Maternal hypercholesterolemia programs dyslipidemia in adult male mouse progeny

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Abstract

As a collection of metabolic abnormalities including inflammation, insulin resistance, hypertension, hormone imbalance, and dyslipidemia, maternal obesity has been well-documented to program disease risk in adult offspring. Although hypercholesterolemia is strongly associated with obesity, less work has examined the programming influence of maternal hypercholesterolemia (MHC) independent of maternal obesity or high-fat feeding. This study was conducted to characterize how MHC *per se* impacts lipid metabolism in offspring. Female ($n=6$ /group) C57BL/6J mice were randomly assigned to: (1.) a standard chow diet (Control, CON) or (2.) the CON diet supplemented with exogenous cholesterol (CH) (0.15%, w/w) throughout mating and the gestation and lactation periods. At weaning (postnatal day (PND) 21) and adulthood (PND 84), male offspring were characterized for blood lipid and lipoprotein profile and hepatic lipid endpoints, namely cholesterol and triglyceride (TG) accumulation, fatty acid profile, TG production, and mRNA expression of lipid-regulatory genes. Both newly weaned and adult offspring from CH mothers demonstrated increased very low-density lipoprotein (VLDL) particle number and size and hepatic TG and n-6 polyunsaturated fatty acid accumulation. Further, adult CH offspring exhibited reduced fatty acid synthase (*Fasn*) and increased diglyceride acyltransferase (*Dgat1*) mRNA expression. These programming effects appear to be independent of changes in hepatic TG production and postprandial lipid clearance. Study results suggest that MHC, independent of obesity or high-fat feeding, can induce early changes to serum VLDL distribution and hepatic lipid profile that persist into adulthood.

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Introduction

An unfavorable *in utero* nutritional and/or metabolic state during pregnancy can adversely affect fetal development and predispose offspring to disease risk throughout life (Friedman 2018, Blin *et al.* 2020). Further still, diet and metabolic health factors of the mother after birth can alter the nutrient and bioactive non-nutritive components (e.g. hormones and inflammatory mediators) of maternal milk to influence the transmission of disease risk in newborns (Fields & Demerath 2012, Isganaitis *et al.* 2019).

Given that an estimated 50% of pregnant women in the United States are overweight or obese (Davis 2020), concerns regarding the developmental origins of health and disease are heavily focused on maternal obesity. Offspring from obese pregnancies are predisposed to a wide range of metabolic complications as adults including obesity (Diaz *et al.* 2020), cardiovascular disease (Gaillard *et al.* 2016), and diabetes (Hussen *et al.* 2015). However, although obesity is often

oversimplified at a population level through indices of excess body weight and adiposity (Neeland *et al.* 2018), it is nonetheless a complex disease state manifesting in multiple metabolic abnormalities including inflammation, insulin resistance, hypertension, adipose tissue dysfunction, and dyslipidemia, among others (Jung & Choi 2014, Goossens 2017). Therefore, from a mechanistic perspective, the confounding of multiple obesity-associated metabolic adaptations during pregnancy make it difficult to discern the specific contribution of individual metabolic insults during early development to later-life disease risk. For instance, although maternal obesity is intricately linked to hypercholesterolemia with dysregulated hepatic cholesterol metabolism (Stahlberg *et al.* 1997, Haeusler *et al.* 2016), small, dense LDL particles (Kulanuwat *et al.* 2015), and low HDL-C, the effects of maternal hypercholesterolemia (MHC) during pregnancy *per se* on offspring development and disease risk have rarely been investigated independent of maternal obesity or high-fat feeding. Still, human studies have reported that

excessive early exposure to cholesterol is associated with multiple adverse health outcomes including fecundity (Schisterman *et al.* 2014), pre-term delivery and low birth weight (Magnussen *et al.* 2011, Maymunah *et al.* 2014), increased childhood BMI and adiposity (Romejko-Wolniewicz *et al.* 2014, Daraki *et al.* 2015), increased blood lipids in the neonatal, adolescent, and adult periods (Morrison *et al.* 2013, Narverud *et al.* 2015, Mendelson *et al.* 2016), and fetal fatty streaks that develop faster in childhood (Napoli *et al.* 1999). Several reports suggest that familial hypercholesterolemic (FH) offspring with maternal inheritance and thus exposure to excessive intrauterine cholesterol have increased CVD risk compared with offspring who inherited FH from their father (van der Graaf *et al.* 2010, Versmissen *et al.* 2011). However, inconsistent results have also been reported (Tonstad *et al.* 2000, Narverud *et al.* 2015), suggesting that other influencing factors related to epigenetics (which is rarely studied) and postnatal environment and lifestyle are incompletely understood.

Although we have previously employed the apolipoprotein E deficient (apoE^{-/-}) mouse model to demonstrate that MHC can program hepatic fat accumulation in male offspring (Dumolt *et al.* 2019a,b), this mouse is genetically predisposed to hypercholesterolemia and atherosclerosis, perhaps limiting the translational significance of this model. Therefore, the current study was conducted to examine if the hepatic programming effects of MHC *per se*, without the confounding influence of maternal obesity, high-fat feeding, or genetic predisposition, could be replicated in WT mice (C57BL/6J).

Materials and methods

Animals and diets

The animals used in this experiment were cared for in accordance with the guidelines established by the Institutional Animal Care and Use Committee (IACUC). All procedures were reviewed and approved by the Animal Care Committee at the University at Buffalo (protocol #PTE16082N).

Experiment 1

This experiment was essentially a replication of our earlier work in apoE^{-/-} mice (Dumolt *et al.* 2019a) to characterize the blood and hepatic lipid phenotype in male offspring (both newly weaned and adults) exposed to cholesterol throughout the gestation and lactation periods, this time in C57BL/6J mice. Two-month-old female ($n=12$) C57BL/6J dams (stock #000664) were purchased from Jackson Laboratory and housed individually at the University at Buffalo in the Laboratory Animal Care Facility in a temperature-controlled room (20°C) with free access to water. Mice were provided an acclimation period of 1 week to adjust to the new environment and research staff prior to initiation of experiments. Dams ($n=6$ /group) were randomly assigned to chow-based isocaloric diets: (1.) a standard chow

diet (Control (CON), Teklad Global 19% Protein Rodent Diet) or (2.) the CON diet supplemented with exogenous cholesterol (CH) (0.15%, w/w, CH, Teklad diet #150003) (Table 1). Dams were fed their respective diets *ad libitum* throughout mating and the gestation and lactation periods. Female mice were timed-mated with CON-fed male breeders by observing the presence of vaginal plugs or observation of spermatozoa in vaginal lavage. To maintain some consistency in exposure to the CH diet, females that did not become pregnant within 1 week were excluded from the study. Male breeders were exposed to the CH diet for the overnight breeding periods only, which averaged ~5 nights. Following parturition on postnatal day 0 (PND), the number of pups born and their weights were recorded. On PND 2, litters were randomly culled to six pups (irrespective of sex) per dam in order to minimize variability in pup growth influenced by litter size. In order to prevent pups from feeding on the experimental diet during lactation, food pellets were placed on raised platforms accessible only to dams and cages were changed daily to remove any diet waste. On PND 21, mothers and one male pup from each litter were killed for blood and liver phenotyping. An additional male pup was weaned onto the CON diet until adulthood (PND 84) for blood and liver lipid phenotyping.

Experiment 2

These experiments were conducted with a separate cohort of breeding female dams to characterize hepatic TG production (newly weaned and adult animals) and postprandial lipid clearance (adults only) in offspring exposed to excessive early cholesterol. Dams ($n=6$ per CON and CH groups, respectively) underwent identical diet and breeding procedures as outlined previously. At weaning, two male pups per litter were weaned onto the CON diet until adulthood (PND 120) to characterize hepatic TG production and postprandial lipid clearance.

Table 1 Diet formulation.

Macronutrients (%) ¹	CON	CH
Crude protein	18.9	18.7
Fat	7.9	7.7
Carbohydrate	51.5	51.3
Crude fiber	2.6	2.6
Ash	5	5
Cholesterol	-	0.15
Energy density (kcal/g)	3.3	3.5
% kcal from:		
Protein	21.4	21.4
Fat	20.1	19.8
Carbohydrate	58.5	58.7

¹Ingredients (in descending order of inclusion) - Ground wheat, ground corn, corn gluten meal, wheat middlings, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, L-lysine, iodized salt, magnesium oxide, choline chloride, DL-methionine, calcium propionate, L-tryptophan, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B12 supplement, folic acid, biotin, vitamin D3 supplement, and cobalt carbonate.

The remaining littermates (four pups, irrespective of sex) were used to examine hepatic TG production at weaning.

Hepatic TG production was measured according to the procedures by (Millar *et al.* 2005). Following a 3-h fast, mice were placed under isoflurane anesthesia (3.5%) for baseline tail vein blood collection (~25–50 µL) using a lancet and microcapillary tube. Mice were then given an intraperitoneal injection (1000 mg/kg or 5 µL/g body weight) of purified P-407, a lipase inhibitor, prepared as a 20% solution in physiological saline by cold-method incorporation (Palmer *et al.* 1998). Blood was then collected from the tail vein at 1, 3, and 6 h following injection for serum separation and TG analysis as described subsequently. Adult TG production studies were conducted in male mice only (one male pup/litter, $n=6$). However, given the volume limitations associated with repeated tail vein collection in newly weaned pups, hepatic TG production studies at weaning were conducted with a mixture of male and female pups using one pup for each time point (four pups/litter).

Postprandial lipid clearance studies were conducted in adult male offspring (PND 84, one male pup/litter, $n=6$) following a 5-h fast. Following baseline tail vein blood collection, mice were administered food grade olive oil with a feeding needle (21 gauge) at a dose of 10 µL/g body weight. At 1, 2, and 3 h post gavage, blood was collected through the tail vein for TG measurement as described subsequently.

Blood lipid analyses

Blood lipids and lipoprotein particle number and size were analyzed by NMR spectroscopy (Liposcience, Raleigh, NC) (Jeyarajah *et al.* 2006). Non-HDL cholesterol was calculated by subtracting HDL-C from total cholesterol. Serum cholesterol panel, including TC, HDL-C, and LDL/VLDL-C, was measured through enzymatic assay (BioAssay Systems, Hayward, CA).

Hepatic lipid analyses

Hepatic cholesterol and fatty acid composition were analyzed using gas chromatography on a Shimadzu GC-17A gas chromatograph with a flame ionization detector using a SAC-5 capillary column (30 m×0.25 mm×0.25 mm, Supelco, Bellefonte, CA) according to our previously published procedures (Rideout *et al.* 2010, Carrier *et al.* 2014). Relative hepatic FA content was calculated by using individual FA peak area relative to the total area and expressed as the percentage of total FA. Frozen pulverized liver tissue (100 mg) was homogenized in 1 mL of aqueous 5% NP-40 solution, heated at 90°C for 10 min, and spun at top speed in a microcentrifuge for 2 min to obtain hepatic extracts for TG analyses (Zenbio, STG-1-NC) (Green & Kehinde 1974).

RNA preparation and real-time RT-PCR

Total RNA was isolated from whole liver tissue using RNA extraction kit (RNeasy Mini Kit, Qiagen). RNA concentration and integrity were determined with spectrophotometry (260 nm) and agarose gel electrophoresis, respectively. RNA preparation and real-time RT-PCR were completed through the one-step

QuantiFast SYBR Green RT-PCR kit (Qiagen Inc.) using a Biorad CFX96 Touch real time PCR. Gene expression was determined through the 2⁻(delta delta Ct) method (Pfaffl 2001). Sequences of sense and antisense primers for target and housekeeping genes were based on previously published reports: β -actin (*Actb*) (Zhou *et al.* 2012): 5'GACAGGATGCAGAAGGAG-ATT3' (forward), 5'TGATCCACATCTGCTGGAAGG3' (reverse); acetyl-coA carboxylase (*Acaca*) (Zhou *et al.* 2008): 5'CAACCACTACGGCATGACTCA3' (forward), 5' CGCAGA-AGCAGCCCATTAATT 3' (reverse); fatty acid synthase (*Fasn*) (Graner *et al.* 2004): 5' CCTCAGTCCTGTTATCACCCGA 3' (forward), 5' GCTGAATACGACCACGCACTA 3' (reverse); sterol regulatory element binding protein 1c (*Srebf1*) (Sim *et al.* 2014): 5' TGG-AGACATCGCAAACAAG 3' (forward), 5' GGTAGACAACAGCCGCATC3' (reverse); Apolipoprotein B-100 (*ApoB100*) (Kanuri *et al.* 2011): 5'TCACCATTGGCCCTCAACCT 3' (forward), 5' CAGGTCAACATCGGCAATCA 3' (reverse); diglyceride acyltransferase (*Dgat1*) (Zhou *et al.* 2008): 5' CAGATGGGGCTGCTGCTACAT 3' (forward), 5' GGCGGCACC-ACAGATTGACAT 3' (reverse); and microsomal triglyceride transfer protein (*Mttp*) (Vrins *et al.* 2013): 5' TCAAGAGAGGCTTGCTAGCTT 3' (forward), 5' GGCCTGGTAGGTCACT-TTACA-ATC 3' (reverse).

Statistical analyses

Based on our previous studies examining the impact of maternal cholesterol during gestation and lactation (Rideout *et al.* 2015, Liu *et al.* 2016), we estimated that we would require a sample size of six mothers/group for our primary endpoint of hepatic cholesterol (effect size, 1.32; power, 0.90; $P<0.05$). Litters from each dam were considered as a single observation ($n=6$ dams per treatment group). Data were checked for normality using the Shapiro-Wilk test and are presented as means $\pm$ s.e. For the hepatic TG production and lipid clearance studies, the area under the serum concentration vs time curve was calculated using the linear trapezoidal rule. Data were analyzed with a general linear model ANOVA using with SPSS 16 for Mac (SPSS Inc). Differences were considered significant at $P<0.05$.

Results

Maternal phenotype

Maternal body weight at baseline and at end of the lactation period did not differ ($P>0.05$) between the two groups throughout the study period (Table 2). Further, gestational weight gains ($P>0.05$) were similar between dams from each treatment group (Table 2). Although both groups of dams displayed nearly identical baseline serum cholesterol, CH dams demonstrated an increase in ($P<0.05$) serum TC (+30%) compared with CON dams (Table 2) by gestation week 1. By week 2 of gestation, both groups had similar ($P>0.05$) cholesterol concentrations that were below that of baseline. By the end of lactation, TC was significantly ($P<0.05$) higher in hypercholesterolemic dams (+38% compared with CON) (Table 2). However, LDL/VLDL-C

Table 2 Maternal phenotype including weight, gestational serum cholesterol and post-lactation serum lipids, and hepatic cholesterol in control (CON) and hypercholesterolemic (CH) dams.

Endpoint	CON	CH
Maternal weight		
Baseline (g)	21.5 ± 1.1	21.8 ± 0.8
Gestational weight gain (g)	16.0 ± 0.9	16.1 ± 0.8
End of lactation weight	28.7 ± 0.65	30.0 ± 1.3
Gestational serum cholesterol (mg/dL)		
Baseline	57.7 ± 2.0	55.0 ± 2.8
Week 1	67.0 ± 3.5	87.5 ± 3.7*
Week 2	35.8 ± 4.6	43.9 ± 4.8
Serum lipids (mg/dL, post-lactation)		
TC	150.4 ± 7.5	208.2 ± 25.5*
LDL/VLDL-C	24.0 ± 1.8	25.6 ± 1.7
HDL-C	140.4 ± 14.0	156.1 ± 19.2
Hepatic lipids (μmol/g, post-lactation)		
Cholesterol	10.1 ± 0.6	25.1 ± 1.2*

Data represent mean ± s.e., $n=6$ per group; groups with an asterisk (*) are significantly different ($P \leq 0.05$). Maternal post-lactation measures were taken at the end of lactation to coincide with postnatal day 21 offspring outcomes.

and HDL-C levels in dams by the end of lactation were not significantly different ($P > 0.05$) between treatment groups. Maternal hepatic cholesterol concentration post-lactation was higher (+147%, $P < 0.05$) in the CH dams compared with CON dams (Table 2).

Offspring phenotype

No difference ($P > 0.05$) was observed in litter birth weights (9.47 ± 0.67 g for CON vs 9.48 ± 0.52 g for CH), litter birth size (7.83 ± 0.60 pups for CON vs 7.33 ± 0.56 for CH), and sex ratio ($53 \pm 5\%$ male for CON vs $58 \pm 8\%$ male for CH after culling). Further, litter growth during lactation and postnatal growth of CON-fed male pups was similar ($P > 0.05$) between offspring from CON and CH dams (Fig. 1).

Serum lipid concentrations, including TC, LDL-C, HDL-C, non-HDL-C, and TG, were not different ($P > 0.05$) in newly weaned pups from CON and CH dams (Fig. 2A). Adult offspring born to CH dams demonstrated higher serum TG (+19%, $P < 0.05$) concentrations compared with offspring from CON dams (Fig. 2B); however, no other changes in serum lipids were noted. Total VLDL particle number was higher ($P < 0.05$) in both newly weaned (+145%) and adult (+29%) offspring from CH vs CON dams (Fig. 3A and C). However, there was no difference ($P > 0.05$) in total LDL or HDL particle number in newly weaned or adult offspring between the two groups. In addition, VLDL particle size was increased ($P < 0.05$) in both newly weaned (+37%) and adult (+65%) offspring from CH vs CON dams (Fig. 3B and D). LDL and HDL size did not differ ($P > 0.05$) between both groups at any stage of development.

Liver weights (mg, normalized to body weight) did not differ ($P > 0.05$) between the chow and CH groups

Figure 1 Litter growth (g) during lactation (A) and growth of CON-fed male offspring in the postnatal period. Data are means ± s.e.; $n=6$ mothers/group.

at weaning (33.3 ± 1.3 for CON vs 33.7 ± 1.2 for CH) or adulthood (50.6 ± 2.3 for CON vs 46.5 ± 1.9 for CH). Newly weaned offspring born to CH dams demonstrated higher ($P < 0.05$) hepatic cholesterol concentration (+34%) compared with CON dams (Fig. 4A). However, this difference did not persist into adulthood (Fig. 4C). Both newly weaned and adult offspring born to CH dams demonstrated elevated hepatic TG compared with CON dams (+153% and +475%, respectively, Fig. 4B and D).

Changes in hepatic TG were associated with altered hepatic fatty acid profile in both newly weaned and adult offspring (Fig. 5). Newly weaned pups from HC dams had lower ($P < 0.05$) total monounsaturated fatty acid (MUFA) and higher ($P < 0.05$) polyunsaturated fatty acid (PUFA) concentration, mainly due to an increase in $n-6$ PUFA species (Fig. 5A). Adult offspring from HC dams demonstrated higher ($P < 0.05$) $n-6$ PUFA and a lower ($P < 0.05$) palmitic to linoleic fatty acid ratio (16:0/18:2n-6) (Fig. 5B).

Although no group differences were observed in hepatic mRNA targets in newly weaned offspring, changes in hepatic lipid profile were associated with a reduction ($P < 0.05$) in *Fasn* (~0.5 fold of CON) and increase in *Dgat1* (~1.5 fold of CON) mRNA expression in adult offspring from CH vs CON dams (Table 3).

Serum TG concentration demonstrated a rapid increase 1 h following administration of P407 and did not return to baseline by the 6-h timepoint (Fig. 6). However, the observed absolute rise in plasma TG and

Figure 2 Serum lipids in newly weaned pups (A) and adult CON-fed offspring (B) born to control (CON) or hypercholesterolemic (CH) dams. Data are means $\pm$ s.e.; $n=6$ mothers/group; * $P<0.05$. $n=6$ mothers/groups; * $P<0.05$.

AUC for TG did not differ ($P>0.05$) in newly weaned (male and female pups, Fig. 6A) or adult male offspring (Fig. 6B) from CH vs CON dams.

Serum TG concentration following an oral fat tolerance test in adult male offspring is presented in Fig. 7. The TG concentration increased from 0 to 3 h post gavage in

Figure 3 Lipoprotein particle number and size in newly weaned (A and B) and adult (C and D) offspring from control (CON) and hypercholesterolemic (CH). Data are mean $\pm$ s.e.; $n=6$ mothers/group; * $P<0.05$.

Figure 4 Hepatic lipids in offspring from control (Con) and hypercholesterolemic (CH) dams. Cholesterol in newly weaned (A) and adult offspring (C). Triglyceride in newly weaned (B) and adult offspring (D). Data are mean $\pm$ s.e.; $n=6$ mothers/group; * $P<0.05$.

Figure 5 Hepatic fatty acid profile in newly weaned pups (postnatal day 21) (A) and adult offspring (postnatal day 84) (B) from control (CON) and hypercholesterolemic (CH) dams. Groups with an asterisk (*) are significantly different ($n=6$, $P<0.05$).

Table 3 Hepatic mRNA expression of lipid regulatory targets in newly weaned pups (postnatal day 21) and adult (postnatal day 84) offspring from control (CON) and hypercholesterolemic (CH) dams.

Gene	CON	CH
Postnatal day 21		
<i>Acaca</i>	1.0 ± 0.10	1.01 ± 0.14
<i>Fasn</i>	1.0 ± 0.22	1.13 ± 0.29
<i>Srebf1</i>	1.0 ± 0.35	0.57 ± 0.04
<i>Apob100</i>	1.0 ± 0.21	1.05 ± 0.12
<i>Dgat1</i>	1.0 ± 0.14	0.98 ± 0.17
<i>Mttp</i>	1.0 ± 0.15	1.40 ± 0.15
Postnatal day 84		
<i>Acaca</i>	1.0 ± 0.12	1.01 ± 0.15
<i>Fasn</i>	1.0 ± 0.19	0.54 ± 0.11*
<i>Srebf1</i>	1.0 ± 0.29	0.68 ± 0.08
<i>Apob100</i>	1.0 ± 0.13	1.18 ± 0.14
<i>Dgat1</i>	1.0 ± 0.10	1.52 ± 0.13*
<i>Mttp</i>	1.0 ± 0.13	1.39 ± 0.19

Data represent mean ± S.E., $n=6$ per group; groups with an asterisk (*) are significantly different ($P \leq 0.05$).

both groups; however, no difference ($P > 0.05$) between offspring from CON and CH dams was observed.

Discussion

Results from this study suggest that MCH, independent of obesity or high-fat feeding, can program early adaptations in lipid metabolism that ultimately lead to dyslipidemia in adult male progeny. Specifically, adult

Figure 6 Time course of serum triglycerides following administration of P-407 in newly weaned (A) and adult (B) offspring born to control (Con) and hypercholesterolemic (CH) dams. Inserts show area under curve analysis of data. Data are mean ± S.E.; $n=6$ mothers/group. **Figure 7** Plasma triglycerides following an oral fat tolerance test in adult offspring born to control (Con) and hypercholesterolemic (CH) dams. Insert show area under curve analysis of data. Data are mean ± S.E.; $n=6$ mothers/group.

male offspring from hypercholesterolemic mothers exhibited: (1.) hypertriglyceridemia and increased VLDL particle number and size; (2.) TG accumulation and modified fatty acid profile in the liver; and (3.) differential hepatic gene profile including reduced *Fasn* and increased *Dgat1* mRNA expression. Our findings further suggest that changes in hepatic lipid content are not associated with alterations in hepatic TG production or postprandial lipid clearance.

The observed increase in the number of VLDL particles in CH offspring (both newly weaned and adults) is noteworthy for several reasons. First, an increase in the number of VLDL particles may have potential cardiovascular implications as they are the direct precursors of LDL, the main causative lipid fraction in atherosclerosis (FERENCE *et al.* 2017). However, we observed no change in the LDL particle profile, which is in contrast to our previous examination of MHC in apoE^{-/-} mice in which newly weaned offspring from hypercholesterolemic mothers exhibited increased VLDL and LDL particle numbers (Juritsch *et al.* 2017). Interestingly, unlike LDL receptor-deficient mice, it has been reported that apoE^{-/-} mice do not demonstrate increased LDL particles, rather accumulation of cholesterol-enriched chylomicron remnants and VLDL remnants (Maeda 2011). These results highlight important model differences and the need to study the effects of MHC in a human population.

Second, previous research has implicated TG-rich lipoproteins including VLDL in atherosclerosis (Oorni *et al.* 2019). A small, dense LDL subfraction is thought to contribute substantially to atherosclerotic plaque development; however, a number of studies suggest that VLDL remnants may also facilitate atherosclerosis progression (Freedman *et al.* 1998, Colhoun *et al.* 2002, Prentner *et al.* 2014). Although the large size of VLDL particles may limit their entry into the arterial intima, VLDL remodeling by cholesterol-ester transport protein

(CETP)-mediated TG transfer to HDL and lipolysis through lipoprotein lipase results in smaller VLDL particles that can be taken up and retained within the arterial wall (Vasile *et al.* 1989, Rutledge *et al.* 2000). Interestingly, although offspring from CH mothers had more VLDL particles, they were also larger in size, suggesting potential protection against arterial infiltration given their lower rate of arterial entrance (German *et al.* 2006). However, similar to LDL particles, larger VLDL particles may enter the arterial intima through leaky paracellular junctions as a result of endothelial dysfunction and associated vascular permeability induced by dyslipidemia (Magalhaes *et al.* 2016, Oorni *et al.* 2019).

The mechanisms underlying hypertriglyceridemia and associated VLDL profile changes in CH offspring are not entirely clear, although potential adaptations to hepatic lipid balance and/or peripheral lipoprotein metabolism are likely involved. As to the former, cholesterol-exposed offspring exhibited increased hepatic cholesterol (newly weaned only) and TG (newly weaned and adult). Furthermore, we observed increased total PUFA (newly weaned) and n-6 PUFA in CH offspring (both newly weaned and adults), which supports previous work reporting higher total PUFA content and n-6 fatty acids in NAFLD patients compared with healthy controls (Puri *et al.* 2007). The lower *Fasn* mRNA expression and reduced 16:0/18:2 ratio (an indirect marker of *de novo* lipogenesis (Hudgins *et al.* 1996)) in adult CH offspring could reflect a protective response to safeguard against excessive lipid deposition. In support of this theory, previous work has reported reductions in hepatic cholesterol synthesis (both HMG-CoA mRNA expression (Tsuduki *et al.* 2016) and activity (Montoudis *et al.* 2003)) and FAS protein abundance (Marseille-Tremblay *et al.* 2007) in response to excessive early cholesterol exposure. Precisely why MHC would lead to increased hepatic lipid synthesis in the first place has not been directly investigated; however, it is possible that excessive maternal-fetal cholesterol transfer could activate lipogenesis through an LXR-mediated pathway (Olkonen *et al.* 2012). Indeed, the increase in hepatic DGAT expression in cholesterol-exposed adult offspring may support this theory and underscore a metabolic explanation for the observed hypertriglyceridemia. Previous work has reported increased levels of DGAT1 mRNA in human livers with NAFLD (Kohjima *et al.* 2007), supporting a direct role of DGAT activity in the pathogenesis of the disease. Hepatic DGAT1 expression has also been shown to be important in VLDL synthesis and secretion (Yamazaki *et al.* 2005), suggesting that, perhaps, increased *Dgat1* expression in adult CH offspring may reflect enhanced VLDL-TG packaging as a means to protect against lipotoxicity. However, we observed no change in the mRNA expression of other VLDL synthesis targets (*Apob100*, *Mttp*) and no difference in hepatic TG production between the two

groups. Although P-407 is widely used to measure hepatic TG production, it is not a direct measure of VLDL secretion and therefore we cannot rule out changes in VLDL secretion to the observed cholesterol-exposed phenotype.

Alternatively, adaptations in peripheral VLDL metabolism, including tissue lipolysis and/or clearance, may also account for the different VLDL profile between control and cholesterol-exposed offspring. However, we did not observe any treatment effect on postprandial lipid clearance, suggesting that early cholesterol exposure did not affect TG tissue uptake, at least in the postprandial period. However, we did not directly measure that VLDL lipolysis and chylomicrons would be the main carrier of TG in the postprandial period, perhaps diluting our ability to detect subtle changes in TG that are part of the VLDL fraction.

This study has several limitations with respect to design and translational application. First, although females were mated with CON-fed males, male breeding partners did have access to the CH-enriched diet during overnight breeding sessions and there is increasing emphasis on paternal factors prior to conception and during sperm development that may be involved in malprogramming of offspring development. Second, our experiments were limited to male offspring, which may have divergent responses to their female counterparts. On this point, given the limitation of sequential blood collection in newly weaned animals, hepatic TG production studies at weaning were done with a mixture of male and female pups. Although the male:female ratio between litters was similar, we cannot rule out potential contribution of sex effects or inter-animal variation in the responses observed, even if offspring were from the same litter. Third, we chose to examine cholesterol exposure throughout both the gestation and lactation periods, as it may be more translatable to the human condition; however, it did not allow us to account for potential period-specific malprogramming effects of excessive cholesterol exposure. Regarding translation, we used the C57BL/6J mouse model, as it is susceptible to diet-induced obesity, hypercholesterolemia, and NAFLD (Anstee & Goldin 2006, Chu *et al.* 2017). Although these mice are often used to model human lipid metabolism, there are well-documented differences including a larger fraction of cholesterol carried in HDL in mice vs humans (Camus *et al.* 1983). The cholesterol supplemented diet was used, as previous work has shown that this level of dietary incorporation (0.15%) results in hypercholesterolemia in rodent models; however, it is unknown how the maternal cholesterol response observed mimics the human trajectory of cholesterol and lipoprotein fractions during pregnancy. Further, although the chow-based diet allowed us to examine hypercholesterolemia without the confounding effects of dietary fat or maternal obesity, its translational value may be limited as it is essentially a plant-based

diet with a variety of bioactive compounds and a high level of dietary fiber.

In summary, results of this study suggest that MHC, independent of obesity or high-fat feeding, can induce early changes to the serum VLDL profile (increased particle number and size) and hepatic TG deposition and FA profile that persist into adulthood. Further, these changes were evident in the absence of a potential exacerbating effect of a high-fat or cholesterol diet in the postnatal period.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J M, S H, and J H D assisted with the animal studies. J M and S H conducted the lab analysis. J M, S H, J H D, M S P, and T C R interpreted the data and collaborated in writing and revising the manuscript. All authors read and approved the final manuscript.

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