 Accepted Manuscript published as REP-23-0024.R1. Accepted for publication: 18-Jul-2023

Maternal age, obesity and hyperglycaemia are associated with a delay in preimplantation embryo development in mouse

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Keywords: High fat diet, obesity, metabolic syndrome, pregnancy, Fertility, Embryo, Blastocyst, Cell fate, Quantitative immunofluorescence.

Word count: 3505

Main Figures: 7

In Brief Statement:

Fertility has decreased due to advanced maternal age and the rising prevalence of the metabolic syndrome. Using quantitative image analyses methods, we show that these factors are associated with delayed preimplantation embryo development in a mouse model

Abstract

Delayed maternal age, obesity and diabetes are associated with reduced fertility. We investigated how age and obesity/metabolic syndrome impact fertility and hypothesized that its decrease is due to defects in preimplantation embryo development.

Three groups of female C57Bl6 mice (12 weeks, 9 months and 1 year old) were fed either a high fat diet for 8 weeks, to induce obesity and the metabolic syndrome, or a control chow diet. Body weight and composition, glucose tolerance and insulin resistance were assessed. Fecundity was evaluated by mating and pregnancy rates, as well as number of embryos. Embryo quality was assessed morphologically, and cell fate composition was analysed in preimplantation embryos by state-of-the-art single cell quantitative confocal image analysis.

The high fat diet was associated with increased adiposity, glucose intolerance and insulin resistance, especially in the older mice. Fecundity was affected by age, more than by the diet. Both age and high fat diet were associated with reduced cell fate allocation, indicating a delay in preimplantation embryo development, and with increased expression of GATA3, an inhibitor of placentation.

These results support that age and the metabolic syndrome reduce fertility through mechanisms which are present at conception very early in pregnancy.

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Introduction:

Lifestyle changes have led to an epidemic increase in obesity and type 2 diabetes, both of which have a negative impact on fertility (Thong *et al.*, 2020) and offspring health (Fleming *et al.*, 2018). Intrauterine exposure to diabetes and obesity is associated with short and long-term health risks, such as congenital anomalies and stillbirth, neurodevelopmental disorders, obesity, heart disease and type 2 diabetes (Ornoy *et al.*, 2015; Godfrey *et al.*, 2017; Faruque *et al.*, 2019).

Furthermore, the age at first pregnancy has been delayed in Europe to 29.4 years in 2019 ('Women in the EU are having their first child later - Products Eurostat News - Eurostat') with consequences on reproductive capacity, embryo quality and even offspring's overall health (Myrskylä and Fenelon, 2012).

Fecundity is defined by the physiological potential to bear offspring while fertility is the actual number of offspring produced (Shenk, 2015). Obesity and glucose dysregulation lead to altered puberty, ovulation and fertility in women (Nandi and Poretsky, 2013). Therefore, women with obesity and/or diabetes have worse outcomes following fertility treatments, usually due to poorer response to gonadotropins and lower yields in harvested oocytes (Silvestris *et al.*, 2018). Moreover, live birth rates can increase up to 9% for every unit decrease in BMI (Grzegorczyk-Martin *et al.*, 2020).

Before implantation, the mammalian embryo develops into a structure known as blastocyst in which three cell types can be identified (Rossant, 2018). The outer cells, the trophectoderm (TE), become the foetal portion of the placenta. The compact inner cells form the inner cell mass (ICM), which will differentiate into

Epiblast (Epi) or Primitive Endoderm (PrE). PrE cells generate the endodermal part of the yolk sac while Epi cells form the proper embryo. TE cells are characterized by the expression of markers like GATA3 or CDX2, while in early embryos ICM cells co-express both NANOG and GATA6. During ICM differentiation in mid blastocysts, cells asynchronously downregulate one of these markers so that Epi progenitors express NANOG, while PrE progenitors express GATA6 (Saiz *et al.*, 2016) followed by other markers such as SOX17, GATA4, or SOX7 (Rossant, 2018). In late blastocysts, PrE cells migrate to form an epithelial layer of cells next to the blastocoele, leaving the Epi cells in an internal localization. These differentiation processes, occurring prior to implantation, can be assessed using quantitative immunofluorescence analysis (QIF) (Fischer *et al.*, 2020, 2023).

The aim of this study was to assess the effect of age and obesity/metabolic syndrome on different aspects related to fertility: fecundability, embryo number and quality and embryo cell type composition. To do this, an established murine model of the metabolic syndrome was used, which underwent further characterisation.

Materials and Methods

C57Bl/6J mice were bred in house at the Universidad de Las Palmas de Gran Canaria (ULPGC) at the Institute for Biomedical and Healthcare Research (IUIBS) animal facility. All mice were housed with controlled room temperature (20-24ºC) and relative humidity (55-72%), and a 12-h light-darkness cycle. All animal studies were conducted following National and European regulations (RD 1201/2005, Law 32/2007, EU Directive 2010/63/EU), were approved by the

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Animal Ethics Committee of the ULPGC and were authorised by the competent authority of the Canary Islands Government (reference number: OEBA-ULPGC_10/2019R1).

On average, female mammals cease to exhibit reproductive cycles by middle age, around 15 months in mouse or 51 years in humans, while reproductive maturity is reached at 42 days in mice and 11.5 years in humans ('Men and mice: Relating their ages | Elsevier Enhanced Reader'). Female mice were divided into three age groups: young adults at the beginning of their reproductive life (Y:12 weeks old), mature adults (M: 9 months old) and middle-late age adults when they were close to reproductive senescence (O: 1 year old). Animals from different cages but with the same date of birth were grouped together and then randomly assigned to one of two diets following ARRIVE guidelines (Percie Du Sert *et al.*, 2020). High fat diet (HFD) in rodents is an established model for the induction of obesity and the metabolic syndrome (Igosheva *et al.*, 2010; Chen *et al.*, 2020; Lilao-Garzón *et al.*, 2020). Half of the animals were fed a standard diet (ND) (Envigo, Global Diet 2014) and the other half a HFD (60% energy from fat, D12492; Research Diets, Brogaarden, Lynge, Denmark) for 8 weeks prior to the target age (Fig. 1). Body weight was measured weekly. At the time of the study, morphometry was performed (head, body and tail length), body mass index (BMI) was calculated (Gargiulo *et al.*, 2014) and body composition measured, by Time Domain Nuclear Magnetic Resonance using a Minispec lean fat analyzer (Bruker Optics, Inc., The Woodlands, TX). Healthy males of up to 6 months of age, from the same colony, were used for breeding purposes.

Oral Glucose Tolerance Test and Intraperitoneal Insulin Tolerance Test

For the Oral Glucose Tolerance test (OGTT), on the eighth week of diet, mice were fasted for 6h and a 2 g/Kg dose of glucose (G7528, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was administered by oral gavage. Blood was sampled from the tip of the tail every 15 minutes up to 1 hour and glucose concentrations were measured and recorded (Glucomen Areo, Menarini Diagnostics). An insulin tolerance test (ITT) was performed by intraperitoneal administration of 0.5 U/Kg of insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark), two days after the OGTT following the same protocol (Brito-Casillas *et al.*, 2016).

Postmortem Analysis

Animals were killed by isoflurane overdose and bled out from the inferior vena cava. Serum was obtained and a full biochemistry panel comprising albumin, alkaline phosphatase, alanine aminotransferase, amylase, blood urea nitrogen, creatinine, globulin, glucose, phosphorus, total bilirubin, total protein, cholesterol and creatine kinase, was performed (PointCare V2 Biochemistry Analyzer, RAL SA, Barcelona).

Liver, pancreas, heart, spleen, reproductive system, white and brown fat, kidneys, and gastrocnemius muscle were dissected during the necropsy. The tissues were weighed and preserved in 4% neutral buffered formalin (Applichem-Panreac, Barcelona), processed routinely, and embedded in paraffin-wax. Sections (5 µm-thick) were stained with haematoxylin-eosin. All histological sections were randomly and blindly evaluated by a board-certified veterinary pathologist (OQC).

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Pancreatic insulin content was evaluated by immunofluorescence. After permeabilization with Triton X-100 0.05%, pancreas sections were incubated with anti-insulin (Santa Cruz, Dallas, TX, USA, sc-9168; 1:80) and anti-Glucagon antibodies (Sigma-Aldrich, G2654, St. Louis, MO, USA, 1:100). Alexa Fluor 488 Tyramide Superboost kit (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA, B40943) was used to increase insulin fluorescent signal following the manufacturer's instructions.

Fecundity Analysis

Animals were mated for up to eight days to allow two consecutive oestrus cycles and pregnancy was confirmed by the presence of a vaginal plug. The ability to mate and get pregnant and the number of embryos per female were used as fecundity indicators. Mating rate was calculated as the proportion of females with a vaginal plug, as an indirect measure of oestrus cycle. Fertilization rate was calculated as the percentage of females with a plug which had embryos in their uteri. Preimplantation embryo quality was assessed by morphological features and classified into 4 categories (A-D) (Gardner and Balaban, 2016).

Preimplantation embryo analysis

After 3.5 days of embryonic development, females were killed by cervical dislocation and preimplantation embryos were flushed from dissected uteri in M2 medium (Embryomax®; Millipore, Ref. MR-015-D, Burlington, MA, EE. UU) and prepared for immunofluorescence as previously described (Nichols *et al.*, 2009). Primary antibodies were anti-NANOG raised in rat (eBioscience, San Diego, CA, USA, Ref.14–5761; 1:200), anti-GATA6 raised in goat (R&D Systems, Minneapolis, MN, USA, AF1700; 1:200), anti-GATA4 raised in rabbit (Santa Cruz,

Dallas, TX, USA, sc-9053; 1:200) and anti-GATA3 raised in rabbit (eBioscience, San Diego, CA, USA, Ref. MA1-028; 1:200). DAPI (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA, D1306; 1:1000) was used to stain the nuclei.

Microscopy and Image Analysis

Both Islets of Langerhans and embryos were imaged using a Zeiss LSM Zeiss LSM700 and a Plan-Apochromat 40x/1.3 Oil DIC (UV) VIS-IR M27 objective, with optical section thickness of 1 µm.

Embryo images were processed and analysed as previously described (Fischer *et al.*, 2020) including the fluorescence decay along the Z axis correction (Saiz *et al.*, 2020). Briefly, using image analysis tools (MINS) (Lou *et al.*, 2014), after immunostaining and confocal imaging, NANOG and GATA6 (GATA4 and/or GATA3) expression levels can be quantified in single cells allowing to monitor the ICM cells differentiation process by custom-made data analyses codes in Mathematica described in (Fischer *et al.*, 2020). Embryos were classified, according to their total cell number, into early (up to 64 cells), mid (65-90 cells) and late (from 91 cells). Cells were then manually classified into TE and ICM. Cells in the ICM were classified using mixture analysis as positive or negative: double negative (DN, negative expression for NANOG and GATA6), Epi progenitors (positive for NANOG and negative for GATA6), PrE progenitors (negative for NANOG and positive for GATA6), and double positive (DP) for uncommitted ICM cells positive for both, NANOG and GATA6. The same classification was based on GATA4 staining instead of GATA6.

Statistics

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Data are presented as mean ± standard error (SEM). Anova with Tukey's multiple comparisons test was used for comparisons between groups and Z-test for frequencies, followed by the Bonferroni correction for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Eight weeks of HFD are associated with altered body composition and glucose metabolism, especially in older animals. Rapid weight gain was observed in the mature and old groups on HFD, whereas no significant effect on body weight or BMI was seen in young animals (Fig. 2a, b). HFD was consistently associated with increased total body fat and reduced lean mass (Fig. 2c).

OGTTs and ITTs were performed in the last week of diet to evaluate the effect of both age and HFD on glucose metabolism (Fig. 1, 2). The mice fed with the HFD showed higher glucose concentrations during the OGTT than those fed with ND, and this was especially evident in the older mice (Fig. 2d). HFD was also associated with decreased glucose-lowering after an insulin injection, reflecting increased insulin resistance (Fig. 2e).

HFD results in increased white fat and reduced muscle mass and high cholesterol and glucose, but no major pathological findings.

Post mortem studies confirmed that animals fed with the HFD had more body fat and less muscle mass than those fed with ND (Fig. 3a, b). A weight increase was also observed in the reproductive system, but this increase was due to the increment of periovaric fat (Supp. Fig. 1). Furthermore, high levels of fat in the diet seem to have a weight curbing effect but not statistically significant on the liver and pancreas as well as in other organs such as heart, kidneys or spleen. In

the blood biochemistry results, animals were grouped according to their diet to increase statistical power. Animals fed with HFD showed higher glucose and total cholesterol concentrations than those fed with ND (Fig. 3c, d). High glucose concentrations were observed even in the blood from animals fed with the ND obtained post-mortem from the inferior vena cava, in agreement with previous reports (Chan *et al.*, 2012; Yu *et al.*, 2020) and is attributed to isoflurane (Horber *et al.*, 1990). No differences were observed in other measurements (Supp. Fig. 2).

Histological findings were consistent with senescence and/or the phenotype of the model (Table 1). In the absence of an infectious agent, the hepatocellular necrosis observed can be considered as background lesion (Percy *et al.*; Hall *et al.*, 1992; Thoolen *et al.*, 2010; Tessitore *et al.*, 2016). Also, the systemic amyloidosis observed is considered a common age-related change in some mouse strains, such as the C57BL6 (Thoolen *et al.*, 2010) and is consistent with the diagnosis of spontaneous systemic amyloidosis (Willard-Mack *et al.*, 2019).

Young groups showed fewer lesions than older groups, but no evident differences were noted between animals fed with ND or with HFD at any age. No qualitative differences were observed in pancreatic insulin or glucagon content comparing age groups or diets (Fig. 3e).

Overall fecundity decreases with age. Mating and fertilization rates were used as descriptive methods to assess fecundity. Old animals fed with HFD showed lower mating and fertilization rates than younger groups (Fig. 4). Only one old ND

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fed dam had embryos and no embryos were obtained in old HFD fed dams. Hence, the old groups were excluded from further analysis.

Embryo quality was assessed according to morphological features. Young ND fed dams had a higher percentage of high quality (category A) embryos and fewer with the worst features (category D) compared with young HFD fed dams (Fig. 4e). Total cell number together with the classification into TE or ICM cells was done, and the ratio ICM/TE calculated. An age effect was observed in HFD fed groups (see Supp. Table 1), indicating altered patterns in embryo cell fate allocation.

Age and HFD are associated with delayed development in preimplantation embryos. Preimplantation embryos flushed from the uteri were stained for GATA3, NANOG and GATA6 and images were analysed by single-cell quantitative methods (Figs. 5-6). Results for early embryos are shown (see classification details in Methods). In general, embryos from HFD fed females showed an increased proportion of uncommitted cells (DP cells) when compared with their ND fed controls. This is especially noticeable in mature groups where Epi progenitor proportion diminished upon HFD feeding (Fig. 6a). Comparing embryos from different ages, despite no change in uncommitted cells, those from mature dams showed a defect in PrE cell fate progression.

Although the population analysis showed more Epi progenitors in embryos from young HFD fed animals, NANOG levels were significantly lower both in DP cells (Fig. 6b) and in Epi progenitors (Fig. 6c). The same was observed in embryos from mature dams regardless of the diet: NANOG levels were significantly lower

in DP cells (Fig. 6b). Epi progenitors showed higher levels of NANOG in embryos from mature HFD fed females (Fig. 6c).

GATA6 levels were significantly lower in embryos from HFD fed females independently of the age in DP cells (Fig. 6d). Embryos from MND fed females showed higher levels of GATA6 than those from YND fed females (Fig. 6d).

Similar results were obtained for the population analysis in mid and late embryos (Supp. Fig. 3) as well as the analysis with the more developmentally advanced PrE marker GATA4 (Supp. Fig. 4).

The higher number of uncommitted cells together with fewer cells fully committed to one cell fate (especially to PrE), and cell fate marker levels indicate that preimplantation embryos developed under an obese environment or from mature females are delayed in their normal development when compared to controls.

Both HFD and advanced maternal age had an increasing effect in the levels of GATA3. GATA3 was analysed in the TE cells where it was almost ubiquitously expressed (Fig. 7a). GATA3 expression increased with age. However, its response to HFD changed in opposite directions in young (increase) and mature (decrease) dams (Fig. 7b).

Discussion

In the present study, we assessed the impact of maternal age and HFD on early embryo development, specifically Epi versus PrE cell fate decision, through state-of-the-art single cell quantitative confocal image analysis (Fischer *et al.*, 2020). In rodent research, the glucose and insulin tolerance tests are the most widely used test to determine whether a mouse is glucose intolerant and diabetic. However, to this date, there is no agreement on which values are considered

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indicative of metabolic syndrome/diabetes (Lilao-Garzón *et al.*, 2021). These is probably due to the many variables such as fasting duration, amount and route of glucose administration, as well as personal training and stress of the animal that greatly impacts on the measurements (Andrikopoulos *et al.*, 2008; Benedé-Ubieto *et al.*, 2020). Indeed, HFD led to increased body fat, glucose intolerance and insulin resistance. To our knowledge, this is the first study to report delayed early embryo development, based on Epi versus PrE cell fate specification, associated with both HFD and age. We also describe alterations in GATA3 levels, showing an effect of age and HFD in TE differentiation. This is significant as it allows us to establish embryo development disorders that could be responsible for embryo loss in the first few days after fertilization, leading to the low fertility described in older and overweight women (Silvestris *et al.*, 2018).

We first confirmed that 8 weeks of HFD in adulthood induced overweight in female mice as shown in previous studies (Picklo *et al.*, 2017; Soleimanzad *et al.*, 2021) but not when started before puberty. Despite similar weight and BMI, the young HFD fed animals showed almost twice as much fat mass as their control group. The OGTT and ITT revealed that HFD induces glucose impairment and insulin resistance, especially in the older groups. However, age itself did not have a direct effect on glucose intolerance, in agreement with a previous study (Soleimanzad *et al.*, 2021).

Frequently, when the effects of childhood obesity in adulthood are assessed, only body weight and BMI are used as indicators (Bentham *et al.*, 2017). Here, we show that in young mice, 8 weeks of HFD did not affect body weight and BMI. However, other parameters that affect overall health such as body composition and glucose metabolism were severely affected.

Furthermore, we validated the 8 weeks HFD in C57Bl6 female mice as a prediabetic mouse model with different levels of obesity and hyperglycaemia depending on age; young adults, mature adults, and middle-late age adults. This strategy allowed us to evaluate how HFD and maternal age affect fecundity.

Selected maternal ages were based on previous studies. Mice are often divided into mature adults (3-6 months), middle aged (10-15 months) and old (18-24 months) (THE JACKSON LABORATORY; Dutta and Sengupta, 2016) with some authors even expanding mouse lifespan up to 36 months (Chaix *et al.*, 2021). In this context, their reproductive senescence is established between 10 to 15 months. Other authors establish the irregular fertility that precedes reproductive senescence at approximately 8 months of age (Brinton, 2012) in accordance with the lower fertility observed in mature groups in the present study.

Infertility (failure to conceive after attempting at least twelve months of natural fertilization) is a rising problem in our society. Life factors such as psychological stress, smoking, drugs or diet and variations in body weight have a substantial effect. Overweight in women is often associated with multiple reproductive disorders such as polycystic ovary syndrome, infertility, increased miscarriage rate, as well as pregnancy complications (gestational diabetes, pre-eclampsia, and macrosomia). Furthermore, overweight (BMI>25 Kg/m^2) and obese (BMI>30 Kg/m^2) women have worse outcomes following fertility treatments than women with normal BMI. They respond poorly to induction of ovulation, require higher doses of gonadotropins and longer treatment courses for follicular development and ovulatory cycles (Ozekinci *et al.*, 2015). When focusing on their blastocyst quality, high BMI is not associated with low embryo quality, though the implantation rate is reduced (Bellver *et al.*, 2010; Larsen *et al.*, 2020; Qi *et al.*,

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2022). Type 2 diabetes is most frequently associated with obesity. However, in the absence of obesity, type 2 diabetes is also associated with reduced fertility and more frequent need for assisted reproduction (Mattsson *et al.*, 2021). Implantation rate is indeed reduced in women with type 2 diabetes, regardless of BMI, as shown by a recent study based on the Danish registry of assisted reproductive techniques (Larsen *et al.*, 2020). Our results showed no significant differences in mating or fertilization rates. Here, we did observe reduced embryo quality in YHFD fed females. Surprisingly, no diet effect was observed in embryos from mature females suggesting that age itself has a bigger impact than obesity. However, the quantitative analyses presented here show a clear diet effect in those embryos. This questions the validity of classical morphological indicators to determine embryo quality. Furthermore, previous epidemiological studies done in humans have shown that the relationship between BMI and fertility is not always clear, especially when maternal age included. Higher BMI has more obvious negative effects on fertility in younger women but these unfavourable effects seem to decrease with age (Sneed *et al.*, 2008; Goldman *et al.*, 2019). Regardless, in our study, we observe that HFD has a greater impact in young females than in mature ones. One possible explanation is that age has such a critical effect on oocyte/embryo quality that those that survive are the best quality ones and are not affected by diet. However, further studies are needed in order to confirm this hypothesis.

During mammalian preimplantation development, two sequential cell fate decisions occur. The first decision induces TE (precursor of the placenta) versus ICM cells. The ICM cells make a further decision, differentiating either into Epi or into PrE (Rossant, 2018). Evidence suggests that inflammation and oxidative

stress can severely affect these cellular events and embryo quality (Miao and Cui, 2022). Moreover, preimplantation embryos cultivated with lipopolysaccharides showed a lower proportion of SOX17 expressing cells, suggesting that the oxidative stress induced by lipopolysaccharides could be impairing the normal development of the embryonic PrE. Furthermore, the correction of the associated oxidative stress, reverted these PrE development impairments (Miao and Cui, 2022).

GATA3 is highly expressed in the invasive murine trophoblast giant cells of the blastocyst and its knockdown enhances placental cell invasion (Chiu and Chen, 2016). Hence, the high levels of GATA3 associated with HFD we observe here might impair placentation. Similarly, embryos treated with lipopolysaccharides also showed an increased expression of TE markers (Miao and Cui, 2022).

A limitation of this study is that we did not measure caspase-3 activity or reactive oxygen species (ROS) in the embryos to assess oxidative stress. However, previous studies showed that oxidative stress is strongly correlated with the prevalence of type 2 diabetes, obesity and aging through different mechanisms such as high ROS production caused by hyperglycaemia and oxidation of fatty acids or by the decrease in antioxidant capacity (Jawerbaum and White, 2010; Fernández-Sánchez *et al.*, 2011; Davalli *et al.*, 2016; Rehman and Akash, 2017). Also, while 8 weeks of HFD feeding may be enough to model changes in embryo development, lifelong female obesity may have more drastic effects.

In summary, we demonstrated that 8 weeks of HFD are enough to model obesity and the metabolic syndrome in mice even at young ages when there are no apparent effects on weight. Age seems to be the most important factor

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determining reduced fecundity, but both HFD and age are associated with delayed preimplantation embryo development that might explain the lower implantation rates observed in women with overweight or type 2 diabetes (Bellver *et al.*, 2010; Larsen *et al.*, 2020).

Contribution statement

JLG, YBC, AMW and SMD conceived and designed the research; JLG, YBC, OQC and SMD performed the research, acquired the data and analysed the results; JLG, YBC, SMD and AMW analysed and interpreted the data. All authors were involved in drafting and revising the manuscript. SMD is the guarantor of all the data.

Disclosure of interest

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

Funding

This work was supported at SMD lab by the ACIISI (CEI2019-02), Programa de Ayudas a la Investigación de la ULPGC, and ACIISI co-funded by FEDER Funds (ProID2020010013). Also at AMW lab by Instituto de Salud Carlos III (PI16/00587, PI20/00846, PMP21/00069) and ACIISI (ProID2021010143) co-funded by European Regional Development Funds, as well as the European Horizon 2020 Programme (101017385) and Fundación Canaria del Instituto de Investigaciones Sanitarias de Canarias (PI19/30, PIFIISC20/16). JLG is supported by the ULPGC predoctoral program. SMD by the “Viera y Clavijo” Program from the Agencia Canaria de Investigación, Innovación y Sociedad de la Información (ACIISI) and the ULPGC.

Acknowledgements

We are grateful to Tina Balayo, Ana Exposito-Montesdeoca and the IUIBS animal facility staff for the technical support. Sabine Fischer for data analysis support.

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Figures

Fig. 1: **Experiment design**. Female mice of different ages were fed either normal diet (ND) or high fat diet (HFD) and their weight was monitored once a week. On the eighth week, oral glucose tolerance test (OGTT), insulin tolerance test (ITT) and body composition assessment were performed. Then, females were mated with young healthy males for up to 8 days and preimplantation embryos were flushed from the uterus 3.5 days after vaginal plugs were detected. A necropsy was performed in a few animals after the 8 weeks of diet.

Fig. 2: **Effect of 8 weeks of high fat diet (HFD) on weight, body composition, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) compared to normal diet (ND)**. (a) Weekly body weight and (b) body mass index. n= 14 YND, 20 YHFD, 14 MND, 17 MHFD, 9 OND and 21 OHFD. (c) Body composition by nuclear magnetic resonance. n= 14 YND, 20 YHFD, 14 MND, 17 MHFD, 9 OND and 20 OHFD. (d, left) OGTT blood glucose. (d, right) AUC calculated from OGTT. (e, left) ITT blood glucose. (e, right) AUC calculated from ITT. n=12 YND, 17 YHFD, 14 MND, 17 MHFD, 9 OND and 20 OHFD. Data are expressed as mean ± SEM, *$p<0.05$ comparing each HFD group with its ND control or between age-groups. YND= Young (12 weeks) Normal Diet (solid blue), YHFD= Young High Fat Diet (patterned blue); MND= Mature (9 months) Normal Diet (solid red), MHFD= Mature High Fat Diet (patterned red); OND= Old (1 year) Normal Diet (solid black), OHFD= Old High Fat Diet (patterned black).

Fig. 3: **Mice were killed and dissected after being fed for 8 weeks with normal or high-fat diet and their organs were weighed**. (a) White fat as the addition of fat from the paragenital fat pads. (b) Gastrocnemius as the addition of the weight of both gastrocnemius muscles from the hind legs. (c-d) For the biochemistry analysis animals were grouped by diet: (c) blood glucose levels and (d) total cholesterol levels at the moment of death. Data are expressed as median, *$p<0.05$ either comparing each HFD group with its ND control or between ages; Each point refers to one animal. (e) Pancreas histological sections immunostained for Insulin (green) and Glucagon (red). Representative maximum projection images from Z stacks for each condition are shown. Scale bar: 20 μm. YHFD= Young High Fat Diet (patterned blue); MND= Mature (9 months) Normal

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Fig. 4: **Effect of 8 weeks of high fat diet on fecundity**. (a) Mating rate. n= 33 YND, 38 YHFD, 41 MND, 48 MHFD, 9 OND, 20 OHFD. (b) Fertilization rate. n= 27 YND, 26 YHFD, 25 MND, 31 MHFD, 5 OND and 1 OHFD. (c) Number of embryos recorded per female. Each dot indicates one litter. No statistical significance was found in any case, *p<0.05 either comparing each HFD group with its ND control or between ages. (d) Representative micrographs of embryos from indicated females. (e) Embryos classified according to their morphological features into four categories (A, B, C, and D, from high to low quality) *p<0.05; YHFD= Young High Fat Diet (patterned blue); MND= Mature (9 months) Normal Diet (solid red), MHFD= Mature High Fat Diet (patterned red); OND= Old (1 year) Normal Diet (solid black), OHFD= Old High Fat Diet (patterned black).

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Fig. 7: **Variations in GATA3 levels in the trophectoderm (TE) assessed by quantitative immunofluorescence.** (a) Population analysis as the percentage of the total number of cells in the TE. (b) Violin plots showing GATA3 expression levels. *p<0.05 either comparing each HFD group with its ND control or between ages. Results showed only for early embryos; YHFD= Young High Fat Diet (patterned blue); MND= Mature (9 months) Normal Diet (solid red), MHFD= Mature High Fat Diet (patterned red).

.

Abbreviations

BMI: Body Mass Index

DN: Double Negative

DP: Double Positive

Epi: Epiblast

HFD: High Fat Diet

ICM: Inner Cell Mass

ITT: Insulin Tolerance Test

MHFD: Mature High Fat Diet

MND: Mature Normal Diet

ND: Normal Chow Diet

OGTT: Oral Glucose Tolerance Test

OHFD: Old High Fat Diet

OND: Old Normal Diet

OQC: Oscar Quesada-Canales

PrE: Primitive Endoderm

QIF: Quantitative Immunofluorescence Analysis

ROS: Reactive Oxygen Species

TE: Trophectoderm

YHFD: Young High Fat Diet

YND: Young Normal Diet

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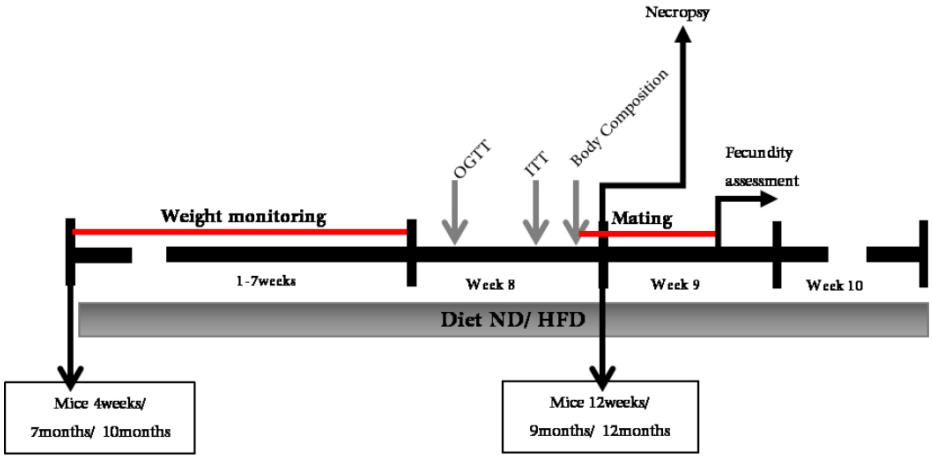

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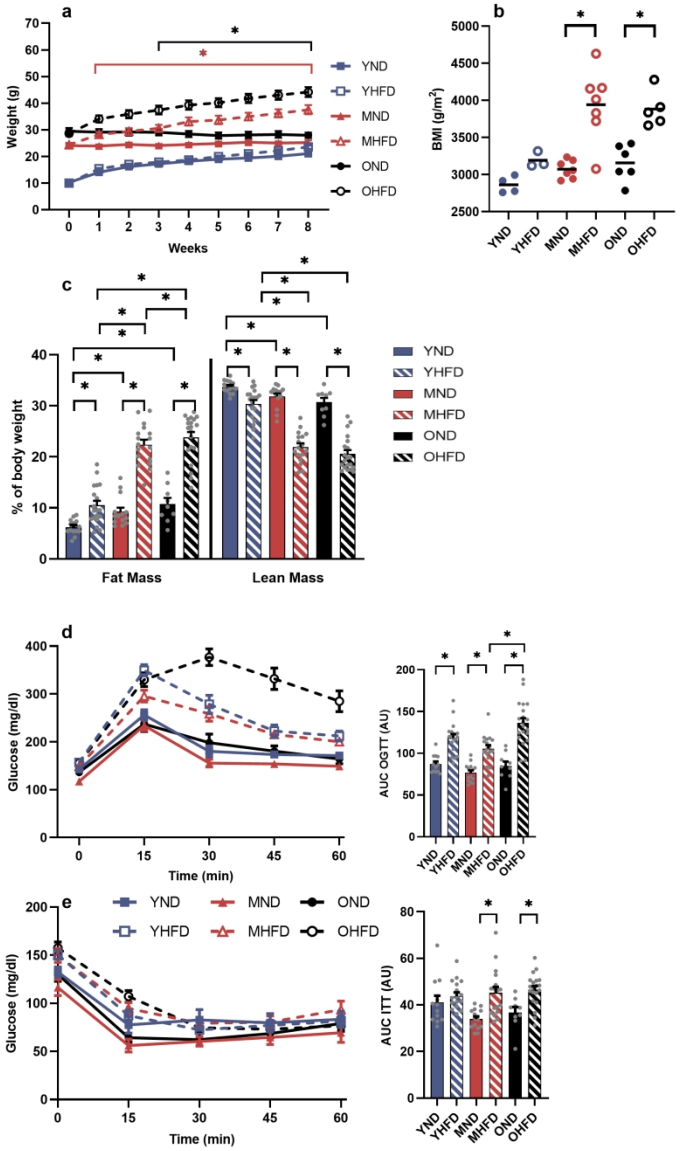

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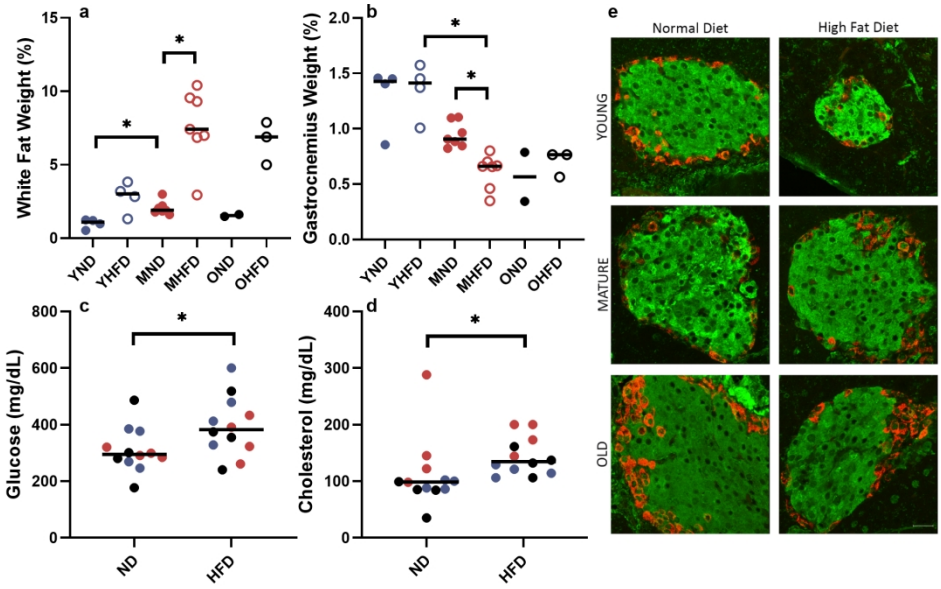

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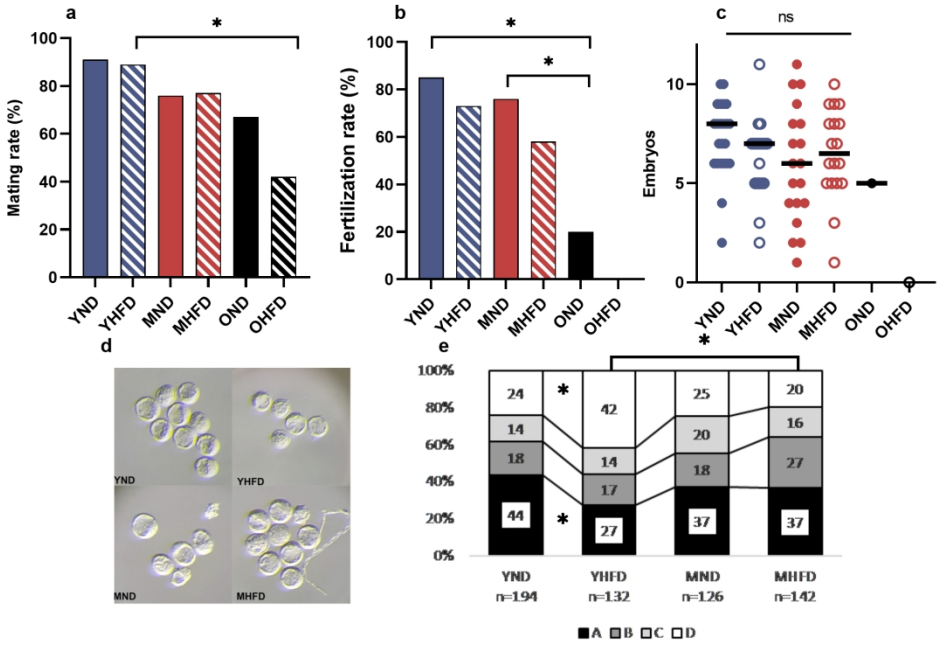

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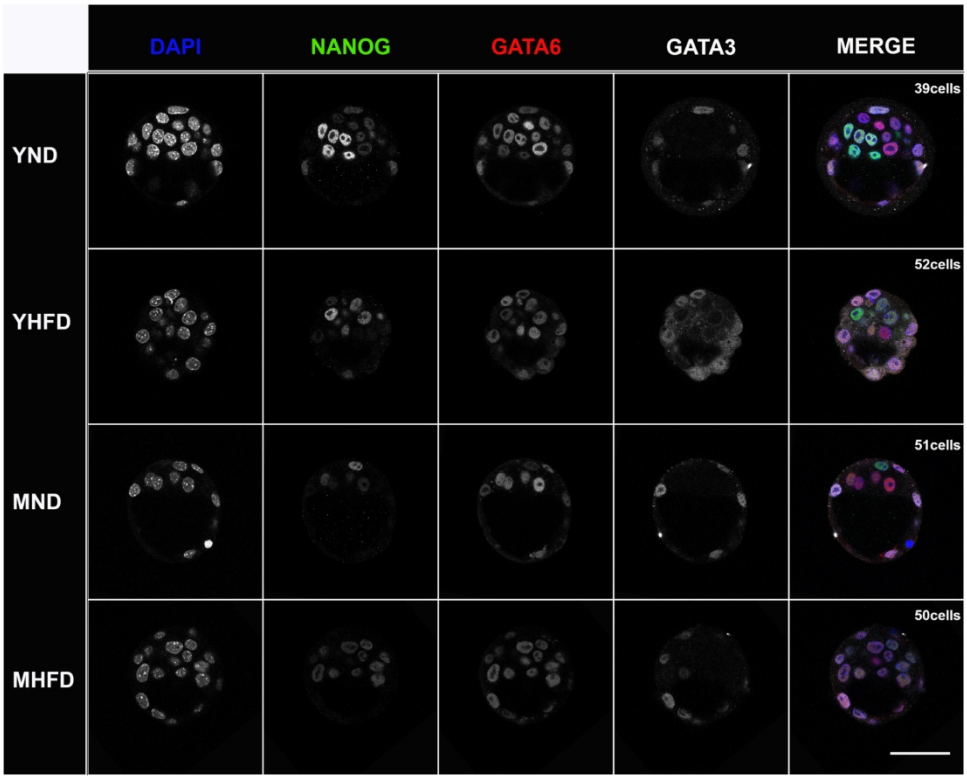

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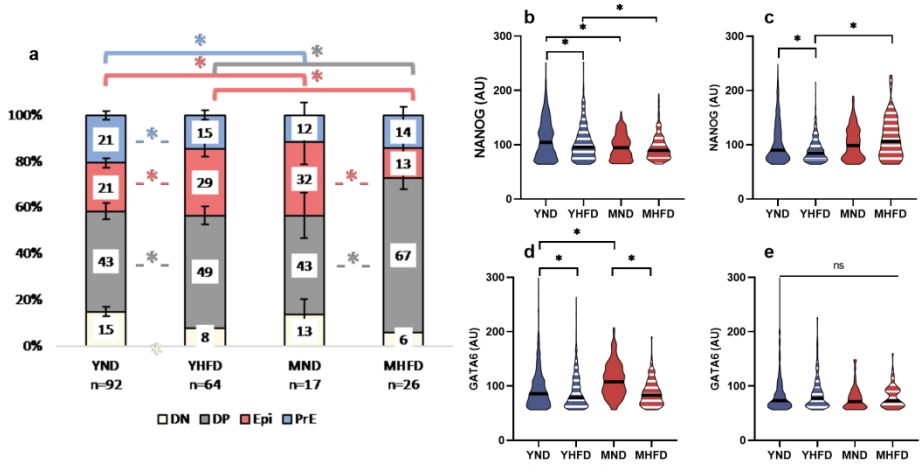

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Accepted Manuscript published as REP-23-0024.R1. Accepted for publication: 18-Jul-2023

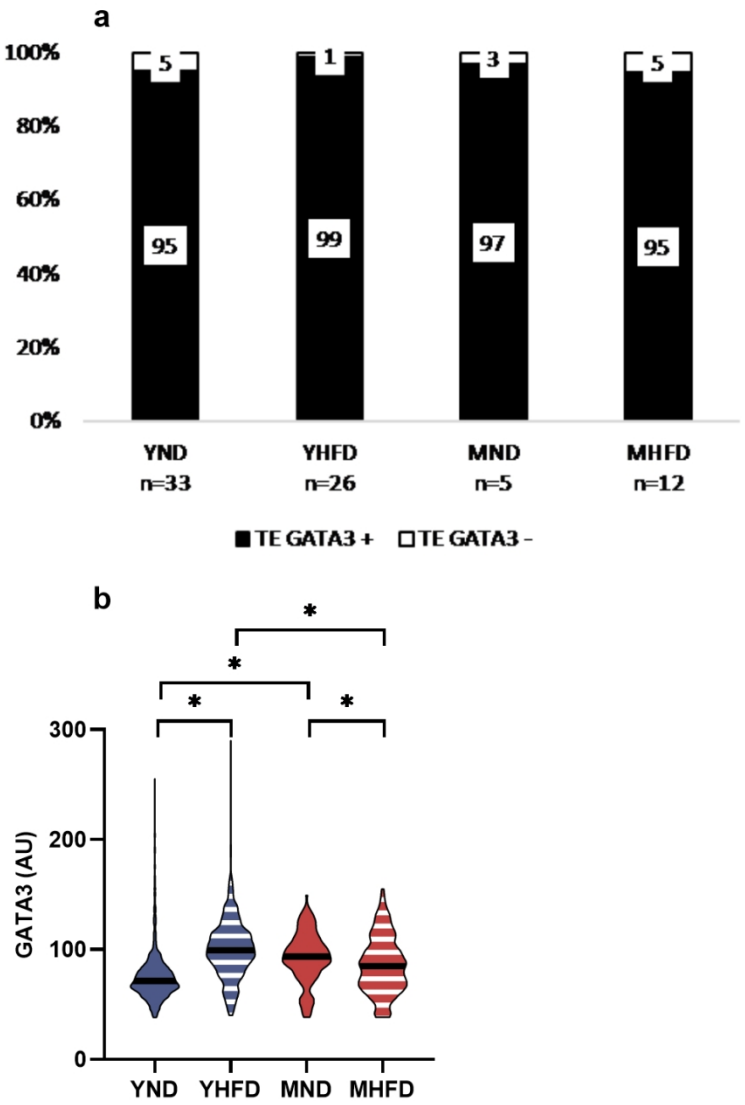

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Table1 Pathology evaluation of tissue samples. Histopathological features found in the respective group and location (number of individuals/total of individuals per group). Only tissues with histopathological features are included in this descriptive table; *n*=24 (4/group).

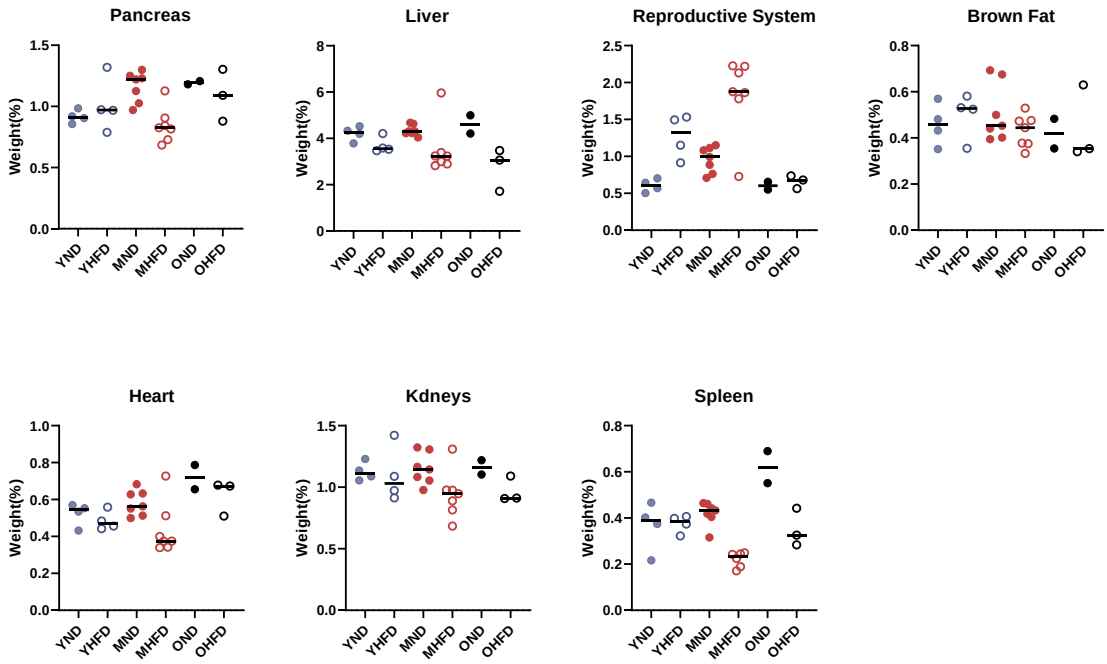
	YND	YHFD	MND	MHFD	OND	OHFD
Liver	Mild, diffuse hepatocellular vacuolization (glycogen accumulation) (4/4)	Mild and diffuse hepatocellular vacuolization (glycogen accumulation) (4/4)	Moderate, diffuse hepatocellular vacuolization (glycogen accumulation) (4/4)	Moderate, diffuse hepatocellular vacuolization (glycogen accumulation) (4/4)	Moderate, diffuse hepatocellular vacuolization (glycogen accumulation), (4/4)	Moderate, diffuse hepatocellular vacuolization (glycogen accumulation) (2/4)
	Randomly, scattered, small aggregates of lymphocytes and rare neutrophils (2/4)	Minimal perivascular infiltrate of lymphocytes and neutrophils. (1/4)	Moderate, diffuse macrovesicular fatty change, (1/4)	Moderate, diffuse macrovesicular fatty change, (2/4)	Mild to moderate perivascular (centrilobular) infiltrate of lymphocytes with fewer neutrophils (2/4)	Mild, multifocal, macrovesicular fatty change (2/4)
		Randomly, scattered small aggregates of lymphocytes and rare neutrophils (3/4)	Mild to moderate perivascular (centrilobular) infiltrate of lymphocytes with fewer neutrophils, (4/4)	Mild to moderate perivascular (centrilobular) infiltrate of lymphocytes with fewer neutrophils, (4/4)	Multifocal, mild-moderate, perivascular (periportal and centrilobular) amyloidosis extending into and expanding adjacent sinusoids (2/4).	Multifocal, mild-moderate, perivascular (periportal and centrilobular) amyloidosis,extending into and expanding adjacent sinusoids (4/4).
			Randomly, minimal aggregates of lymphocytes, with rare neutrophils, (4/4)	Randomly, minimal aggregates of lymphocytes, with rare neutrophils, (1/4)		Mild to moderate perivascular (centrilobular) infiltrate of lymphocytes with fewer neutrophils (4/4)
						Randomly, occasional and discrete foci of hepatocellular necrosis with cellular debris and rare neutrophils (1/4)
Spleen	NPF	NPF	Moderate, multifocally disseminated, golden brown pigment-laden macrophages (4/4)	Moderate, multifocally disseminated, golden brown pigment-laden macrophage (4/4)	Moderate, multifocally disseminated, golden brown pigment-laden macrophage (2/4)	Severe amyloidosis (4/4)
			Multifocal lymphoid hyperplasia. (4/4)	Multifocal lymphoid hyperplasia (4/4)	Severe amyloidosis (2/4)	Multifocal lymphoid hyperplasia (1/4)
					Multifocal lymphoid hyperplasia (4/4)	
Heart	NPF	NPF	Mild, multifocal amyloidosis (1/4)	NPF	Severe, multifocal amyloidosis (1/4)	Severe, multifocal amyloidosis (2/4)
					Mild, multifocal amyloidosis (1/4)	
Kidneys	NPF	NPF	Mild-moderate, locally extensive medullary tubular mineralization (1/4)	Mild-moderate, multifocal perivascular infiltration of lymphocites in the cortex (1/4)	Mild, multifocal, interstitial amyloidosis (1/4)	Mild, multifocal, interstitial amyloidosis (1/4)
					Multifocal, severe, interstitial lymphocytic nephritis (1/4)	Multifocal, severe, interstitial lymphocytic nephritis (2/4)
						Mild, multifocal perivascular infiltration of lymphocites in the cortex (3/4)
Pancreas	NPF	NPF	NPF	NPF	Moderate, multifocal, perivascular amyloidosis (1/4)	Moderate, multifocal, perivascular amyloidosis (2/4)
						Mild, multifocal periductal infiltrate of lymphocytes, (1/4)

NPF= Non pathological findings; YND= Young Normal Diet, YHFD= Young High Fat Diet, MND= Mature Normal Diet, MHFD= Mature High Fat Diet, OND= Old Normal Diet, OHFD= Old High Fat Diet.

 Accepted Manuscript published as REP-23-0024.R1. Accepted for publication: 18-Jul-2023

Online Supplemental Materials

SuplementaryFigure1



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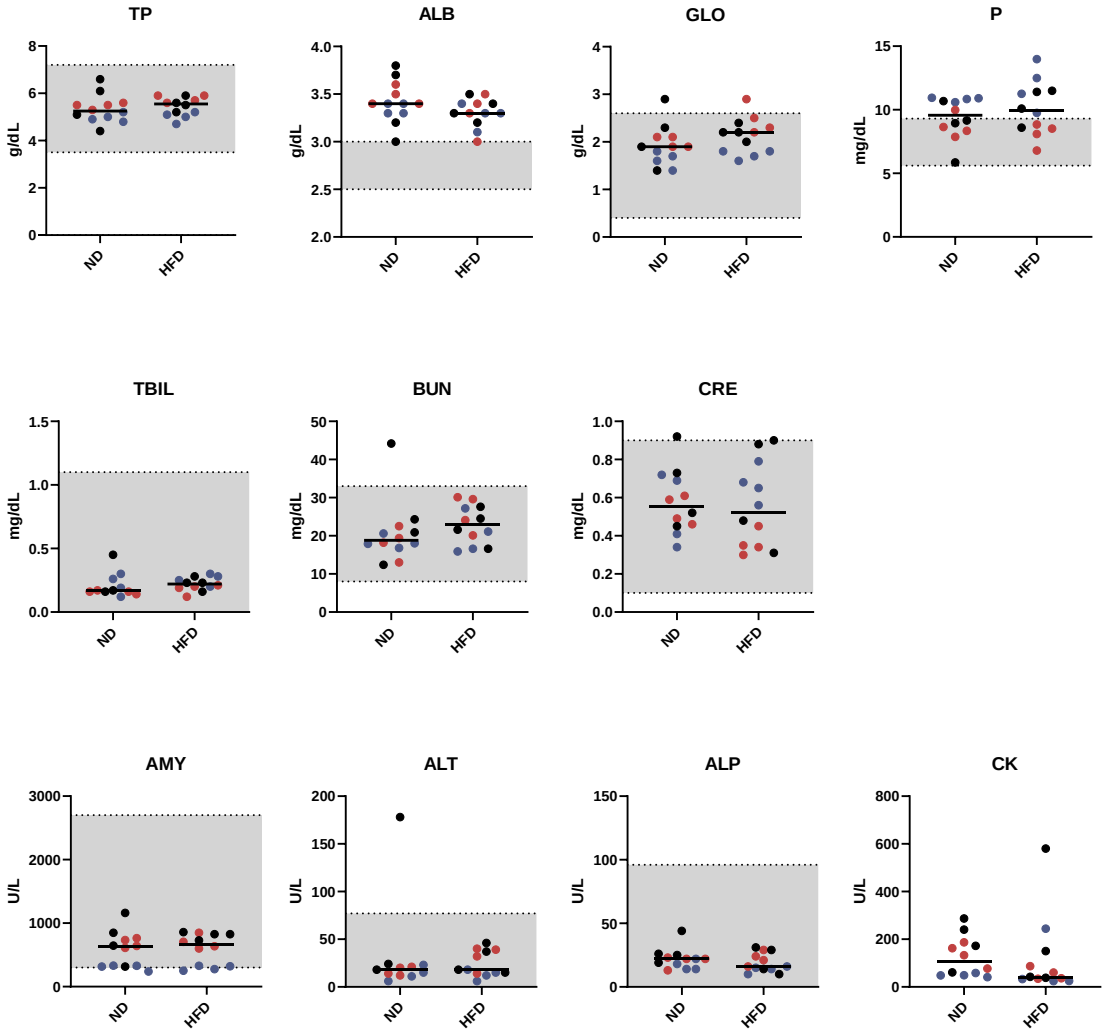
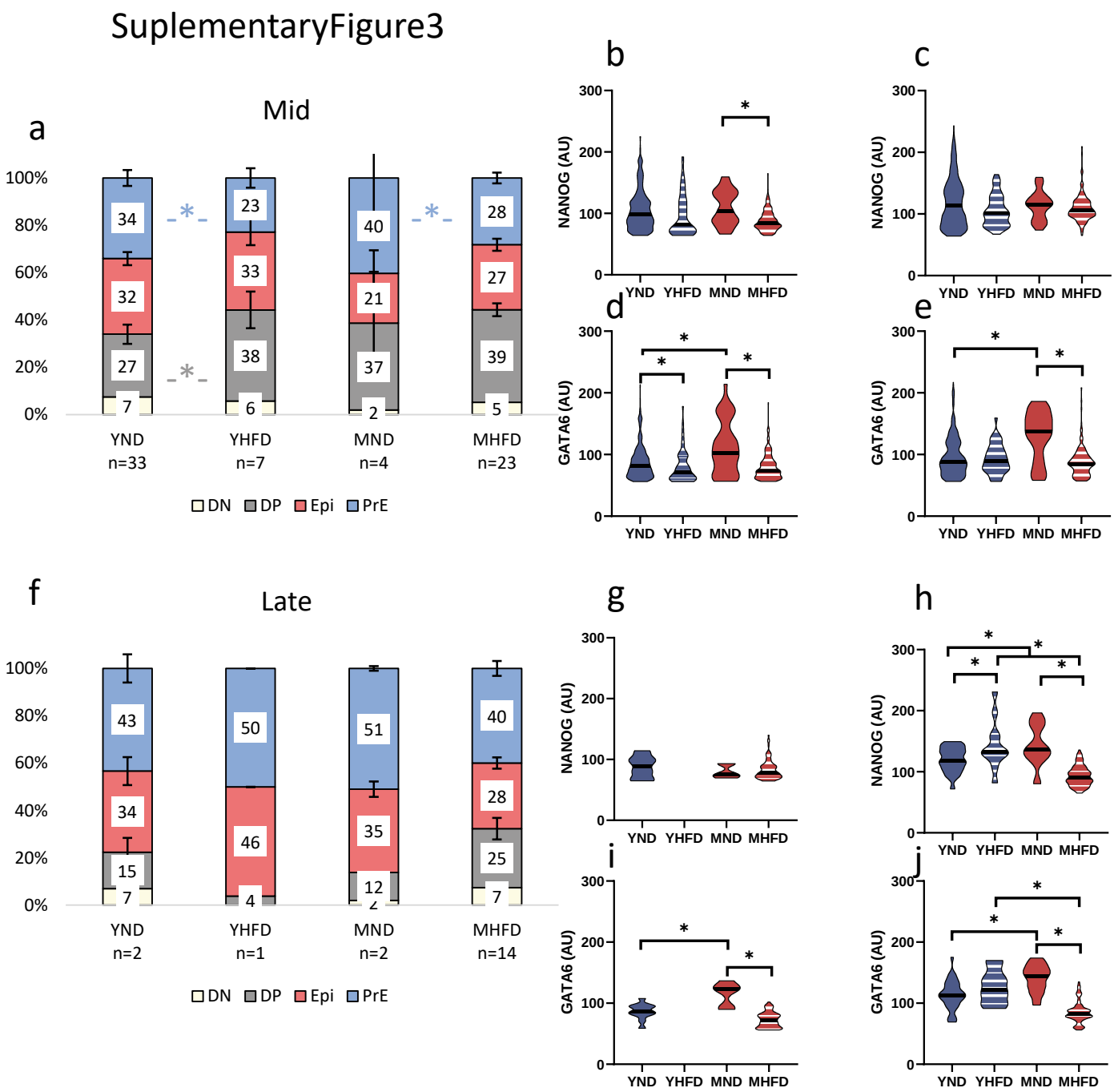
 Accepted Manuscript published as REP-23-0024.R1. Accepted for publication: 18-Jul-2023

SuplementaryFigure2



Sup. Fig. 2: Biochemistry analysis. Animals were grouped by diet: total protein (TP), albumin (ALB), globulin (GLOB), phosphorus (P), total bilirubin (TBIL), blood urea nitrogen (BUN), creatinine (CRE), amylase (AMY), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and creatine kinase (CK). Data are expressed as median, *p<0.05 comparing each HFD group with its ND control; Each point refers to one animal; 12 weeks (blue), 9 months (red) and 1 year old (black). YND= Young Normal Diet, YHFD= Young High Fat Diet, MND= Mature Normal Diet, MHFD= Mature High Fat Diet, OND= Old Normal Diet, OHFD= Old High Fat Diet. Gray areas indicate physiological ranges according to the manufacturer.



Sup. Fig. 3: Variations in embryo developmental stage assessed by quantitative immunofluorescence. (a, f) Population analysis as the percentage of the total number of cells in the ICM for Mid (a) and late (f) embryos; Violin plots showing fate marker expression levels: (b, g) NANOG levels in single DP cells and (c, h) in Epi progenitors. (d, i) GATA6 levels in single DP cells (e, j) and in PrE progenitors. Data are expressed as mean ± SEM, *p<0.05 comparing each HFD group with its ND control or between age-groups; YND= Young Normal Diet, YHFD= Young High Fat Diet, MND= Mature Normal Diet, MHFD= Mature High Fat Diet, OND= Old Normal Diet, OHFD= Old High Fat Diet.

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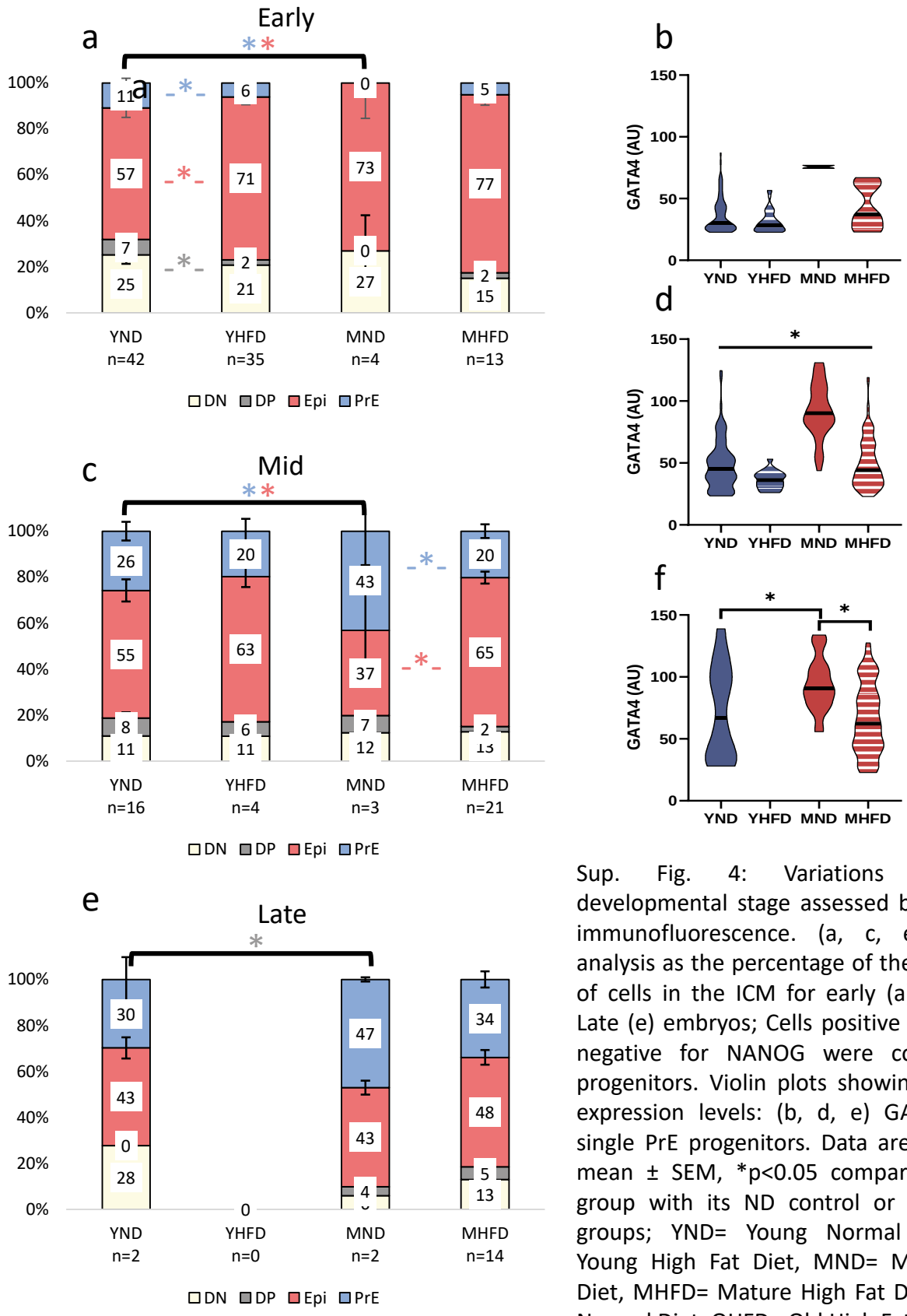
SuplementaryFigure4



Sup. Fig. 4: Variations in embryo developmental stage assessed by quantitative immunofluorescence. (a, c, e) Population analysis as the percentage of the total number of cells in the ICM for early (a), Mid (c) and Late (e) embryos; Cells positive for GATA4 but negative for NANOG were considered PrE progenitors. Violin plots showing fate marker expression levels: (b, d, e) GATA4 levels in single PrE progenitors. Data are expressed as mean ± SEM, *p<0.05 comparing each HFD group with its ND control or between age-groups; YND= Young Normal Diet, YHFD= Young High Fat Diet, MND= Mature Normal Diet, MHFD= Mature High Fat Diet, OND= Old Normal Diet, OHFD= Old High Fat Diet.

Supplementary Table 1

			Total cell number		Total TE number		Total ICM		ICM/TE Ratio	
	Stage	N Embryos	Average	SE	Average	SE	Average	SE	Average	SE
YND	Early	92	54.18	1.06	33.93	0.71	20.25	0.56	0.61	0.02
	Mid	33	72.12	1.07	48.82	1.23	23.30	0.68	0.49	0.03
	Late	2	92.00	1.00	68.50	3.50	23.50	2.50	0.35	0.06
YHFD	Early	64	52.47	1.39	32.45	1.02	20.02	0.61	0.64*	0.02
	Mid	7	71.57	2.22	45.86	3.26	25.71	1.60	0.59	0.07
	Late	1	91.00	NA	65.00	NA	26.00	NA	0.40	NA
MND	Early	17	45.35	3.39	28.47	2.19	16.88	1.51	0.60	0.04
	Mid	4	79.00	3.54	53.00	4.02	26.00	1.08	0.50	0.06
	Late	2	93.00	0.00	67.50	0.50	25.50	0.50	0.38	0.01
MHFD	Early	26	44.84	2.61	29.24	1.63	15.60	1.12	0.53*	0.03
	Mid	23	75.09	1.91	48.04	1.82	27.04	0.76	0.58	0.03
	Late	14	97.86	1.62	69.21	1.97	28.57	1.45	0.42	0.03
*p<0.05 only YHFD vs MHFD										

Supplementary Table 1: **Embryo analysis by quantitative immunofluorescence**. Staging criteria: Early (up to 64 cells), Mid (65-90 cells) and Late (from 91 cells). YND= Young Normal Diet, YHFD= Young High Fat Diet, MND= Mature Normal Diet, MHFD= Mature High Fat Diet. TE= trophectoderm cells, ICM= inner cell mass cells.