RESEARCH PAPER

Obesogenic diet in mice compromises maternal metabolic physiology and lactation ability leading to reductions in neonatal viability

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Abstract

Aims: Diets containing high-fat and high sugar (HFHS) lead to overweight/obesity. Overweight/obesity increases the risk of infertility, and of the pregnant mother and her child for developing metabolic conditions. Overweight/obesity has been recreated in mice, but most studies focus on the effects of chronic, long-term HFHS diet exposure. Here, we exposed mice to HFHS from 3 weeks prior to pregnancy with the aim of determining impacts on fertility, and gestational and neonatal outcomes.

Methods: Time-domain NMR scanning was used to assess adiposity, glucose, and insulin tolerance tests were employed to examine metabolic physiology, and morphological and proteomic analyses conducted to assess structure and nutrient levels of maternal organs and placenta.

Results: Fertility measures of HFHS dams were largely the same as controls. HFHS dams had increased adiposity pre-pregnancy, however, exhibited exacerbated lipolysis/hyper-mobilization of adipose stores in late pregnancy. While there were no differences in glucose or insulin tolerance, HFHS dams were hyperglycemic and hyperinsulinemic in pregnancy. HFHS dams had fatty livers and altered pancreatic islet morphology. Although fetuses were hyperglycemic and hyperinsulinemic, there was no change in fetal growth in HFHS dams. There were also reductions in placenta formation. Moreover, there was increased offspring loss during lactation, which was related to aberrant mammary gland development and milk protein composition in HFHS dams.

Conclusions: These findings are relevant given current dietary habits and the development of maternal and offspring alterations in the absence of an increase in maternal weight and adiposity during pregnancy, which are the current clinical markers to determine risk across gestation.

KEYWORDS
adiposity, diet, fetus, lactation, metabolism, obesity, pregnancy
1 | INTRODUCTION

A “Western style” diet high in fat and sugar is contributing to a pandemic of raised body mass index (BMI) and obesity in developed countries.1–4 As a result, the majority (52.7%) of women are overweight (BMI >25 kg/m²) or obese (BMI >30 kg/m²) at the point of conception5–7 leading to problems in both achieving,8 and maintaining a healthy pregnancy. For example, increased BMI is associated with an increased risk of pregnancy loss and compromised fetal development and wellbeing.9,10 It is also linked to a greater risk of the infant developing health problems during childhood,11 as well as hypertension, heart disease, diabetes, and obesity in adult life.12,13 As a result, obstetric health care costs are 23% and 37% higher for overweight and obese mothers compared to healthy weight mothers, respectively.14 Moreover, when obese women also struggle to conceive, the cost of each livebirth is 70% higher for ovulatory and 100% higher for anovulatory obese compared to healthy weight women who struggle to conceive.15 Combined with the programming effects of obesity on offspring disease risk later in life, this renders maternal obesity not just an increasing strain on national health services, but a global health threat.16

The impact of high maternal BMI on fertility and pregnancy outcomes may be due to a number of reasons. Women who are overweight/obese can have menstrual cycle disturbances with anovulatory cycles.17 Furthermore, there is reduced oocyte quality18,19 and decreased chances of implantation8 due to endometrial disturbances in decidualization and gene expression.20–22 This leads to a greater proportion of women with raised BMI relying on assisted reproductive technology services. Within fertility treatment, 10% fewer obese anovulatory women will achieve pregnancy through fertility treatment than healthy weight anovulatory women.15 If pregnancy is achieved, early pregnancy loss (<20 weeks) is significantly higher (OR 1.67) in overweight versus healthy weight women23,24 regardless of conception method.

Raising BMI also increases the risk of a number of maternal pregnancy complications, such as gestational hyperglycemia, gestational diabetes mellitus (GDM), and non-alcoholic fatty liver disease.25–28 In part, these poor maternal outcomes may reflect aberrant maternal metabolic physiology in pregnancy. Normal pregnancy involves the development of hyperinsulinemia, insulin resistance, hyperglycemia, and hyperlipidemia in the mother to allow for nutrient partitioning to the fetus for growth and development.27–29 Metabolic adaptations in pregnancy require careful orchestration of changes in insulin sensitivity by maternal metabolic organs, including adipose and liver, as well as alterations in insulin production by the pancreas. When the mother is obese, these adaptations in metabolic physiology may be mis-appropriately induced or become exaggerated, and likely contribute to the development of gestational diabetes and fetal growth disturbances, namely large for gestational age (LGA) infants.30 However, in a subset of obese women, infants may also be born normal weight or can instead be small for gestational age (SGA) or show fetal growth restriction (FGR).31–33 Moreover, there are data to suggest that obesity leads to impaired breastfeeding24 which could impact neonatal development and growth.35

A “Western style” high-fat, high sugar (HFHS) diet has been recreated in mouse models replicating weight gain, maternal metabolic maladaptations in pregnancy, abnormal fetal growth, and offspring adult health programming.31 However, the majority of studies have focused on long-term exposure to an obesogenic diet (from anywhere between 4 weeks to 14 weeks prior to pregnancy) with established maternal obesity as characterized by significant increases in body weight.36,37 By contrast, the effects of shorter-term consumption of such diets on fertility, pregnancy and offspring outcomes, and contributions of changes in key maternal organs to these outcomes, are far less explored.38 In particular, whether there may be changes in maternal hepatic nutrient handling, pancreas insulin production, or mammary lobuloalveolar development, as these could impact metabolic health in pregnancy and the provision of resources for fetal and neonatal growth and survival. This is important for understanding the impact of mothers changing their dietary habits just before and during pregnancy and who may not show overt changes in body weight, rather than chronically obese mothers who may also have other underlying problems, like insulin resistance.

This study aimed to determine the effect of a HFHS diet from 3-weeks prior to pregnancy and known maternal adiposity on fertility, gestational, and neonatal outcomes in mice. In particular, we examined the ability to achieve and maintain pregnancy, placental structure, fetal growth and maternal metabolic physiology, lactational indices and neonatal survival in mice fed a HFHS diet compared to controls.

2 | RESULTS

2.1 | Greater increase in maternal adiposity with HFHS diet pre-pregnancy

At 6 weeks of age, female C57Bl/6J mice were randomized to remain on the control (RM3) chow or provided with a customized high-fat high sugar (HFHS) diet (n = 20/group) for 3 weeks prior to, and during,
pregnancy (study design; Figure S1). As determined by weight gain monitoring and time-domain nuclear magnetic resonance (TD-NMR) scanning, no differences were seen in initial (pre-diet allocation) maternal body weight, adiposity, and lean mass between chow and HFHS fed mice (Table 1). There was also no effect of a HFHS diet on pre-pregnancy body weight and change (delta) in body weight since commencing the diet 3 weeks earlier. However, HFHS mice exhibited a greater increase in adiposity, but lower gain of lean mass over those 3 weeks pre-pregnancy, when compared to chow-fed counterparts (Table 1, p < 0.001).

2.2 Increased maternal adiposity due to a HFHS diet does not have a notable impact on fertility

Markers of fertility were measured based on mating attempts and rates of pregnancy. Rates of infertility (inability to conceive), days to plug, and plug to pregnancy rate were not different between control and HFHS diet-fed mice (Table 1). The number of HFHS mice that plugged on the first night of being paired with a male was almost half of those fed a control diet (17.5% vs 30.0%) although this did not reach statistical significance (p = 0.1487). Despite this, there was a small, but significant reduction in the number of mating attempts required to achieve a pregnancy in the mice fed the HFHS diet (p < 0.05).

2.3 HFHS diet-fed dams show a hyper-mobilization of adiposity across pregnancy

Although both control and HFHS mice gained weight across gestation (Figure 1A), those fed a HFHS diet gained significantly less weight between embryonic days (E) 0.5 and 17.5 (Figure 1B). This difference may, in part, be due to differences in maternal adipose deposition in pregnancy. Mice fed a control diet, on average, gained ~10.5% adiposity by E7.5 (Figure 1C) and, although they showed a lower percentage of adiposity by E17.5 (6.12%) due to increasing gravid body weight, on average had a net gain in adiposity across pregnancy (between E0.5 and 17.5 Figure 1D). Despite starting with higher adiposity levels, mice fed the HFHS diet exhibited a drastic reduction in adiposity as a percentage of body weight (20.7% at E0.5 to 6.0% at E17.5; Figure 1C), and exhibited a net loss of percentage adiposity between E0.5 and 17.5 (Figure 1D). This may indicate a hyper-mobilization of adipose depots in HFHS-fed mice between E7.5 and 17.5 of pregnancy.

### Table 1 Maternal body composition and fertility parameters

| Body composition          | Control (n = 40) | HFHS (n = 41) | p Value |
|---------------------------|-----------------|--------------|---------|
| Body weight               |                 |              |         |
| Initial                   | 17.81 ± 0.33    | 17.71 ± 0.29 | NS      |
| Pre-pregnancy             | 21.58 ± 0.39    | 22.22 ± 0.46 | NS      |
| Delta                     | 3.76 ± 0.28     | 4.52 ± 0.43  | NS      |
| Adiposity (g)             |                 |              |         |
| Initial                   | 2.018 ± 0.08    | 2.14 ± 0.08  | NS      |
| Pre-pregnancy             | 2.067 ± 0.08    | 4.95 ± 0.29  | <0.0001 |
| Delta                     | 0.07 ± 0.08     | 2.77 ± 0.27  | <0.0001 |
| Adiposity (%)             |                 |              |         |
| Initial                   | 11.22 ± 0.43    | 12.31 ± 0.43 | NS      |
| Pre-pregnancy             | 9.59 ± 0.32     | 21.86 ± 0.95 | <0.0001 |
| Delta                     | −0.67 ± 0.59    | 9.61 ± 0.95  | <0.0001 |
| Lean (g)                  |                 |              |         |
| Initial                   | 13.12 ± 0.33    | 12.64 ± 0.26 | NS      |
| Pre-pregnancy             | 15.82 ± 0.29    | 13.84 ± 0.18 | <0.0001 |
| Delta                     | 2.68 ± 0.21     | 1.18 ± 0.25  | <0.0001 |
| Lean (%)                  |                 |              |         |
| Initial                   | 72.53 ± 0.73    | 71.27 ± 0.68 | NS      |
| Pre-pregnancy             | 73.33 ± 0.26    | 62.90 ± 1.00 | <0.0001 |
| Delta                     | 0.84 ± 0.70     | −8.32 ± 1.06 | <0.0001 |

### Fertility measure

- **Pregnancy success rate**
  - # plugs: 41 vs 36
  - # plugs resulting in pregnancy: 36 vs 33
  - %All mating attempts: 56.3% vs 52.6%
  - %All plugs: 87.1% vs 87.7%
  - Days to plug (mean ± SEM): 2.86 ± 0.28 vs 3.06 ± 0.24

- **Missed plug**
  - # NPFs: 24 vs 20
  - # Missed plug: 2 vs 4
  - %All mating attempts: 3.08% vs 7.14%
  - %All NPFs: 8.33% vs 20.0%
  - # mating attempts-pregnancy (mean ± SEM): 1.78 ± 0.14 vs 1.38 ± 0.11

- **Rate of infertility n (%)**
  - 3 (7.5%) vs 2 (4.8%)

*Note: Initial = at point of diet allocation (6 weeks of age); Pre-pregnancy = −3 weeks post-diet allocation and immediately prior to mating; plug = evidence of copulation plug; NPFs = no plugs found during mating period; missed plug - pregnancy = no plug found during mating period but resulted in pregnancy; %All mating attempts = as a proportion of all mating periods with male regardless of if plug was found or pregnancy resulted; %All plugs = as a proportion of all matings that resulted in a plug regardless of whether it resulted in a pregnancy; Day to plug – number of days paired with male until a plug was found that resulted in a pregnancy; # mating attempts – pregnancy = number of mating periods with male required before a pregnancy was achieved, infertility = either three plugs found that did not result in pregnancy, or five mating attempts that did not result in pregnancy regardless of whether plugs were found. Data analyzed by Student’s t-test or Fisher’s exact for continuous and categorical data, respectively; significance at p < 0.05. The bold values are to indicate the significant p values.*

Abbreviation: NS, not significant.
2.4 | Maternal insulin and glucose handling are not overtly altered, but circulating lipids are decreased in HFHS diet-fed pregnant dams

As indicated by an insulin tolerance test, dams fed the HFHS diet did not exhibit alterations in maternal insulin sensitivity on E17.5 (Figure 2A and area above the curve [AAC] mean±SEM: 38.63±6.83 vs 46.34±7.22 mmol/L/min in control vs HFHS, respectively, p = 0.6905). Similarly, as informed by a glucose tolerance test, maternal glucose handling on E17.5 was not significantly different between HFHS and control dams (Figure 2B and area under the curve [AUC] mean±SEM: 61.15±24.95 vs 112.9±33.95 mmol/L/min in control vs HFHS, respectively, p = 0.1508). Maternal blood glucose concentrations were significantly higher in the fed, but not fasted state (fasted for 4 h) in the HFHS compared to the control pregnant dams (p = 0.0239, Figure 2C).

Circulating insulin was lower in fasted HFHS mice versus controls (p = 0.0051, Figure 2D), but this was not the case for insulin concentrations in the fed state. Both circulating triglycerides and free fatty acids were significantly lower in HFHS diet-fed mice compared to controls at E18.5 (fed state, p = 0.0159 and 0.0317, respectively, Figure 2E,F). Both circulating triglycerides and free fatty acids showed strong negative correlations to pre-pregnancy percentage adiposity and positive correlations to the change in percentage adiposity between pre- and late pregnancy (Figure 2G–J).

2.5 | Maternal organ weights and hepatic and pancreatic morphology are altered in HFHS diet fed pregnant dams

At E18.5, maternal whole and hysterectomised body weight were not altered by a HFHS diet (Table S1).
as a proportion of hysterectomised body weight, mice fed a HFHS diet had greater retroperitoneal adipose depot mass compared to control diet-fed mice, although renal and gonadal fat depots were not different between dietary groups (Table S1). In mice fed the HFHS diet, the weight of multiple maternal organs was increased; brain, heart,
liver, and mammary gland (all \( p < 0.05 \), except for a statistical tendency for mammary gland; \( p = 0.06 \)), and pancreatic weight decreased when compared to controls (Table S1).

Further investigations revealed that the increase in liver weight in HFHS diet-fed dams is related to an increase in liver fat content (10% vs 19% fat content, \( p = 0.0079 \), Figure 2K,L). Histological analysis of the maternal liver, showed an increase in fat droplet diameter \( (p<0.0001, \text{Figure 2M}) \), indicating a degree of steatosis in the liver of mice fed a HFHS diet. As determined by an enzymatic assay, liver glycogen content was however not different between dietary groups (Figure 2N). In addition, histological investigation of the maternal pancreas revealed that, although number and area of islets and \( \beta \)-cell mass were not altered (Figure 2O–R), there was a reduction in the number of small islets with a diameter of 20–40 \( \mu \text{m} \) in dams fed the HFHS diet \( (p<0.0001, \text{Figure 2S}) \). Islets of that size (20–40 \( \mu \text{m} \)) are the most abundant in both control and HFHS diet-fed pregnant mice (Figure 2T).

### 2.6 Fetal weight is not affected, even though fetal glucose and insulin concentrations are elevated in dams fed a HFHS diet

No differences were seen in the number of total conceptuses (resorptions + viable fetuses), resorptions, or viable/live fetuses between control and HFHS diet-fed dams at E18.5 (Figure 3A). Moreover, there was no overall significant change in fetal weight distribution or average fetal weight between control and HFHS diet-fed mice (Figure 3B,E). When data were split by fetal sex, the proportion of fetuses that fall below the 5th centile of the control population (hence, classified as small for gestational age; SGA) doubled from 5.4% to 11.4% in males (Figure 3C), and increased by 62% (from 7.3% to 11.8%) in female fetuses of HFHS diet-fed mice (Figure 3D). Moreover, the proportion of males falling above the 95th centile of the control group (hence, LGA) was halved in mice fed the HFHS diet (Figure 3C). However, the frequencies of SGA or LGA for each fetal sex were not significantly different between control and HFHS diet-fed dams. There was also no evidence of asymmetric fetal growth in HFHS dams, as indicated by the ratio of the fetal brain and liver to body weight, and the ratio of brain to liver weight in either sex (Figure 3F–H). However, in dams fed a HFHS diet, fetal blood glucose (Figure 3I) and insulin (Figure 3J) concentrations were increased in both sexes compared to controls; with pairwise comparisons revealing that the effect that was stronger for females. Fetal triglyceride concentrations were not affected in either sex by a maternal HFHS diet (Figure 3K).

### 2.7 Placental morphology is altered in dams fed a HFHS diet concentrations are elevated in dams

To provide information on the mechanisms contributing to the lack of alterations in fetal growth despite fetal hyperglycemia and hyperinsulinemia in HFHS dams, placental size, and morphology were determined. A maternal HFHS diet did not alter placental weight or placental efficiency (fetal to placental weight ratio), although overall, females had slightly smaller and more efficient placentas than males at E18.5 (Figure 4A,B). As informed by exhaustive placental sectioning, hematoxylin and eosin staining and unbiased stereological analysis, a maternal HFHS diet also did not affect the volume of the maternal decidua basalis (Db), endocrine junctional zone (JZ), and transport labyrinth zone (LZ) in either fetal sex (Figure 4C–E). However, as indicated by periodic acid Schiff staining, a maternal HFHS diet overall increased placental glycogen (Figure 4F,G). Measurement of the size of individual glycogen cells in the JZ revealed there were no differences in the average size or size frequency distribution of JZ glycogen cells in the placenta between control and HFHS diet-fed mice (Figure 4H,J,K). Instead, a greater area of glycogen staining was related to a greater number of glycogen cells within the placenta JZ of HFHS mice (Figure 4I); an effect most pronounced in female fetuses. Using double-label immunohistochemistry and stereology, the morphology of the LZ was also determined (Figure 4L). This showed that there was a tendency for a maternal HFHS diet to reduce the volumes of fetal capillaries (FC), trophoblasts (TB), and maternal blood space (MBS) within the LZ (Figure 4M). In addition, the surface area of the FC and MBS for substrate exchange was reduced in male but not female fetuses of HFHS dams (Figure 4N). There was no effect of a maternal HFHS diet on interhaemal membrane barrier thickness, fetal capillary length, or diameter in the placental LZ either fetal sex (Figure 4O–Q). However, there was a significant interaction of maternal diet and fetal sex on the specific diffusing capacity of the placenta, whereby it was lower in males, but higher in females of HFHS diet-fed dams compared to their respective controls (Figure 4R).

### 2.8 Offspring survival is reduced through lactation period in pups from HFHS diet-fed dams

In separate dams allowed to deliver naturally and continued their respective diet, we assessed impacts of a maternal HFHS diet on post-partum body composition and
offspring outcomes (up until weaning, at 3 weeks of age). Maternal body weight at weaning and change in body weight between pre-pregnancy and weaning was not altered by the HFHS diet (Table 2). Post-partum, HFHS diet-fed dams had greater adiposity levels at weaning (both in g and % body weight; Table 2). However, by comparing to pre-pregnancy adiposity levels, post-partum HFHS dams exhibited a loss in adiposity over that period, unlike the control dams which instead showed a gain (Table 2). This pattern of change in maternal adiposity is likely due to
the hyper-mobilization of adipose depots in HFHS dams during pregnancy. Lean mass (g and %) remained less in
post-partum HFHS dams, however, the percentage gain in
lean mass from pre-pregnancy to weaning was greater in
HFHS-fed mice compared to control (Δ% lean mass,
\( p = 0.0061 \); Table 2).

There was no difference in gestation at delivery be-
tween HFHS and control diet-fed dams (Table 2). Total
litter size (live and dead pups) and live litter size at birth/
postnatal day (D)0 and live litter size at weaning were
lower in dams fed a HFHS diet compared to those fed a
control diet (Table 2). There was no relationship between
litter size and pre-pregnancy body weight. However,
when both dietary groups were combined, total litter size
at D0 was shown to negatively correlate with maternal
pre-pregnancy adiposity and change (delta) in adiposity
across pregnancy (total litter size shown in Figure 5A–C),
but positively correlated with maternal pre-pregnancy
lean mass (Figure 5D). Offspring demise was significantly
greater at D3 (15.5% vs 6.3%), D7 (16.6% vs 8.2%), and D21
(17.1% vs 8.8%), in litters from HFHS dams compared to
control, respectively (Table 2). Average percentage loss
per litter was also greater in HFHS compared to control
dams (16.9% vs 9.1%). Although not significant, a greater
proportion of HFHS diet-fed dams exhibited a total litter
loss by weaning as compared to control dams (11.5% vs
3.2%, respectively; Table 2). The number of pups lost by
D21 showed a tendency to correlate with change in matern
al adiposity between pre-pregnancy and weaning, with
a greater loss in adiposity over this period resulting in a
greater litter loss by weaning (Figure 5E).

2.9 Mammary development during pregnancy is advanced in dams fed a
HFHS diet

To gain information on the mechanisms contributing to poor
offspring outcomes during lactation observed in HFHS diet-
fed dams, mammary glands were obtained from near-term
dams (E18.5), and lobuloalveolar development was assessed
in hematoxylin and eosin-stained sections using stereology
(Figure 5F,G). Alveoli were larger in size (Figure 5H,I), with
greater luminal area (Figure 5K,L) and reduced epithelial
thickness in HFHS diet-fed mice (Figure 5J), indicating
more advanced lobuloalveolar development when com-
pared to control diet-fed mice. Furthermore, assessment of
lipid droplet location showed lipid droplets located closer
to the mammary gland epithelial luminal surface in HFHS
diet-fed mice compared to control (Figure 5M), suggesting
advanced milk production and secretory capacity. The abun-
dance of milk proteins in homogenized mammary glands
as assessed by mass spectrometry revealed that HFHS diet-
fed dams had increased α-S1-casein, and α-lactalbumin but
decreased ɛ-casein and H-FABP (heart fatty acid binding
protein) levels compared to control diet-fed mice (Table 3).
There were no significant differences in the other mammary
gland milk proteins assessed (Table 3).

3 | DISCUSSION

This study in mice shows that a HFHS diet from 3-weeks
prior to pregnancy increases adiposity without overt im-
acts on body weight or fertility. Despite this, maternal
metabolic physiology, and placental development during
pregnancy, as well as neonatal outcome are negatively
impacted in HSHF mice. In particular, the HFHS dams
exhibited enhanced adipose tissue mobilization in late
pregnancy in association with altered circulating lipids,
and changes in key maternal metabolic organs, namely
the pancreas with reduced insulin secretion and the liver
with excessive fat accumulation. There was the reduced
formation of placental labyrinth zone structures in both
sexes, with decreased placental diffusional capacity spe-
cifically in males. Postnatally, there was a significant
reduction in litter size at birth for dams fed the HFHS
diet and a greater number of their pups did not survive
during the lactational period. Postnatal demise seemed
to be correlated to changes in maternal adiposity across
the pregnancy and weaning period and aberrant devel-
opment of mammary lobuloalveolar structures and milk
protein production. These data are highly relevant given
that women at reproductive age often consume HFHS
diets and although they may not necessarily show gross alterations in body weight, these women could have metabolic problems during pregnancy with developmental consequences for their child.

It is well established that obesity negatively impacts the ability of a woman to fall pregnant. Infertility is threefold more common in obese women than those with a normal BMI and 15% of women seeking assisted reproductive
therapies (ART) fall into the obese category.\textsuperscript{39,40} Women with raised BMI have a longer latency to pregnancy period partly due to irregular menstrual cycles and diminished ovarian function and oocyte quality.\textsuperscript{8,41–43} In our mouse model, we saw that a maternal HFHS diet (~3x fat and ~5x sugar than control diet) for 3 weeks prior to mating did not significantly increase the number of mating attempts needed to result in pregnancy or reduce the number of mice exhibiting a successful mating (copulation plug) on the first night when they were paired with a stud male. Previous studies showed that mice fed a very high-fat diet (~6x control) exhibit altered estrous cycles only after 2 weeks on the high-fat diet when they also show enhanced body weight.\textsuperscript{44} Thus, it is not surprising that no overt changes in fertility were seen in our model after 3 weeks of exposure to a high-fat (and sugar) diet.

| Post-partum maternal body composition | Control (N = 31) | HFHS (N = 39) | p Value |
|--------------------------------------|-----------------|--------------|---------|
| Body weight (g)                      | 26.16 ± 0.54    | 26.59 ± 0.44 | NS      |
| Delta                                | 4.92 ± 0.50     | 4.70 ± 0.31  | NS      |
| Adiposity (g)                        | 3.19 ± 0.31     | 5.34 ± 0.34  | <0.0001 |
| Delta                                | 1.09 ± 0.30     | 0.20 ± 0.25  | 0.0302  |
| Adiposity (%)                        | 12.03 ± 0.83    | 19.50 ± 1.05 | <0.0001 |
| Delta                                | 2.18 ± 0.85     | −2.78 ± 1.03 | 0.0005  |
| Lean (g)                             | 19.12 ± 0.31    | 17.65 ± 0.28 | 0.0012  |
| Delta                                | 3.38 ± 0.29     | 3.78 ± 0.22  | NS      |
| Lean (%)                             | 72.80 ± 0.97    | 66.00 ± 0.89 | <0.0001 |
| Delta                                | −0.28 ± 1.07    | 4.19 ± 1.13  | 0.0061  |

| Offspring survival                   | (N = 19, n = 159) | (N = 26, n = 181) |
|--------------------------------------|-------------------|-------------------|
| Gestation at delivery (day)          | 20.19 ± 0.08      | 20.13 ± 0.10      | NS      |
| Total litter size (D0)               | 8.44 ± 0.23       | 7.19 ± 0.43       | 0.0014  |
| Live litter size (D0)                | 7.89 ± 0.48       | 6.65 ± 0.43       | NS      |
| Live litter size at weaning (D21)    | 7.56 ± 0.58       | 5.77 ± 0.56       | 0.0370  |

| Pup loss by D3                       |                   |                   |
|--------------------------------------|-------------------|-------------------|
| # litters with pup loss              | 2 (10.5%)         | 8 (30.8%)         | NS      |
| # pups lost                         | 10                | 28                | 0.0121  |
| % loss                              | 6.3%              | 15.5%             |         |

| Pup loss by D7                       |                   |                   |
|--------------------------------------|-------------------|-------------------|
| # litters with pup loss              | 4 (21.0%)         | 10 (38.5%)        | NS      |
| # pups lost per litter               | 13                | 30                | 0.0307  |
| % loss                              | 8.2%              | 16.6%             |         |

| Pup loss by D21                      |                   |                   |
|--------------------------------------|-------------------|-------------------|
| # litters with pup loss              | 5 (26.3%)         | 11 (42.3%)        | NS      |
| # pups lost per litter               | 14                | 31                | 0.0358  |
| % loss                              | 8.8%              | 17.1%             |         |

| Average % loss/litter TLL            |                   |                   |
|--------------------------------------|-------------------|-------------------|
| # litters                           | 1                 | 3                 | NS      |
| % litters                           | 3.2%              | 11.5%             |         |

Note: Post-partum maternal body composition was determined within 1 week of weaning offspring. Data expressed at mean ± SEM unless stated otherwise and analyzed by Student’s t-test or Fisher’s exact for continuous and categorical data, respectively; significance at p < 0.05. The bold values are to indicate the significant p values.

Abbreviations: D, postnatal day; delta, change between pre-pregnancy and post-weaning; N, number of dams; n, number of pups; NS, not significant; TLL, total litter loss (no pups surviving by D21).
FIGURE 5 Offspring survival and lactational capacity assessed in dams fed HFHS versus control diet. (A–D) Litter size (day 0 post-partum; D0 PP) correlated with pre-pregnancy maternal body weight in A, pre-pregnancy adiposity in B, change in adiposity during pregnancy in C and pre-pregnancy lean mass in D. (E) The number of pups lost per litter at D21 PP correlated with change in maternal adiposity (%) from pre-pregnancy to post weaning. Correlations assessed by Spearman Rank Correlation, r² quoted when significant (p < 0.05, dietary groups combined). (F and G) Representative images of mammary tissue from control and HFHS fed dams at E18.5 of pregnancy (n = 6/group) at 40x (scale bar = 50 μm). (H and I) Average area and size frequency of alveoli. (J) Epithelial thickness. (K and L) Luminal area and size distribution and (M) Lipid droplet location (proximity to lumen or epithelium) in alveoli. (N–Q) Relative abundance of milk proteins in mammary tissue from control and HFHS fed dams at E18.5 of pregnancy as determined by mass spectrometry (n = 10/group). Data presented as individual values and/or mean ± SEM and were analyzed by t-test except for frequency distributions, which were analyzed by Two-way ANOVA (diet and size frequency).
pregnancies, found that most poor outcomes (including preeclampsia, gestational diabetes, and LGA) increased with weight gain of just one or two BMI units between pregnancies, independent of if the mother was overweight or not. 50 Another study found that raised maternal BMI in a first pregnancy with uncomplicated outcome still increased the risk of poor outcome in the second pregnancy, even if these women have normal BMI in second pregnancy. 51 These data together suggest there may be an effect of having a first pregnancy with sub-optimal maternal adiposity that causes physiological adaptations leading to a higher risk of poor outcome in the subsequent pregnancies, regardless of BMI, further accentuating the importance of studying and intervening with mid-risk (not overtly overweight or obese) women in their first pregnancies.

Interestingly, mouse dams fed a HFHS diet who had greater adiposity at the start of pregnancy showed a greater loss of adiposity across late gestation compared to controls. As a result, HFHS diet-fed dams ended pregnancy with similar body adiposity levels to control diet-fed dams (only weight of the retroperitoneal fat store, not renal, or gonadal was greater in HFHS dams). Compared to the anabolic state in early-mid pregnancy, late gestation represents a catabolic state when the pregnant mother exhibits increased lipolytic activity and raised maternal free fatty acids and triglyceride levels 52 to provide fuel for fetal growth and development, as well as maternal parturition and lactation. 53 This catabolic state may be exaggerated in our HFHS diet-fed mouse dams, implying increased lipolytic activity of adipose stores that results in their re-sizing/greater loss in the lead up to term. This may also explain the increase in fat content of the liver, as circulating maternal lipids are filtered by the liver and can accumulate in this ectopic site. 38 Circulating triglycerides and free fatty acids were both reduced in our HFHS diet-fed dams, which has been seen previously in other similar rodent models. 38 This could reflect a greater storage or usage of lipids by the mother, or increased utilization by the developing conceptuses. Furthermore, a less pronounced increase in triglycerides at the end of pregnancy in preparation for parturition and lactation has been seen in obese women. 52 Future work could look at longitudinal changes in maternal lipid levels and lipid handling using lipid tolerance tests throughout pregnancy in our HFHS diet-fed dams. This would inform on timing of the change from anabolic and catabolic states of pregnancy and whether there was a peak in circulating maternal lipids earlier in gestation (between E12.5–17.5) when maternal adiposity declined extensively in the HFHS-fed dams. Interestingly, maternal body weight gain during pregnancy is often monitored clinically, as increased gestational weight gain is associated with an elevated risk for LGA and gestational diabetes 54–56. Interventions that improve maternal diet

**Table 3** Milk composition at E18.5

| Milk composition | Control (N = 10) | HFHS (N = 10) | p Value |
|------------------|------------------|---------------|---------|
| α-S1-casein      | 8359 ± 1153      | 14 528 ± 2012 | 0.0159  |
| ε-casein         | 0.70 ± 0.05      | 0.40 ± 0.03   | 0.0003  |
| α-lactalbumin    | 0.15 ± 0.04      | 0.35 ± 0.08   | 0.0406  |
| H-FABP           | 24.72 ± 1.85     | 15.97 ± 1.17  | 0.0009  |
| b-casein         | 0.60 ± 0.09      | 0.50 ± 0.07   | NS      |
| k-casein         | 0.23 ± 0.02      | 0.22 ± 0.02   | NS      |
| γ-casein         | 1.69 ± 0.28      | 2.49 ± 0.41   | NS      |
| Lactotransferrin | 0.06 ± 0.02      | 0.03 ± 0.003  | NS      |
| Whey acidic protein | 0.15 ± 0.03    | 0.18 ± 0.02   | NS      |
| Serum albumin    | 6.03 ± 1.06      | 4.31 ± 0.61   | NS      |

Note: Data presented as mean ± SEM of peak area/mg tissue. Data were analyzed by student t-test or Mann–Whitney U test; significance at p < 0.05. The bold values are to indicate the significant p values.

Abbreviation: NS, not significant.
and limit maternal weight gain, therefore, have positive impacts on reducing rates of LGA and gestational diabetes.\(^5^7\) While limiting excessive gestational weight gain reduces rates of LGA in overweight/obese women, gestational weight loss is not advised due to the increased risk of delivering an SGA infant.\(^5^8\)

Mice fed a HFHS diet were found to have fewer of the most abundant but small-sized islets (20–40\(\mu\)m) and reduced fasting insulin. Similar findings have been seen in other models of short-term pre-pregnancy obesogenic diet exposure.\(^5^9\) It is well established that during normal pregnancy, there is an increase in proliferation and size of \(\beta\)-cells.\(^6^1\) There may also be \(\beta\)-cell neogenesis in the mother during pregnancy.\(^6^2\) \(\beta\)-Cells adaptations enable enhanced insulin production to control for the insulin resistance state of the mother during pregnancy.\(^2^9\) Our findings may therefore indicate an impaired ability of the mother to undergo \(\beta\)-cell proliferation, hypertrophy, and/or islet neogenesis during pregnancy when she is consuming a HFHS diet.\(^2^9\) While dams fed the HFHS diet did not show overt alterations in insulin or glucose handling as determined by in vivo tolerance testing in late pregnancy, the subtle alterations in islet morphology likely contributed to the reduced fasted insulin and increased fed glucose levels seen and together, suggest a reduced \(\beta\)-cell function in HFHS dams.\(^6^3\) Moreover, the difference in the area under the curve for the glucose tolerance test and variation between animals were large, suggesting that the lack of significance between the control and HFHS groups may have been due to limited sample size, which should be verified in future work. Prolonged exposure to high glucose levels can lead to \(\beta\)-cell glucotoxicity, oxidative stress, apoptosis, and insulin insufficiency.\(^6^4\) Future work should therefore employ pancreatic islet cultures to study insulin secretion responses of the \(\beta\)-cells and to dissociate the endocrine and metabolic environment from HFHS dams.

Dams fed the HFHS diet had livers with higher fat content and a greater number of large lipid droplets (8–20\(\mu\)m diameter), which are indicative of steatosis and non-alcoholic fatty liver disease (NAFLD).\(^6^5\) This may be due to the exaggerated mobilization of adipose stores in HFHS diet-fed dams in the second half of pregnancy and molecular changes in the liver. Previous work in rodents fed HFHS diets has reported an altered abundance of insulin signaling and lipid uptake and lipogenic proteins in the maternal liver in pregnancy.\(^3^8\) Obesity and insulin resistance are the most prevalent risk factors for hepatic steatosis, which can lead to NAFLD.\(^6^5 \text{,}6^6\) In turn, NAFLD increases the risk of gestational diabetes,\(^6^8\) hypertensive disorders of pregnancy including, preeclampsia,\(^7^0\)\(7^1\) and HELLP syndrome, and enhances the risk of post-partum hemorrhage and maternal death.\(^7^1\) Further work is therefore required to understand the pathways contributing to the hepatic steatosis in our HFHS dams in pregnancy.

There was no significant effect of maternal HFHS diet consumption on fetal weight by the end of pregnancy (only non-significant increases in the rate of SGA for males and females were observed). Work in several previous animal studies has shown that the impacts of an obesogenic diet on fetal growth vary greatly and depend on the specific nutrient composition, time of onset, and duration of the diet (recently reviewed\(^7^2\)). Despite the lack of effect on fetal growth, fetal glucose, and insulin levels were both increased in response to maternal HFHS diet exposure. Placental transport and fetal glucose levels closely correlate with maternal glucose concentrations, which were also raised (in fed state) in dams fed the HFHS diet. Previous work has shown that dams fed a HFHS diet exhibit increased placental glucose transport in vivo.\(^7^4\) In addition, chronic fetal hyperglycemia has been shown to result in reduced insulin sensitivity, diminished protein synthesis, and fetal growth restriction.\(^7^7\) These data may provide some explanation for the lack of significant changes in fetal growth despite elevated fetal glucose and insulin concentrations in our dams fed a HFHS diet. Indeed, other work has shown that fetuses of women who are obese show insulin resistance in utero.\(^7^8\)

No significant differences were seen in gross placental weight or placental zone volumes between HFHS diet and control dams in either sex. However, there was a moderate reduction in the volumes of maternal blood space, trophoblast, and fetal capillaries in the placental labyrinth zone in both sexes with maternal HFHS diet exposure. Placentas of male fetuses from HFHS diet-fed mice also showed a reduction in fetal capillary and maternal blood space surface areas and decreased diffusional capacity (a slight increase in diffusing capacity was instead seen in females). These data suggest a sexually dimorphic response of placental LZ development to a maternal HFHS diet. Junctional zone glycogen cell area and number were increased in placentas from both male and female fetuses of mice fed a HFHS diet, however, the magnitude of the effect was stronger in the females. Other work in rodent models has also reported greater effects on the junctional zone of females, compared to male counterparts.\(^7^9\) Placental glycogen is a key store of glucose, and increased glycogen stores may reflect an attempt to limit fetal exposure to glucose in hyperglycemic conditions.\(^8^0\) This may be most important given that both the HFHS diet-fed dams and her fetuses were hyperglycemic in our study. While the definitive role of placental glycogen is elusive, the release of placental glycogen stores in late pregnancy is thought to be important for meeting placental and fetal fuel demands.\(^8^0 \text{–}8^2\) Thus, it is possible that the increase in placental glycogen stores in HFHS diet-fed dams may also reflect an inability to release
glucose reserves with important implications for neonatal outcomes.83

Indeed, significant reductions in offspring survival were seen in HFHS diet-fed dams. Despite no differences in live litter size at E18.5, there was a significant decrease in litter size at birth. Since the process of parturition was not observed, we do not know if there was an increase in peripartum stillbirth rates – as these are known to be increased by maternal obesity,11 or whether pups may have been cannibalized by the dam before litter size was recorded. The incidence of offspring demise continued throughout the post-partum period, and particularly in the first 7 days.84 Our studies are consistent with the increased mortality seen within the first 6 days after the birth of litters born from dams with diet-induced obesity and previous work has shown that this was not related to differences in feeding behavior or endocannabinoid systems in the offspring (important food intake and energy balance).85 Interestingly, previous work showed that offspring who survived/fared better in the first weeks of life were more likely to be males than females from obese mothers.85 As we wanted to limit handling of the neonates and minimize any effect on neonatal survival rates, we did not obtain information on sex ratio at birth or during lactation, so we do not know whether the reduction in neonatal survival was equal for both females and males. However, interestingly, the reduction in litter size and rate of litter loss was correlated with maternal percentage adiposity pre-pregnancy and the change in adiposity between pre-pregnancy and end of pregnancy. Furthermore, surviving litter size at weaning correlated with adiposity change between pre-pregnancy and post-weaning, with the greatest adiposity loss over this time resulting in greater offspring demise post-partum.

Obese women have lower rates of breastfeeding and shorter time to breastfeeding cessation than lean counterparts.34 While reasons for this may be behavioral and cultural, there are data that also suggest this could be due to delayed onset of lactogenesis II (postnatal milk production).86 Interestingly, characteristics of more advanced mammary gland development, such as greater alveoli area, greater luminal area, thinning of epithelial wall, and migration of lipid droplets from epithelial cytoplasm to the luminal space,87 were seen in our HFHS diet-fed mice. Similar results have been seen in other studies investigating mammary development in rabbits fed a high-fat diet from pre-pregnancy.88 In our study, the concentrations of key milk proteins,89 was altered in HFHS dams; namely α-S1-casein and α-lactalbumin were increased and ε-casein and H-FABP were decreased. The physiological significance of changes in milk protein composition for offspring growth and development has been previously highlighted.90 However, the significance of the specific changes in milk protein levels found in our HFHS diet-fed dams with regards to the reduced neonatal survival requires further investigation. Indeed, postnatal pup weight, other aspects of lactation, such as the ability of the mother to provide milk to her pups, maternal nurturing behavior, and the ability for pups to suckle effectively,91 necessitate study in future work.

It must be noted that lean mass of dams fed a HFHS diet was reduced both pre-pregnancy and end of pregnancy, which has been seen in other mouse models fed an obesogenic diet.92 In our study, females were fed the HFHS diet from 6 weeks of age, which is soon after sexual maturity, and before they are fully grown. Thus, it is possible that the timing of feeding explains the lack of change in body weight and lower gain of lean mass (as well as greater reduction in adiposity during late pregnancy) in our study. Seeing as lean mass is strongly correlated with fat mass, it is difficult to distinguish the individual effects of these maternal body composition parameters on our findings. Maternal low protein diet has been known to impact fetal growth and placental function,93 as well as offspring health and behavior.44,95 We are also not able to determine whether the pre-pregnancy diet exposure and/or the pregnancy and lactation exposure to a HFHS diet was responsible for the poor neonatal outcomes in our model. Previous work using embryo transfers from high fat-fed to a control diet-fed mice found that pre-gestational exposure to a high fat diet is sufficient to impact prenatal growth.96 However, prenatal development was also reduced in the embryos from control diet fed females implanted into high fat diet-fed dams, and the gestational exposure to a maternal high-fat diet was responsible for alterations in offspring obesity and glucose metabolism postnatally.96 With these important data in mind, we, therefore, are unable to determine if alterations in offspring outcomes seen in our study are due to pre, peri, or post-natal exposure to the maternal HFHS diet.

In summary, this study uses a highly integrative approach combining in vivo imaging and metabolic testing, with morphological, biochemical, and proteomic analyses to assess the impact of a maternal western-style HFHS diet on pregnancy physiology and lactation ability (Figure S2). In particular, mice fed a HFHS diet from 3 weeks prior to pregnancy show altered adiposity, lipid handling, metabolic organ morphology, and circulating insulin and glucose levels in pregnancy. This was associated with dysmorphic placentas, aberrant mammary gland development, and milk composition, as well as increased rates of neonatal loss. These findings may be relevant for the portion of mothers that change their culinary habits during pregnancy (short exposures to obesogenic just before and during pregnancy). Our findings also highlight the inaccuracy of relying on pre-pregnancy maternal body weight to predict pregnancy risk
of metabolic disorders that impact prenatal development and offspring health and wellbeing.

4 METHODS

4.1 Mice and husbandry

All animal experimentation was carried out under the UK Home Office Animals (Scientific Procedures) Act 1986, following ethical review by the University of Cambridge. All mice used were housed under 12:12 h dark/light photocycles with ad libitum access to water and standard rodent diet (RM3, Special Dietary Services [SDS], Witham, UK) (11% fat, 7% simple sugars of energy contribution [%kcal]) from weaning. At 6 weeks of age, female C57Bl/6J mice (who are homozygous for the Igf2Flox transgene but have been on a C57Bl/6J background for >10 generations, as part of a larger study and do not affect these pregnancies) were randomized to remain on the control (RM3) chow or provided with a customized high-fat high sugar (HFHS) diet (n = 20/group). The HFHS diet contained high-fat diet pellet (D12451 diet, Research Diets Inc, Denmark) and condensed milk (Carnation, Nestle, Gatwick, UK) in a 1.5:1.0 ratio, that was blended in a food processor with water and baked into patties at 55°C for 46–48 h, as described previously.97 The resulting nutritional composition was 38% fat, and 33% simple sugars of energy contribution (%kcal). Mice had ad libitum access to food and water, and the HFHS diet was replaced every other day to maintain palatability.

Mouse weight gain was monitored and a TD-NMR scanner (LF50H Minispec, Bruker, Coventry UK) was used to determine longitudinal changes in body composition (lean and fat mass) during the study, as well as to refine our study groups (Figure S1). Female mice were TD-NMR scanned at diet assignment, 3 weeks later at time-mating with a male (see below), as well as at E7.5, 12.5, and 17.5 of pregnancy. The subset of dams that provided litters for neonatal studies were also TD-NMR scanned after weaning. Only control and HFHS diet-fed mouse dams with pre-pregnancy adiposity (% of body weight) of ≤12.4% and ≥12.5%, respectively, were mated for this study, to ensure the two groups were sufficiently separated. Using these criteria, 15 controls and 14 HFHS-fed dams were excluded.

4.2 Mouse mating and fertility assessments

As briefly mentioned above, after 3 weeks on the control or HFHS diet, female mice were time-mated with a wildtype C57Bl/6J proven stud male. Females were with a male for no more than 5 days or until copulation plug was seen. Mice were then weighed to assess if pregnant (regardless of whether a plug was found) and then re-mated if not pregnant. The presence of a copulation plug was considered E0.5. Females on HFHS diet had access to control diet while housed with males for time-mating only. Female mice who had plugged but did not result in a pregnancy were re-mated for a maximum of three attempts. Female fecundity was noted by tracking plug rates, time to plug and pregnancy rates (proportion of mating attempts that resulted in pregnancy). Mating attempts equal the number of times a female was put with a male until a pregnancy resulted.

A subset of pregnant control and HFHS mice underwent either metabolic testing at E17.5 with terminal collection of maternal and feto-placental tissues at E18.5 or were allowed to litter and attend to their young for a 21-day lactation period (Figure 1). For the latter cohort, the dams remained on their respective diet and gestational age at delivery, litter size, and offspring death/survival during the lactation period were recorded.

4.3 Maternal glucose and insulin handling in pregnancy

Mice at E17.5 were fasted from 10:00 am for 4 h and either underwent an insulin tolerance test (ITT) or glucose tolerance test (GTT) (n = 5/group/test) by administering an intraperitoneal bolus dose of 0.25 U/kg insulin (Actrapid, Henry Schein, UK) or 1 g/kg of glucose (Dechra Veterinary Products, UK), respectively. Serial blood sampling was done through a tail nick at 0, 15, 30, 45, 60, and 120 min to measure blood glucose using a OneTouch Select Plus® glucose meter (Lifescan, Uxbridge, UK). During the GTT, maternal blood samples collected at 0 and 15 min were centrifuged at 3000 rpm for 10 min to collect serum for determining maternal fasted and glucose-stimulated insulin concentrations. Insulin concentrations were determined using Mouse Insulin ELISA (Mercodia, Sweden) following the manufacturer’s protocols. Insulin concentrations were calculated interpolated from a standard curve using cubic spline regression in Prism (USA, average intra-assay %CV 3.89).

4.4 Pregnancy outcome indicators and collection of maternal tissues

After the ITT or GTT, dams were immediately returned to a cage for ad libitum access to their respective diet (control or HFHS). The following morning (E18.5), dams were either anesthetized (1:1:0.16 Fentanyl (Fentadon, Dechra
Veterinary Products UK), Midazolam (Hypnovel®, Roche, UK), and Ketamine (Ketavet, Zoetis, UK) diluted in 3.26 parts water and injected i.p. with 10 μl/g for collection of cardiac blood into an EDTA tube (GTT animals), or immediately schedule 1 killed by cervical dislocation (ITT animals). Fetuses were removed by laparotomy and decapitated. Fetal blood was also collected from the animals that had undergone a GTT the previous day. Fetuses were sexed by eye and sex was then confirmed by SRY genotyping. The male and female pup with the closest weight to the average for the litter per sex was selected and fetal blood was collected immediately following decapitation. Maternal and fetal blood were centrifuged at 12000 rpm to obtain plasma and serum fractions, respectively, and samples were then stored at −20°C until analysis. Maternal organs and fetuses and placentas were weighed. From a sub-set of mice, maternal liver, pancreas, and right abdominal mammary gland, and placenta per sex per litter were fixed in 4% paraformaldehyde (PFA) and processed into paraffin wax for sectioning and histological analysis. From a different subset of mice, maternal liver, and left abdominal mammary gland was snap frozen and stored at −80°C for biochemical analysis.

Pregnancy outcome was assessed by total litter size, number of viable fetuses, and presence of resorptions within the litter, fetal, and placental weight, and fetal weight distribution using <5th, 5–95th, and >95th centiles of the control diet group as clinically relevant markers of small for gestational age (SGA), appropriate for gestational age (AGA) and large for gestational age (LGA). Fetal brain and liver weights and blood glucose were also determined from a subset of fetuses within each litter.

4.5 | Plasma and serum analysis

Maternal plasma and fetal serum samples were analyzed by the Core Biochemical Assay Laboratory (CBAL, Cambridge University Hospital NHS Foundation Trust) to determine the concentrations of insulin (Multiplex ElectroChemical Luminescence Immunoassay, Mesoscale Discovery, USA, inter-assay %CV 4.4–4.7%), triglycerides (Lipase Enzyme Assay, Siemens Healthcare, Germany, inter-assay %CV 3.4–5.5%), and non-esterified free fatty acids (maternal plasma only, Roche Free Fatty Acid Kit (Sigma-Aldrich, inter-assay %CV 15.0–12.5%).

4.6 | Maternal liver assessments

4.6.1 | Fat content assay

Maternal liver fat content was assessed in 100 mg of tissue homogenized in 1 ml Folch mixture (chloroform:methanol 2:1), mixed with water and centrifuged at 13200 rpm for 10 min to obtain the lipid phase. The weight of lipid content was assessed by dry extracting fats at 37°C overnight and presented as a percentage of wet tissue mass.

4.6.2 | Glycogen content assay

Glycogen content of the maternal liver was assessed as previously described. In brief, 100 mg/ml of tissue in distilled H₂O (dH₂O) was incubated at 55°C for 10 min with acetate buffer, then a further 10 min with or without 70 U/mg amyloglucosidase enzyme in duplicate. 0.3 M zinc sulfate and 0.3 M barium hydroxide (Sigma-Aldrich) were added to de-proteinise the samples. After centrifugation at 3000 rpm for 10 mins, glucose content of the supernatant was quantified on a YSI Analyzer (YSI Inc., USA). Glucose concentrations were compared between assays in the presence and absence of the amyloglucosidase to quantify the conversion of glycogen to glucose as the indicator of glycogen content and presented as mg/g of wet liver tissue.

4.6.3 | Liver steatosis

Hematoxylin and eosin (H&E) stained 7 μm sections of the left lateral lobe of maternal livers were imaged in a systematic, random fashion to obtain ten 20x images representative of the whole tissue. Images were processed using ImageJ (NIH Image, USA) and a developed macro that had been previously validated, to assess lipid droplets size as a marker of steatosis. Briefly, the original H&E image was converted to 8-bit gray-scale image, color inverted, and a threshold of 150–255 gray levels applied to identify the lipid droplets. Particle analysis was then configured to count particles with circularity between 0.5 and 1.0 AU and a diameter between 0.1 and 50 μm. The macros created a database with each droplet diameter and area in each image, which were compiled for the 10 images per sample from which actual and relative distributions of lipid droplet size could be assessed.

4.7 | Maternal pancreas assessments

4.7.1 | Islet morphology by immunohistochemistry

Three non-adjacent 5 μm sections (100μm apart in depth) of paraffin-embedded maternal pancreas were assayed to ensure sampling throughout the entire tissue was stained by standard immunohistochemical techniques. Briefly, rehydrated sections were treated with 10% hydrogen peroxide...
(Fisher Chemical, USA) in methanol for 15 min to quench endogenous peroxidases. Antigen retrieval was conducted in citrate buffer (pH 6.0) in an 80°C water bath for 30 min. Sections were then blocked using blocking buffer (2% BSA, 1% skimmed milk powder, 0.1% Tween in dH2O) including 10% goat serum for 1 h. Sections were then incubated overnight at 4°C with either rabbit anti-mouse insulin (1:1000, #4590 Cell Signaling) diluted in the blocking buffer. Bound antibody was detected using biotinylated goat anti-rabbit secondary (1:1000, ab6720, Abcam) and then streptavidin-horseradish peroxidase (1:500, S000-03, Rockland Immunochemicals) each for 1 h at room temperature. Bound antibody was then visualized using 3, 3′-diaminobenzidine (DAB, Sigma) and slides were counterstained with Nuclear Fast Red (Vector Laboratories). Washes throughout the procedure were done with Tris-buffered saline (TBS) or TBS-tween (0.05%) where appropriate. Whole sections were imaged by Nanozoom slide scanner (Hamamatsu, Japan). Insulin-stained sections were analyzed for β-cell and islet area/diameter by manually drawing around insulin-positive stained areas. β-Cell mass was presented as % area and mass in mg by multiplying percentage area by total pancreatic wet weight.

### 4.8 Maternal mammary gland assessments

#### 4.8.1 Mammary gland histology

Three non-adjacent 5 μm mammary gland sections per sample were stained with Masson’s Trichrome to stain collagen and muscle fibers. Samples were deparaffinized and hydrated. Sections were placed in Bouin’s Fluid at 56°C for 1 h, then in working Weigert’s iron hematoxylin stain for 10 min followed by 10 min in Biebrich Scarlet-Acid Fuchsin Solution and 5 min in phosphotungstic-phosphomolybdic acid solution. Finally, samples were stained in aniline blue stain solution for 10 min, placed in acetic acid for 1 min and dehydrated. Once scanned by Nanozoom slide scanner (Hamamatsu, Japan), ten fields of view captured at 20x magnification from representative areas of each section were analyzed by Image J (NIH Image). For mammary gland area, an ImageJ macro was developed. Briefly, scale was adjusted to 2.22 pixels/μm. The picture was converted to 8-bit and a threshold with cut-off values of 0–215 gray levels was applied. Alveolar development was analyzed on single mid-tissue sections per sample. Using NDP view, alveolar and luminal area/diameter were quantified by manually drawing around 50 randomly selected alveoli (5 per lobule over 10 lobules) per section at 80x magnification. Mean epithelial thickness was calculated as; alveoli diameter – luminal diameter. Average area of cytoplasmic lipid droplets (CLD) was analyzed using Adobe Photoshop CC 2017 of ten 80x magnification images from single sections per sample and was presented as both a mean and a frequency distribution and location in alveoli (epithelial/luminal adjacent/luminal).

#### 4.8.2 Characterization of mammary tissue milk proteins

Mammary tissue was powered and then 13-20 mg was homogenized in 250μl of 6 M guanidine hydrochloride (Sigma-Aldrich) with Lysing Matrix D (MPbio) in a FastPrep-24 homogenizer. Proteins were extracted by adding 75% (v/v) acetonitrile (ACN) (Fisher Scientific) to 150μl of lysate, vortexing to mix, and centrifuged (12000 g, 10 min, 4°C) to obtain the aqueous phase. The aqueous phase was collected and dried under oxygen-free nitrogen (40°C) on a SPE Dry evaporator system (Biotage, Upsalla, Sweden). Extracts were re-suspended in 500μl of 0.1% (v/v) formic acid in water and loaded to a Waters HLB μElution solid-phase extraction plate (Waters, Milford, MA) as described previously. Following reduction and alkylation, 10 μl trypsin (100 μg/ml) was added and incubated overnight at 37°C. The following day, 20 μl 1% formic acid was added.

LC–MS/MS was performed on a M-Class UPLC system (Waters, Milford, USA) with a TQ-XS triple quadrupole mass spectrometer with an IonKey interface (Waters). Digested sample (5 μl) was loaded onto a NanoEase 0.3 × 50 mm, BEH C18 trap column (Waters), at 15 μl/min for 3 min. Peptides were then separated on a 0.15 × 100 mm HSS T3 iKey (Waters) at 3 μl/min. The load and initial conditions were 90% A (0.1% formic acid in water v/v) and 10% B (0.1% formic acid in ACN v/v) rising to 55% B over 13 min, before a 3-min flush at 85% B and returning to initial conditions for a 20-min run time. Peptide transitions that were used to monitor for selected proteins are included in Table S2. Peak areas for the proteins were generated in the TargetLynx XS program (Waters). The whole procedure was performed by the Peptidomics and Proteomics Core at the Wellcome-MRC Institute of Metabolic Sciences, Metabolic Research Laboratories.

### 4.9 Placental assessments

#### 4.9.1 Gross placental structure

Paraffin-embedded placentas were exhaustively and serially sectioned at 8 μm with one section in every 20 mounted onto slides for H&E staining. Nanozoomed
scans of stained sections were used to assess gross placental structure using ImageJ and point counting to determine the volume density/proportion of decidua basalis (DB), junctional zone (JZ), or labyrinth zone (LZ).

4.9.2 | Analysis of placental junctional zone glycogen cells

Periodic acid Schiff staining was performed on one 5 μm mid-sagittal section of each placenta. Rehydrated sections were incubated in Periodic Acid solution for 5 min at RT, rinsed in distilled water, and then immersed in Schiff’s reagent for 15 min at RT. Sections were counterstained with hematoxylin (10 min). Mounted sections were scanned and then NDP.view was used to draw around 100 (25/edge, 50/central) randomly selected glycogen cells within the JZ. The distribution of glycogen cell area and total area of glycogen cells in the JZ was then calculated.

4.9.3 | Placental labyrinth zone morphology

Cytokeratin and isolectin dual staining was performed on one 5 μm mid-sagittal section per placenta as described previously. In brief, following rehydration and blocking of endogenous peroxidase activity, antigen retrieval was conducted in 0.04% Pepsin in 0.01 M HCl at 37°C for 10 min then blocked with blocking buffer including 10% goat serum (as described above for the pancreas immunohistochemistry). A biotinylated antibody against lectin (Isolectin B4, B-1205, Vector Laboratories, 1:250) was applied for 90 min at 37°C and visualized using streptavidin and DAB. Subsequently, sections were re-blocked with blocking buffer including 10% goat serum and then a rabbit anti-rabbit antibody-alkaline phosphatase secondary antibody for 1 h at room temperature then developed using NBT/BCIP (Thermofisher) in the dark. Sections were counterstained with Nuclear fast red, then dehydrated and mounted for imaging as described above. Sections were analyzed in NDP.view using random systematic sampling as described previously.

4.10 | Statistics

Data were analyzed using GraphPad Prism (La Jolla, USA). Temporal maternal adiposity and blood glucose concentrations were analyzed by Two-way ANOVA with multiple comparisons. Area above the curve (AAC) or area under the curve (AUC) was calculated for temporal blood glucose concentrations during ITT and GTTs, respectively. The distribution of data was assessed by D’Agostino & Pearson test. When data showed normal distribution it was analyzed by student t-test or Two-way ANOVA, as appropriate. When data were not normally distributed, it was analyzed Mann–Whitney U test. Correlations were determined by the Spearman-rank test. Raw distributions were initially assessed by Kolmogorov–Smirnov test. The 5th and 95th percentiles of fetal weight from control populations were calculated using Z critical values of −1.649 and 1.649, respectively using the formula: (Z critical value × standard deviation) + mean for either whole population or separated sexes where appropriate. Proportion of population categorized as SGA or LGA was analyzed by Fisher’s Exact test. Where multiple values for one parameter were obtained in the litter (namely fetal and placental weights), the closest to average per sex per litter was used in statistical analysis. All data were considered significant at p < 0.05, and a statistical tendency at p < 0.1.

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CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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