Maternal Obesogenic Diet Attenuates Microbiome-Dependent Offspring Weaning Reaction with Worsening of Steatotic Liver Disease

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The mechanisms by which maternal obesity increases the susceptibility to steatotic liver disease in offspring are incompletely understood. Models using different maternal obesogenic diets (MODEs) display phenotypic variability, likely reflecting the influence of timing and diet composition. This study compared three maternal obesogenic diets using standardized exposure times to identify differences in offspring disease progression. This study found that the severity of hepatic inflammation and fibrosis in offspring depends on the composition of the maternal obesogenic diet. Offspring cecal microbiome composition was shifted in all MODE groups relative to control. Decreased α-diversity in some MODE offspring with shifts in abundance of multiple genera were suggestive of delayed maturation of the microbiome. Next, the weaning reaction typically characterized by a spike in intestinal expression of $\text{Tnfa}$ and $\text{Ifng}$ was shown to be attenuated in MODE offspring in an early microbiome-dependent manner using cross-fostering. Cross-fostering also switched the severity of disease progression in offspring dependent on the diet of the fostering dam. These results identify maternal diet composition and timing of exposure as modifiers in mediating transmissible changes in the microbiome. These changes in the early microbiome alter a critical window during weaning that drives susceptibility to progressive liver disease in offspring. (Am J Pathol 2023, 191: 1–16; https://doi.org/10.1016/j.ajpath.2023.11.006)

Steatotic liver disease (SLD) affects approximately 25% of the world’s population, making it the most common liver disease throughout a person’s lifespan.1,2 Genetic and environmental factors play important roles in the initiation and progression of SLD. Emerging interest focuses on the role(s) of early life exposures programming later-life SLD. Maternal obesity now has been well established as an important determinant of SLD in offspring (reviewed by Thompson3). Multiple birth cohorts have shown an association between maternal prepregnancy body mass index $\geq$30 and offspring SLD, with multivariable logistic regression supporting its role as an independent predictor of this outcome.4,5 Animal models have shown that maternal obesogenic diet exposure (MODE) worsens SLD in offspring.6–8 However, the mechanisms and mediators of offspring SLD are incompletely understood, and preclinical models have failed to provide clarity, likely reflecting the composition and duration/timing of the experimental maternal diet, which in turn impact the severity of disease in offspring.

We reported that maternal high-fat/high-sucrose diet (HF/HS) exposure promotes increased hepatic steatosis in offspring, but not inflammation or fibrosis when those offspring are fed a SLD-inducing diet starting at 6 to 8

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weeks. However, those findings are at variance with other studies showing that maternal obesity associated with feeding a fibrogenic high-fat/fructose/cholesterol—containing diet (HFFC) or with maternal high-fat diet (HFD) exposure exacerbated hepatic inflammation and fibrosis in offspring, in offspring weaned to a fibrogenic (HFFC) diet. Those phenotypic differences in progressive necroinflammatory injury likely reside in a combination of the composition and timing of introduction of the various obesogenic dietary regimens and further emphasize the need for more rigorous analysis of the pathways involved.

Among the potential mechanisms of intergenerational liver injury associated with maternal obesity is transmission of an altered microbiome at birth. MODE alters the offspring microbiome in rodents, nonhuman primates, and humans in patterns that causally are involved in altering bile acid homeostasis and steatosis. The mechanisms that underlie MODE-induced shifts in the microbiome and development of offspring SLD have not been elucidated and it is unclear whether early or sustained shifts in the microbiome drive this phenotype. Recent studies have shown that maturation of the early microbiome is critical for intestinal immune system development, termed the “weaning reaction.” Disruption of the weaning reaction in neonatal mice is associated with increased susceptibility to inflammatory conditions and infections in adulthood.

The present study compared three different maternal obesogenic diets to assess the direct effect on offspring progressive liver disease and explore potential mechanisms accounting for phenotypic variations in liver injury in the offspring. These findings resolve prior discrepancies in phenotypic variation that were associated with the timing and composition of the maternal obesogenic diets and suggest that early changes in the offspring gut microbiome modify the weaning reaction and exacerbate liver disease in adult offspring fed a fibrogenic diet.

### Materials and Methods

#### Mouse Breeding Scheme, Feeding Paradigm, and Metabolic Analysis

All procedures in this study were approved by the Animal Studies Committee at Washington University School of Medicine and conformed to National Institutes of Health guidelines and reporting consistent with ARRIVE guidelines. Four-week-old female C57Bl/6J mice were fed one of three obesogenic diets or a control diet (Table 1). Tissue and cecal contents were collected at necropsy at 2, 3, 4, and 5 weeks of age. A subset of offspring was weaned to HFFC diet for 7 weeks to induce metabolic dysfunction—associated steatohepatitis. The number of offspring and different litters represented are noted in each figure legend. For these studies, at least five litters were represented in each group. Body weights were measured for all offspring weekly during feeds, and body and liver weight measurements were measured at necropsy. For all breeding, potential dams were staged to identify the most likely interval for successful mating. The sire was placed in the cage for only 24 hours to limit co-housing effects.

#### Cross-Fostering Mice

The day they were born, offspring from control (CON) dams were cross-fostered with an HFFC dam and offspring from HFFC dams were cross-fostered with a CON dam. Cross-fostering was performed synchronously with donor and recipient dams giving birth on the same day. The litter to be fostered was placed in a clean cage and mixed with nestlets from the recipient dam’s cage. The pups then were placed in the recipient dam’s cage and mixed with bedding before returning the recipient dam to the cage. Cages were monitored closely for the next 72 hours to identify evidence of rejection by the recipient dam. Necropsy tissue from cross-fostered offspring were collected at 2, 3, and 4 weeks of age. A subset of cross-fostered offspring was weaned to HFFC diet (7 weeks for males, 13 weeks for females) to induce metabolic dysfunction—associated steatohepatitis.

#### Histology, Stains, and Immunohistochemistry

Tissues fixed in 10% formalin were embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin and picrosirius red (PSR), as reported previously by Thompson et al. For immunohistochemistry, sections were rehydrated by passing through xylene, graded alcohol, and distilled water, and then stained with select primary antibodies (CD45, Mac-2, and α-smooth muscle actin) and the Vectastain Elite ABC-AP kit (Vector Laboratories, Hamburger im Herzogtum Sachsen, Germany).
Burlingame, CA) protocol. Antigen retrieval was performed by heating sections (15 minutes in citrate buffer, pH 6.0) in a pressure cooker. Endogenous peroxide activity then was blocked and tissues were incubated with the primary antibody (approximately 20 hours, 4°C). Sections then were washed and incubated with biotin-conjugated secondary antibody (30 minutes) (Vector Laboratories), washed, incubated with the ABC-AP reagent, washed, and incubated with the alkaline phosphatase red substrate kit (Vector Laboratories). All washings used phosphate-buffered saline with Tween. Sections were counterstained with hematoxylin and cover-slipped using Cytoseal XYL (Richard Allen Scientific, Kalamazoo, MI). PSR staining, CD45 immunohistochemistry, and Mac-2 immunohistochemistry was quantified using ImageJ software version 1.54d (NIH, Bethesda, MD; https://imagej.net/) to calculate the percentage area of the section that was positive. Three representative images were taken (objective, 10×) for each mouse, with one image coming from each of three different liver lobes. For all quantitative graphs for staining, each data point represents a single mouse. Slides were scored by a pathologist (S.J.B.) who was blinded to the dietary intervention of each mouse.

Quantitative PCR Analysis

Total hepatic RNA was extracted and cDNA was prepared using an ABI high-capacity cDNA reverse transcription kit with 1 μg total RNA. Real-time quantitative PCR used cDNAs from at least six animals per group and was performed in duplicate on an ABI 7500 sequence detection system using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA) and appropriate primer pairs (Table 2). Relative mRNA abundance is expressed as the fold-change compared with the CON group after normalization to glyceraldehyde-3-phosphate dehydrogenase.

RNA Sequencing Analysis

For 3-week-old offspring from each group, RNA was isolated and submitted to the Genome Technology Access Center at the McDonnell Genome Institute at Washington University School of Medicine. RNA integrity was determined using an Agilent Bioanalyzer or 4200 Tapestation. Library preparation was performed with 5 to 10 μg total RNA with a Bioanalyzer RIN score greater than 8.0. rRNA was removed by poly-A selection using Oligo-dT beads (mRNA Direct kit, Life Technologies). mRNA then was fragmented in reverse-transcriptase buffer and heated (94°C, 8 minutes). mRNA was reverse transcribed to yield cDNA using the SuperScript III RT enzyme (Life Technologies), per the manufacturer’s instructions, and random hexamers. A second-strand reaction was performed to yield double-stranded cDNA. cDNA was blunt ended, had an A base added to the 3’ ends, and then Illumina sequencing adapters were ligated to the ends. Ligated fragments then were amplified for 12 to 15 cycles using primers incorporating unique dual index tags. Fragments were sequenced on an Illumina NovaSeq-6000 using paired end reads extending 150 bases. All significantly different genes are listed (Supplemental Table S1).

Pathway analysis was performed on the Consensus Path Database for all significantly up-regulated and down-regulated genes between groups using an unadjusted P value < 0.05. The top 10 pathways are presented in graphic form. A full listing is provided (Supplemental Table S2).

16S Sequencing for Gut Microbiome Analysis

The cecal contents were collected and sent to MR DNA (Shallowater, TX) for 16S rRNA gene sequencing and bioinformatics analysis. The Q25 sequence data derived from the sequencing process were processed using the MR DNA ribosomal and functional gene analysis pipeline.
Figure 1  Worse steatotic liver disease (SLD) in maternal obesogenic diet exposure (MODE) offspring with variability in disease progression depending on maternal diet. A: Schematic representation of MODE model with weaning to high-fat/fructose/cholesterol-containing (HFFC) diet to induce SLD in offspring. Created with BioRender.com (Toronto, Canada). B: Representative photomicrographs of hematoxylin and eosin (H&E), Mac-2, picrosirius red and α-SMA.
Sequences were depleted of primers, short sequences <150 bp were removed, and sequences with ambiguous base calls were removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. Unique sequences were identified and PCR point errors were removed, followed by chimera removal, thereby providing a denoised sequence or zOTU. Final zOTUs were classified taxonomically using BLASTn (NCBI) against a curated database derived from NCBI and compiled into each taxonomic level into both counts and percentage files.

η-Diversity and β-diversity analyses were viewed through Qiime 2. Taxonomic data are presented as abundance relative to all counts.

Statistical Analysis

Two-way analysis of variance with post hoc comparison or unpaired t-tests with Welch’s correction were used when appropriate using GraphPad prism software and noted in the figure legend. Data are presented as means (±SD or SEM), with two-tailed \( P < 0.05 \) representing significance and two-tailed \( P > 0.05 \) and < 0.10 representing a statistically insignificant trend.

All authors had access to the data and have reviewed and approved the final manuscript.

Results

SLD Progression in Offspring Reflects the Composition and Timing of Maternal Diet Exposure

To directly compare the effect of the four maternal diet models, offspring from all groups were weaned to the HFFC diet for 7 weeks to induce metabolic dysfunction-associated steatohepatitis (Figure 1A). All groups had similar body and liver weights, and liver weight to body weight ratios (Supplemental Figure S1, A–C). HFFC and HFD offspring showed increased steatosis compared with CON offspring (Figure 1B). Concentrations of hepatic triglycerides were increased in HF/HS and HFD offspring (Figure 1C). SLD steatosis scores identified a shift in the distribution to more stages 1 to 3 steatosis in HFFC offspring, whereas HFD offspring showed a steatosis score profile similar to CON offspring (Figure 1G). To evaluate macrophage expansion after HFFC feeding, mac-2 staining was performed. Maternal HF/HS offspring did not show greater mac-2 staining than CON offspring (Figure 1, B and D), whereas HFFC and HFD offspring did have greater mac-2 staining (Figure 1, B and D). SLD scoring for inflammation (PSR), and α-smooth muscle actin (α-SMA) staining in offspring from each group. C: Hepatic triglyceride concentrations in liver of offspring. D: Quantitation of Mac-2 staining in liver of offspring. E: Quantitation of PSR staining in liver of offspring. F: Quantitation of α-SMA staining in liver of offspring. G: Steatosis score distribution among offspring after 7 weeks of HFFC diet. H: Inflammation score distribution among offspring after 7 weeks of HFFC diet. Percentage of offspring with each steatosis score. I: Total NAS score distribution among offspring after 7 weeks of HFFC diet. J: Fibrosis score distribution among offspring after 7 weeks of HFFC diet. K: Comparison of the DE genes increased between HFFC and CON yield increased primarily pathways involved in lipid metabolism (Figure 2C). Comparison of the DE genes increased between HFFC and CON yield increased primarily pathways involved in the cell cycle (Figure 2E). Comparison of the DE genes increased between HFFD and CON yield increased primarily pathways involved in fibrosis (Figure 2G). These findings indicate that the type of maternal diet specifies gene expression profiles in the offspring liver. SLD relevant genes that were increased on RNA sequencing in all MODE groups included Scd1, Ppala5, Fgl21, Elovi5, and Fhbp (Figure 2, D, F and H).
Figure 2 The hepatic transcriptome is altered in weaning-age offspring in a maternal diet-dependent manner. A: Principal component (PC) analysis plot of RNA sequencing data. B: Venn diagram of the number of differentially expressed genes on RNA sequencing between each maternal diet compared with control (CON) offspring at 3 weeks of age. C: Pathway analysis of up-regulated genes in high-fat/high-sucrose (HF/HS) offspring compared with CON. D: Select genes up-regulated in HF/HS offspring compared with CON. E: Pathway analysis of up-regulated genes in high-fat/fructose/cholesterol-containing (HFFC)
Quantitative PCR confirmed shifts in expression of Fapb4, Fg21, Cd36, Elov16, Col1a1, and Col3a1 (Figure 3A). Fapb4 and Fg21 were increased significantly or trended toward an increase in all three MODE groups compared with CON. Although the pathways that up-regulated gene enrichment in each group varied, comparison of the DE genes down-regulated by MODE yielded similar results on pathway analysis (Supplemental Figure S2). Quantitative PCR for other genes involved in lipid metabolism, inflammation, and fibrosis did not show differences in hepatic expression based on maternal diet (Figure 3B). These findings highlight that the hepatic transcriptome in offspring is affected by their maternal diet, and that multiple genes involved in hepatic lipid metabolism are up-regulated.

MODE Alters the Early Offspring Microbiome

To identify the effect of the different maternal diets on the early offspring microbiome, 16S sequencing was performed of cecal contents from each group of offspring at 3 weeks of age. Principal component analysis revealed a shift in β-diversity in HFD and HFFC offspring relative to CON (Figure 4A). HF/HS appeared to separate from CON, but the difference did not reach statistical significance. α-Diversity was decreased in HFFC and HFD offspring, with HFFC offspring showing the greatest decline in Shannon diversity (Figure 4, B and C). At the phylum level, a distinct pattern of bacterial abundance depended on the type of maternal diet (Figure 4D). The CON offspring microbiome consisted primarily of Bacteroidetes and Firmicutes, whereas all three MODE groups had a microbiome that more predominantly was Firmicutes and less Bacteroidetes. HFFC offspring had an increased abundance of Veerrucocmicribia whereas the HFD offspring microbiome predominantly was Firmicutes. Differences in abundance were observed at the genera level between all four groups (Figure 4E). Lactobacillus were increased in abundance in HFFC offspring compared with CON offspring (Figure 4F), and Bacteroides were decreased in all three MODE groups compared with CON offspring (Figure 4G). Akkermansia were increased in HFFC offspring compared with the other three groups, accounting for the increase in Veerrucocmicribia observed at the phylum level (Figure 4H). Lactococcus were increased in HFFC offspring compared with CON offspring (Figure 4I). These findings highlight that the early microbiome depends on the composition of the maternal diet. In particular, maternal exposure to either HFFC or HFD feeding (but not HF/HS) delayed diversification of the early microbiome in offspring with a sustained predominance of Lactococcus and Lactobacillus.

MODE Affects the Weaning Reaction in Offspring

The early microbiome is essential for establishment of the intestinal immune system. Specifically, the presence of the early microbiome induces a brief period of inflammation in the intestine with increased expression of proinflammatory cytokines (Tnfa and Ifng) and development of Ror1 regulatory T cells. Germ-free mice lack this "weaning reaction" and are more susceptible to inflammatory diseases. Based on these findings and the changes in microbial taxa detailed above, whether MODE-induced shifts in the early microbiome affects the weaning reaction and, ultimately, the long-term health of the offspring was investigated. To evaluate the weaning reaction in the maternal obeseogenic diet models, tissues were collected from 2- to 5-week-old offspring after MODE (Figure 5A).

Shells in body weight and liver weight/body weight emerged between 2 and 4 weeks of age (Supplemental Figure S3). The study measured the expression of Tnfa and Ifng in the ileum of offspring from all four groups (CON, HF/HS, HFFC, and HFD). As previously reported, an increase in ileal Tnfa expression was observed in CON offspring at 3 weeks of age that normalized by 5 weeks of age (Figure 5B). In offspring exposed to MODE, there was a variable response that was dependent on the type of maternal diet. HF/HS, HFFC, and HFD offspring had no increase in Tnfa expression at 3 weeks of age relative to CON. HF/HS and HFD offspring showed a peak in Tnfa expression at 4 weeks of age, but the peak was blunted relative to the CON peak. HFFC offspring had a peak a week later at 5 weeks, although it was not increased significantly relative to baseline expression. A similar pattern was observed in ileal Ifng expression (Figure 5C). CON offspring showed peak Ifng expression at 3 weeks of age, whereas ileal Ifng expression in HF/HS, HFFC, and HFD offspring did not show a similar increase in expression. These findings suggest that the choreographed weaning reaction is attenuated in MODE offspring.

Maternal Diet—Induced Shifts in the Early Microbiome Regulate the Offspring Weaning Reaction

To determine if early microbiome shifts that depend on maternal diet regulate the offspring weaning reaction, CON and HFFC groups were cross-fostered. Within 24 hours after birth, CON offspring were cross-fostered to an HFFC dam (CON→HFFC) and HFFC offspring were cross-fostered to a CON dam (HFFC→CON) (Figure 6A). The ileum was collected between 2 and 4 weeks of age to assess Tnfa and Ifng expression as a surrogate marker of
the weaning reaction. CON offspring cross-fostered to HFFC dams showed no spike in ileal expression of Tnfa or Ifng between 2 and 4 weeks of age, consistent with a loss of the weaning reaction (Figure 6, Band C). Conversely, HFFC offspring cross-fostered to CON dams showed a spike in expression of both Tnfa and Ifng at 3 weeks of age, suggesting the weaning reaction was reestablished (Figure 6, B and C). These findings show that cross-fostering replicates the weaning reaction phenotype between different MODE groups and highlights the importance of early microbiome exposure mediated through maternal diet.

Early Maternal Diet Effects on the Microbiome Are Sufficient to Explain MODE Worsening of Offspring SLD

To determine if early microbiome shifts that depend on maternal diet are involved mechanistically in MODE-
associated exacerbation of offspring SLD, cross-fostered offspring from both groups were weaned to HFFC diet: males for 7 weeks (Figure 7A) and females for 13 weeks (Figure 8A) to induce progressive metabolic dysfunction–associated steatohepatitis. An extended duration of exposure was chosen for female mice because female mice are more resistant to the development of metabolic dysfunction–associated steatohepatitis than male mice. In male offspring, cross-fostering did not affect body weight after HFFC (Figure 7B), but there was a trend toward decreased liver weight and the liver weight to body weight ratio in CON offspring fostered by an HFFC dam (Figure 7, C and D). Histologic analysis showed more widespread steatosis in CON–HFFC offspring than HFFC–CON male offspring, despite similar hepatic triglyceride concentrations (Figure 7, E and F). There was increased staining for CD45 and Mac-2 in CON–HFFC male offspring (Figure 7, E, G, and H). Staining for PSR also was increased in CON–HFFC offspring, although the staining was localized primarily to the perportal area compared with the panzonal staining observed in baseline HFFC offspring (Figure 7, E and I). Staining for α-smooth muscle actin was greater in CON–HFFC than in HFFC–CON male offspring (Figure 7, E and J).

Figure 4  Cecal microbiome of weaning-age offspring shifts based on type of maternal obesogenic diet. A: Bray-Curtis plots for β-diversity of cecal microbiome from offspring. Table with comparisons and P values noted below. B: Shannon diversity in cecal microbiome of offspring at 3 weeks of age. C: Observed OTUs in cecal microbiome of offspring at 3 weeks of age. D: Relative abundance of each bacterial phylum in cecal microbiome of offspring. E: Relative abundance of the top 20 genera of bacteria present in cecal microbiome of offspring. F: Relative abundance of 4 select genera of bacteria that were significantly different between groups. Quantitative data are presented as means ± SD. n = 6 in each group and 3 separate litters represented in each group. Male and female offspring are included in each graph. *P < 0.05, **P < 0.01, and ***P < 0.001. CON, control; HFD, high-fat diet; HFFC, high-fat/fructose/cholesterol-containing; HF/HS, high-fat/high-sucrose.
In female offspring, cross-fostering did not affect body weight, liver weight, or the liver weight to body weight ratio after HFFC (Figure 8, B–D). There was more diffuse steatosis in liver of CON offspring cross-fostered with HFFC dams than HFFC offspring cross-fostered with CON dams, without differences in hepatic triglyceride concentrations (Figure 8, E and F). Mac-2 and CD45 immunohistochemistry showed increased staining for macrophages and monocytes in CON offspring cross-fostered to HFFC dams (Figure 8, E, G, and H). PSR staining for collagen also was increased in CON offspring cross-fostered to HFFC dams compared with HFFC→CON offspring (Figure 8, E and I). α-Smooth muscle actin also was increased in CON→HFFC female offspring, although the increase was not statistically significant (Figure 8, E and J) (P = 0.052). These findings support that cross-fostering is sufficient to increase SLD progression in CON offspring and lessen progression in HFFC offspring, highlighting the importance of the early microbiome in programming susceptibility to disease progression. These differences are greater in male offspring, but also significant in female offspring, suggesting an impact on both sexes.

**Discussion**

The key findings of this study of phenotypic variability in progressive liver disease in offspring (Figure 9) are as follows: i) the composition and timing of maternal diet dictates the severity of disease progression in offspring; ii) the composition of maternal diet differentially shifts the weaning age microbiome and hepatic transcriptome; iii) MODE alters the early offspring microbiome, which attenuates the offspring weaning reaction; and iv) MODE-induced transmissible shifts in early offspring microbiome are sufficient to accelerate SLD progression in the offspring. Prior work has established that SLD in offspring can be programed developmentally by exposure to the maternal obesogenic diet, with shifts in the microbiome involved in the pathophysiology of intergenerational transmission.6,9,12,16,18 Most studies have shown increased steatosis and inflammation in the liver of offspring exposed to a SLD-inducing diet after MODE,9,12,16 and some studies have shown worsened fibrosis.6,10 Indeed, it previously was reported by Thompson et al that offspring exposed to maternal HF/HS feeding developed more severe steatosis, although they did not develop advanced fibrosis when fed a fibrogenic diet.

This study replicated earlier findings of unchanged fibrosis in HF/HS offspring, but increased fibrosis was observed in offspring from dams exposed to either HFFC or HFD feeding. These findings are consistent with prior reports and confirm that the composition of the maternal diet (ie, obesogenic versus fibrogenic) affects both the development and severity of SLD and fibrosis observed in offspring.6,10 Evaluation of the hepatic transcriptome of...
weaning-age offspring identified shifts in gene expression that also were dependent on the composition of the maternal diet. Notably, HFD offspring already had increased expression of fibrosis-related genes at 3 weeks of age, and HFFC offspring had increased cell-cycle related genes. These changes could be related causally to the hepatic response of chronic lipotoxic injury leading to fibrosis, and enhanced proliferation leading to tumorigenesis. Indeed, maternal HFD exposure promotes hepatocarcinogenesis in offspring.20 Similarities also were present in the response of the offspring hepatic transcriptome to the different maternal diets. This occurred primarily in genes involved in hepatic lipid metabolism. These findings highlight the importance of maternal diet selection when attempting to study a specific liver phenotype end point in these models.

Offspring primarily acquire their pioneering microbiome at birth and in the perinatal period, and thus a variety of factors that can impact the maternal microbiome could have an immediate and long-term impact on the offspring’s health.21 Although mechanistic analysis is evaluated primarily in offspring after they have been weaned or through the use of a microbiome transplant into mice after they have been weaned, the early microbiome also plays important roles through the weaning reaction that defines some components of the gut immune system.14 It was hypothesized that MODE alters the early microbiome dependent on the type of maternal diet and that this is associated with disease severity in the offspring. All three groups showed shifts in the microbiome compared with control offspring. Notably, there was significant variability depending on the type of maternal diet. HFFC and HFD offspring showed a decrease in α-diversity, which could represent delayed diversification of the gut microbiome. As offspring age, their gut microbiome becomes increasingly diverse until it achieves a stable degree of diversity driven by environment and diet. Delayed diversification of the gut microbiome significantly could affect development of the offspring’s intestinal immune system. If the lack of gut microbiome diversity

Figure 6 Maternal obesogenic diet exposure (MODE)-induced attenuation of offspring-weaning reaction is dependent on the early microbiome. A: Schematic representation of cross-fostering between high fat/fructose/cholesterol (HFFC) and control (CON) offspring. Created with BioRender.com (Toronto, Canada). B: Body weight and liver weight/body weight ratio of offspring at 3 weeks of age. C: Relative expression of Tnfa in offspring ileum between 2 and 4 weeks of age in cross-foster offspring. D: Relative expression of Ifng in offspring ileum between 2 and 4 weeks of age in offspring. Quantitative data are presented as means ± SD. n ≥ 7 in each group and ≥5 separate litters represented in each group. Male and female offspring are included in each graph. *P < 0.05 compared with CON→HFFC at 2 weeks. #P < 0.05 compared with CON→HFFC at 3 weeks. **P < 0.01.
weakens or ablates the weaning reaction, the propensity for tissue injury from a second insult would increase. We previously reported an increase in Akkermansia abundance in 6-week-old HF/HS offspring relative to CON,13 as have other studies in MODE offspring.22 The current study also identified an increased abundance of Akkermansia at 3 weeks of age in HFFC offspring. In each of these studies, increased abundance of Akkermansia was associated with worse metabolic and inflammatory outcomes in the offspring. This runs contrary to human and animal studies showing reduced Akkermansia during obesity and development of metabolic disease.25–27 Interestingly, Akkermansia increases after bariatric surgery but is not associated with metabolic improvement.26 Supplementation with Akkermansia in obese patients increases insulin sensitivity and slightly decreases body weight.28 The

Figure 7  Cross-fostering shifts offspring susceptibility to steatotic liver disease (SLD) progression in male offspring. A: Schematic representation of the maternal obesogenic diet exposure (MODE) model with cross-fostering and weaning to a high fat/fructose/cholesterol (HFFC) diet for 7 weeks to induce SLD in male offspring. Created with BioRender.com (Toronto, Canada). B: Body weight of offspring. C: Liver weight of offspring. D: Liver weight/body weight of offspring. E: Representative photomicrographs of hematoxylin and eosin (H&E), Mac-2, picrosirius red (PSR), and α-smooth muscle actin (α-SMA) staining in offspring from each group. F: Hepatic triglyceride concentrations in liver of offspring. G: Quantitation of CD45 staining in liver of offspring. H: Quantitation of Mac-2 staining in liver of offspring. I: Quantitation of PSR staining in liver of offspring. J: Quantitation of α-SMA staining in liver of offspring. Quantitative data are presented as means ± SD. n ≥ 4 in each group and ≥3 separate litters represented in each group. All data are from male and female mice. *P < 0.05. Scale bars = 50 μm. CON, control; TG, _______.
A paradoxical association between Akkermansia and worse metabolic outcomes in MODE models and the extensive literature linking this genus to a reduced risk of metabolic complications prompts speculation that age-related responses of the host to this taxon exist, based on epithelial maturation and intestinal permeability. An alternative explanation is that Akkermansia defined by 16S rRNA sequencing, as in this and many other studies, fails to capture the genomic diversity of this genus in the human gut.29 Bacterial genomic content could correspond to the effect on the host. Future work should focus on specific host (mouse) and bacterial factors and processes that differentiate hepatic outcomes.

Although the studies implicated early shifts in the microbiome as a causal factor in developmental programming of SLD by maternal diet, the specific maternal source has not yet been defined. A maternal–infant dyad studied identified that 58.5% of the infant microbiome is attributed to maternal sources and that all maternal sources can seed the infant, including the vagina, feces, skin, breast milk, saliva, and nasopharynx.21 The source of seeding was dependent on the mode of birth (vaginal versus C-section).
In the model used in this study, it is likely that many of these maternal sources are contributing to the alterations in the offspring microbiome. This also includes direct exposure to maternal feces present in the bedding because mice are coprophagic. The contribution of maternal milk in driving early development of the offspring microbiome also is important to consider. Maternal factors can shift the composition of breast milk and, ultimately, affect the breastfeeding offspring’s microbiome. A maternal HFD affects the composition of human milk oligosaccharides, a critical nutrient source for molding the offspring microbiome. Future studies will be important to define the source of maternal microbes that are seeding the offspring in the setting of maternal obesity, as well as a deeper understanding of the factors in maternal milk that contribute to the offspring microbiome as it develops during the weaning period.

Coincident with delayed diversification of the gut microbiome, a delayed weaning reaction was observed in MODE offspring as defined by an increase in intestinal Tnfa and Ilng expression that occurs at 3 weeks of age (age of weaning for mice). This event was defined previously by comparing germ-free mice with controls, in which germ-free mice had attenuation of an increase in these two genes at 3 weeks of age unless colonized with a control microbiome before 2 weeks of age. Although MODE attenuates the weaning reaction, all three groups experienced some degree of increased, possibly compensatory, expression of the two genes at a later time point. Using cross-fostering, the phenotype between CON and HFFC offspring could be switched. This suggests that the maternal diet–induced shifts in the microbiome that offspring acquire from their dams in the early perinatal period is critical for this early programming event. Recent studies have identified that delayed maturation/diversification of the microbiome is detrimental to early immune system development. Maternal HFD and HFFC exposure also notably delayed microbiome development in the offspring in this study. Previous studies have identified sex differences in the response to a maternal obesogenic diet and the development of offspring SLD. In this study, both male and female offspring were included in the evaluation, but significant inflammation and fibrosis did not occur in females, suggesting a need for more prolonged exposure to the HFFC diet to assess disease progression phenotypes. These studies had several limitations. The design focused on early life microbiome changes (3 weeks of age) and did not evaluate the effect of sustained changes in the microbiome as functions of the maternal diet. However, multiple studies have shown that MODE drives sustained microbiome changes in adult offspring. This study also showed that transfer of microbiome from adult offspring to antibiotic-treated mice induces shifts in bile acid metabolism and increases fructose-induced hepatic steatosis. Hence, the deleterious shifts in this study are believed to be durable. In addition, the role of epigenetics was not evaluated, which likely also are involved in developmental programming of steatotic liver disease. Finally, as noted above, the genic content of putatively proinflammatory/profibrotic Akkermansia was not determined.

In summary, MODE alters both the weaning reaction and the early microbiome in offspring. These changes depend on the type of maternal diet consumed. The study also showed variability in the severity of developmentally programed SLD depending on the type of maternal diet. Overall, maternal HFFC and HFD drive more severe liver disease in the offspring compared with maternal HF/HS, suggesting that those diets may be more appropriate to study developmentally programed disease progression. Through cross-fostering, it has been shown that the early perinatal period is critical for programming these phenotypes and thus is an important timeframe in which to focus interventions.
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work will be important to translate these findings to human studies and begin to develop approaches to intervene in early life.

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**Disclosure Statement**

None declared.

**Supplemental Data**

Supplemental material for this article can be found at http://doi.org/10.1016/j.japh.2023.11.006.

**References**


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