



Gene expression and DNA methylation as mechanisms of disturbed metabolism in offspring after exposure to a prenatal HF diet^S

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Abstract Exposure to a prenatal high-fat (HF) diet leads to an impaired metabolic phenotype in mouse offspring. The underlying mechanisms, however, are not yet fully understood. Therefore, this study investigated whether the impaired metabolic phenotype may be mediated through altered hepatic DNA methylation and gene expression. We showed that exposure to a prenatal HF diet altered the offspring's hepatic gene expression of pathways involved in lipid synthesis and uptake (SREBP), oxidative stress response [nuclear factor (erythroid-derived 2)-like 2 (Nrf2)], and cell proliferation. The downregulation of the SREBP pathway related to previously reported decreased hepatic lipid uptake and postprandial hypertriglyceridemia in the offspring exposed to the prenatal HF diet. The upregulation of the Nrf2 pathway was associated with increased oxidative stress levels in offspring livers. The prenatal HF diet also induced hypermethylation of transcription factor (TF) binding sites upstream of lipin 1 (*Lpin1*), a gene involved in lipid metabolism. Furthermore, DNA methylation of *Lpin1* TF binding sites correlated with mRNA expression of *Lpin1*.^{Fig} These findings suggest that the effect of a prenatal HF diet on the adult offspring's metabolic phenotype are regulated by changes in hepatic gene expression and DNA methylation.—Rouschop, S. H., T. Karl, A. Risch, P. A. van Ewijk, V. B. Schrauwen-Hinderling, A. Opperhuizen, F. J. van Schooten, and R. W. Godschalk. **Gene expression and DNA methylation as mechanisms of disturbed metabolism in offspring after exposure to a prenatal HF diet.** *J. Lipid Res.* 2019. 60: 1250–1259.

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The parents' diet before and during pregnancy significantly determines their offspring's risk of developing diseases later in life (1–7). Observational studies have shown that maternal consumption of a high-fat (HF) diet and obesity during pregnancy and lactation predispose offspring to developing various metabolic disorders, such as obesity and type 2 diabetes, and cardiovascular diseases (8–11). In mice, a maternal HF diet leads to increased body weight and fat mass and decreased energy expenditure in offspring (12, 13). Similarly, a paternal HF diet before conception is able to increase the offspring's body weight and fat mass and impair its lipid and glucose metabolism (14, 15). In addition, both maternal and paternal HF diet have been shown to induce nonalcoholic fatty liver disease in mouse offspring, indicated by increased liver size, excessive accumulation of cholesterol and triglycerides

Abbreviations: Aacs, acetoacetyl-CoA synthetase; Cd163, cluster of differentiation 163; FDR, false discovery rate; GSEA, gene set enrichment analysis; GTRD, Gene Transcription Regulation Database; HF, high-fat; Hmgcr, HMG-CoA reductase; Il1r1, interleukin 1 receptor, type I; KEGG, Kyoto Encyclopedia of Genes and Genomes; LF, low-fat; Lpin1, lipin 1; NES, normalized enrichment score; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Pla2g16, phospholipase A2 group XVI; qPCR, quantitative PCR; Saa1, serum amyloid A1; TF, transcription factor; TG, triglyceride; TSS, transcription start site.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Rouschop et al., 2019) and are accessible through GEO Series accession number GSE123009 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123009>).

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(TGs), and eventually hepatic inflammation (13, 14). Moreover, reports studying the influence of both paternal and maternal diet showed that the combination of these diets has an additive effect on mouse offspring health, leading to more prominent negative effects in offspring compared with only a paternal or maternal HF diet (14, 16).

Although the effects of a prenatal HF diet on the offspring phenotype have been studied thoroughly, it is less known through which mechanisms these long-lasting effects are transmitted. The responsible molecular mechanisms for this have been suggested to involve epigenetics, because both human and animal studies demonstrated that prenatal exposures alter DNA methylation in the offspring (12, 13, 17, 18). In addition, both maternal and paternal HF diets have been shown to modify gene expression, predominantly affecting pathways related to metabolism and inflammation (12, 13, 17). Altering these metabolic and inflammatory pathways contributed to the impaired metabolic phenotype in the offspring of HF diet-fed mice. Altogether, this leads to the hypothesis that a prenatal HF diet changes DNA methylation in offspring, which subsequently alters gene expression, thereby affecting the offspring's phenotype.

To test this hypothesis, we studied the effect of a combined paternal and maternal HF diet on hepatic DNA methylation and gene expression in mouse offspring. In this study, both parent mice received a HF or low-fat (LF) diet before and during pregnancy and throughout lactation, after which offspring were weaned onto a HF diet. Feeding both parents the same diet and weaning offspring onto a HF diet better approximates the current Western situation in which the diet is characterized by a HF content, and eating patterns are shared by individuals from the same family (19). Only male offspring were included for analyses, because sex differences are known to occur in the response to a prenatal HF diet (12, 20–23). Previous work showed that, at 12 weeks of age, the offspring displayed postprandial hypertriglyceridemia, which related to an impaired clearance of lipids from the blood into the liver after a HF meal (24).

To study the mechanisms underlying the impaired metabolic phenotype after prenatal exposure to a HF diet, hepatic genome-wide gene expression was studied using whole transcript microarrays. In addition, hepatic DNA methylation of selected genes was assessed by bisulfite pyrosequencing. This work shows that exposure to a prenatal HF diet alters gene expression of pathways involved in hepatic lipid synthesis and uptake, oxidative stress response, and cell proliferation. Furthermore, the prenatal HF diet induces hypermethylation of transcription factor (TF) binding sites upstream of lipin 1 (*Lpin1*), a differentially expressed gene involved in lipid metabolism.

MATERIALS AND METHODS

Study design and workflow

A schematic overview of the study design and workflow is presented in Fig. 1.

Animal model

Animal experiments were approved by the Institutional Ethics Committee on Animal Welfare of Maastricht University. Mice were housed under standard conditions at 25°C with a 12-12 h light-dark cycle with ad libitum access to water and feed. Specific pathogen-free C57Bl/6J (Charles River, Saint-Germain-Nuelles, France) parent mice were randomly assigned to either a HF diet [45% kcal fat (31% saturated, 36% monounsaturated, and 33% polyunsaturated fatty acids), 20% kcal protein, and 35% kcal carbohydrate; Research Diets, New Brunswick, NJ) or a standard chow LF diet (9% kcal fat, 33% kcal protein, and 58% kcal carbohydrate; ssniff Spezialdiäten GmbH, Soest, Germany). The diet started 6 weeks before mating and continued throughout gestation and lactation. Maternal food intake and bodyweight were measured weekly (24). At weaning, all offspring received a HF diet, thereby creating the experimental groups HF/HF and LF/HF, with the first two letters indicating the parental diet and the last two letters indicating the offspring diet. Offspring was weaned onto a HF diet, because this experimental setup provides a better representation of the Western diet, which is predominantly characterized by a HF content. Litter size and offspring food intake were similar for both experimental groups (24). Only male offspring were included for further experiments. Phenotypic outcomes in offspring between 12 and 28 weeks of age were previously reported (24). To study how these effects of the prenatal HF diet were transmitted, livers were obtained from the offspring at 12 weeks of age ($n = 7$ per group) and 28 weeks of age ($n = 9$ per group) and were used for subsequent measurements in gene expression, oxidative stress, and DNA methylation.

Gene expression microarray

Total RNA from offspring livers was extracted using an RNeasy Mini kit (Qiagen, Venlo, The Netherlands). RNA concentration and purity were assessed using a Nano Drop ND-1000 spectrophotometer (Isogen, IJsselstein, The Netherlands). RNA quality was measured on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands). All RNA samples had an RNA integrity number >8.0 . RNA (100 ng) was labeled with whole transcript sense target assay and hybridized to Affymetrix Mouse Gene 1.1 ST arrays (Affymetrix, Santa Clara, CA). Microarrays were analyzed using MADMAX pipeline for statistical analysis of microarray data (25). Signal intensities were normalized with the robust multichip average method and probes were annotated (26). Microarray data are available through the Gene Expression Omnibus database (accession number GSE123009).

Gene set enrichment analysis

Functional data analysis of microarray data was performed with gene set enrichment analysis (GSEA) (27). GSEA focuses on gene sets (groups of genes that share common biological function or regulation) rather than individual genes. For GSEA, annotated gene sets were used from the databases, BioCarta (28), Kyoto Encyclopedia of Genes and Genomes (KEGG) (29), Reactome (30), and WikiPathways (31). Significantly affected gene sets were selected on a false discovery rate (FDR) q -value of <0.05 and were ranked by the normalized enrichment score (NES).

Selection of candidate genes for DNA methylation analysis

Individual genes from the microarray were defined as differentially expressed when comparison of the normalized signal intensities showed a P -value <0.05 in two-tailed paired intensity-based moderated t -statistics and an absolute fold-change >1.2 (32).

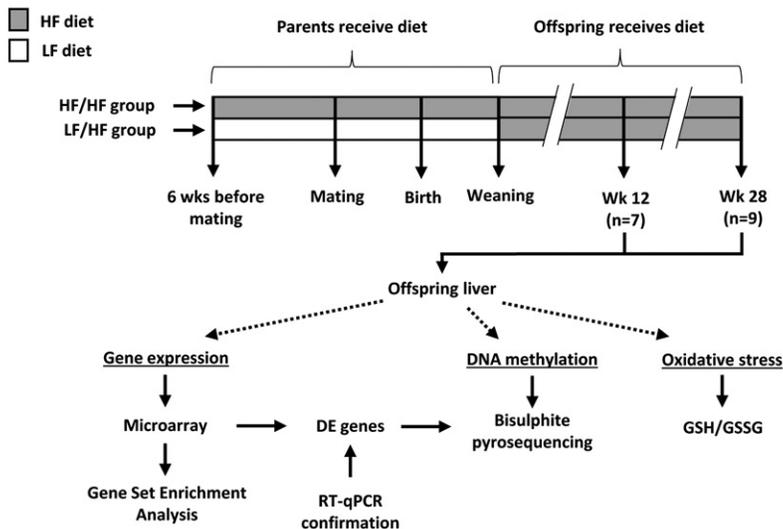


Fig. 1. Overview of study design and workflow. Parent mice received a HF or LF diet starting 6 weeks before mating and continuing during pregnancy and lactation. Offspring were weaned onto a HF diet, thereby creating the experimental groups LF/HF and HF/HF, with the first two letters indicating the parental diet and the last two letters indicating the offspring diet. At 12 and 28 weeks of age, offspring livers were collected and analyzed. Gene expression was assessed using microarrays and GSEA. Selected differentially expressed (DE) genes were confirmed with real-time qPCR. DNA methylation of TF binding sites upstream of differentially expressed genes was measured using bisulfite pyrosequencing. Oxidative stress was measured with the GSH/GSSG assay.

Differentially expressed genes were ranked by fold-change (HF/HF versus LF/HF at week 12) and filtered for involvement in lipid metabolism or inflammation. A selection was made for the top two genes that were differentially expressed at both week 12 and week 28 [cluster of differentiation 163 (*Cd163*) and HMG-CoA reductase (*Hmgcr*)] and the top five genes that were differentially expressed at only week 12 and not at week 28 [acetoacetyl-CoA synthetase (*Aacs*), *Lpin1*, phospholipase A2 group XVI (*Pla2g16*), serum amyloid A1 (*Saa1*), and interleukin 1 receptor type I (*Il1r1*)].

Gene expression real-time quantitative PCR

To confirm differential expression of candidate genes for DNA methylation, real-time quantitative (q)PCR was used. First, RNA was reverse transcribed into cDNA using iScript (Bio-Rad, Venendaal, The Netherlands). Real-time qPCR was performed on a Bio-Rad CFX384 real-time PCR system using iQ SYBR Green Supermix (Bio-Rad). Primer sequences (supplemental Table S1) were obtained from PrimerBank (33) and qPrimerDepot (34). Housekeeping genes, *Gapdh* and *Actb*, were used for normalization. Relative expression levels were quantified with the $\Delta\Delta C_t$ method (35).

Selection of TF binding regions

Candidate genes of which differential expression was confirmed with real-time qPCR were used for subsequent DNA methylation analysis. For this purpose, CpGs were selected with the potential to regulate expression of the genes, *Cd163*, *Hmgcr*, *Aacs*, *Lpin1*, *Saa1*, and *Il1r1*. Regions with a high density of TF binding sites were identified <2,000 bp upstream of the transcription start site (TSS) of each gene (Fig. 2A) by using the Gene Transcription Regulation Database (GTRD; version 17.04) (36). GTRD is a database of TF binding sites identified by ChIP-seq experiments (36). Genomic locations of the selected TF binding regions and CpGs within those regions are shown in Fig. 2B. Methylation of CpG sites within the identified TF binding regions could potentially influence TF binding and thus regulate gene transcription. Therefore, methylation of these CpGs was measured using pyrosequencing.

Pyrosequencing

To assess DNA methylation levels of selected CpG sites, a pyrosequencing approach was used. For this, DNA from offspring livers was extracted using the DNeasy blood and tissue kit (Qiagen) and subjected to bisulfite conversion with an EZ

DNA methylation kit (Zymo Research, Irvine, CA) and subsequently used as a template for amplification of the selected TF binding regions. PCR was performed on an Eppendorf Mastercycler nexus (Eppendorf, Nijmegen, The Netherlands) using HotStarTaq DNA polymerase (Qiagen) and dNTP mix (Thermo Fisher Scientific, Eindhoven, The Netherlands). Biotin-labeled amplicons were captured with Streptavidin Sepharose beads (GE Healthcare, Vienna, Austria) and denatured and annealed to the sequencing primer using the PyroMark Q24 vacuum workstation (Qiagen). Amplicons were sequenced on a PyroMark Q24 (Qiagen), using PyroMark Q24 Advanced CpG reagents (Qiagen). Methylation of each CpG was quantitatively assessed using the PyroMark Q24 Advanced software. Assays and primers for PCR and pyrosequencing (supplemental Table S2) were designed with PyroMark Assay Design software.

GSH and GSSG assay

To assess oxidative stress in offspring livers, GSH and GSSG were measured according to Rahman, Code, and Biswas (37). In short, liver homogenate was mixed with 5-sulfosalicylic acid to prevent further oxidation of GSH to GSSG. Supernatant of this mixture was used for both GSH and GSSG assessment. GSH was measured by oxidizing it with DTNB to form TNB, which is measurable with a spectrophotometer. For GSSG measurement, samples were first preincubated with 2-vinylpyridine to deplete all GSH. Subsequently, samples were incubated with NADPH and glutathione reductase, which reduces GSSG into GSH, and simultaneously incubated with DTNB to quantify the amount of GSH through TNB formation. GSH and GSSG concentrations were normalized to the starting amount of liver tissue.

Statistical analysis

For GSH, GSH/GSSG ratio, real-time qPCR, and DNA methylation, differences between groups were tested by two-tailed independent samples Student's *t*-test. For DNA methylation, *P*-values were corrected for multiple testing by computing FDR *q*-values using the Benjamini-Hochberg procedure (38). For GSSG, differences between groups were tested by two-tailed Mann-Whitney U test. The relation between the DNA methylation level of TF binding regions and mRNA expression was described using the Pearson correlation coefficient with log transformed mRNA expression levels. *P* < 0.05 or *q* < 0.05 was considered significant. All statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria).

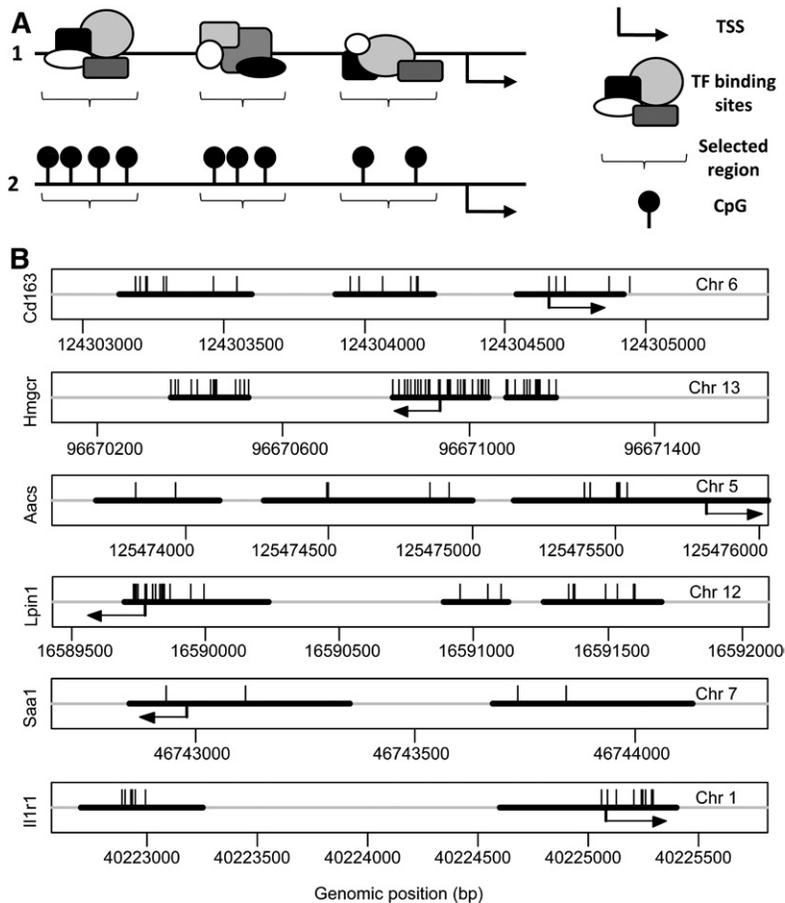


Fig. 2. Identification of TF binding regions and selection of CpG sites for pyrosequencing. A1: Regions with a high density of TF binding sites were identified <2,000 bp upstream of the TSS of a selected gene, based on GTRD. A2: CpG sites within the selected TF binding regions were selected for DNA methylation analysis. B: Genomic positions of TF binding regions (indicated by black horizontal lines) and CpGs within these regions (indicated by vertical lines), which were selected for DNA methylation analysis. Arrows mark TSS.

RESULTS

A prenatal HF diet alters hepatic expression of gene sets involved in lipid metabolism, oxidative stress response, and cell proliferation

Previous work showed that, at 12 weeks of age, HF/HF offspring displayed postprandial hypertriglyceridemia and an impaired clearance of lipids from the blood into the liver after a HF meal (24). To study whether this impaired metabolic phenotype in HF/HF offspring was related to alterations in hepatic gene expression, a microarray was performed on offspring livers at 12 and 28 weeks of age. As a result, a total of 492 genes were found to be differentially expressed in 12-week-old HF/HF offspring, compared with LF/HF offspring. Moreover, GSEA revealed that the prenatal HF diet significantly affected expression of four different pathways in offspring livers at 12 weeks of age, with two being upregulated and two downregulated (**Table 1**). The two downregulated pathways were related to hepatic lipid biosynthesis and uptake (i.e., *Activation of gene expression by SREBP*) and cellular movement (i.e., *Striated muscle contraction*), whereas the upregulated pathways were linked to oxidative stress response [i.e., *nuclear factor (erythroid-derived 2)-like 2 (Nrf2) targets*] and intracellular transport (i.e., *Kinesins*).

In 28-week-old offspring, expression of 534 individual genes was altered in HF/HF offspring compared with LF/HF offspring. Furthermore, the prenatal HF diet significantly affected expression of 63 pathways of which two

pathways were upregulated and 61 pathways were downregulated. The two upregulated pathways were both related to Sema4D signaling. Of the downregulated pathways, however, the majority were involved in cell proliferation, having functions in mitosis, cell cycle, proteasomal degradation, Wnt signaling, and Hedgehog signaling. In addition, hepatic lipid biosynthesis and uptake was still downregulated in 28-week-old HF/HF offspring, including gene sets *Activation of gene expression by SREBP*, *Cholesterol biosynthesis*, and *Steroid biosynthesis*.

Altogether, a prenatal HF diet altered hepatic expression of gene sets involved in lipid biosynthesis and uptake, oxidative stress response, and cell proliferation.

A prenatal HF diet increases oxidative stress in offspring livers

Because the prenatal HF diet upregulated hepatic expression of the Nrf2 pathway in 12-week-old offspring, we next examined whether livers of these mice also presented increased levels of oxidative stress, which was done by measuring GSH and GSSG. The results show that GSH levels were similar in all four groups, whereas GSSG levels were significantly increased in HF/HF offspring compared with LF/HF at 12 weeks of age (**Fig. 3**). Consequently, the GSH/GSSG ratio was significantly decreased in HF/HF offspring compared with LF/HF at 12 weeks of age, indicating an increased exposure to oxidative stress in the livers of HF/HF mice. In addition, there was a tendency ($P = 0.06$) for decreased GSH/GSSG ratio in 28-week-old

TABLE 1. Pathways significantly affected by prenatal HF diet in offspring livers at 12 weeks (n = 7 per group) and 28 weeks (n = 9 per group) of age

Biological Function of Pathway	Pathway	NES	FDR
12 weeks			
Cellular movement	Striated muscle contraction ^a	-2.34	0.004
Lipid biosynthesis/uptake	Activation of gene expression by SREBP ^b	-2.32	0.002
Oxidative stress response	Nrf2 targets ^b	2.60	<0.000
Intracellular transport	Kinesins ^c	2.08	0.015
28 weeks			
Lipid biosynthesis/uptake	Cholesterol biosynthesis ^b	-2.29	0.002
	Activation of gene expression by SREBP ^b	-1.95	0.006
Mitochondrial translation	Steroid biosynthesis ^c	-1.91	0.009
	Mitochondrial translation initiation ^b	-2.28	0.001
	Mitochondrial translation elongation ^b	-2.28	0.001
Antigen processing/presentation	Mitochondrial translation termination ^b	-2.20	0.001
	Cross-presentation of soluble exogenous antigens (endosomes) ^b	-2.22	0.001
	Antigen processing-cross presentation ^b	-1.77	0.028
Mitosis/cell cycle	ER-phagosome pathway ^b	-1.84	0.016
	Autodegradation of Cdh1 by Cdh1:APC/C ^b	-2.20	0.001
	APC/C:Cdc20-mediated degradation of mitotic proteins ^b	-2.18	0.001
Proteasomal degradation	APC/C:Cdc20-mediated degradation of Securin ^b	-2.18	0.001
	Proteasome ^c	-2.15	0.001
	Proteasome pathway ^d	-2.11	0.001
HIV-host interaction	Proteasome degradation ^a	-1.99	0.005
	Vpu mediated degradation of CD4 ^b	-1.93	0.008
	Vif-mediated degradation of APOBEC3G ^b	-1.93	0.008
Regulation of transcription and translation	Ribosome biogenesis in eukaryotes ^c	-2.00	0.005
	Eukaryotic transcription initiation ^a	-1.91	0.009
	RNA transport ^c	-1.89	0.011
Wnt signaling	Degradation of axin ^b	-1.89	0.011
	Asymmetric localization of PCP proteins ^b	-1.88	0.011
	Degradation of DVL ^b	-1.80	0.022
Hedgehog signaling	Degradation of DVL ^b	-1.80	0.022
	Hedgehog ligand biogenesis disease ^b	-1.80	0.022
	Degradation of GLI1 by the proteasome ^b	-1.79	0.023
Sema4D signaling	Processing defective Hh variants are degraded by the proteasome ^b	-1.78	0.027
	Sema4D in semaphorin signaling ^b	2.11	0.040
	Sema4D-induced cell migration and growth cone collapse ^b	2.06	0.038

For each biological function, the top three (based on NES) significant pathways are shown. A negative or positive NES indicates that the pathway was downregulated or upregulated, respectively, in HF/HF offspring compared with LF/HF offspring. A complete overview of all significantly affected pathways is listed in supplemental Table S3.

^aWikiPathways database.

^bReactome database.

^cKEGG database.

^dBioCarta database.

compared with 12-week-old LF/HF offspring, indicating that oxidative stress tended to increase over time in the control group.

A prenatal HF diet induces hypermethylation of TF binding sites upstream of *Lpin1*

To examine whether the observed effect of a prenatal HF diet on hepatic gene expression is regulated by alterations in DNA methylation, further analysis was performed on the candidate genes, *Cd163*, *Hmgcr*, *Aacs*, *Lpin1*, *Pla2g16*, *Saa1*, and *Il1r1*. These genes were selected based on their involvement in lipid metabolism (*Hmgcr*, *Aacs*, *Lpin1*, and *Pla2g16*) or inflammation (*Cd163*, *Saa1*, and *Il1r1*) and their significant differential expression in the microarray. This differential expression was verified by real-time qPCR, which showed that the prenatal HF diet significantly altered hepatic mRNA expression of *Cd163* and *Hmgcr* at 12 and 28 weeks of age, whereas expression of *Aacs*, *Lpin1*,

Saa1, and *Il1r1* was only significantly changed at week 12 (Fig. 4). For *Pla2g16*, differential expression could not be confirmed with real-time qPCR, and it was therefore not included for further analyses.

For each of the confirmed genes, methylation was measured at CpGs within TF binding sites, thereby having the potential to regulate expression of that gene. Analysis showed that, at 12 weeks of age, hepatic DNA methylation of *Lpin1* TF binding region 2 (Chr12: 16,590,890–16,591,130 bp) was significantly increased ($\Delta = 7.1\%$, $q = 0.002$) in HF/HF offspring compared with LF/HF offspring (Fig. 5). At 28 weeks of age, there was no longer a significant difference, with *Lpin1* region 2 DNA methylation levels of HF/HF offspring partly being restored to the level of LF/HF offspring. No significant changes were observed for *Lpin1* TF binding regions 1 and 3 or for any TF binding region upstream of *Cd163*, *Hmgcr*, *Aacs*, *Saa1*, and *Il1r1* at 12 or 28 weeks of age (supplemental Fig. S1).

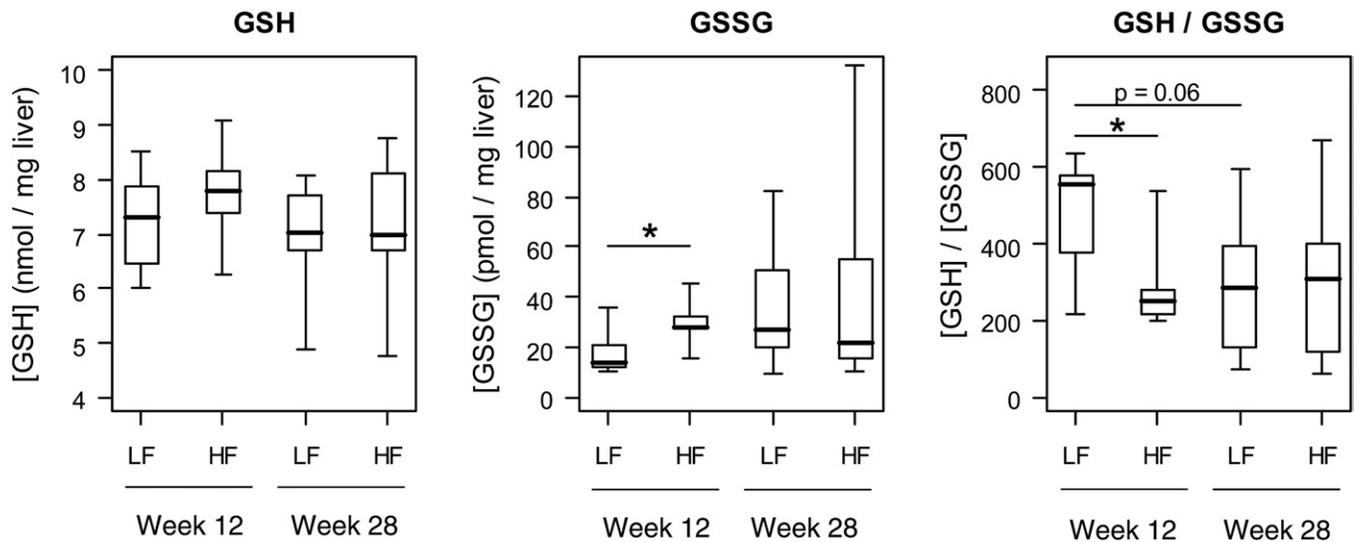


Fig. 3. GSH and GSSG concentrations and the ratio between GSH and GSSG concentrations in 12-week-old ($n = 7$ for LF and $n = 6$ for HF) and 28-week-old ($n = 9$ per group) offspring livers after both parent mice received either a prenatal LF or HF diet. Boxplots represent minimum, first quartile, median, second quartile, and maximum. * $P < 0.05$, based on two-tailed independent samples t -test (GSH and GSH/GSSG) or two-tailed Mann-Whitney U test (GSSG).

mRNA expression of *Lpin1* correlates with DNA methylation of *Lpin1* TF binding sites

The observed hypermethylation of *Lpin1* TF binding region 2 in 12-week-old HF/HF offspring was concurrent with a decrease in mRNA expression of *Lpin1*, indicating a negative relation between DNA methylation and gene expression for this locus. Existence of a correlation, however, would provide a stronger indication for DNA methylation to be involved in regulation of gene expression. Therefore, Pearson correlation was assessed for mean DNA methylation levels of TF binding regions versus microarray expression levels of the corresponding gene. This analysis showed that, at 12 weeks of age, *Lpin1* mRNA expression correlated significantly with DNA methylation of *Lpin1* TF binding region 2 ($r = -0.54$, $P < 0.05$). Furthermore, *Saa1* mRNA expression and DNA methylation of *Saa1* TF binding region 1 also correlated significantly ($r = 0.53$; $P < 0.05$).

At 28 weeks of age, both of these correlations were no longer significant. However, there was a significant correlation between *Lpin1* mRNA expression and DNA methylation of *Lpin1* TF binding region 3 ($r = 0.50$, $P < 0.05$) at 28 weeks of age. Interestingly, this *Lpin1* region 3 is a reported binding site for both of the TFs, Nrf2 and SREBP-1 (obtained from GTRD, version 17.04).

DISCUSSION

While the phenotypic effects of a prenatal HF diet on offspring have been studied thoroughly, it is less well-known through which mechanisms these long-lasting effects are transmitted. Therefore, we studied the effect of a combined paternal and maternal HF diet on hepatic DNA methylation and gene expression in adult mouse offspring.

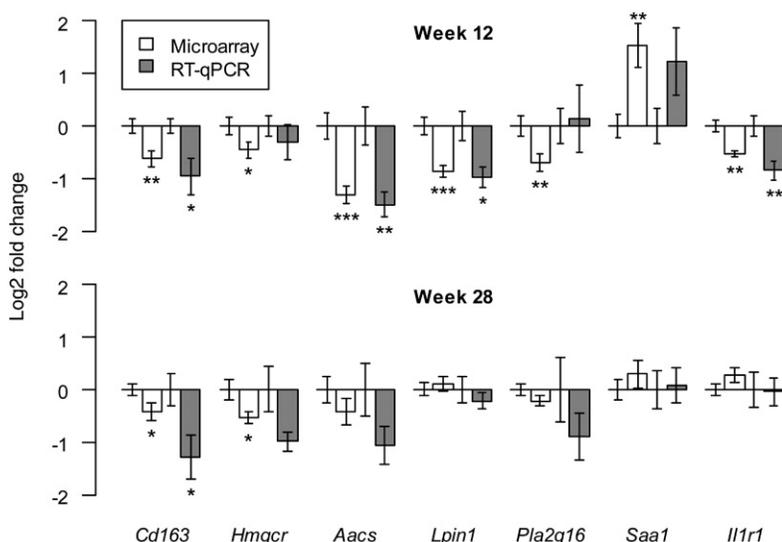


Fig. 4. Effect of prenatal HF diet on hepatic mRNA expression of *Cd163*, *Hmgcr*, *Aacs*, *Lpin1*, *Pla2g16*, *Saa1*, and *Il1r1* in 12-week-old ($n = 7$ per group) and 28-week-old offspring ($n = 9$ per group) analyzed by microarray and real-time qPCR. Expression was normalized against *Actb* and *Gapdh* and presented relative to control (LF/HF) expression (controls have Log₂-fold change of 0). Bars indicate mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, based on two-tailed paired intensity-based moderated t -statistics (microarray) or two-tailed independent samples t -test (real-time qPCR).

Lpin1, region 2

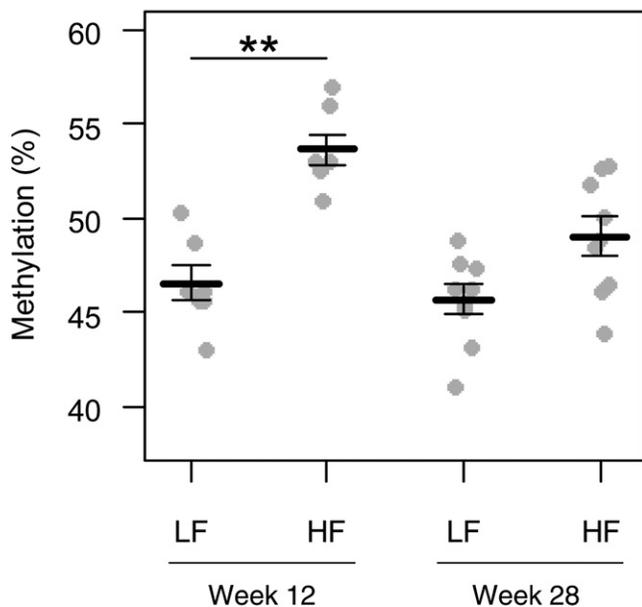


Fig. 5. Effect of prenatal HF diet on offspring hepatic DNA methylation of *Lpin1* TF binding region 2 (Chr12: 16,590,890–16,591,130 bp) at 12 weeks ($n = 7$ per group) and 28 weeks of age ($n = 9$ per group). Lines and error bars indicate mean \pm SEM. **FDR q -value < 0.01 , based on two-tailed independent samples t -test followed by correction for multiple testing using the Benjamini-Hochberg procedure.

We showed that this prenatal HF diet altered hepatic DNA methylation and gene expression of pathways involved in lipid metabolism, oxidative stress response, and cell proliferation. We hypothesize that these changes underlie the previously reported postprandial hypertriglyceridemia and decrease hepatic lipid clearance in offspring exposed to a prenatal HF diet (24).

First, we showed that a prenatal HF diet consistently downregulated the gene set *Activation of gene expression by SREBP* at both 12 and 28 weeks of age. SREBP represents a group of TFs that regulate expression of genes involved in fatty acid and cholesterol synthesis and uptake (39–44). Because SREBP targets are involved in lipid uptake, the observed downregulation of hepatic SREBP signaling in HF/HF offspring may contribute to the impaired postprandial hepatic lipid clearance, as was previously reported in these offspring mice (24). The observed downregulation of SREBP signaling is in agreement with two previous studies that examined the effect of a maternal HF diet on offspring livers. These studies reported that SREBP gene and protein expression were diminished and that this regulated the observed decrease in mRNA expression of genes involved in fatty acid, cholesterol, and steroid biosynthesis, and other SREBP-related functions (45, 46). In contrast, two other studies found an increase in mRNA expression of *Srebp1c* and some of its downstream genes involved in de novo lipogenesis in response to a prenatal HF diet (13, 14). Although the reasons for these opposite effects on SREBP expression in similar experimental settings remain unclear, these reports do underline the involvement of SREBP in the effects of a prenatal HF diet on offspring livers.

Next to a decreased hepatic lipid uptake induced by SREBP downregulation, alterations in the metabolic phenotype of HF/HF offspring can also be a result of an increased export of lipids from the liver into the circulation. Such an increased export may be facilitated by the upregulation of the pathway *Kinesins* in HF/HF offspring. Kinesins are motor proteins that are, among other processes, required for intracellular transport of TG-rich lipid droplets to the smooth ER in hepatocytes (47). This transport enables lipid droplets to be catabolized and TGs to be available for VLDL assembly and subsequent secretion from the liver. Upregulation of these kinesin motors could therefore contribute to enhanced VLDL-mediated export of lipids from the liver to the blood, thereby adding to the previously reported trend for decreased fasted hepatic lipid content in HF/HF offspring (24).

Besides the *Kinesins* pathway, the prenatal HF diet also increased the expression of the gene set *Nrf2 targets* in 12-week-old offspring. Nrf2 is a TF that regulates expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation (48–50). To examine whether these mice also displayed increased levels of oxidative stress, intracellular levels of GSSG and GSH were measured in offspring livers. This showed that at 12 weeks of age, oxidative stress levels were indeed significantly increased in HF/HF offspring livers compared with LF/HF. At 28 weeks of age, however, GSSG and GSH levels, as well as expression of the Nrf2 pathway, were no longer different between HF/HF and LF/HF offspring. Instead, both groups displayed elevated levels of oxidative stress, similar to the levels observed in 12-week-old HF/HF offspring. These findings suggest that the postweaning HF diet led to a gradual increase in oxidative stress levels in livers, irrespective of the parents' diet. If the parents were additionally exposed to a HF diet, the increased levels of oxidative stress were already reached at 12 weeks of age, thereby prolonging the time that offspring livers were exposed to oxidative stress. This link between a HF diet and oxidative stress response has been indicated before by studies showing that pharmacologic or transgenic activation of Nrf2 protects mice from the effects of a HF diet, such as obesity, insulin resistance, and liver steatosis (51, 52). Furthermore, it has previously been reported that exposure to a prenatal HF diet affected not only hepatic mRNA levels (17, 53) but also DNA methylation of Nrf2-related genes (17), implying that epigenetics may regulate the effects of a prenatal HF diet on oxidative stress response.

To further investigate the involvement of epigenetics in the effects of a prenatal HF diet, DNA methylation was measured at CpGs within TF binding sites of selected differentially expressed genes. We found that the prenatal HF diet hypermethylated a TF binding region upstream of *Lpin1*. In addition, DNA methylation of two *Lpin1* TF binding regions correlated significantly with *Lpin1* gene expression. The observed correlations suggest that, in the current study, *Lpin1* mRNA expression was (at least partly) regulated by DNA methylation. The Lpin1 protein has several biological functions, depending on the tissue in which it is expressed (54–56). In liver, Lpin1 mainly functions as a

transcriptional coactivator and its knockdown leads to increased VLDL secretion and elevated plasma TGs (55). Therefore, hypermethylation of *Lpin1* TF binding regions and subsequent downregulation of *Lpin1* expression may explain the phenotypic changes in HF/HF offspring, including increased circulatory TGs and decreased hepatic lipids.

In addition to *Lpin1*, a correlation was also observed between a TF binding region and mRNA expression for *Saa1*. *Saa1* is an acute phase protein mainly produced by hepatocytes in response to inflammation and tissue damage. Normally, the liver is able to recover from tissue damage (57–59). In the current study, however, pathway analysis of microarray data showed that the prenatal HF diet downregulated expression of various gene sets involved in mitosis, cell cycle, proteasomal degradation, Wnt signaling, and Hedgehog signaling in offspring livers. Although we do not have phenotypical data to further support this, these results are an indication that cell proliferation is reduced in HF/HF offspring, possibly leading to impaired recovery from liver damage. These findings are in line with a previous study, which reported that rat offspring of mothers fed a HF diet showed hepatic cell cycle inhibition, together with related changes in gene expression and DNA methylation (60). Similarly, many genes involved in cell growth, differentiation, proliferation, and development were both differentially expressed and differentially methylated in the livers of offspring of HF diet-fed mothers (17). Furthermore, it has been shown that exposure to HF diet not only before birth but also after birth results in decreased liver proliferation, indicated by impaired liver regeneration after partial hepatectomy in mice (61, 62). Therefore, alterations in tissue damage and cell proliferation in the liver after a prenatal HF diet deserve further attention in future research.

In comparison to previous reports, our study has a few advantages. First, whereas most studies only examined the effects of a prenatal HF diet at one specific offspring age, we performed measurements in offspring at 12 and 28 weeks of age. This enabled us to analyze alterations in gene expression and DNA methylation over time. Second, many studies only focus either on the phenotypic outcome of a prenatal HF diet or on mechanisms underlying these effects. In our experiment, previously published effects on the metabolic phenotype of offspring were now linked to alterations in gene expression, DNA methylation, and oxidative stress. Finally, most studies examine the effects of a prenatal HF diet by combining a maternal HF diet with a paternal LF diet or vice versa. However, in our study, both father and mother were fed the same diet to better mimic the current Western dietary situation in which family members share eating patterns (19). In addition, combining a maternal and paternal HF diet can have additive effects on the offspring's metabolic health, resulting in more prominent health effects in offspring when compared with separate maternal or paternal HF diets (14, 16).

With the current study design, we did not assess the effect of a postweaning LF diet. All offspring was weaned onto a HF diet, because this experimental setup provides a

better model for the current Western type of diet, which is characterized by a HF content. Furthermore, it has been shown that a postweaning HF diet may aggravate the negative effects of the prenatal HF diet (15). Another study limitation is that the analyses were limited to male offspring. Sex differences occur in response to a prenatal HF diet, with male offspring having a larger increase in bodyweight and plasma TG (23), whereas female offspring have higher blood pressure and leptin levels (20, 21). Likewise, exposure to a prenatal HF diet affects hepatic gene expression differently in male and female offspring (23). Major contributors to these sex dimorphisms are estrogens and androgens (63, 64). In females, these effects depend on the hormonal cycle, making female mice more prone to variation in metabolic outcomes. Therefore, only male offspring were included in present study.

In summary, we showed that a combined maternal and paternal HF diet alters hepatic gene expression of pathways related to lipid synthesis and uptake, oxidative stress response, and cell proliferation. The downregulation of lipid uptake could account for the previously observed decreased postprandial hepatic lipid uptake and hypertriglyceridemia in HF/HF offspring. Furthermore, the upregulation of the Nrf2-mediated oxidative stress response was confirmed by assessment of GSH/GSSG ratios in the livers of offspring. In other words, the changes in gene expression due to prenatal HF diet were mechanistically linked to phenotypic alterations. We propose that the observed effects on the offspring's metabolic phenotype and gene expression are driven by alterations in DNA methylation, as indicated by the hypermethylation of TF binding sites upstream of *Lpin1* and the correlation between DNA methylation of *Lpin1* TF binding sites and gene expression of *Lpin1*. Society may have to invest in early life nutrition to prevent common diseases occurring in offspring at older ages. More knowledge is needed to unravel the molecular mechanisms, including DNA methylation and other epigenetic mechanisms that underlie the negative effects of a prenatal HF diet on offspring health. 

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