are shown as 100× (large images) and 200× (small images) magnifications (A). Values are number of mice diagnosed with varying degrees of hepatic steatosis based on the evaluation results of NAFLD activity scoring. Labeled groups without a common letter differ, \( P < 0.05 \) (B). Values are means ± SDs. \( n = 6 \). *Different from corresponding postweaning CD group, \( P < 0.05 \) (C). ALT, alanine transaminase; AME, adverse maternal environment; CD, control diet; Con, control; H&E, hematoxylin and eosin; WD, Western diet.

(Figure 3C). Furthermore, AME-WD offspring had significantly higher expression of the P2 and P3 transcripts compared with that of Con-WD and AME-CD (\( P \) values < 0.05), but no significant interaction between WD and AME was found (\( P \)-interactions ≥ 0.05). Promoter 1 transcripts were expressed very low and unaltered by either AME or WD. These findings in mRNA variants indicate that WD and AME activated promoters 2 and 3 to increase transcription initiated from P2 and P3. Together with the altered distribution pattern of CD36+ hepatocytes (Figure 3B) in the AME-WD livers, we speculated that AME-WD had DNA hypomethylation in promoters 2 and 3, but not in promoter 1 region.

**AME or WD decreased DNA methylation around Cd36 promoters 2 and 3**

As expected, both WD and AME had significant effects on the DNA hypomethylation across all the studied CpG sites on both promoters 2 and 3 (\( P \) values < 0.05) (Figure 4A). Importantly, AME-CD was significantly hypomethylated compared with Con-CD in 1 of 2 CpG sites in P2 and 4 of 6 CpG sites in P3 (Figure 4A), indicating AME alone resulted in DNA hypomethylation in Cd36 promoters 2 and 3 in the offspring. This AME effect on promoter hypomethylation was to a similar extent to that of postweaning WD (\( P \) values ≥ 0.05 across all the CpG sites between AME-CD and Con-WD). AME-WD further decreased DNA methylation in 2 of 2 CpG sites of P2 and 4 of 6 CpG sites of P3 compared with the 2 corresponding control groups (Con-WD and AME-CD) (\( P \) values < 0.05) (Figure 4A), indicating postweaning WD and AME had additive effects on Cd36 promoter hypomethylation (\( P \)-interaction ≥ 0.05).

These effects of WD and AME on DNA hypomethylation on P2 and P3 of Cd36 are locus specific because neither WD nor AME had altered CpG methylation in the P1 region (Figure 4A) and the intron 1 region (Supplemental Figure 2A).

Promoters 2 and 3 were hypomethylated in CD36+ hepatocytes, vice versa.

To verify if the P2 and P3 regions studied were critical for the Cd36 promoter activity, we quantified DNA methylation of the laser-capture microdissected CD36+ and CD36- hepatocytes. We expected high methylation in the CD36- cells but low methylation in the CD36+ cells if the CpG sites are critical to the activity of the corresponding promoters (49, 55).
**Discussion**

The primary finding of this study is that an adverse maternal environment together with postweaning WD results in hepatic steatosis in adult male offspring. In this model the severity of steatosis increases in parallel with elevated hepatic CD36 mRNA and protein expression, altered lobular distribution pattern of CD36-expressing hepatocytes, and perturbed intercellular epigenetic heterogeneity of CD36 promoter methylation. Additionally, we found that adverse maternal environment alone predisposes the offspring to mild hepatic steatosis concurrent with DNA hypomethylation on the promoters 2 and 3 of CD36 and this predisposition is not altered by a low-fat postweaning diet. These findings indicate that adverse maternal environment set the epigenetic background of the offspring’s hepatic CD36 gene to effectively place the offspring at greater risk for NAFLD when experiencing the ever-common WD later in life.

WD alone has been associated with the increased risk of adult NAFLD in human (56, 57) and animal models (24–30). Additionally, maternal HFD together with HFD in offspring has also been found to result in the onset of NAFLD in offspring (5–9, 31). However, the role of the postweaning diet following maternal stress and diet-induced AME on the development of NAFLD has not been explored. Likewise, the molecular changes associated with AME-induced NAFLD have, to our knowledge, not been explored previously.

Our data demonstrate that an adverse maternal environment coupled with a postweaning WD have an additive effect and cause the development of hepatic steatosis. Importantly, dietary intervention of a low-fat control diet following weaning does not fully correct the development of steatosis. In fact, mice exposed to AME alone develop an extent of steatosis similar to that of mice on a postweaning WD alone. These findings indicate the power and lasting effect of an adverse early life environment.

The relation of CD36 mRNA and protein expression and adult hepatic steatosis has been reported in both human and animal studies (19–21, 58). Upregulation of CD36 membrane protein is positively correlated with hepatic steatosis (11, 20, 59) and elevates cellular uptake of fatty acids (11, 59). Inhibition of CD36 mRNA (60) and protein ablated the accumulation of lipid in vitro and in vivo (60, 61). Liver-specific CD36 knockout attenuates steatosis in mouse models of NAFLD (4, 12). We report that AME alone upregulated hepatic CD36 mRNA expression, which is correlated with the presence of mild steatosis. More importantly, this AME-upregulation of CD36 mRNA is correlated with the hypomethylation of CD36 promoter regions.

The differentially methylated CpG sites in promoters 2 and 3 of CD36 found in this study may be important loci for regulating CD36 transcription. Both promoters 2 and 3 have been reported to have promoter activities in mice and humans (the CD36 gene is conserved between mouse and human) (4, 62, 63). The promoter 2 CpG sites examined here are immediately upstream of the active promoter reported by Sato, et al. (63). The CpG sites we have studied in promoter 3 region are in proximity of the binding sites of well-known steatosis-promoting transcription factors, including liver X receptor, progamine X receptor, and PPARγ (4). Studies have shown that these transcription factors promote hepatic steatosis through CD36 activation (4, 64, 65). Importantly, we have revealed 2 regions of environmentally induced epigenetic heterogeneity within CD36. Our findings of environmentally driven DNA hypomethylation of hepatic CD36 coupled with increased expression and severity of steatosis are
important for the molecular understanding of AME-related NAFLD.

Caution is always necessary when translating the findings of a mouse model to human pathophysiology. We acknowledge that although C57BL/6J mice are useful to study diet-induced obesity and IR, they are not the ideal animal model to investigate the role of inflammation in the development of hepatic steatosis. No single animal model can completely define a human condition considering the diversity of genetics and experience that are intrinsic to humanity.

NAFLD is a pathological state that is attributed to imbalance of multiple factors, including fatty acid influx, fatty acid oxidation, de novo lipogenesis, and triglyceride secretion. This study focuses on the outcome of AME and postweaning WD on CD36 expression and its epigenetic characteristics in offspring. Further studies are needed to understand the pathways by which these environmental conditions mediate changes in CD36 methylation.

CD36 has complex functions across multiple tissues. This complexity attributes to a broad range of ligands it interacts with. Therefore, protein interactions and post-transcriptional regulation are also very important for further elucidating the role of CD36 in the pathogenesis of NAFLD.

We recognize that further pursuit of mechanisms affecting the relation between promoters 2 and 3 methylation in Cd36 and hepatic steatosis is necessary. We also recognize that we do not know whether AME in conjunction with WD induced similar molecular and phenotypical changes in female offspring, who do not suffer as severe hepatic steatosis relative to the male.
counterparts. Further studies would be necessary to elucidate sex differences in the future.

NAFLD is a common disease with a growing worldwide prevalence and burden. Understanding how environmental factors like adverse maternal environments contribute to the disease is of increasing importance. Additionally, identifying molecular targets, such as changes in Cd36 expression and methylation, is critical in the path to ameliorate disease burden.

Acknowledgments
The authors’ responsibilities were as follows—AVM, RHL, and QF: designed research; QF, AVM, XK, KAF, and YWH: conducted research; PEN: evaluated NAFLD activity score; QF and AVM: analyzed and interpreted data; QF and AVM: drafted the article; AVM and RHL: critically revised the article; and all authors read and approved the final manuscript.

FIGURE 4 DNA CpG methylation of Cd36 promoters 1, 2, and 3 regions in livers of d120 mouse from the Con and AME maternal groups and on the postweaning diet of CD or WD (n = 6) (A) and in laser-capture micro-dissected CD36− and CD36+ hepatocytes (n = 3–4) (B). The negative number below each CpG site indicates the number of base pair upstream relative to the transcription start site of the corresponding exon, respectively. Values are means ± SDs. *Different from corresponding postweaning CD group, \( P < 0.05 \); $Different from corresponding maternal Con group, \( P < 0.05 \); \#Different from maternal environment interaction was not significant, \( P > 0.05 \); $Different between CD36− and CD36+ hepatocytes, \( P < 0.05 \). AME, adverse maternal environment; CD, control diet; Con, control group; WD, western diet.

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