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Maternal body composition and the placental-fetal unit under maternal high-fat feeding partially improve by metformin treatment or lifestyle interventions during pregnancy in a mouse model

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ABSTRACT

Introduction: Studies showed that metabolic imbalances during pregnancy in obesity impair the maternal-fetal axis, resulting in fetal growth disturbances with higher risk later in life. Since research has linked many of maternal and fetal sequelae to placental dysfunction, we tested if treatment with an anti-diabetic drug (metformin) or lifestyle interventions improve maternal body weight, placental status or fetal growth.
Methods: Mice were either fed a control or high-fat diet. After mating, high-fat diet mice were split into 4 subgroups, 1 received no intervention while the other 3 received either metformin treatment, a nutritional intervention (NI) or an exercise intervention (RUN). At gestational day 15.5, mice were sacrificed.
Results: All interventions improved body weight of high-fat diet dams, but only NI and RUN maintained fetal growth compared to HFD. Investigation of the placenta showed that (i) NI reduced lipid accumulation in the labyrinth zone (LZ) but neither NI nor RUN attenuated calcification and oxidative stress. Endothelial gamma-H2AX staining was decreased in the LZ by both NI and RUN. (ii) Metformin did not attenuate lipid accumulation in the LZ, placental calcification and oxidative stress, however, protein levels of the endothelial cell marker CD31 were restored in whole placenta lysates. No changes were detected for fetal vessel capillary surface or length of the LZ under any intervention group.
Discussion: Although all tested interventions had beneficial effects on the mother and the placental-fetal unit, NI seems to be most promising. Our findings highlight that preventive strategies for women with obesity should aim for pre-counseling advisory service. In particular, NI and RUN protected from endothelial stress response and metformin was vasculo-protective, offering strategies to preserve the physiological placental-fetal unit and materno-fetal nutrient supply.

1. Introduction

Overweight and obesity is a worldwide epidemic, affecting 43 % (BMI 25–30 kg/m^2) or 16 % (BMI >30 kg/m^2) of adult world population respectively [1]. This leads to an increasing number of overweight or obese women at reproductive age and hence, to an increasing number of

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overweight or obese pregnant women [2]. Overweight or obesity increases the risk for the expectant mother to suffer from pregnancy-related complications like gestational diabetes, necessity for a caesarean section and high blood pressure. As for the unborn child, maternal overweight or obesity is a risk factor for intrauterine death, prematurity, disturbed fetal growth, asphyxia, sepsis and increased mortality during the first year of life [3]. Moreover, offspring of overweight or obese mothers have an increased risk of being overweight or obese in later life, causing a vicious circle [4].

Several strategies exist as potential therapy during obese pregnancies to reduce the risk of pregnancy complications and long-term health consequences for mother and child. Lifestyle interventions are often recommended, like changing eating habits or performing more exercise. However, these kinds of interventions need good compliance, which is often low. An alternative to lifestyle interventions might be drug treatment. Here, metformin is emerging as a prominent candidate. Metformin is an established drug for treating diabetes type 2. It is known as insulin-sensitizer, reduces gluconeogenesis in the liver and increases glucose uptake in peripheral cells (like muscle cells) [5].

Placental dysfunction has been linked to many pregnancy complications and under maternal obesity, amongst others, altered placental vascularity has been described both in human placenta samples [6–8] and in placenta samples from animal studies [9–13]. We showed in a previous study that in a mouse model of high-fat diet induced maternal obesity, placental vasculature is impaired and accompanied by fetal growth restriction at gestational day (G)15.5 [14]. Proper development of the placental vasculature is essential for fetal nutrient and oxygen supply and impaired placental vascularization has been linked to e.g. intrauterine growth restriction [15,16], preeclampsia [17] and stillbirth [18]. As a possible cause of placental vascular changes, increased oxidative stress has been discussed [19,20]. Obesity itself can induce increased oxidative stress in the placenta [9,19,21], probably due to lipid accumulation causing lipotoxicity [22].

The aim of this project was to determine if metformin or a lifestyle intervention (switching to a control diet or performing physical exercise) during an obese pregnancy can improve maternal body composition and fetal growth through prevention of a placental stress response, including lipid accumulation, calcification or lipid peroxidation, as well as impaired placental vasculature. A mouse model was selected for this purpose, as it is a well-established animal model for the study of high-fat diet-induced obesity. Besides allowing for controlled experimental diet and interventional settings, the murine placenta shares similarities with the human placenta, allowing a partial transfer of knowledge gained in this study.

2. Material and methods

2.1. Animal handling and experimental procedures

All procedures were in accordance with german animal law and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV; grant number 84–02.04.2016.A046). Mice were housed at the decentral animal facility of the University Hospital of Cologne under a 12h light/12h dark cycle.

Female mice (C57Bl6N, Janvier labs, France) were fed either a standard diet (SD, R/M − H, sniff, Germany) containing 9 kJ% fat, or a high-fat diet (HFD, C1057 modified, altromin, Germany) containing 60 kJ% fat, ad libitum from week 3 on until mating. The average age of dams at time of mating was: SD = 13.9 weeks, HFD = 15.8 weeks, HFD-MF = 15.4 weeks, HFD-NI = 15.0 weeks, HFD-RUN = 15.4 weeks. Mating took place over night with male mice and next day was defined as G0.5. After mating, the control group kept feeding the SD until the end of the experiment, while the HFD group was split randomly into 4 subgroups: one group received HFD only without any additional intervention. Another group kept receiving HFD, but additionally received drinking water supplemented with 5 mg/ml metformin (Alfa Aesar, Germany) ad libitum until the end of the experiment (HFD-MF). The third HFD-subgroup was switched to a SD after mating until the end of the experiment and represents the nutritional intervention group (HFD-NI). A last HFD subgroup was supplemented with a running wheel in the cage, that was used on a voluntary basis (HFD-RUN). An overview of the mouse groups is depicted in Fig. S1. At G15.5, mice were sacrificed for blood samples and organ (perigonadal white adipose tissue (pgWAT), placenta, fetuses) removal. More details can be found in the supplements.

2.2. Detection of metformin serum level

Detection of metformin in maternal serum was performed as described previously [23]. Shortly, serum samples (diluted in acetonitrile) were analyzed via liquid chromatography-mass spectrometry (LC-MS) and metformin concentration was calculated using a calibration curve of 0–50 ng/ml metformin.

2.3. Placental Oil Red-O and Alizarin-Red staining and quantification

The procedure for Oil Red-o staining to detect lipid droplets has been described in detail before [24]. Briefly, 5 µm cryosections were stained with Oil Red-O (Sigma-Aldrich, Germany) and counterstained with Mayer's hematoxylin (Carl Roth, Germany).

For detection of calcification, 3 µm thick paraffin sections (close to the umbilical cord) were stained with Alizarin-Red. Details on the staining procedure can be found in the supplements.

The labyrinth zone (LZ) was manually defined and within this area, Oil Red-O or Alizarin-Red positive pixels were detected via a trained pixel classifier and calculated as positive area/LZ area [%].

2.4. Immunohistological stainings and analyses of placenta samples

The procedures for CD31 and gammaH2AX staining have been described in detail before [14]. Briefly, 3 µm placenta sections (close to the umbilical cord) were incubated with the anti-CD31 antibody (ab28364, rabbit polyclonal, 1:300, abcam, Cambridge, UK) and re-stained with the anti-phospho S139-gammaH2A.X antibody (ab11174, rabbit polyclonal, 1:4000, abcam, Cambridge, UK) according to the Tyramide SuperBoost™ kit with Alexa Fluor™ 555 (B40923, 1:500, Invitrogen, Eugene, OR, USA) manual. In digitized sections, all CD31 positive endothelial cells and gammaH2AX positive endothelial cells were manually counted in the LZ. Additionally, all CD31 positive endothelial cells and were counted in the LZ and shown as percent of all cells (DAPI positive).

Malondialdehyde (MDA) was detected as marker for lipid peroxidation and hence oxidative stress. Briefly, 3 µm thick placenta sections (close to the umbilical cord) were incubated with the anti-MDA antibody (ab243066, mouse monoclonal, 1:100, abcam, Cambridge, UK), followed by an incubation with ZytoChem Plus (HRP) One-Step Polymer anti-Mouse/Rabbit (ZUC053-100, Zytomed Systems, Berlin, Germany). MDA positive area was determined in digitized slides and calculated as positive area/LZ area [%]. A detailed description of the staining and analysis procedure can be found in the supplements.

2.5. Stereological analysis of placenta samples

The procedure has been described in detail before [14] and more details can be found in the supplements. Sections of whole placenta samples were used. The organ was randomly oriented and 7 µm thick sections were prepared on a rotary microtome (Leica RM2). Every 40 sections, a total of 3 consecutive sections was used for staining and analysis. CD31 and hematoxylin staining was performed and after digitization of stained slides, surface and length of fetal capillaries in the LZ was determined by using the method of stereology.

2.6. Histological analysis of fat cell area

5 μm sections of paraffin-embedded pgWAT samples were stained with hematoxylin & eosin (H&E; Carl Roth, Germany), and digitized (SCN400, Leica, Germany). By using QuPath (University of Edingburgh, Great Britain, version 0.2.0 m2) and the ImageJ Adiposoft plug-in (CIMA, Universidad de Navarra, Spain; version 1.16), fat cell area [μm^2] was measured. More details on the method can be found in Ref. [23] and in the supplements.

2.7. Total RNA isolation, reverse transcription and quantitative real-time PCR (RT-qPCR) analysis

Total RNA from pgWAT and placenta was reverse transcribed into cDNA as described in detail previously [25]. To analyse mRNA expression levels of the adipokine leptin, the senescence markers p16 (CDKN2a) and p21 (CDKN1a) and the nutrient transporters GLUT-1, GLUT-4, FABP-1 and FABP-4 and the housekeeping genes 18s rRNA and beta-actin, a RT-qPCR method was applied and relative mRNA expression levels were calculated by using the DeltaDeltaCT method [26]. More details can be found in the supplements.

2.8. Western blot analysis

Placental tissue was lysed in modified RIPA buffer (see supplements) and 30 μg protein per sample was used for SDS-PAGE, transferred to nitrocellulose membrane and antigen detection via first and secondary antibodies was carried out by standard procedures using the following antibodies: CD31 (ab28364, rabbit polyclonal, 1:1000, abcam), beta-actin (#3700, mouse monoclonal, 1:5000, Cell Signaling Technology, Germany), anti-rabbit-HRP (#7074, 1:2000, Cell Signaling Technology), anti-mouse-HRP (#7076S, 1:2000, Cell Signaling Technology). Signals were detected via the ChemiDoc™ XRS+Imager (BioRad Laboratories, Germany) and densitometric quantification was performed using the Image Lab Software v5.2.1 (BioRad Laboratories).

2.9. Statistical analyses

For statistical analyses, GraphPad Prism 10.1.2 (San Diego, CA, USA) was used. All data are presented as mean ± SEM. Normal distribution of data was tested via the D'Agnostio & Pearson test to choose the appropriate statistical test. Since within the data sets from all analyses at least one data set of a group did not show a normal distribution, a Kruskal-Wallis test was carried out for all analyses. If a significant difference was indicated in the Kruskal-Wallis test, a Mann-Whitney test was performed as post-hoc test. We compared the groups SD vs HFD, HFD vs HFD-MF, HFD vs HFD-NI and HFD vs HFD-RUN. The analyzed number of samples and information about the statistical test applied is indicated in the figure legends.

3. Results

Daily metformin intake of dams in the HFD-MF group was 432 mg/kg body weight. Dose estimation was based on the metformin dose in drinking water and the average body weight and corresponds to a human equivalent dose of about 35 mg/kg body weight metformin per day (calculated as described in Ref. [27]). Metformin levels in maternal serum at G15.5 were about 2.15 μg/ml (Table 1).

Table 1
Metformin data for the HFD-MF group. Based on average body weight of the mice and water intake, daily mean metformin intake was calculated (for n = 7 dams). After sacrifice at G15.5, metformin level in maternal serum was measured by HPLC and displayed as μg/ml (for n = 10 dams). Mean ± SEM.

daily metformin intake [mg/kg body weight]	432.13 ± 13.25
metformin level in serum at G15.5 [μg/ml]	2.15 ± 0.31

Pregnant mice from the HFD-RUN group run approximately 4.12 ± 0.55 km per day with a mean speed of 1.42 ± 0.04 km per hour.

3.1. Metformin and lifestyle interventions improve maternal body weight

First, we investigated the impact of 3 intervention strategies on maternal body weight during gestation and pgWAT weight. Dams from the HFD, the HFD-MF, the HFD-NI and the HFD-RUN group were significantly heavier at mating day (G0) when compared to the control group receiving SD, as this was a requirement of our experimental setting (Fig. 1a). At G15.5, HFD-MF, HFD-NI and HFD-RUN mice were significantly lighter compared to mice from the HFD group (Fig. 1b). This was, however, not true when comparing maternal body weights per viable litter size (Fig. S2a). Gestational body weight gain from G0 to G15.5 was significantly lower in all interventional groups compared to the HFD group, while gestational body weight gain in SD and HFD was about the same (Fig. 1c). This was confirmed when comparing gestational body weight gain per viable litter size (Fig. S2b).

Similar to body weight, pgWAT weight was significantly higher in HFD compared to SD mice at G15.5. All interventions induced a loss in pgWAT weight when compared to HFD. In addition, analysis of adipocyte size revealed that the mean fat cell area was significantly increased in HFD compared to SD, and significantly decreased in HFD-NI samples compared to HFD. Both HFD-MF and HFD-RUN showed a similar trend towards smaller adipocytes compared to HFD (Fig. 1d and e). In addition, leptin mRNA expression, an adipokine known to be increased under obesity, was significantly downregulated in pgWAT after both a metformin (HFD-MF) and a nutritional (HFD-NI) intervention compared to the HFD mouse group (Fig. 1f).

A glucose tolerance test (GTT), performed at G13.5, showed impaired glucose clearance for HFD dams compared to SD dams. Contrary to our expectations, metformin treatment of obese dams did not improve glucose tolerance compared to the HFD-group (Fig. S3). For the other experimental groups, a GTT was not performed.

3.2. A nutritional intervention reduces lipid deposits in the placental labyrinth zone

As we associated maternal high-fat feeding to increased lipid accumulation in the placenta in our diet-induced obesity mouse model [14], we next tested whether any of the interventions can reduce placental metabolic stress response, indicated by lipid storage. To this end we assessed lipid deposits using Oil Red-O staining on placental cryo-sections. The analysis revealed that only the nutritional intervention (HFD-NI) was able to significantly reduce lipid accumulation in the LZ compared to HFD alone (Fig. 2a and b).

Next, calcification of placental tissue in the LZ was measured by Alizarin-Red staining. Placentas from the HFD group showed a higher calcification rate in the LZ compared to SD that was in part prevented in the HFD-NI group ($p = 0.0749$) (Fig. 2c and d).

Furthermore, we aimed to check for oxidative stress particularly in the LZ, as Napso et al. [9] found higher calcification rates to be associated with higher oxidative stress in this placenta zone. As marker for oxidative stress/lipid peroxidation, we detected and quantified the MDA positive area within the LZ. As depicted in Fig. 2e and f, MDA positive zones were significantly more abundant in placentas from the HFD group when compared to placentas from the SD group. None of the tested interventions was able to reduce oxidative stress/lipid peroxidation in the LZ compared to HFD alone.

3.3. Metformin restores placental CD31 levels, while lifestyle interventions reduce DNA damage in ECs

Prior studies showed that maternal HFD reduces placental fetal vessel surface and length in the LZ when compared to SD. This was

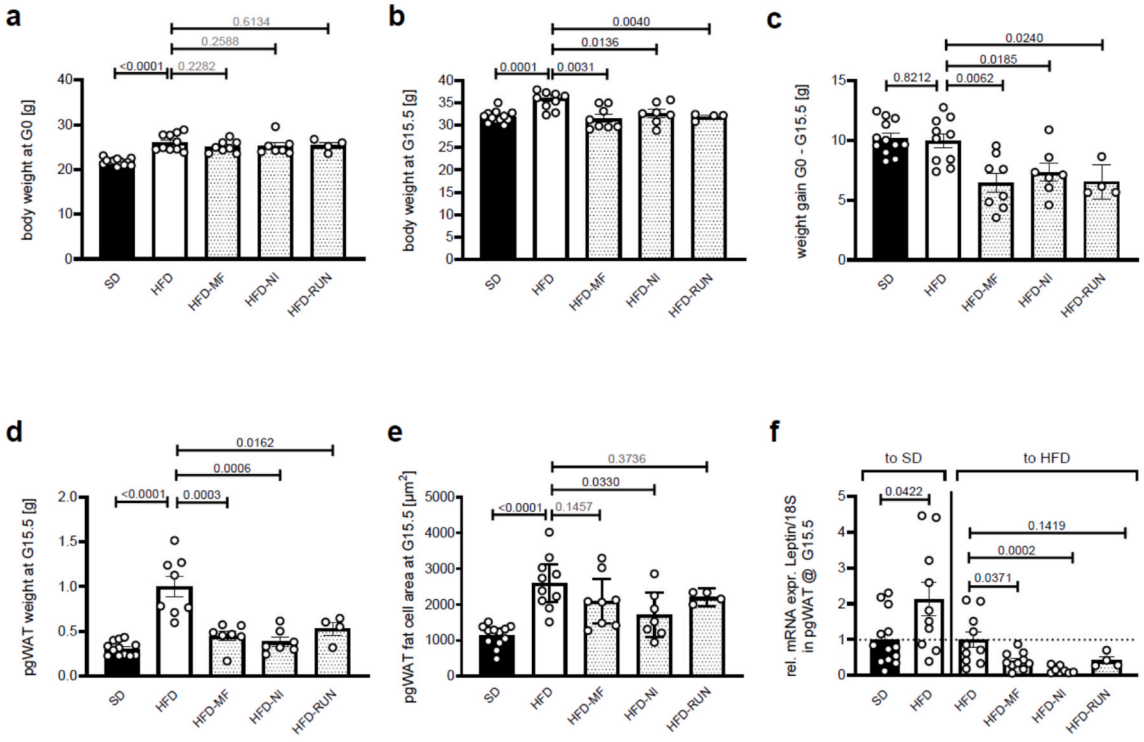

Fig. 1. Body weight of dams at G0 and G15.5, and weight and cell size of maternal perigonadal white adipose tissue at G15.5.
a) Before mating (G0), body weight [g] was determined. SD: n = 12; HFD: n = 29. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
b) Body weight [g] was also determined after sacrifice (G15.5). SD: n = 12; HFD: n = 10; HFD-MF: n = 8; HFD-NI: n = 7; HFD-RUN: n = 4. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
c) Weight gain between G0 and G15.5 [g] was calculated and plotted in the graph. SD: n = 12; HFD: n = 10; HFD-MF: n = 8; HFD-NI: n = 7; HFD-RUN: n = 4. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
d) After sacrifice at G15.5, pgWAT fat pad was dissected and weight [g] was determined. SD: n = 12; HFD: n = 8; HFD-MF: n = 7; HFD-NI: n = 7; HFD-RUN: n = 4. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
e) Fat cell area [μm^2] was measured in pgWAT paraffin sections stained with H&E. SD: n = 12; HFD: n = 10; HFD-MF: n = 8; HFD-NI: n = 7; HFD-RUN: n = 4. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
f) rel. mRNA expression level for Leptin/18S in pgWAT samples. SD n = 13; HFD n = 10; HFD-MF n = 10; HFN-NI n = 8; HFD-RUN n = 4. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
Data for SD, HFD and HFD-MF have been partially published before [23].

probably caused by increased DNA damage in endothelial cells in fetal capillaries and was accompanied by a decreased placental endothelial cell marker (CD31) [14].

Metformin treatment of obese dams (HFD-MF) protected from the reduction of CD31 protein levels in whole placenta lysates when compared to the HFD group and reached CD31 levels similar to those of the SD group. This metformin-mediated beneficial effect however, was not observed for placentas of the other interventional groups HFD-NI and HFD-RUN (Fig. 3a and b). To understand if changes in CD31 levels on the protein level are based on the number of endothelial cells or the intensity of CD31, we determined the percentage of endothelial cells in the LZ (relative to all DAPI positive cells). The number of endothelial cells did not differ between the SD and the HFD group, and also not between the HFD any of the HFD-interventional groups (Fig. 3c).

The pronounced decrease of the endothelial cell marker CD31 in placentas of HFD dams might indicate an adverse metabolic or inflammatory effect on endothelial cells. Assessment of gammaH2AX in fetal capillaries of the LZ as an indicator of DNA damage showed a marked increase in placental endothelial cells of HFD dams when compared to SD dams. This increase was attenuated by metformin and completely prevented in the HFD-NI and HFD-RUN group (Fig. 4a).

Analysis of fetal vessel structures in the LZ did not reveal any improvement after treatment of obese dams with metformin (HFD-MF) or performing a lifestyle intervention (HFD-NI, HFD-RUN) when compared to HFD alone (Fig. 4b and c).

3.4. Fetal body weight improves under lifestyle interventions

To determine whether beneficial effects of interventions on the placenta described above have a positive impact on fetal growth, we measured fetal body weight at G15.5. As depicted in Fig. 5a, fetuses of HFD dams display a significant growth restriction at G15.5 when compared to those of lean dams (SD). Metformin treatment of obese dams (HFD-MF) during pregnancy did not improve mean fetal weight. In contrast, both lifestyle interventions (HFD-NI, HFD-RUN) significantly improved fetal weight when compared to fetuses of obese dams without any intervention (HFD). This was, however only true when fetal body weights were individually included in the statistical analysis. When comparing mean fetal weights per viable litter size, only the growth restriction in the HFD group compared to the SD group remained. For the HFD-NI group, an improved fetal body weight compared to the HFD group was visible by trend ($p = 0.0702$), while for the other two interventional groups no significant differences compared to the HFD group were detected (Fig. 5b).

Fetal body weight was also used to calculate the rate of fetuses below the 5th and between the 5th - 10th weight percentile of control SD fetuses (Fig. 5c). Rates of fetuses below the 10th growth percentile (sum of rates <5th and 5th-10th) were highest for the HFD-MF group (28.81 %), followed by the HFD group (20.51 %). The SD group (10.3 %) and the HFD-RUN group (13.33 %) showed similar rates of fetuses below the 10th percentile, while the rate was lowest in the HFD-NI group (1.82 %).

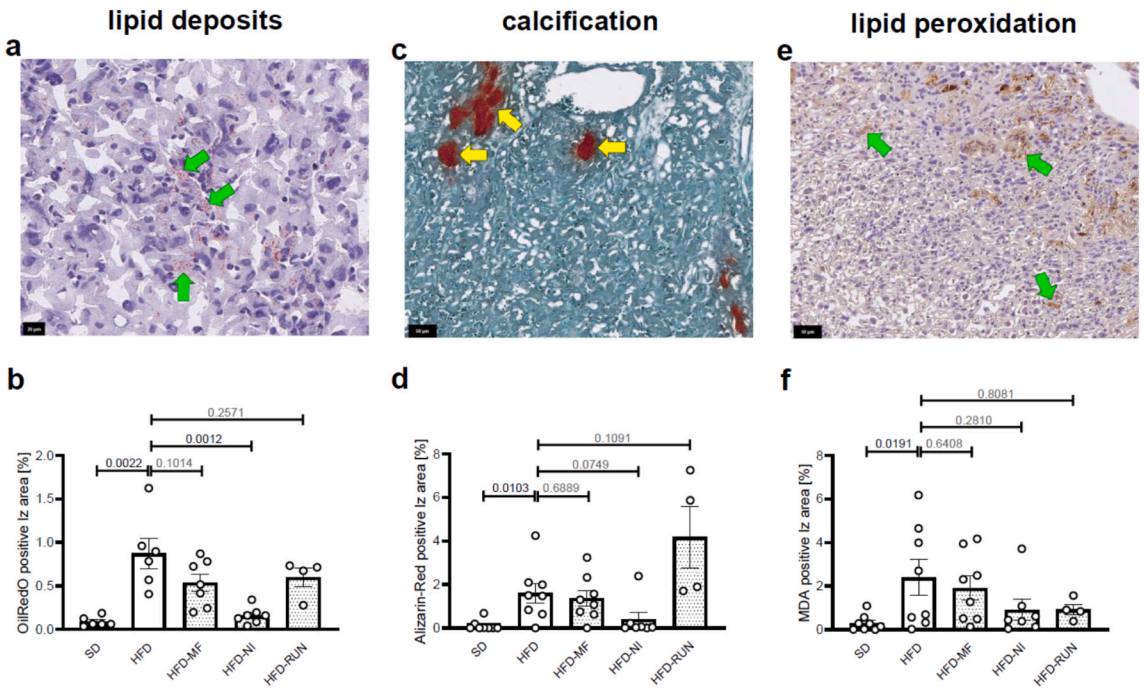

Fig. 2. Lipid accumulation, calcification and lipid peroxidation in the LZ of murine placentas.
a) Placenta cryo sections were stained with Oil Red-O and hematoxylin. The green arrows point to lipid deposits (Oil Red-O positive area) in the LZ of a placenta from the HFD group. Scale bar 20 μm
b) Oil Red-O staining of placenta cryo sections were digitized and the positive area in the whole LZ was measured and calculated as % of positive area/total LZ. SD: n = 6 placentas/6 dams; HFD: n = 6 placentas/6 dams; HFD-MF: n = 7 placentas/7 dams; HFD-NI: n = 7 placentas/7 dams; HFD-RUN: n = 4 placentas/4 dams. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
c) Placenta paraffin sections were stained with Alizarin-Red and Fast Green. The yellow arrows indicate calcified (Alizarin-Red positive) areas in the LZ of a placenta from the HFD group. Scale bar 50 μm
d) Placenta paraffin sections were stained with Alizarin-Red and digitized. The Alizarin-Red positive area of the labyrinth section was measured and is shown as % of positive area/total LZ. SD: n = 8 placentas/8 dams; HFD: n = 8 placentas/8 dams; HFD-MF: n = 8 placentas/8 dams; HFD-NI: n = 7 placentas/7 dams; HFD-RUN: n = 4 placentas/4 dams. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
e) Placenta paraffin sections were stained with an anti-MDA antibody and counterstained with hematoxylin. The green arrows point to MDA-positive (brown signal) areas in the LZ of a placenta from the HFD group. Scale bar 50 μm
f) Placenta paraffin sections were stained with an antibody against MDA and digitized. The MDA positive area of the labyrinth section was measured and is shown as % of positive area/total LZ. SD: n = 8 placentas/8 dams; HFD: n = 8 placentas/8 dams; HFD-MF: n = 8 placentas/8 dams; HFD-NI: n = 7 placentas/7 dams; HFD-RUN: n = 4 placentas/4 dams. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Viable litter size and the resorption rate, which corresponds to a miscarriage in humans, was not significantly altered, neither when comparing HFD to SD nor any interventional group to HFD (Fig. 5d and e).

4. Discussion

In our mouse model of high-fat diet induced maternal obesity, metformin treatment, switching to a healthy diet and exercise during pregnancy had beneficial effects on maternal body composition. Placental lipid deposits, calcification and oxidative stress marker were partially improved by a nutritional intervention. The endothelial cell marker CD31 was restored by metformin treatment, while endothelial cells in the LZ displayed decreased DNA damage marker under a nutritional and an exercise intervention. Although none of the interventions restored fetal capillaries in the LZ, fetal growth restriction was ameliorated by lifestyle interventions.

4.1. Effects on maternal body composition and glucose metabolism

Prevention of high gestational weight gain is a positive effect, as it reduces the risk for adverse perinatal outcomes and also helps to start with lower body weight into a subsequent pregnancy. According to the 2009 guidelines by the Institute of Medicine and National Research Council (IOM and NRC) [28], recommended weight gain during pregnancy decreases with increasing pre-pregnancy BMI. In this study, all tested interventions have a positive effect on maternal body composition, while the metformin and nutritional intervention have the strongest effect. Our data for the metformin treated group are in accordance with data published by Hufnagel et al. who showed in a mouse model of maternal obesity that metformin intervention during pregnancy lowers maternal fat mass at G18.5 [29]. In human obese patients with and without GDM, lower gestational weight gain was reported in women treated with metformin [30,31]. Data on a nutritional intervention in mouse models of maternal obesity are scarce. A previous study showed no differences in maternal body weight at G17.5 after switching HFD-fed obese dams to standard chow after mating [32]. Gimpfl et al. reported lower maternal body weight after modified high calorie diet before and during pregnancy, where the high-saturated fatty acids were replaced by medium chain fatty acids (MCFAs) and a reduced n-6:n-3 long chain polyunsaturated fatty acid (LC-PUFA) ratio (original ratio 7.2:1 was reduced to 2.2:1) [33]. Controversial data exist about the effects of a nutritional intervention during pregnancy regarding gestational weight gain in obese women [34,35]. In a mouse model, physical exercise during pregnancy had no positive effect on gestational weight gain [36]. Observational studies with pregnant women have shown that physical activity during pregnancy can reduce the risk of high gestational weight gain [37–39] or has no positive effect on high gestational weight gain

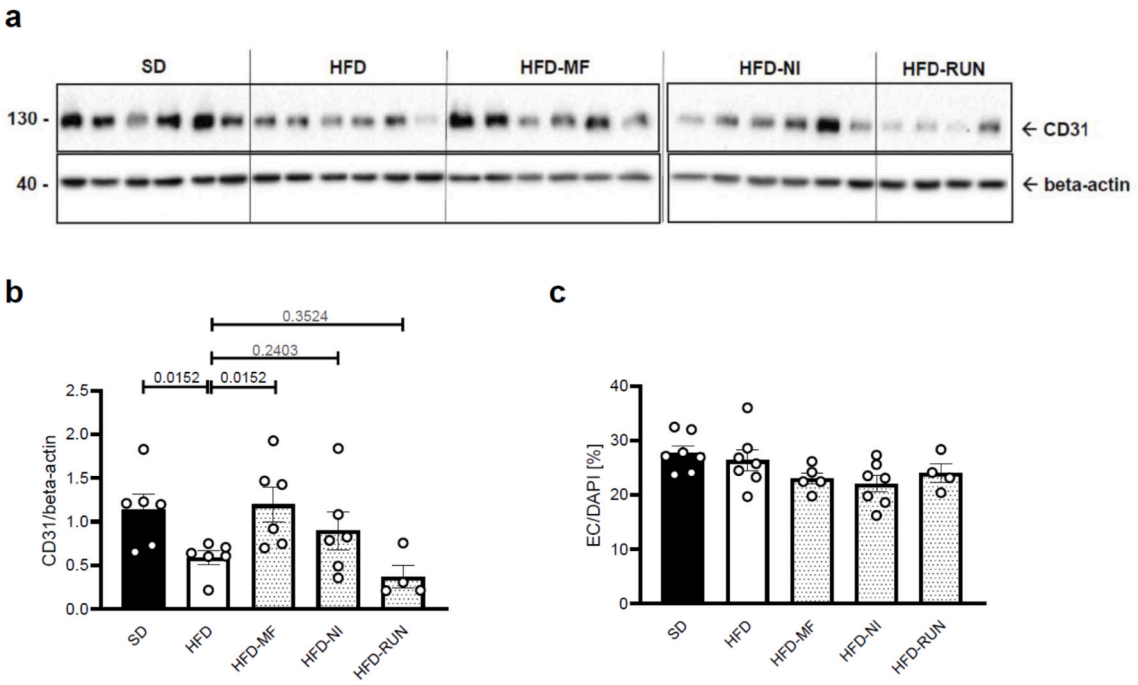

Fig. 3. Total placental CD31 level and number of CD31 positive cells in the placental LZ.
a) Western Blot analysis of the endothelial cell marker CD31 and beta-actin for normalization in whole placenta lysates. SD: n = 6 placentas/6 dams; HFD: n = 6 placentas/6 dams; HFD-MF: n = 6 placentas/6 dams, HFD-NI: n = 6 placentas/6 dams, HFD-RUN: n = 4 placentas/4 dams. The Western blot was rearranged for this figure to put the test groups into a logical order. This is indicated in the picture by rectangulars placed around the Western blot parts. The full uncropped Western blot images can be found in supplementary Fig. S7.
b) Densitometric analysis of the Western blot shown in a). Relative protein level of CD31 were normalized to beta-actin. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
c) Via immunofluorescence staining, endothelial cells (CD31, red) and nuclei (DAPI, blue) in the placental LZ were detected. Endothelial cells (EC, CD31 positive) and all cells (DAPI positive) were determined in the placental LZ and results (EC/DAPI) are illustrated in the graph. SD: n = 7 placentas/7 dams; HFD: n = 7 placentas/7 dams; HFD-MF: n = 5 placentas/5 dams; HFD-NI: n = 7 placentas/7 dams; HFD-RUN: n = 4 placentas/4 dams. As the Kruskal Wallis test showed no significant differences, no post-hoc test was performed and hence no p-values are indicated in the graph.

[34,40]. This discrepancy for human data regarding lifestyle interventions most likely result from inconsistent settings within the trials. Finally, it must be considered that interventions inducing reduction of fat mass might induce lipolysis and hence, lead to increased circulating fatty acids in maternal blood, as has been reported for a nutritional intervention during pregnancy in a human trial [34].

In our setting, the metformin treatment did not ameliorate glucose tolerance of obese mice. In contrast, other mouse models of maternal obesity/HFD feeding using a metformin intervention during pregnancy showed improved glucose tolerance, however experimental settings of the different studies were not the same and might have led to conflicting data: Zhao et al. fed C57Bl6 mice with a HFD (45 kcal% fat) 1 week prior to mating and until G18.5 and treated mice with 300 mg/kg*day metformin from E11.5 – E17.5 via gavage, while the GTT was performed at E16.5 [41]. Wu et al. fed C57Bl6J mice a HFD (60 kcal% fat) from week 4–19. Then mice were mated and from E0.5–10.5 received 200 mg/kg*day metformin via drinking water. At day E8.5, a GTT was performed [42]. Finally, Hufnagel et al. fed C57Bl6/J mice with an obesogenic diet (20 % animal fat, 10 % simple sugars) together with condensed milk (55 % simple sugars, 8 % fat) until the end of the experiment. 1 week prior to mating and throughout pregnancy, metformin was applied via condensed milk at 255.2 + 48 mg/kg*day. The GTT was performed at E17.5 [29]. Also, women with GDM that are treated with metformin during pregnancy often need supplemental insulin, indicating that metformin alone is sometimes not sufficient to meet glycemic targets [43].

4.2. *Placental stress response*

Lipid deposits can be found in placentas of patients with and without obesity [44]. Increased lipid deposits in the placenta might be a sign of lipid spillover of the system, as excessive fatty acids are not stored in fat tissue but also in other organs, a phenomenon known as lipotoxicity. Maternal obesity leads to higher triglyceride levels in human placenta samples [45], while triglycerides dominate in lipid droplets [46]. If augmented lipid deposits in the placenta can negatively affect organ or fetal development, is unknown. In our mouse setting, we previously demonstrated that a HFD feeding prior and during pregnancy significantly elevated lipid accumulation in the LZ compared to the LZ of placentas from mice fed a control diet [24], a phenomenon that was also seen in other studies [47]. The nutritional intervention reduced the amount of lipid accumulation in the LZ, although the effect did not reach statistical significance. This was probably due to an outlier in the HFD-NI group, but this is only speculative as we did not test for significant outliers due to the low n-number in the experiments. The metformin and exercise intervention had no effect. As it is known that lipotoxicity is connected to oxidative stress and endothelial dysfunction regarding cardiovascular diseases [48], it is tempting to speculate that placental endothelial cells might be negatively affected by increased lipid storage in this organ. Interestingly, higher neutral storage lipids have been found in preeclamptic placentas from a human cohort [49], a pregnancy related disorder that is related to altered placental vascularization. To detect oxidative stress in the LZ of murine placentas in this study, we stained for MDA, a lipid peroxidation marker. Indeed, HFD-induced maternal obesity resulted in higher amounts of oxidative stress in the LZ when compared to placentas from lean dams. Neither

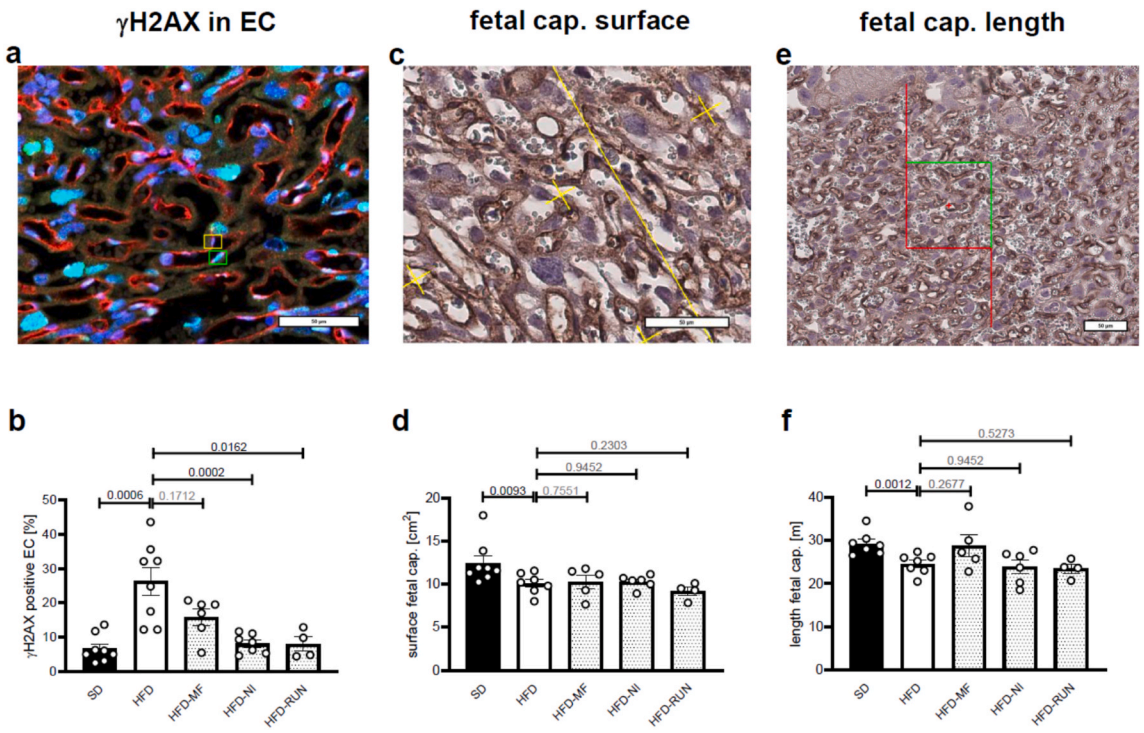

Fig. 4. Endothelial cell DNA damage and fetal vessel structure of the placental LZ.
a) Via immunofluorescence staining, endothelial cells (CD31, red), DNA damage (gammaH2AX, green) and nuclei (DAPI, blue) in the placental LZ were detected. The green rectangle marks a gammaH2AX positive EC, the yellow rectangle a gammaH2AX negative EC. Scale bar 50 μm
b) GammaH2AX positive endothelial cells [% of all endothelial cells] in the placental LZ were determined and results are illustrated in the graph. SD: n = 7 placentas/7 dams; HFD: n = 7 placentas/7 dams; HFD-MF: n = 5 placentas/5 dams; HFD-NI: n = 7 placentas/7 dams; HFD-RUN: n = 4 placentas/4 dams. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
c) Endothelial cells (CD31, brown) were detected via immunohistochemistry and counterstained with hematoxylin (blue). Yellow lines and crosses were added to the digitized images to assess fetal capillary surface area via stereology. Scale bar 50 μm
d) The surface of fetal capillaries [cm^2] in the placental LZ was analyzed via stereology and results are shown in the graph. SD: n = 8 placentas/8 dams; HFD: n = 7 placentas/7 dams; HFD-MF: n = 5 placentas/5 dams; HFD-NI: n = 6 placentas/6 dams; HFD-RUN: n = 4 placentas/4 dams. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.
e) Endothelial cells (CD31, brown) were detected via immunohistochemistry and counterstained with hematoxylin (blue). Rectangles (red and green lines) were added to the digitized images to enable evaluation of fetal capillary length. Scale bar 50 μm
f) By using the stereology method, fetal capillary length [m] in the LZ of placentas was determined and results are illustrated in the graph. SD: n = 8 placentas/8 dams; HFD: n = 7 placentas/7 dams; HFD-MF: n = 5 placentas/5 dams; HFD-NI: n = 6 placentas/6 dams; HFD-RUN: n = 4 placentas/4 dams. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

metformin treatment nor any lifestyle intervention (HFD-NI, HFD-RUN) was able to reduce oxidative stress in the placental LZ, although we could show that a nutritional intervention (by trend) lowered lipid accumulation. Napso et al., 2022 [9] found increased calcification in the LZ of male placentas, and this was associated with higher lipid peroxidation (MDA immunohistochemical staining). Hufnagel et al. [29] recently demonstrated that the labyrinthine structure, as seen in CD31 immunohistological stainings of murine placenta sections, is damaged in calcified regions of obese dams. A metformin intervention in obese dams was not able to prevent high calcification rates. In the setting used here, calcification in the LZ was also increased under HFD and partially mitigated by a nutritional intervention by trend ($p = 0.075$).

Previous work by us on the mouse model of maternal obesity revealed decreased levels of the endothelial cell marker CD31 in whole placenta lysates, increased gammaH2AX positive endothelial cells as well as a decreased surface and length of fetal capillaries in the LZ of placentas from the HFD group [14]. Metformin treatment of obese dams during pregnancy restored CD31 levels, but had no effect on the rate of gammaH2AX positive endothelial cells or the surface area and length of fetal capillaries. Since the percentage of endothelial cells in the LZ is not changed, it is likely that endothelial cells display downregulated CD31 levels under maternal obesity and upregulated CD31 protein levels under metformin treatment. Not much is known about regulation of CD31 levels in placenta samples, but Shen et al. showed that both in human samples complicated by preeclampsia and also in a preeclampsia mouse model, CD31 expression is downregulated in placental endothelial cells probably due to deregulated angiogenetic factors [50]. As CD31 levels were measured in whole placenta lysates, we cannot say if endothelial cells of the LZ or of vessels in other placental zones benefited from increased CD31 levels. This should be addressed in future research by e.g. laser dissection of the LZ from cryo-sections of placental tissue for generating Western blot samples. In contrast, both a nutritional and exercise intervention did not have any effect on CD31 levels but led to decreased rates of gammaH2AX positive endothelial cells in the LZ. GammaH2AX is a marker for DNA damage, but in the absence of a proliferative signal it has been proposed to be a useful marker for cellular senescence [51]. Analysis of the proliferation rate of endothelial cells in the LZ in our samples did not shown any differences between the five groups (Supplementary Fig. S4). Hence, our data imply that endothelial cell senescence in the LZ is increased under maternal obesity and can be reduced by a nutritional or exercise intervention. A qPCR-analysis of total placenta lysates however did not show any differences for the two senescence markers p21 and p16 between the SD and HFD group nor between the HFD and any interventional group

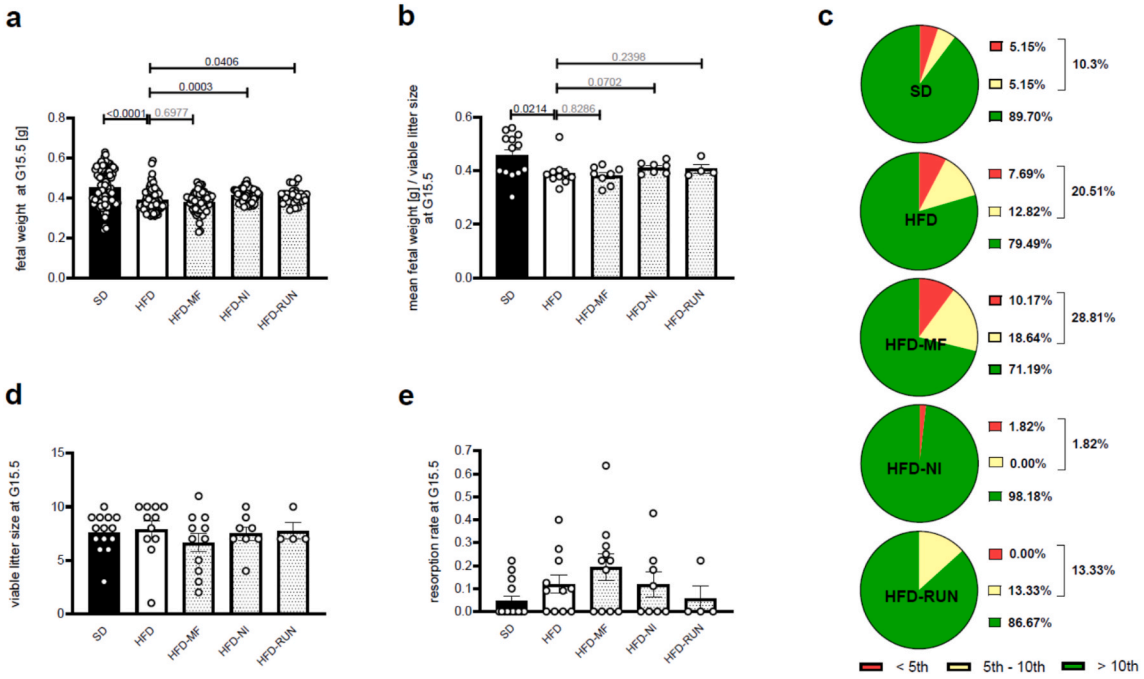

Fig. 5. Fetal body weight and resorption rate at G15.5.

a) fetal body weight [g] at G15.5 was measured. SD: n = 97 (13); HFD: n = 78 (10); HFD-MF: n = 59 (8); HFD-NI: n = 55 (7); HFD-RUN: n = 29 (4), with n = number of fetuses (from n dams). A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.

b) mean fetal body weight [g] per viable litter size at G15.5 is plotted in the graph. SD: n = 13; HFD: n = 10; HFD-MF: n = 8; HFD-NI: n = 7; HFD-RUN: n = 4. A Kruskal-Wallis test with a Mann-Whitney test as post-hoc test was performed.

c) rate of fetuses below the 5th, between the 5th – 10th and below the 10th weight percentile of control SD fetuses at G15.5. The diagrams show the % of fetuses below the 5th (red), between the 5th – 10th (yellow), and above the 10th (green) percentile for fetuses from the experimental groups SD, HFD, HFD-MF, HFD-NI and HFD-RUN. The % of fetuses below the 10th percentile is also shown (sum of fetuses below the 5th and between the 5th – 10th growth percentile).

d) viable litter size at G15.5 was determined and is plotted in the graph. SD: n = 14; HFD: n = 11; HFD-MF: n = 11; HFD-NI: n = 8; HFD-RUN: n = 4. As the Kruskal Wallis test showed no significant differences, no post-hoc test was performed and hence no p-values are indicated in the graph.

e) resorption rate at G15.5 per litter is displayed in the graph. SD: n = 14; HFD: n = 11; HFD-MF: n = 11; HFD-NI: n = 8; HFD-RUN: n = 4. As the Kruskal Wallis test showed no significant differences, no post-hoc test was performed and hence no p-values are indicated in the graph.

Data for SD and HFD have been partially published before [64]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. S5). Again, additional experiments need to be performed to further clarify the senescence status specifically of endothelial cells in the LZ. Also, reduced DNA damage marker rates in endothelial cells of the LZ under a nutritional and exercise intervention have no positive impact on neither fetal capillary surface nor length. The prevention of genomic instability through NI or RUN does not provide evidence for improved endothelial function or angiogenic capacity. Effective angiogenesis not only depends on preventing DNA damage in endothelial cells, but also on the presence of a pro-angiogenic microenvironment, including growth factors (e.g., VEGF, apelin) and cell–cell interactions (e.g., between pericytes, endothelial cells, and mesenchymal cells). The absence of such factors may contribute to the impaired vessel formation observed under HFD conditions, despite NI and RUN, and should be explored in future studies. In summary, our data show that the tested interventions may have some benefits on placental endothelial parameters but are not able to restore fetal capillaries.

4.3. Fetal outcome

Fetuses exhibit a growth restriction in the HFD group compared to the SD group. Treatment of obese dams with metformin during pregnancy had no effect on fetal weight. This is in accordance with data from Hufnagel et al., who showed no positive effect of a metformin intervention in obese mouse pregnancies on fetal weight [29]. In contrast to metformin treatment, both lifestyle interventions ameliorated fetal weight compared to the HFD group. Improvement of fetal weight by lifestyle interventions was further corroborated by decreased rates of fetuses below the 5th and 10th growth percentile compared to the HFD group. However, when comparing mean fetal birth weights, only the nutritional intervention showed a tendential but not significant improvement (p = 0.0702). Mean fetal weights of the HFD-RUN group were not improved compared to the HFD group.

To reason, damaged fetal vessels in the LZ seem not to be the (sole) cause for the observed fetal growth restriction, as this feature is not improved under the lifestyle interventions but fetal body weight is. Fetal supply via the feto-maternal transfer zone (LZ) does not only depend on an intact fetal vessel structure, but also on the activity of nutrient transporter [52] and improvement on this level of action has to be proven. A RT-qPCR analysis of two glucose transporters (GLUT-1, GLUT-4) and two fatty acid binding proteins (FABP-1, FABP-4) in whole placenta lysates did not show upregulated expression levels by lifestyle interventions compared to HFD (Fig. S6). A more detailed analysis of placental nutrient transporters, e.g. at the protein level, or the performance of in vivo experiments on placental transfer capacity were not carried out, as this was not the focus of this work. Although maternal obesity has been connected to fetal growth restriction in human pregnancies [53–55], it has to be mentioned that, in contrast to our mouse model, maternal obesity mainly results in a higher risk of large for gestational age (LGA) or macrosomic babies [56]. Metformin treatment of obese pregnant women without GDM had no effect on birth weight [57]. Also, lifestyle interventions usually do not show any improvement when it comes to reducing the risk for LGA or macrosomia. Harreiter et al. showed that birth weight was not affected, neither by switching to healthy eating nor to physical activity during pregnancy [34]. A

systematic Cochrane review published in 2015 showed that both diet and exercise interventions have no clear effect on the odds ratio for having a macrosomic infant, however pregnant women of any BMI were included. In contrast, a subgroup analysis of 'high-risk women' did show a reduced risk of giving birth to a macrosomic baby when mothers received both a diet and exercise intervention during pregnancy [58]. Catalano et al. postulated that the missing positive effect of lifestyle interventions on fetal body weight might be due to interventions starting too late, when placental and maternal metabolic impairments have been initiated/manifested already [59]. In our study, mice started earlier with a nutritional or physical exercise intervention directly after mating, which might be the reason why we did observe a positive effect on fetal body weight.

5. Limitations

The most serious limitation of the study is that data were not analyzed regarding fetal sex, as it is well known that maternal obesity does have a sex-dependent impact on the placenta [9,60–62]. This might also, at least in part, explain the high variability in some data sets represented in this study. Also, metformin levels in our mouse model were above the levels detected in human pregnant patients (1.5 μg/mL + 0.5 (mean +SD); [63], which might have induced effects that could not be seen under 'therapeutic' metformin levels. Referring to the analyses of the HFD-RUN group, it has to be mentioned that the number of analyzed animals (n = 4) is very low. This is due to the facts that either mice did not use the wheel installed in their cage, and that running mice did show a low pregnancy rate. Moreover, the GTT was not performed for the HFD-NI and the HFD-RUN group. Finally, not all analyses were carried out specifically for the LZ. This concerns the Western blot and qPCR analyses, making it difficult to combine certain results and drawing conclusions.

6. Conclusion

Maternal obesity has negative impacts on the placenta and fetal growth. Metformin treatment and lifestyle interventions (nutritional or exercise intervention) during an obese pregnancy can reduce maternal body weight gain during pregnancy, while placental abnormalities mostly profit from switching to a healthy diet. For the developing fetus, metformin treatment during pregnancy does not bring any benefit on the examined outcomes, however lifestyle interventions are able to ameliorate fetal growth restriction, again seeing the strongest effect for a nutritional intervention. It has to be considered that in our study, all interventions started right after mating before the beginning of mouse pregnancy. This is usually challenging for a human pregnancy. Hence, a later start of the interventions in our mouse model would reflect the clinical situation much better and should be investigated in subsequent studies. Additionally, metformin-associated benefits resulting from positive endothelial effects, should be considered in further studies.

CRediT authorship contribution statement

Tobias Kretschmer: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Eva-Maria Turnwald:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis. **Antje Thiele:** Investigation, Formal analysis. **Ciara Kallage:** Investigation, Formal analysis. **Lena Neweling:** Investigation, Formal analysis. **Merlin Kammerer:** Investigation, Formal analysis. **Ruth Janoschek:** Writing – review & editing, Writing – original draft, Investigation. **Peter Zentis:** Writing – review & editing, Writing – original draft, Formal analysis. **Marion Handwerk:** Investigation. **Maria Wohlfarth:** Writing – review & editing, Investigation. **Simone Kalis:** Methodology, Investigation, Formal analysis. **Eva Nüsken:** Writing – review & editing, Writing – original draft. **Kai-Dietrich Nüsken:** Writing – review & editing, Writing – original draft. **Inga Bae-Gartz:** Writing – review & editing. **Angela Köninger:** Writing – review & editing, Conceptualization. **Alexandra Gellhaus:** Writing – review & editing, Conceptualization. **Dirk Gründemann:** Writing – review & editing, Methodology, Formal analysis. **Eva Hucklenbruch-Rother:** Writing – review & editing. **Jörg Dötsch:** Writing – review & editing, Writing – original draft. **Miguel A. Alejandre Alcazar:** Writing – review & editing, Writing – original draft. **Sarah Appel:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no competing interests.

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LIST OF ABBREVIATIONS

BMI	body mass index
CD31	cluster of differentiation 31
DAPI	4′,6-diamidino-2-phenylindole
EC	endothelial cell
G	gestational day
gammaH2AX	phosphorylated form of *H2A histone family member X*
HFD	high-fat diet
LZ	labyrinth zone
MDA	malondialdehyde
MF	metformin
NI	nutritional intervention
pgWAT	perigonadal white adipose tissue
RUN	physical exercise
SD	standard diet

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2025.09.016.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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