ABOUT THE COVER

The figure on the cover is a transmission electron microscopic image from a primordial follicle of a female Swiss mouse at birth (age less than one hour).

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Universiteit Antwerpen

Department FBD-Veterinary Physiology

The interaction of pre- and postnatal obesogenic environments in affecting daughter's metabolic health and oocyte quality: fundamental insights for sustainable advice

Dissertation submitted for the degree of Doctor in Veterinary Sciences at the University of Antwerp, to be defended by Xhonneux Inne.

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List of abbreviations

ALT = alanine amino transferase
ATP = adenosine triphosphate
AUC = area under the curve
BMI = body mass index
BMP15 = bone morphogenetic protein 15
C = control
C»C = offspring born to a control mother and fed a control diet
C»OB = offspring born to a control mother and fed an obesogenic diet
COC = cumulus oocyte complex
DAG = diacyl glycerol
DOR =diminished ovarian reserve
Drp1 = dynamin-related protein 1
ER30 = elimination rate the first 30 minutes after insulin injection
ETC = electron transport chain
F_0 = maternal generation
F_1 = first generation offspring
FFA = free fatty acids
Fis1 = fission 1
FSH = follicle stimulating hormone
FOXO = forkhead transcription factors
GDF9 = growth differentiation factor 9
GDM = gestational diabetes mellitus
GnRH = gonadotropin releasing hormone
HDL = high-density lipoproteins
HF/HS = high-fat, high-sugar
HPG = hypothalamic-pituitary gland
HSL = hormone sensitive lipase
Hsp = heat shock protein
I = interaction
IL = interleukins
IMF = intermyofibrillar
ITT = insulin tolerance test
iV = integral volume
LDC = lipid droplet content
LDL = low-density lipoproteins
LH = luteinizing hormone
LHON = Leber hereditary optic neuropathy
Mfn2 = mitofusin 2
MMP = mitochondrial inner membrane potential
mRNA = messenger RNA
mtDNA = mitochondrial DNA
NAFLD = non-alcoholic fatty liver disease
NEFAs = non-esterified fatty acids
OB = obesogenic
OB»C = offspring born to an obesogenic mother and fed a control diet
OB»OB = offspring born to an obesogenic mother and fed an obesogenic diet
OPA1 = autosomal dominant optic atrophy
OXPHOS = oxidative phosphorylation
PCOS = polycystic ovarian syndrome
PGC-1α = Peroxisome proliferator-activated receptor-gamma
PI3K = phosphatidylinositol-3 kinase
PINK1 = PTEN-induced kinase 1
PIP3 = Phosphatidylinositol (3,4,5)-trisphosphate
POI = premature ovarian insufficiency
PPAR = peroxisome proliferator)activated receptor
PTP = protein tyrosine phosphatase
ROS = reactive oxygen species
rRNA = ribosomal RNA
SAM = S-Adenosyl methionine
SIRT3 = sirtuin 3
SS = subsarcolemmal
T2DM = type 2 diabetes mellites
TEM = transmission electron microscopy
TG = triglycerides
TIM23 = mitochondrial inner membrane receptor subunit
Acetyl-CoA = acetyl-coenzyme A
TGFβ = transforming growth factor beta
TNFα = tumor necrosis factor alpha
TOM20 = mitochondrial outer membrane receptor subunit
tRNA = transcriptional RNA
VLDL = very low-density lipoproteins
WHO = World Health Organizatio

Chapter 1 Introduction

1.1 Obesity

1.1.1 Prevalence of (female) obesity in adults and children

Obesity has reached pandemic proportions globally, with over 2.8 million people dying every year as a result of being overweight or obese (WHO, 2022). Obesity is defined by the World Health Organization (WHO) as an abnormal or excessive fat accumulation in the body leading to a body mass index (BMI) of more than 30 kg/m^2, which can negatively affect the health and fertility of the individual. When the BMI is between 25 and 30 kg/m^2, this is considered as overweight, while the BMI of normal weight people is between 18.5 and 24.9 kg/m^2 (WHO, 2022). The prevalence of obesity is increasing worldwide, mediated by the modern sedentary lifestyle and the intake of high-fat and high-sugar containing diets (Hu et al., 2003; Roberto et al., 2015). Socioeconomic determinants such as income, employment status and place of residence also contribute to inequalities in the prevalence of overweight and obesity when comparing specific society cohorts (Hebebrand et al., 2017). Previously, undernutrition was a major health concern and obesity would only affect high-income countries. However, these days an increasing amount of people in both high- and low-income countries are suffering from obesity with a prevalence of over 2.5 billion adults being overweight in 2022. Of these people, 890 million were obese (equal to 1 in 8 people) (WHO, 2022). In the WHO European regions, almost 60 % of adults are overweight, of which 23 % is obese (WHO Regional Office for Europe, 2022). In Belgium, almost 50 % of the adult population is overweight, of which 16 % is obese (Sciensano, 2018).

Obesity, type 2 diabetes mellitus (T2DM, a metabolic disease where the response to insulin is diminished) and other metabolic disorders linked to an obese phenotype affect more women, having poorer prognostic outcome, major psychological and social burdens compared to men (Bentley-Lewis et al., 2007; Westerman and Kuhnt, 2022). Obesity in women is problematic because it not only impacts their own health and fertility, but also increases the risk of metabolic disorders and obesity in the next generation (Eriksson et al., 2014; Contreras et al., 2016). In Western countries it was noticed that up to 30 % of woman suffered from overweight or obesity during gestation, implying a major risk for the ongoing pregnancy and for the health of their offspring (Gaillard, 2015; Boutari and Mantzoros, 2022). The Helsinki Birth Cohort (where 13.345 people were included) associated an increased maternal BMI with an increased risk of diseases in adult offspring, where cardiovascular diseases and T2DM were some of them (Eriksson et al., 2014). On top, it has been shown that children often copy the lifestyle of their parents (Pfledderer et al., 2021). Therefore, children raised in a sedentary environment where unhealthy diets high in fat and/or high in sugar are common, are more likely to follow the same unbalanced diets, having an increased risk of suffering from the same metabolic conditions as their parents. This makes it sometimes difficult for researchers to dissect prenatal from postnatal origins of metabolic diseases. Recent data show that the prevalence of overweight and obesity among children and adolescents has risen dramatically from just 4 % in 1975 to over 18 % in 2016 (WHO, 2016). Worldwide, about 390 million children and adolescents aged between 5 and 19 years were overweight, of which 160 million were obese. Up to 37 million children under the age of 5 were overweight in 2022 (WHO, 2022). In Europe, 30 % of the children aged 5 to 9 years old are living with overweight of which 12 % are obese, and even 8 % of the children under 5 years of age were overweight or obese in 2020 (WHO Regional Office for Europe, 2022). In Belgium, 19 % of children aged 2-17 years were overweight and almost 6 % were obese in 2018 (Sciensano, 2018). These numbers are alarming, because childhood obesity not only affects the children's current health, but has also implications later in life since children with obesity at young age have an increased risk of developing

metabolic and cardiovascular diseases at adulthood, among other disorders (Godfrey and Barker, 2000; Eriksson et al., 2014; Contreras et al., 2016; Stacy et al., 2019).

1.1.2 Obesogenic diet consumption and metabolic stress

1.1.2.1 *What is an obesogenic diet?*

An obesogenic (OB) diet is an unbalanced diet that promotes excessive weight gain and obesity. It typically consists of foods high in calories, such as fats and sugars. This type of diet often leads to an imbalance between caloric intake and energy expenditure, contributing to the development of obesity and related health issues (Siino et al., 2021; Smits et al., 2021a). Typical examples of OB diets are highly processed foods, fast foods, ready-to-eat meals and sugar-rich beverages and snacks, with high fat and sugar content and low overall nutritional quality (Jensen et al., 2014).

OB diets often contain saturated fatty acids such as stearic (SA, C18:0) and palmitic acid (PA, C16:0). These fatty acids contain only single bonds between carbon atoms and are associated with the development of obesity, cardiovascular diseases and T2DM (Agraib et al., 2023). The WHO and the Dietary Reference Intakes (DRI) recommend an overall human total daily dietary fat intake between 20 % and 35 % of the total daily calories. A minimum of 20 % is advised to ensure sufficient intake of total energy, essential fatty acids, and fat-soluble vitamins. The upper limit of 35 % is recommended to limit saturated fat intake and to avoid excessive weight gain (Liu et al., 2017). The WHO also suggests that no more than 10 % of our daily energy intake consists of free sugars (WHO, 2022). As explained below, OB diet consumption causes a shift in the systemic blood lipid fraction, leading to dyslipidemia with an increase in non-esterified fatty acids (NEFAs) or free fatty acids (FFAs), and hypercholesterolemia associated with adverse systemic conditions and significant health problems (Klop et al., 2013). Harmful effects of OB diets occur prior to the development of the OB phenotype. Therefore, the consumption of OB diets as such is sufficient to induce pathology (Williams and Goulding, 2009; Moorkens et al., 2022; Moorkens et al., 2023; Ojeda et al., 2023).

Studies investigating the effect of OB diet consumption on the individual's health and/or fertility often use mouse models. Panchal and Brown (2011) studied the different diets needed to mimic metabolic syndrome in rodent models. They concluded that a high carbohydrate-high fat diet mimics the human diet the most closely, as rodents fed this diet develop similar symptoms present in human metabolic syndrome (Panchal and Brown, 2011). Mostly, a 45 % dietary fat intake is used to induce obesity in mice and when the fat content is further increased, obesity occurs more rapidly (Speakman, 2019). A daily sugar intake of 20 % in rodents can be seen as a high sugar intake that closely resembles 2-3 soda cans a day in humans (Ruff et al., 2013). The most common carbohydrates added to human OB diets are sucrose and fructose (Panchal and Brown, 2011; Wong et al., 2016), with mainly high fructose related to the development of metabolic disorders (Aydin et al., 2014; Pereira et al., 2017). In previous research performed at our lab, we indeed confirmed that feeding outbred Swiss mice a diet with 60 % of fat and 20 % of fructose for 7 weeks resulted in a significantly reduced sensitivity to insulin, and a reduced oocyte quality (Smits et al. 2022).

1.1.2.2 *Dyslipidemia and lipotoxicity*

Triglycerides (TGs) are the primary storage form of fat in the body, serving as an important energy source when needed (Karpe et al., 2011). After ingestion, dietary TGs are mixed with bile salts and emulsified in the duodenal lumen, then further hydrolyzed by pancreatic enzymes into free fatty acids (FFAs) and monoglycerides in preparation for absorption across the intestinal wall (Karpe et al., 2011). Consequently, FFAs are taken up by enterocytes and packed into chylomicrons along with cholesterol-esters, the latter defined as dietary cholesterol absorbed and converted into cholesterol-esters within enterocytes (Klop et

al., 2013; Hussain, 2014). In the systemic circulation, chylomicrons engage with the lipoprotein lipase (LPL) bound to the luminal surface of vascular endothelial cells in different tissues, to initiate intracellular lipolysis, a process controlled by insulin. After the engagement of chylomicrons with LPL, they are digested into FFA and chylomicron remnants. FFAs released from chylomicrons are used as energy source, or taken up by adipocytes and re-esterified into TG for immediate storage. The chylomicron remnants are transported to the liver undergoing breakdown into FFAs, cholesterol, glycerol, and amino acids (Klop et al., 2013), where very-low-density lipoproteins (VLDLs) are produced through a multi-step process involving the packaging of hepatic fats into transport vesicles. This process is essential for the export of endogenous lipids from the liver and their distribution to peripheral tissues for energy utilization or storage (Chen et al., 2024). VLDL facilitate fatty acid supply during fasting to muscles, and chylomicrons primarily provide fatty acids to adipose tissue (Klop et al., 2013; Hussain, 2014). Hormone-sensitive lipase (HSL) is active in various tissues, including adipose tissue, and provides FFA to the circulation after lipolysis of TGs when the necessity arises to mobilize the stored fat in adipose tissue. HSL is activated by extracellular hormones such as glucagon and is inhibited by insulin. The released FFA are insoluble in water and bind to albumin in the bloodstream to be used in peripheral tissue as a source of energy during fasting and inter prandial periods (Chen et al., 2024).

Adipocytes store energy as fat (TGs) (Snider and Wood, 2019). The number of adipocytes is established early in life and remains more or less stable throughout adulthood. Notably, childhood obesity is characterized by an increased number of adipocytes, significantly elevating the risk for the development of obesity in later life (Horwitz and Birk, 2023). Increased nutrient intake and increased systemic lipid circulation without adequate energy expenditure eventually results in adipose hypertrophy, increasing the volume of pre-existing adipocytes, increasing the risk of metabolic diseases (Horwitz and Birk, 2023). Adipocyte hypertrophy will first occur in the subcutaneous fat depots, then in visceral fat depots and eventually also in non-adipose tissue when the obesogenic state continues (Horwitz and Birk, 2023). Adipocyte hypertrophy is associated with a reduced sensitivity to insulin. Hypertrophic adipocytes are less responsive to anti-lipolytic insulin compared to normal-sized adipocytes, leading to further increased systemic FFA in obese patients due to increased HSL activity and upregulated lipolysis (Yang et al., 2012). Consequently, consumption of OB diets causes a shift in the systemic blood lipid fraction, also referred to as dyslipidemia (Klop et al., 2013), and manifests as an elevated cholesterol, TG and FFA concentration in the blood system (Zakai et al., 2022). This results in alleviated lipotoxic FFA provision in peripheral tissue leading to an uptake of FFA also in non-adipose tissue. The uptake of FFAs by non-adipose tissues, negatively affecting their function is called lipotoxicity (Lewis et al., 1993).

As adipocytes enlarge, they enter a state of hypoxia when adipose tissue outgrows its blood supply, accompanied by an increased cellular stress and production of pro-inflammatory adipokines (Snider and Wood, 2019). Pro-inflammatory pathways are activated and infiltration of white blood cells in the fat tissue occurs, attracting immune cells, mainly macrophages, to the adipose tissue. This inflammation, originating in the adipose tissue, will eventually reflect in the blood and the entire body, resulting in a chronic state of low-grade systemic inflammation, associated with increased pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. Pro-inflammatory cytokines interfere with insulin signaling pathways, further increasing the resistance to insulin (Ellulu et al., 2017).

The increased FFAs in the blood of obese patients can either be oxidized (and used as energy source) or re-esterified and stored within cells as (ectopic) lipid droplets (Arner, 2005) when the FFA supply exceeds the metabolic needs. Cytosolic acyl-coA synthetase converts fatty acids to acyl-CoA to be transported into the mitochondrial matrix via carnitine palmityl transferases 1 and 2 (CPT1-CPT2 shuttle). Consequently, acyl-CoA undergoes β-oxidation, a series of reactions that cleave two-carbon units from the fatty acid chain (Wan et al., 2020), thereby producing acetyl-CoA. Acetyl-CoA is metabolized through the tricarboxylic acid (TCA) cycle (Longo et al., 2016), supplying electron carriers such as NADH and $FADH_2$, of which the electrons are transported over the inner mitochondrial membrane to produce ATP. This process

is called the oxidative phosphorylation or OXPHOS (Tang et al., 2020). However, when the acetyl-CoA production from the increasing FFA provision surpasses the capacity of the TCA to metabolize acetyl-CoA, a cascade of events occur, involving an inhibition of the acyl-CoA transport in the mitochondria that will lead to cytoplasmic acyl-CoA accumulation. The excess of cytosolic acyl-CoA is first esterified into lipids such as TG, stored in intracellular lipid droplets. However, eventually, also this conversion will exceed its capacity, leading to an accumulation of FFA in the cytosol (He et al., 2012; Lipke et al., 2022).

The surplus of cytosolic FFAs are eventually metabolized into toxic substrates such as diacyl-glycerols (DAGs) and ceramides (Sergi et al., 2019), associated with increased cellular stress, mitochondrial dysfunction and impairing insulin signaling and glucose uptake (Bergman and Ader, 2000). DAGs, important for proper function of cell membranes and involved in cell signaling pathways, are synthesized from glycerol-3-phosphate and FFAs through enzymes such as glycerol-3-phosphate acyltransferase that are overexpressed in obese patients, particularly in muscle and liver tissues (Szendrödi et al., 2011). Ceramide synthases also incorporates different acyl-CoAs as it is composed of sphingosine and a fatty acid joined by an amide bond (Siskind et al., 2005). Ceramids play a role in cell differentiation, cell signaling and apoptosis (Vanlerberghe, 1996), but elevated ceramide levels in tissues are associated with impaired insulin signaling by inhibiting the activity of phosphatidylinositol-3 kinase (PI3K) and disrupting glucose metabolism, further exacerbating insulin resistance in obese patients (Sokolowska and Blachnio-Zabielska, 2019).

A simplified overview of the lipotoxicity mechanism is presented in Figure 1.

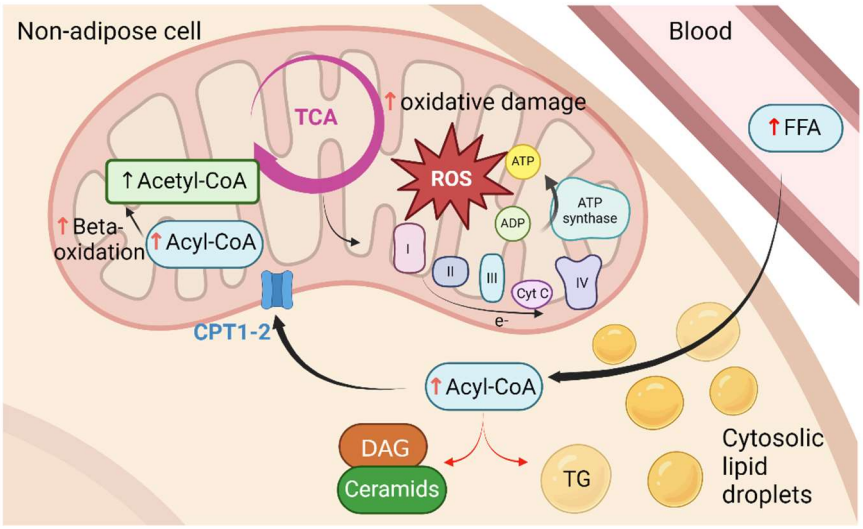

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Figure 1. Simplified overview of cellular lipotoxicity. Increased free fatty acids (FFA) in the blood will lead to increased β-oxidation and increased oxidative stress and damage, eventually resulting in an accumulation of cytosolic Acyl-CoA. The increased cytosolic FFA will be stored as triglycerides (TG) in lipid droplets and lipotoxic metabolites such diacyl-glycerols (DAGs) and ceramides.

1.1.2.3 *Mitochondrial functions and the impact of dyslipidemia*

Mitochondria produce ATP as cellular energy substrate and are, amongst others, also needed for calcium activation, signal transduction and stress responses (Heuer, 2021). Mitochondria have circular double stranded DNA, containing 37 genes with 22 tRNAs (transcriptional RNA needed for the translation of messenger RNA (mRNA) to proteins) and 2 rRNAs (ribosomal RNA, important for protein synthesis), of which 13 genes encode for the electron transport chain (ETC, where electrons are transported over the inner mitochondrial membrane to produce energy). Of the 1500 proteins needed for mitochondrial function, only 13 are encoded within the mitochondrial DNA (mtDNA) (Van Blerkom, 2008a; Heuer, 2021). Therefore, effective communication and coordination between the nucleus and the mitochondria are imperative for proper mitochondrial function (Jastroch et al., 2010; Kim et al., 2019a; Kim et al., 2019b; Tang et al., 2020).

Mitochondria have an outer and inner membrane with an intermembrane space, surrounding a mitochondrial matrix (Heuer, 2021). The mitochondrial inner membrane is increased in size due to several protrusions, the cristae, where OXPHOS takes place. The end product of glycolysis and β-oxidation is acetyl-CoA that enters the TCA cycle, supplying NADH or $FADH_2$ to be oxidized to NAD+ or FAD through OXPHOS (Fernandez-Vizarra et al., 2022). During OXPHOS, ATP is produced after transporting electrons over 4 different complexes (I-IV) of the mitochondrial electron transport chain (ETC). Meanwhile, protons are pumped into the intermembrane space (mainly at complex I, III and IV) to generate a proton gradient, causing force driven generation of ATP by ATP synthase in the fifth complex (V) (Bergman and Ben-Shachar, 2016). An overview of the mitochondrial ETC and corresponding ATP production is shown in Figure 2.

During this transport of electrons across the mitochondrial inner membrane, reactive oxygen species (ROS) are also generated, mainly at complex I and III (Bergman and Ben-Shachar, 2016). As electrons pass through the ETC a small fraction of electrons escapes, resulting in ROS. ROS are often oxygen-derived molecules containing a free radical or an unpaired electron and may act as aggressive free radicals, capable of harming other tissues when exceeding a certain threshold. However, importantly, at physiological concentrations, ROS also have important functions serving as a second messenger system (Pi et al., 2007) enhancing for example insulin sensitivity (Loh et al., 2009a; Cheng et al., 2010; Barquissau et al., 2017b). In addition, ROS as such are crucial for optimal immune function and steroidogenesis (Hussain et al., 2021). They also play a role in many processes in fertility such as oocyte maturation by acting as signaling molecules regulating meiosis, and in luteolysis by promoting apoptosis in luteal cells for corpus luteum breakdown (Agarwal et al., 2005). ROS is also involved in the process of embryonic implantation by for example regulating endometrial remodeling and vascularization (Chen et al. 2018; Gupta et al., 2008). In addition, ROS controls cellular differentiation, proliferation (Gupta et al., 2008), and signaling pathways needed for organ development during embryogenesis (Hussain et al., 2021). In the context of ROS, a proper balance between oxidants and anti-oxidants, molecules that neutralize unpaired electrons, is required. The amount or concentration of ROS will play a role in the balance between physiological and pathophysiological circumstances (Pi et al., 2007).

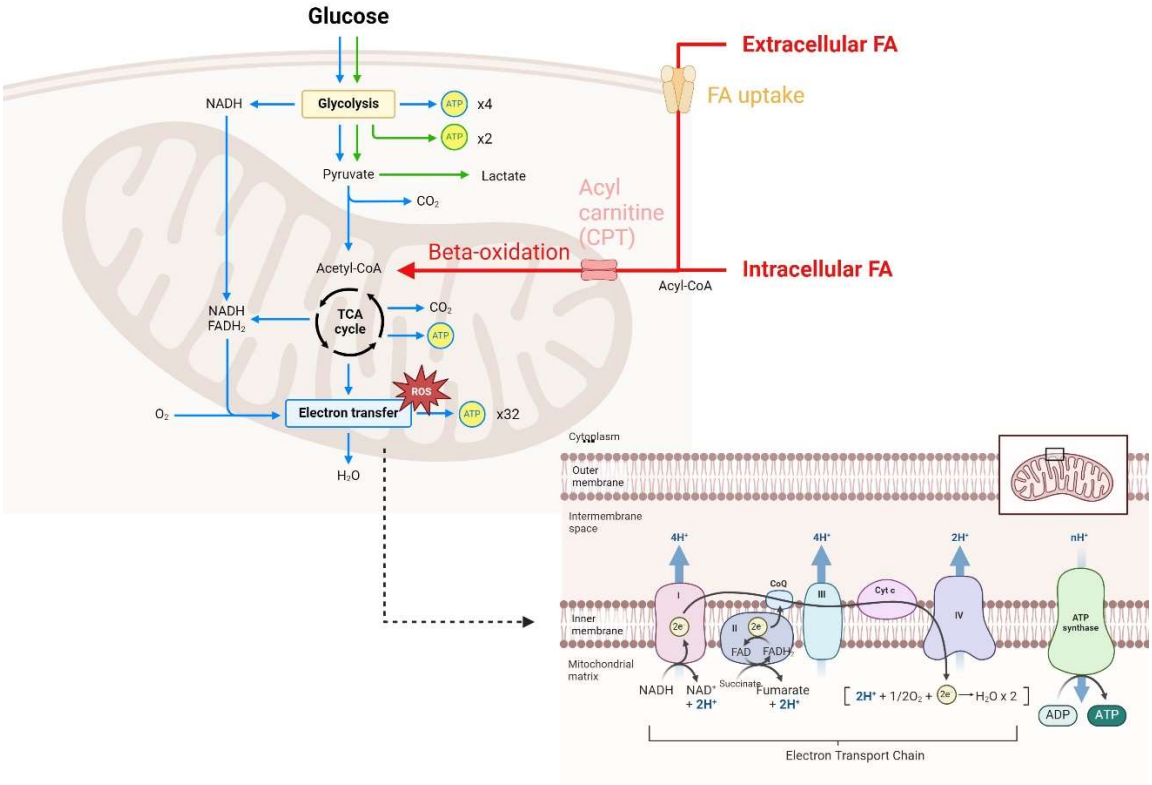

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Figure 2: Mitochondrial ATP production in a somatic cell. A schematic overview of the main energy production pathways in the mitochondria. Blue lines represent the aerobic respiration. Green lines represent the anaerobic respiration in absence of oxygen. Red lines represent the oxidation of fatty acids. All lines display energy substrate delivery to the mitochondria, producing energy in the form of ATP.

Mitochondrial β-oxidation depends on several factors including the nutritional status. In case of dyslipidemia and increased FFAs due to obesity, the mitochondrial β-oxidation will increase, leading to an elevated accumulation of ROS and high oxidative stress (Serra et al., 2013). An overload of such ROS accumulation can oxidize lipids present in the mitochondrial membrane, forming lipid peroxides that harm, amongst others, the mitochondrial membranes (Ademowo et al., 2017). Inner mitochondrial membrane uncoupling proteins are activated and cause a reduction in the proton gradient on the mitochondrial inner membrane by facilitating the proton flow from the mitochondrial inner membrane space to the mitochondrial matrix (Cater and Bombek, 2022). The reduced membrane potential triggers the activation of mitochondrial unfolded protein responses (mtUPR), the first molecular line of stress defense (Shpilka and Haynes, 2018). Next, the transcription factor associated with stress-1 (ATFS-1) is activated and translocates to the nucleus, stimulating gene expression of chaperone proteins such as Hsp70 (heat-shock protein 70) (Weng et al., 2020). Chaperones ensure protein folding, prevent protein aggregation and facilitate damaged protein degradation (Jovaisaite et al., 2014). At low levels of stress, the mtUPR restores cellular homeostasis. However, higher stress levels stimulate mitochondrial dynamics as a second organellar line of defense against increasing stress, enabling maintenance of optimal mitochondrial function (Seli et al., 2019).

Mitochondrial dynamics are well described in somatic cells, and involve a balance between fission and fusion (Scott and Youle, 2010). Mitochondrial fission is orchestrated by proteins such as Dynamin-Related Protein 1 (Drp1) and Fission 1 (Fis1), and divides single mitochondria into two, allowing isolation of impaired mitochondrial segments, thereby maintaining bioenergetic capacity (Chiaratti et al., 2018). Dysregulation of mitochondrial fission, leading to mitochondrial fragmentation, is implicated in the

pathogenesis of several metabolic diseases, including obesity, diabetes, and cardiovascular disease by reducing cellular respiration and growth (Yoon et al., 2011). Fusion merges two mitochondria into one, enabling one mitochondrion to compensate for functional deficiencies in others by exchanging mitochondrial matrix components such as RNA or proteins, thereby maintaining a healthy and functional mitochondrial network (Scott and Youle, 2010). Fusion also allows distribution of metabolites throughout the entire mitochondrial compartment to increase the bioenergetic efficiency by homogenously dividing the fatty acids within lipid droplets, and is coordinated by proteins such as Optic Atrophy 1 (OPA1) and Mitofusin 2 (Mfn2) (Rambold et al., 2015). In addition, mitophagy, controlled by for example Serine/threonine PTEN-induced putative kinase 1 (PINK1) and Parkin, selectively removes damaged mitochondria by autophagy and lysosomal degradation (Lenaers et al., 2012; Yan et al., 2013; Straub et al., 2016; Widlansky and Hill, 2018; Scaini et al., 2022). Altogether, mitochondrial dynamics are crucial for optimal mitochondrial function, adapting to changing bioenergetic conditions and eliminating damaged mitochondria. These processes are characterized by ultrastructural changes in the shape of mitochondria (Scott and Youle, 2010).

When stress levels further increase, the necessity for cellular elimination arises (Ashrafi and Schwarz, 2013). For stress levels that cannot be tolerated, mitochondria will initiate cell apoptotic pathways via cytochrome-C, activating caspases, and via apoptosis inducing factors (AIF). (Lenaers et al., 2012; Yan et al., 2013; Straub et al., 2016; Widlansky and Hill, 2018; Scaini et al., 2022). An overview of the different defense mechanisms is shown in Figure 3.

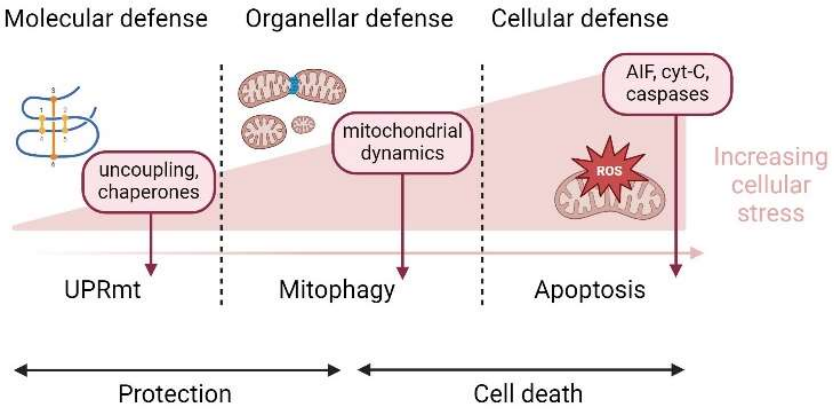

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Figure 3. Different levels of defense against cellular stress, mediated by the mitochondrial unfolded protein response, mitochondrial dynamics and mitophagy, leading to apoptosis.

1.1.2.4 *Reduced insulin responsiveness*

Insulin is a pancreatic hormone with a crucial role in the regulation and homeostasis of systemic blood glucose. Insulin facilitates the cellular glucose uptake particularly in myocytes and adipocytes, as it stimulates glucose transporter type 4 (GLUT4) translocation to the cell membrane, thereby increasing the glucose uptake from the bloodstream into cells (Cignarelli et al., 2019). Insulin also promotes glycogen and fat storage and suppresses gluconeogenesis, the production of glucose from non-carbohydrate sources in the liver (Divya et al., 2015). Insulin enhances glycogen synthesis, while glucagon is a pancreatic hormone that stimulates glycogen breakdown (Cheng et al., 2010). Glycogen, stored in the liver, provides glucose to the bloodstream in case of hypoglycemia, while glycogen in skeletal muscles serves as a direct energy source for muscle cells during contraction (Figure 4) (Adeva-Andany et al., 2016).

When blood glucose increases, insulin is released in the bloodstream and binds to insulin receptors (IRS), thereby activating the PI3K/Akt pathway. Phosphoinositide 3-kinase (PI3K) converts phosphatidylinositol-4,5-bisphosphate (PIP2) in the cell membrane to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits and activates protein kinase B (Akt) (Shaw, 2011). In addition, Akt activates the mammalian target of rapamycin (mTOR) pathway, for protein synthesis and cell growth (Fingar et al., 2004) and inhibits the expression of gluconeogenic genes in the liver (Divya et al., 2015). Forkhead transcription factors, e.g. FOXO1, important for mitochondrial genome regulation and function, are phosphorylated by Akt as well, implying a close interaction between insulin signaling and mitochondrial function in different organs and tissues encompassing liver, skeletal muscle, adipose tissue and brain (Jerome et al., 2022).

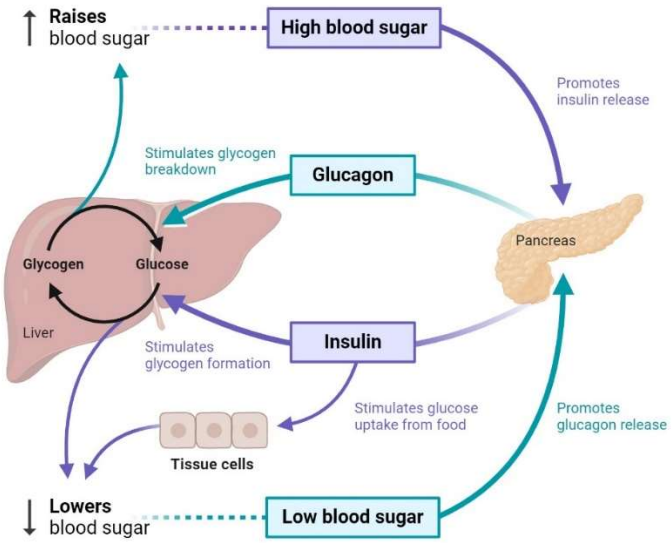

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Figure 4. Regulation of the blood glucose. Green lines represent a low blood glucose level where glucagon synthesis stimulates glucose release into the bloodstream. The purple lines represent a high blood glucose level where insulin is responsible for glycogen synthesis.

Reduced insulin sensitivity or insulin resistance, is a condition where the body's cells become less responsive to insulin, resulting in hyperglycemia, both in fasting and postprandial states (Sergi et al., 2019). IR is a key feature of T2DM, obesity and metabolic syndrome (Rahman et al., 2021). A crucial factor in modulating insulin sensitivity is the increased adiposity (particularly visceral fat) and dyslipidemia, along with the associated inflammatory responses and increased circulating proinflammatory cytokines, oxidative stress and toxic substrates such DAGs and ceramides (Kahn and Flier, 2000). However, ROS, more specifically H_2O_2 originated from the ETC, can also enhance insulin signaling via second messenger systems (Loh et al., 2009b). Protein tyrosine phosphatases (PTPs, e.g. PTPB1) can dephosphorylate PIP3 to terminate the pathway activated by insulin (Loh et al., 2009b). However, a certain threshold exists in the balance between the insulin sensitivity enhancing effects and the insulin sensitivity suppressive effects of ROS. While ROS generated by physiological stimuli like insulin can be beneficial, high levels of ROS accumulation linked to hyperglycemia and/or hyperlipidemia in obesity and diabetes may contribute to insulin resistance (Loh et al., 2009b; Barquissau et al., 2017a).

Striated muscle mitochondrial dysfunction and insulin resistance are closely related (Mu et al., 2019) (Figure 5). Since skeletal muscles account for 60-70 % of the insulin-stimulated glucose uptake, they are described as the primary determinant of metabolic disorders (Turpin et al., 2009; Montgomery et al.,

2017; Mikovic and Lamon, 2018). The ceramide and DAG build-up in skeletal muscles impairs the insulin signaling pathway by phosphorylating insulin receptor substrate 1 (IRS-1) leading to a reduced insulin sensitivity (Sergi et al., 2019). In addition, ceramides inhibit Akt, diminishing the downstream effects of the normal insulin response. Also, the expression of Peroxisome Proliferator-activated Receptor-γ Coactivator (PGC-1α), a key player in mitochondrial biogenesis is decreased in obese patients due to the ceramid-induced downregulation of SIRT3 (Cheng et al., 2010; Sergi et al., 2019), and the mitochondrial respirasome formation in skeletal muscle (supercomplex; I, III and IV combined) that allows myocytes to autonomously respire in absence of ETC electron carriers such as ubiquinone and cytochrome C (Sergi et al., 2019), is decreased in patients with T2DM. The oxidative capacity of muscle cells or the ability to produce energy through aerobic metabolism, determined by the ETC complex expression and the number and size of mitochondria (Conley et al., 2000), is also decreased in patients with T2DM, in association with hypermethylation of muscle mitochondrial proteins. In addition, mitochondrial dynamics increase in response to long chain FFAs that are present in most OB diets (Sergi et al., 2019). Patients suffering from insulin resistance show reduced oxidative phosphorylation and downregulated genes involved in oxidative metabolism and mitochondrial respiration in skeletal muscle tissue (Sergi et al., 2019). Such genes involved in oxidative metabolism and mitochondrial respiration are even downregulated in the skeletal muscle tissue of patients having a family history of T2DM (Mootha et al., 2003; Patti et al., 2003), **showing the importance of the parental metabolic background in the offspring's disease susceptibility**.

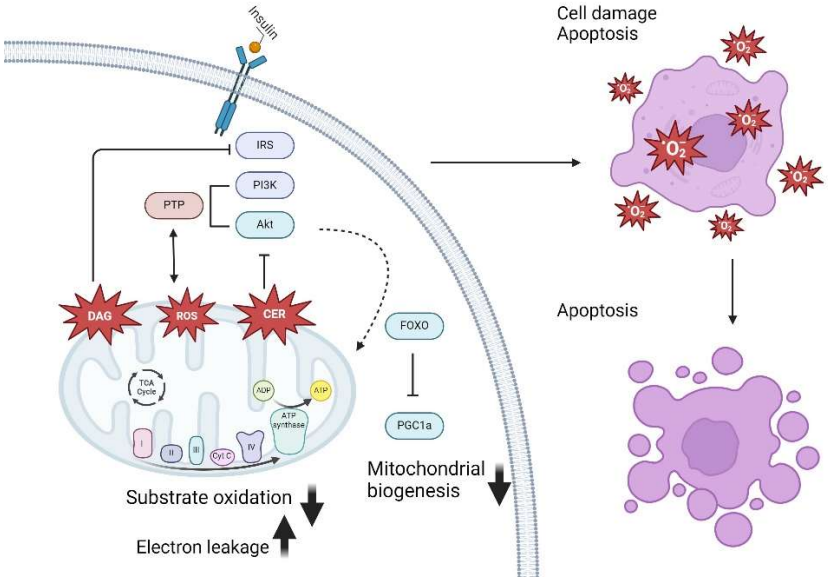

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Figure 5: Simplified overview of the link between reduced insulin sensitivity and mitochondrial dysfunction in striated muscle.

1.1.3 Obesity and the impact on female fertility: the story of the oocyte

Fertility encompasses precisely regulated endocrine, cellular, and molecular processes that ideally culminate in the birth of a healthy offspring. Maternal metabolic disorders are acknowledged for their impact not only on health, but also on reproductive physiology, ultimately leading to compromised fertility. Subfertility or a reduced fertility indicates the inability to naturally conceive a clinical pregnancy within the first 6 menstrual cycles with intercourse in the fertile peri-ovulatory phase, while infertility is defined as the inability to conceive within one year (Gnoth et al., 2005). Obesity coincides with subfertility, resulting in up to three times higher rates of infertility in obese women compared to those with normal weight (Grodstein et al., 1994; Richedwards et al., 1994; Pfeifer et al., 2015). Epidemiological studies

reveal a 5 % reduction in the chance of spontaneous conception in ovulatory women with each increased BMI unit (van der Steeg et al., 2008).

Subfertility is a multifactorial problem due to the complex multivarious interplay of biological, environmental and lifestyle factors (Emokpae and Brown, 2021). Although obesity causes hormonal dysregulation and coincides with alterations in the ovary, the oviduct and the uterus, complicating isolation of the most critical factor (Bollig and Dolinko, 2024), a reduced oocyte quality plays a crucial role in the pathogenesis of diet-induced subfertility (Leroy et al., 2022). Clinical studies show that embryo transfer from healthy and normal weight oocyte donors restored pregnancy success rates in obese women, clearly highlighting and confirming the importance of reduced oocyte quality in the pathogenesis of subfertility in metabolically compromised mothers (Luke et al., 2011). Furthermore, the disappointing assisted reproduction technology (ART) outcome as clinically reported in overweight and obese women, underlines the specific importance of reduced oocyte quality in the pathogenesis of subfertility (Pandey et al. 2010).

1.1.3.1 *Oocyte quality*

Oocyte quality refers to the ability of an oocyte to support proper fertilization, embryonic development, and ultimately, a healthy pregnancy (Coticchio et al., 2004; Leroy et al., 2022). The ability of a mature oocyte to be fertilized and sustain the initial phases of embryonic development until the blastocyst stage is referred to as the oocyte developmental capacity, and it is a critical factor in determining the success of IVF (Innocenti et al., 2022). Acquisition of oocyte developmental competence is a cumulative process that takes place in the ovarian follicle during oocyte growth and maturation (Coticchio et al., 2004). Therefore, the mechanisms by which obesity can impact oocyte quality can be initiated either during the final stages of maturation in the preovulatory follicle, or earlier, during preantral folliculogenesis.

1.1.3.1.1 **Folliculogenesis and acquisition of oocyte developmental competence**

During early embryonic development in mammals, primordial germ cells migrate towards the gonadal ridges in the embryo and multiply during their migration (Paulini et al., 2014; Findlay et al., 2015). Upon arrival in the gonadal ridges, they become mitotically active and are called oogonia. Later on, the mitotic divisions cease and the oogonia enter into meiosis I, becoming primary (immature) oocytes (Paulini et al., 2014; Findlay et al., 2015). From shortly before birth until after puberty, primary oocytes are arrested at the diplotene stage of the first meiotic division. The chromosomes are encapsulated in the nucleus, also called the germinal vesicle (GV). These primary oocytes are surrounded by a single layer of flattened (pre-)granulosa cells and form a primordial follicle, with a basal lamina deposited on the surface of the granulosa cells, isolating these newly formed primordial follicles from the stroma (Aerts and Bols, 2010b). In humans, primordial follicles are formed in the fetal ovaries between the sixth and ninth month of gestation, a process that is completed at birth (Fortune et al., 2010). In mice, this process continues until a few days after birth (Wear et al., 2016). The enclosed oocytes within the primordial follicles form a limited stock, also referred to as the dormant primordial follicle pool, and remain in a resting phase until puberty (Aerts and Bols, 2010b).

From sexual maturity onwards, primordial follicles are continuously recruited in cohorts to initiate folliculogenesis, a process mediated by follicle stimulation hormone (FSH) that takes around three to six months in humans but only three weeks in mice (Lussier et al., 1987; Gougeon, 2010; Clarke, 2017). This follicle recruitment leads to major follicle differentiation with some resulting in an ovulated oocyte, while other follicles degrade at various developmental stages before reaching the pre-ovulatory phase (Scaramuzzi et al., 2011).

While primordial follicles are generally considered to be dormant or in a resting state, the oocytes within are, albeit limited, transcriptionally active and regulate their own activation (McLaughlin and McIver, 2009; Ernst et al., 2017). Consequently, they are not immune to the effects of various stressors, which can influence their survival and potential activation. An active bi-directional relationship exists between the oocyte and the granulosa cells, and the oocyte expresses growth factors such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), that are picked-up by granulosa cells via transforming growth factor β (TGFβ) superfamily receptors for inducing follicle activation (Scaramuzzi et al., 2011). In addition, follicle activation is mediated by the PI3K-AKT-FOXO3 pathway within the oocyte (John et al., 2008). The crucial role of FOXO3, a transcription factor for mitochondrial biogenesis and mitochondrial dynamics (Cheng, 2022), implies that mitochondria are also involved in the process of follicle activation (John et al., 2008). During oogenesis, the number of mitochondria within the ooplasm gradually increases (Van Blerkom, 2008a). After primordial follicle activation the oocyte enlarges, a process accompanied by a further increase in numbers of mitochondria, smooth endoplasmic reticulum, ribosomes and lipid droplets (Nagamatsu et al., 2019).

After primordial follicle activation, the granulosa cells surrounding this young oocyte transform from flattened to cuboidal in shape. Together with the primary oocyte, they are now called primary follicles (Aerts and Bols, 2010b). Next, a layer of glycoproteins, the zona pellucida, is deposited around the oocyte (Aerts and Bols, 2010b). The number of granulosa cells further increases and when over two layers of granulosa cells are present, the follicle becomes a secondary follicle (Edson et al., 2009). The transcriptional activity of the oocyte increases from the secondary stage onwards, thereby synthesizing and accumulating RNA, ribosomes and proteins that are vital to resume and complete maturation, support fertilization and initiate embryo development (Fair, 2003).

The follicle is devoid of any vascular supply, which necessitates the intercellular contact of the oocyte with neighboring granulosa cells via gap junctions. The presence of these gap junctions allows a bidirectional communication between the oocyte and granulosa cells and among granulosa cells. Gap junctions facilitate the transfer of ions, amino acids, glucose metabolites and nucleotides to the oocyte, necessary for growth and maturation (Ackert et al., 2001; Oktem and Oktay, 2008; Marchais et al., 2022). Transition to the tertiary follicle is characterized by the continued proliferation and differentiation of granulosa cells into the granulosa cells that line the follicle wall having a steroidogenic role, and the cumulus cells that form an intimate life-support association with the oocyte as the cumulus-oocyte-complex (COC) (Scaramuzzi et al., 2011). Cumulus cells become essential for the oocyte's required energy provision, as oocytes lack phosphofructokinase activity to convert glucose to pyruvate and lactate (Warzych and Lipinska, 2020). After the granulosa cell differentiation, several fluid-filled patches appear in the granulosa cells that coalesce into a single cavity, the antrum. This process is driven by FSH (Smitz and Cortvrindt, 2002). Together with antrum formation, nuclear maturation occurs with the resumption of meiosis, comprising the disintegration of the nucleus membrane thereby releasing the nuclear contents into the cytoplasm (germinal vesicle breakdown (GVBD)). During GVBD, mitochondria accumulate close to the perinuclear region while after GVBD, mitochondria distribute throughout the ooplasm (Durinzi et al., 1995).

Subsequently, luteinizing hormone (LH) peaks trigger final oocyte maturation in the dominant follicle (Drummond and Findlay, 1999; Findlay and Drummond, 1999) and an increase in lipid content, a reduction in the Golgi compartment and relocation of cortical granules to the peri cortical region of the oocyte occur in the cytoplasm (Paulini et al., 2014; Vogt et al., 2019). During meiosis I, mitochondria localize again near the spindle (Van Blerkom, 2011), and prior to ovulation, the oocyte progresses through the final stages of meiosis I and arrests at metaphase II with the extrusion of the first polar body (Fair, 2003; Aerts and Bols, 2010a). Here, the nucleus of the now called secondary oocyte is reorganized to an inactive, transcriptionally quiescent state, referred to as pre-maturation or capacitation of the oocyte, preparing

the oocyte for fertilization and subsequent embryonic development (Hyttel et al., 1997; Dieleman et al., 2002).

1.1.3.1.2 Role of Mitochondria in oocytes and embryos

As the most abundant organelles in the mature oocyte and early embryo, mitochondria are of capital importance to guarantee the oocyte's quality and developmental competence, acting as the main energy source (Van Blerkom, 2004). The amount of mitochondria, estimated by the mtDNA copy number, increases enormously during oocyte development (Figure 6). Primordial germ cells hold approximately 200 copies of mtDNA, whereas a fully grown murine oocyte contains over 100 000 copies in mice, and even more in other species (Van Blerkom, 2008a; Kirillova et al., 2021b). Oocyte mitochondria are reported to be immature and are, compared to somatic cells, in a state of low oxidative activity. This low oxidative activity is suggested to be maintained by suppressing the activity of complex I (Rodríguez-Nuevo et al., 2022), thereby reassuring the oocyte's lifespan.

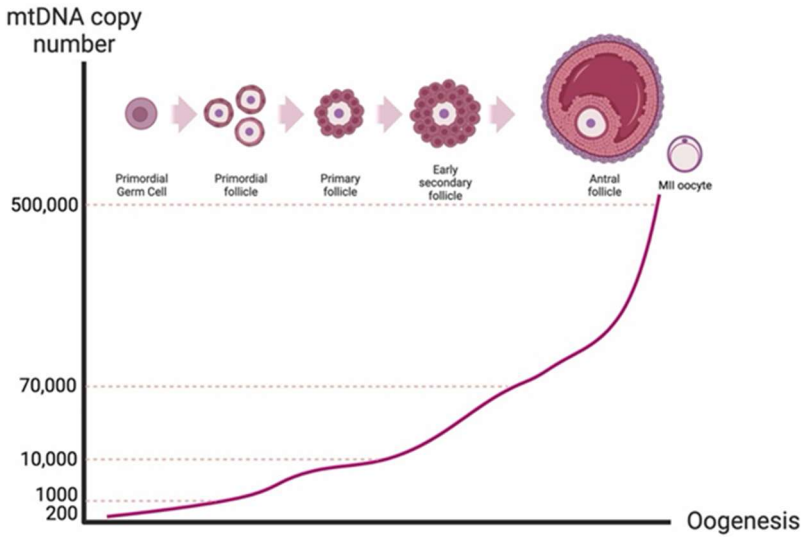

Figure 6. mtDNA replication during folliculogenesis and oogenesis. Primordial germ cells hold around 200 copies of mtDNA, whereas a fully mature oocyte can reach hundreds of thousands mtDNA molecules, depending on the species (Yildirim and Seli, 2024).

Mitochondria are important for calcium signaling. Oscillations in mitochondrial membrane potential are associated with cellular calcium release or calcium uptake in response to cytoplasmic and extracellular signals (Harris, 1979; Loew et al., 1994). For example, the penetration of the spermatozoa during fertilization and the following mitochondrial regulation of polyspermy prevention is calcium dependent (Van Blerkom and Davis, 2007; Van Blerkom, 2008b).

As mentioned earlier, mitochondria are pivotal for meiotic resumption, shown by a major increase in ATP production during polar body expulsion (Kirillova et al., 2021a). The distribution patterns of mitochondria throughout the ooplasm fluctuate in response to cellular and metabolic needs. For example, mitochondria translocate to the peri-nuclear region during spindle-formation (Madan et al., 2022). In human studies, mature oocytes lacking a proper meiotic spindle had significantly lower mtDNA copy number, associated with significantly reduced fertilization rates (Van Blerkom, 2008a), and spindle-lacking oocytes coincide with abnormal preimplantation embryonic development (Zeng et al., 2007; Van Blerkom, 2008b). Consequently, mitochondrial dysfunction is associated with an increase in aneuploidy, the presence of an abnormal number of chromosomes in the cell, also associated with reduced implantation potential and

increased miscarriages (Greaney et al., 2018; Zielinska et al., 2019; Mikwar et al., 2020). Also, mitochondria tend to translocate towards the cortical regions prior and after fertilization to support embryonic cleavage and cortical granule extrusion, and to allow equal division of the mitochondria among the blastomeres (Wang et al., 2009). Abnormal patterns of mitochondrial aggregation and distribution in mature human oocytes coincided with poor developmental competence and outcome after IVF (Vanblerkom and Runner, 1984; Meriano et al., 2001). Due to the diverse range of essential mitochondrial functions required for oocyte viability, mitochondrial dysfunction, for example induced by OB diet consumption, will significantly impair oocyte quality.

1.1.3.2 *The impact of OB diet consumption on oocyte quality*

1.1.3.2.1 Impact on the preovulatory follicle, during oocyte maturation

The upregulated lipolysis in obese patients will lead to systemic dyslipidemia with increased FFAs. This metabolic profile will also be reflected in the follicular fluid (FF), the microenvironment of the oocyte (Robker, 2008; Cardozo et al., 2011; Antunes et al., 2014; Leary et al., 2015). For example, the FF is an ultrafiltrate of the blood, mediated by the blood follicle barrier. The size of molecules determines their passage through this barrier and thus, small molecules such as FFAs can reach the FF, whereas chylomicrons and VLDLs cannot (Aardema et al., 2018). In addition, FFAs can reach the FF via granulosa cell secretion (Dunning et al., 2014). It was noted that the FF of obese women indeed had increased concentrations of FFAs, along with elevated levels of glucose, hormones and pro-inflammatory cytokines, which can impact the quality of the oocyte (Carrell et al., 2001; Robker et al., 2009; Valckx et al., 2014). Although FFAs can be taken up by the cumulus cells surrounding the oocyte and converted into mono-unsaturated fatty acids via stearoyl-CoA desaturase for lipid droplets storage as a protective mechanism for the oocyte (Aardema et al., 2017), this storage capacity is limited. The excess of FFA can still reach the oocyte via gap-junctions, creating a nutrient overload in oocytes (del Collado et al., 2017). Also in oocytes, lipid droplets are formed, but the biochemical changes of the oocyte's microenvironment and the ectopic lipid storage eventually results in a dysregulation of the oocyte's metabolism, leading to high levels of oxidative stress and mitochondrial dysfunction (Boudoures et al., 2017b; Zhao et al., 2017; Bradley and Swann, 2019; Marei et al., 2020; Smits et al., 2021a; Leroy et al., 2022).

1.1.3.2.2 Specific effects of obesogenic diet consumption on mitochondrial functions of mature (ovulated) oocytes

Lipotoxic conditions in both *in vivo* murine studies and bovine *in vitro* models caused increased lipid droplet accumulation in the oocytes, altered mitochondrial activity (evidenced by changes in mitochondrial membrane potential (MMP)), and led to high levels of oxidative stress due to increased ROS accumulation (Igosheva et al., 2010; Marei et al., 2020). In animal models, OB diet consumption alters mtDNA copy numbers and reduces the oocyte ATP content (Reynolds et al., 2015; Marei et al., 2020). Eventually, such alterations result in abnormal transcriptomic and proteomic profiles, in association with phenotypic functional abnormalities of the corresponding embryo (Van Hoeck, 2013; Wu et al., 2015; Marei et al., 2019). As mentioned earlier, OB diet consumption in mice is also associated with spindle malformation and abnormal chromosomal segregation. This leads to aneuploidy due to abnormal mitochondrial distribution, which affects the energy supply needed for proper spindle formation. This has serious consequences on pregnancy and offspring health (Luzzo et al., 2012b; Adhikari et al., 2022).

While normal mature oocyte mitochondria are egg-shaped or spherical having a dense matrix with or without the presence of a vacuole, the mitochondrial morphology changes in mice fed an OB diet. The presence of rose petal shaped mitochondria (or so-called onion-rings), dumbbell-shaped or elongated mitochondria, but also mitochondria with affected outer and inner membranes and degenerated mitochondria increases in oocytes of OB diet fed mice (Marei et al., 2020) (Figure 7). Mitochondrial

morphology often serves as a proxy for mitochondrial function. Changes in mitochondrial shape (elongation, dumbbell) are associated with affected fission/fusion machinery (Amartuvshin et al., 2020), while changes in membrane structure may imply alterations in OXPHOS and proper function of ATP synthase (Paumard et al., 2002; Vincent et al., 2016).

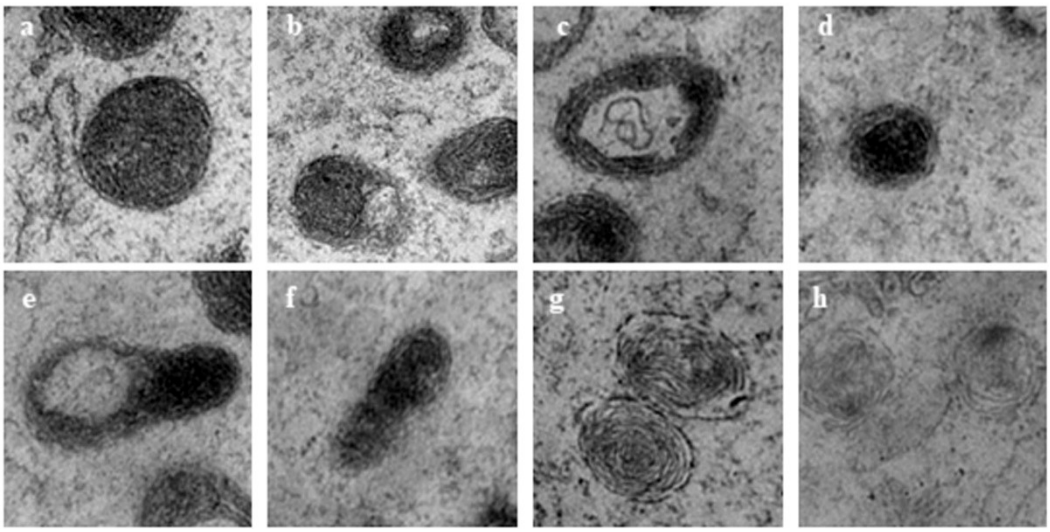

Figure 7. Oocyte mitochondrial ultrastructure of adult mice, presented by Marei et al. (2020). **Normal oocyte mitochondria** classified as**:** spherical (a), regular vacuoles (b). **Abnormal mitochondria** classified as: membranous vacuoles (c), electron dense foci (d), dumbbell-shaped (e), elongated (f), rose petal-shaped (g), degenerated (h).

1.1.3.2.3 Potential impact on earlier stages of folliculogenesis

Britt (1992) noticed that cows having severe body condition score (BCS) loss during early lactation had lower progesterone with reduced pregnancy rates 80 days later, compared to cows without weight loss or with weight gain (Britt. 1992). These findings led to the suggestion that the primordial follicle pool may already be affected by adverse maternal metabolic conditions, having long-term effects on the quality of the mature oocyte upon breeding. Recent data from studies using dairy cow models focusing on optimal cow fertility to reach high milk yield are also in line with this notion. Cows selected for high milk yield and kept under suboptimal management conditions often suffer from extreme BCS loss due to excessive mobilization of fat to the milk, leading to hyperlipidemia and a state of lipotoxicity that is similar to obesity (Leroy et al., 2022). By the time artificial insemination (AI) takes place, the cows BCS and systemic lipid profile is restored, yet the pregnancy rates after AI remain low (Leroy et al., 2014). Cows with major BCS loss from 3 weeks before until 3 weeks after calving (the transition period) still have low pregnancy per AI outcomes, coinciding with reduced embryo quality (Carvalho et al., 2014). In addition, the metabolic and antioxidant status during this transition period was associated with changes in the granulosa cell transcriptomic profile of preovulatory follicles several weeks later (Marei et al., 2022). This again strongly suggests that young follicle pools may already be affected by maternal lipotoxic conditions.

Similar suggestions that preantral follicles may be affected by maternal OB diet consumption were also made in murine studies. Smits et al. (2021b) showed prolonged effects of a long-term OB diet on oocyte quality in an obese Swiss mouse model. In this latter study, female OB diet fed mice still suffered from a reduced oocyte quality even after several weeks of diet normalization before gestation. The oocytes of

OB fed mice still showed increased oocyte lipid droplet content and increased MMP after 6 weeks of being fed a 30 % caloric restriction diet, while the metabolic profile was already improved after 2 weeks of preconception care intervention (PCCI) (Smits et al., 2021a). This suggests that also in mice the dormant follicle pool may indeed be affected by OB diet consumption, since folliculogenesis in mice takes around 2 to 3 weeks (Chen et al., 2021). **However, the hypothesis that oocytes of primordial follicles are already affected by OB diet consumption has never been thoroughly investigated since most studies only focus on the mature ovulated oocytes.** Yet, investigating the effect of an OB diet on primordial follicles is of pivotal importance, as an affected oocyte already in the primordial follicle pool may lead to an increased follicle depletion rate with diminished ovarian reserve (DOR), and premature ovarian aging, the latter defined as an unusually low ovarian reserve or a low number of good quality oocytes (Subrat et al., 2013).

1.2 Prenatal and early postnatal impact of maternal obesogenic diet consumption on offspring health and fertility

1.2.1 The impact of maternal obesogenic diet consumption on offspring health

The concept of Developmental Origins of Health and Disease (DOHaD) states that events during embryonic-fetal development and during early childhood can influence the risk of developing various diseases later in life, including cardiovascular diseases, metabolic conditions (e.g. maternal obesity), and neurodevelopmental disorders in daughters (Cinzori and Strakovsky, 2022; Muhammad et al., 2023). A variety of factors have been investigated, ranging from maternal malnutrition, stress, and exposure to environmental toxins on fetal and early childhood development (Wojtyla, 2011; Junien et al., 2016; Hagemann et al., 2021). For example, maternal emotional stress increases the risk of childhood obesity due to a maternal corticotrophin induced catch-up growth of the offspring (Matvienko-Sikar et al., 2021).

In (Western) countries where a sedentary lifestyle is common and high-fat and high-sugar diets comprise a large part of the diet, maternal obesity is increasing and can contribute to the development of offspring disorders later in life (Lacagnina, 2020). In humans, maternal obesity is associated with an increased risk of obesity development in their offspring, having altered body fat composition (estimated by dual-energy X-ray absorptiometry), increased blood pressure, adverse lipid profile, coronary heart disease and stroke, T2DM, asthma, allergic reactions and neurobehavioral disorders and poorer cognitive performance in their offspring (Godfrey et al., 2017; Kankowski et al., 2022). In mice, an association has been shown between maternal obesity and a higher offspring weight gain with adipocyte hypertrophy, endothelial dysfunction and hypertension, including increased risk of hyperglycemia, hypertension and fatty liver disease. In addition, maternal OB diet consumption coincided with reduced insulin sensitivity and reduced skeletal muscle mass in their offspring at adult age (Samuelsson et al., 2008). Also in mice, maternal OB diet consumption was associated with cardiac dysfunction in fetuses, having prolonged effects up until adult age (Vaughan et al., 2022). Even neurological disorders were detected in offspring born to obese dams (Bordeleau et al., 2022). Therefore, prenatal care for the mother is extremely important, as ensuring optimal maternal health and nutrition may contribute to long-term health of their offspring (Newnham, 2007; Lumpkins and Saint Onge, 2017).

1.2.2 The impact of maternal obesogenic diet consumption on offspring fertility

Human studies indicate that maternal obesity coincides with poor offspring reproductive outcomes. Disrupted external genital development and precocious puberty was noticed in sons and earlier menarche and PCOS in daughters born to obese mothers (Cinzori and Strakovsky, 2022; Muhammad et al., 2023). In female mice born to mothers fed an OB diet, the ovarian blood flow was modified and the ovarian development was impaired, with a reduced amount of primordial and antral follicles and upregulated genes involved in follicular apoptosis (Cheong et al., 2014; Puttabyatappa and Padmanabhan, 2018). Léveillé et al. (2014) noted that the number of atretic follicles was increased in offspring born to mothers (rabbits) fed a diet high in fat for 17 weeks. In C57BL/6 mice born to mothers fed a diet high in fat and sugar for 6 weeks (Aiken et al., 2016), ovarian gene analysis showed increased mitochondrial antioxidant defense mechanisms via upregulation of superoxide dismutase (MnSOD), glutathione peroxidase and lipoxygenase, together with an increase in mitochondrial biogenesis via an upregulation of TFAM (Aiken et al., 2016). In Winstar albino rats, adult (age 11 weeks) control diet fed females born to mothers fed an OB diet for 3 months showed signs of obesity, reduced insulin sensitivity and hyperlipidemia, in association with an upregulation of markers related to inflammation and oxidative stress and a downregulation of markers related to mitochondrial biogenesis and antioxidant responses in their ovaries. In addition, histological examination of the ovaries showed the presence of numerous degenerated and atretic follicles, together with vascular congestion, perivascular oedema and mononuclear inflammation in the interstitial ovarian tissue of OB-born females (Ramadan et al., 2023). In

conclusion, maternal obesity and maternal OB diet consumption have long-lasting effects on their offspring's health, including increased risks of various diseases and altered reproductive health outcomes.

1.2.3 What mediates the intergenerational effects of maternal obesity?

Offspring born to OB fed mothers have increased risk of developing several metabolic diseases such as obesity and T2DM, and maternal OB diet consumption is associated with subfertility in their offspring daughters (Cinzori and Strakovsky, 2022; Muhammad et al., 2023). This can be, amongst other factors, inherited through the female germline, due to epigenetic regulation of the offspring, and/or due to *in utero* metabolic reprogramming and adaptation of the offspring. Furthermore, *de novo* mutations occurring early postnatal and during early life may also play an important role.

1.2.3.1 *The female germline*

Oocyte mitochondrial dysfunction affects the subsequent embryo as well, because mitochondria are only inherited through the female germline (Van Blerkom, 2008a). Sperm mtDNA typically carries a higher mutation rate and is more susceptible to damage from ROS compared to oocytes, which could potentially result in the inheritance of mitochondrial diseases (Rojansky et al., 2016). Therefore, maternal mitochondrial health is of the utmost importance, as only the maternal mitochondria will be transmitted to the next generations of offspring (Van Blerkom, 2009), implying a significant risk for their offspring to inherit damaged mitochondria when the mother suffers from obesity.

In an inbred mouse study, mitochondrial alterations were noted in the oocytes and muscles of female offspring born to mothers fed an OB diet (Saben et al., 2016b). Such mitochondrial abnormalities were even found in the muscle tissue up until the third generation of the adult offspring (Saben et al., 2016b). Afterwards, it has been suggested by Boudoures et al. (2017a) that these diet induced mitochondrial aberrations persevered during early embryo development due to a lack of mitophagy mechanisms in oocytes that can repair mitochondrial damage. A similar phenomenon was suggested by Jin et al. (2022) in aging oocytes, where an age-related increase in proteins such as PINK1 contributed to the accumulation of damaged mitochondria in oocytes, leading to meiotic arrested oocytes. Therefore, it is suggested that maternal OB diet consumption can cause long lasting carry-over effects on the following generations via the germline transmission of dysfunctional mitochondria. However, several mechanisms preclude the transfer of damaged mitochondria to the offspring, such as the mitochondrial self-repair mechanisms via fission and fusion as mentioned earlier, the bottleneck phenomenon (Lee et al., 2012; Cox et al., 2021) and strict germ cell selection (Lei and Spradling, 2016). Therefore, this germline transmission of aberrant mitochondria remains questionable.

During early embryo development, epigenetic regulation takes place, a process that is strongly influenced by maternal metabolic health conditions (Meulders et al., 2023). Epigenetic regulation involves reversible changes in the function of genes that are inheritable through mitosis and/or meiosis, but do not modify DNA sequencing. This process implicates DNA methylation and histone acetylation and methylation (Weinhold, 2006). During DNA methylation, a methyl group (CH_3) is added to the DNA molecule in gene promotor regions, mediated by DNA methyltransferases (DNMTs). Consequently, the DNA becomes less accessible for transcription, and gene expression is reduced. Histone acetylation involves the addition of an acetyl group ($COCH_3$) to histone proteins, improving transcription and promoting gene expression (Lee et al., 2020). During gametogenesis the first epigenetic reprogramming event takes place. Primordial germ cells are demethylated genome-wide, and maternal imprints are established during the final stages of gametogenesis (Dvoran et al., 2022). The second event is during early embryo development, causing extensive reprogramming that results in non-genomic hereditary information that can be transmitted to the offspring and may affect its phenotype (Xavier et al., 2019).

Mitochondrial metabolites (e.g. Acetyl-CoA, alpha ketoglutarate, S-adenosyl methionine (SAM), NAD+ and beta-N-acetylglucosamine) play a crucial role in regulating such epigenetic processes (Xu et al., 2021). An *in vitro* bovine study investigated the direct link between mitochondrial functions and epigenetic regulation (Meulders et al., 2023). Bovine oocytes were *in vitro* exposed to pathological concentrations of PA during oocyte maturation and embryo culture, resulting in increased DNA methylation and histone acetylation, associated with mitochondrial dysfunction. These results imply a direct link between epigenetic mechanisms and mitochondrial functions in oocytes and embryos (Meulders et al., 2023). It has been shown that oocyte maturation under lipotoxic conditions alters mitochondrial functions and DNA methylation in the resulting pre-implantation embryos (Desmet, 2019). This may lead to significant epigenetic modifications in imprinted and developmentally important genes with long-lasting effects for offspring born to OB mothers (Singh and Sinclair, 2007; Arnaud, 2010).

Murine embryo transfer studies investigated the impact of maternal obesity during solely the periconceptional period on offspring metabolic health, and showed that exposure to maternal obesity before conception resulted in fetal growth restriction that persisted until the birth of the offspring (Luzzo et al., 2012a; Sasson et al., 2015). However, in real life, this affected embryo will further grow and develop within the reproductive tract of the same obese or metabolically compromised mother. This reproductive tract and its micro-environment will be affected by the maternal OB diet effects, which may further aggravate the already hampered embryo during subsequent development.

1.2.3.2 *The oviduct*

The oviduct plays a vital role in facilitating gamete transport, fertilization, and early embryonic development during the first 4-6 days in humans (Kim and Kim, 2017) and the first 3 days in mice (Yoshinaga, 2013), creating a supportive and regulated environment for successful reproduction (Barton et al., 2020). In the oviduct, embryonic genome activation takes place (also called maternal-to-zygote transition), an event where the zygotic genome starts to control the embryonic development instead of the maternal genome (Lee et al., 2013). Embryonic genome activation occurs at the 2-cell stage (Schultz, 1993) in mice, while in humans this event arises around the 8-cell stage (Yuan et al., 2023).

The oviductal fluid is positively correlated with the FFA concentrations in serum (Jordaens et al., 2017a). Therefore, maternal diets can significantly change the oviductal environment, impacting reproductive outcomes. For example, food deprivation in sows caused disruption of oviductal motility in association with increased prostaglandins and decreased oestradiol concentration, resulting in a delayed oviductal-uterine oocyte transport (Mwanza et al., 2000). In mice it was noticed that oviductal epithelial cells showed distinct gene patterns involved in acute stress response already after a 3 day dietary intake of an OB diet (Moorkens et al., 2022), in combination with changes in the lipidomic profile of these oviductal epithelium cells, the latter gradually increasing in association with the length of the dietary intake (Moorkens et al., 2023). *In vivo* modified bovine oviductal epithelial cells exposed to FFAs showed altered apoptotic and anti-oxidative gene expression of BCL2 and SOD1 respectively, in combination with an upregulated lipid metabolism (Jordaens et al., 2017b), and was associated with reduced cleavage rates and blastocyst formation (Akca et al., 2012). Recently, it was found in a murine study that adult male and female offspring born to mothers fed a HF/HS diet only during the first 3 days of pregnancy, coinciding with the preimplantation period, showed altered gene expression of glucose transporters, adiponectin, leptin and insulin receptors in their hippocampus (Ojeda et al., 2023). In addition, the preimplantation period of embryo development represents a critical period during which mitochondrial regulation of epigenetics can have substantial short- and long-term impacts on the health of the resulting offspring (Harvey, 2019).

1.2.3.3 *The uterus*

The morula (16 cells) is transported to the uterine lumen via oviductal cilia, and transforms into a blastocyst, possessing an inner cell mass that will become the embryo, and the trophectoderm surrounding the inner cell mass and the blastocoel (Biggins et al., 2015). Trophectoderm cells will develop into supporting structures such as the amnion, the yolk sac and the foetal placenta (Marikawa and Alarcón, 2009). In the second week of human development (Kim and Kim, 2017) and on day 4 in mice (Paria et al., 1993), the blastocyst embeds into the endometrial lining of the uterus.

The uterus provides a nurturing environment for the developing embryo after implantation. However, this nurturing environment can also be affected by maternal obesity. A direct and strong correlation was found between an increase in maternal BMI and the prevalence of pre-eclampsia (Bodnar et al., 2005; Alba et al., 2018). Pre-eclampsia is a specific human pathology that is characterised by hypertension, oedema and proteinuria during pregnancy, due to abnormal placentation and reduced placental perfusion, leading to abnormal vasoconstriction impacting embryonic nutrient availability, and resulting in intrauterine growth restriction, premature birth, and even maternal and foetal morbidity (Lopez-Jaramillo et al., 2018). The risk of pre-eclampsia doubled with a BMI of 26 kg/m^2 and almost tripled when the BMI exceeded 30 kg/m^2 (Bodnar et al., 2005; Alba et al., 2018). Pre-eclampsia-born children are more prone to the development of vascular and pulmonary diseases, kidney defects, and alterations in the development of the important hypothalamic-pituitary (HPG) axis (Lu and Hu, 2019). The HPG axis controls the reproductive system through endocrine signaling, originating with hypothalamic secretion of gonadotropin-releasing hormone (GnRH) that stimulates the pituitary gland to synthesize and release FSH and LH, needed for optimal ovarian function (Mikhael et al., 2019).

Maternal OB diet consumption during pregnancy induces placental inflammation, hypoxia and impaired placental development (Wallace et al., 2019). Furthermore, maternal obesity leads to an intrauterine increase of FFAs, hyperglycaemia, increased placental glucocorticoids, anabolic hormones such as IGF-1 and insulin, resulting in an increased ROS. These changes in the uterine environment can impact both short-term and long-term health outcomes of the offspring (Garcia-Vargas et al., 2012). Long-term complications among offspring exposed to GDM include insulin resistance, impaired glucose tolerance, and T2DM, increased risk of obesity, high blood pressure and kidney failure (Garcia-Vargas et al., 2012). An increased placental transfer of nutrients such as glucose and lipids also cause adaptations in appetite and energy metabolism of offspring and is defined as the 'fetal overnutrition hypothesis', serving as an underlying cause of the increased prevalence of obesity and cardiovascular diseases in offspring born to obese mothers (Lawlor et al., 2007).

In addition, maternal OB diet consumption can trigger epigenetic alterations that predispose the offspring to various diseases, such as obesity and metabolic disorders (Peral-Sanchez et al., 2022). Indeed, adult rat offspring born to mothers having had increased fructose supply (10 %) via the drinking water solely during pregnancy, demonstrated affected cholesterol plasma levels (increased in males offspring, decreased in female offspring) and altered methylation patterns in genes associated with lipolysis (Rodrigo et al., 2018). Similarly, in mice exposed to a gestational maternal diet containing 60 % fat, hypermethylation of adiponectin and leptin receptor genes occurred, which may contribute to the development of metabolic syndrome in offspring (Masuyama and Hiramatsu, 2012; Wankhade et al., 2017). One week of maternal OB diet feeding from the start of pregnancy already increased the maternal fasting basal glucose levels compared to control-fed mothers. By the age of six weeks, an age representing early fertile age in mice, the offspring of OB-fed dams had an increased fat pad weight and showed signs of hyperinsulinemia (Yokomizo et al., 2014). In a study conducted by Nguyen et al. (2017) it was noted that a HF diet intake of pregnant C57BL/6 mice from gestational day 10-20 even coincided with an increased risk for mammary cancer in their offspring (Nguyen et al., 2017). This highlights again the importance of an optimal maternal diet before, but also during pregnancy, for the health of the offspring. The early phases of peri-

implantation embryo development seem to be very vulnerable to the effects of maternal OB diet consumption.

1.2.3.4 *Developmental plasticity*

The foundation of the DOHaD paradigm was laid by Hales and Barker (1992) with the Thrifty Phenotype Hypothesis, which states that "*the epidemiological associations between poor fetal and infant growth and the subsequent development of type 2 diabetes and the metabolic syndrome result from the effects of poor nutrition in early life, which produces permanent changes in glucose-insulin metabolism*." It was suggested that, during periods of maternal under- or malnutrition, fetuses undergo adaptive responses to increase their chances of survival (Hales and Barker, 2013). However, such adaptations are not always beneficial for the individual's health later in life when there is a mismatch between the conditions before and after birth.

In the winter of 1944-1945 during the second world war, people living in the Netherlands suffered from a sever famine, also known as the Dutch famine or "the Hunger Winter". Many years after the war ended, researchers were able to investigate the disease and mortality rates in a retrospective way, since the food intake during this period was restricted and well documented. The medical records were also documented, and during this severe famine, an association was noted between undernutrition of gestational women and long-term implications on their offspring's health, involving an increased risk of developing vascular diseases, kidney failure, and airway diseases in later adult life. Children born to famine-exposed mothers were also significantly heavier at birth, had increased risk of being overweight at young adulthood (19 years of age) and had increased risk of developing T2DM and even young mortality (Lumey and Van Poppel, 1994).

Many researchers have studied the implications of the thrifty phenotype hypothesis, leading to, amongst others, the Predictive Adaptive Response hypothesis (PAR), suggested by Bateson et al. (2014). The PAR hypothesis implies that during early development, organisms can make anticipatory adaptations based on impulses from the maternal environment. This suggests that early life stressors, predominantly stemming from the maternal environment, significantly influences an individual's phenotype development that normally would be adapted to similar environmental conditions later in life. The PAR hypothesis was further refined into a general concept of match and mismatch between maternal and offspring environment, suggested by Holt (2023). This concept stated that the environmental developmental plasticity, the ability of an organism to react to an environmental input, allows species to quickly adapt to changes over following generations. Here, the importance of match or mismatch between maternal and offspring's environment was clearly highlighted, since adverse phenotypes may occur if the offspring's actual environment diverges from the maternally predicted environment. All these different hypotheses have the same general conclusion which is now widely accepted: an optimal maternal environment is extremely important for the health of the following generation, since offspring may adapt to an environment similar to that of their mothers (Dey et al., 2016; Holt, 2023).

1.2.3.5 *Post-natal effects*

A maternal OB diet can further impact offspring health even after gestation. In mice, as well as in humans, skeletal muscles, liver, adipose tissue and the pancreas are not fully developed after birth (Ellsworth et al., 2018). Thus, organ differentiation will continue also after gestation, making postnatal nutrition a critical factor in the long-term metabolic programming of offspring (Ellsworth et al., 2018). The 'lactocrine hypothesis' was first introduced by Bartol et al. (2008), and states that changes in early transmission of signalling molecules may influence offspring development with both short-term and long-term consequences for the offspring. As such, the relaxin supply from milk is shown to be crucial for the

development of the uterus and endometrium of porcine newborns, highlighting the critical importance of a healthy post-natal maternal environment (Bagnell et al., 2005; Bartol et al., 2008; Bartol et al., 2017).

While breastfeeding in general decreases the risk of obesity in the offspring and avoids infant overfeeding since the infant's demand is matched with the milk production of the mother (Williams et al., 2014), some adverse effects occur in case of maternal obesity. For example, maternal obesity is associated with changes in the macronutrient concentration of milk that may affect the weight gain of the offspring (Isganaitis et al., 2019). In a study monitoring a cohort of women to pregnancy or during their first trimester, milk samples from 2 weeks to 6 months post-partum were investigated and their children were monitored (Saben et al., 2020). The milk composition of mothers having a BMI > 30 kg/m^2 had elevated concentrations of monosaccharides and sugar alcohols or polyols, compared to their lean counterparts, which coincided with an increased risk of childhood obesity in the first 6 months of their offspring's life (Saben et al., 2020). In another study it was noticed that the milk fat content and lactose concentrations were increased in mature milk of obese mothers, but reduced in transitional milk (the transition from colostrum to mature milk), while the protein content remained unaffected (Leghi et al., 2020), compared to lean mothers.

The effect of the lactational period on its own can be investigated in animal models via cross-fostering, the transfer of newborn pups between mothers. In a study using cross-fostering, it was observed that offspring exposed to maternal obesity only during lactation had increased body weight, plasma insulin, leptin and liver malfunction in adult life compared with offspring of lean or obese dams, suckled by lean dams (Oben et al., 2010). Furthermore, pups fed high-carbohydrate milk formula immediately after birth resulted in the development of obesity in female offspring during adulthood (Srinivasan et al., 2008). Gorski et al. (2006) cross-fostered murine offspring of diet-resistant mothers to OB mothers during lactation and noticed an increase in obesity and insulin resistance in the offspring and offspring born to OB mothers but fostered by diet-resistant mothers showed improved insulin sensitivity (Gorski et al., 2006).

Interestingly, a mouse model cross-fostering healthy control-born pups to obese mothers during lactation showed that maternal obesity specifically during lactation partially protects offspring from the effects of a high fat diet later in life (Monks et al., 2018), supporting the PAR theory. Possibly, by cross-fostering pups between obese and lean mothers during lactation, a mismatch between the prenatal and postnatal environment is created, which may be detrimental for offspring that were not 'programmed' for a high energy availability later in postnatal life (Holt, 2023). Moreover, cross-fostering as such may increase the level of stress for offspring, which may affect the metabolic and cardiovascular functions later in the offspring's adult life, and may induce bias in studies focusing on intergenerational disease transmission (Matthews et al., 2011; Sasaki et al., 2013).

Clearly, maternal obesity can trigger or adapt the offspring's development in many ways. The affected oocyte develops in an altered oviductal and uterine environment. Furthermore, the corresponding OB-born offspring will be fed milk with different nutrient composition, compared to milk from lean mothers. This may imply an additive effect over time, that can developmentally reprogram the offspring (Hashemi-Nazari et al., 2020) and may lead to a different response to the effects of an OB diet. **However, it remains unknown if such offspring developmental reprogramming due to maternal OB diet consumption will increase or reduce the offspring's sensitivity to an OB diet.**

1.3 Gaps in knowledge

1.3.1 The maternal x offspring diet interaction

The 'First 1000 Days' campaign states that the nutritional environment during the fetal, as well as the neonatal period until about 2 years after birth, dictates developmental plasticity (Cumerlato et al., 2023). Poor nutrition, both maternal nutrition during embryonic development and lactation, and offspring nutrition from birth until the second birthday can cause irreversible damage to a child's development and can set the stage for later obesity and diabetes (Olney et al., 2019; Thomas, 2022). For example, large cohort studies performed on data from UK Biobank found that individuals with a birth weight of less than 2,85 kilograms had a 50 % increased risk of coronary heart disease compared to normal birth weight individuals (Smith et al., 2016; Liang et al., 2021). Maternal diabetes during pregnancy is associated with a 61 % increased risk of obesity in offspring by the age of 7 (Baptiste-Roberts et al., 2012). In addition, a longitudinal human study showed that 83 % of overweight youth remained overweight as adults (Herman et al., 2009), and children who develop obesity at young age are more prone to the development of metabolic and cardiovascular diseases later in life (Godfrey and Barker, 2000; Eriksson et al., 2014; Contreras et al., 2016; Stacy et al., 2019).

Children are more likely to become obese if one or both of their parents are obese. This increased risk can be even up to 90 % and is directly linked to the BMI of the mother before conception (Cuda and Censani, 2019). Moreover, family lifestyle is mostly persistent, and the maternal dietary preferences and lifestyle are directly correlated with children's dietary choices (Pfledderer et al., 2021), implying that children born to OB mothers have an increased risk of the same OB dietary exposure as their mother. Therefore, when looking at the human situation, not only the maternal diet, but also the offspring's diet are important factors that may affect the health of the offspring. However, in most studies, only the diet of the mother is addressed. Consequently, the increased risk of obesity reported in humans can be, at least in part, due to consuming an OB diet in early life. In this context, the extent by which maternal obesity per se contributes to the continued propagation of the risk of obesity and metabolic disorders across generations, and its interaction with the offspring response to an OB diet, are not clearly defined.

In studies using rodent models, aberrant mitochondria were noticed in oocytes of offspring fed a standard control diet but born to obese mothers (Saben et al., 2016a). Offspring born to OB mothers also showed exacerbated obesity, and showed maldevelopment of the female offspring's reproductive system (Cheong et al., 2014; Ramadan et al., 2023; Wei et al., 2023). However, in these studies offspring were fed only a standard control diet. Thus, only the effect of the maternal OB diet was investigated and a possible interaction with the effect of the offspring's diet remained overlooked. According to Barker's thrifty phenotype hypothesis that is previously described, fetuses are programmed to adapt to the environment they expect to be born in (Barker, 1997; Godfrey and Barker, 2000), and Bateson et al. (2014) suggested with the PAR that early life signals influence an individual's phenotype development, normally adapted to similar conditions later in life. When anticipated and actual environments diverge, this may lead to adverse consequences for both Darwinian fitness and future health and fertility of this individual (Bateson et al., 2014). Consequently, some of the reported effects of maternal obesity on their offspring that are reported earlier, may occur due to the mismatch created by feeding offspring born to obese mothers a control diet after weaning**. It remains unknown, yet crucial, whether there is an additive, diminished or adapted effect of the daughter's own dietary choices, when she is born to an obese mother. This may lead to an increased or a reduced risk of developing metabolic diseases and even subfertility in daughter's born to obese mothers. However, the effect of the mother x daughter diet interaction on daughter's health and fertility, remains largely underexplored in research.**

Obesity and OB diet consumption are associated with subfertility (Leroy et al., 2022) that requires intensive and expensive fertility treatments and ART (Koning et al., 2010). The response to such

treatments varies among individuals and is largely dependent on the women's metabolic health. Preconception care interventions (PCCI) do not always fully restore the quality of the oocyte in metabolically compromised women, leading to disappointing results after fertility treatment and ART (Pandey et al. 2010). However, it is possible that the dietary history of the woman's mother needs to be taken into account to optimize such PCCI or ART, since maternal OB diet consumption can induce long-term effects on the daughter's health and fertility that may affect the overall response of the oocyte to environmental stressors. Understanding the effect of such interaction between the maternal and offspring exposure to OB diets is therefore important to develop tailored strategies for reducing the risk of metabolic diseases and improving the health and fertility in offspring born to OB mothers. This fundamental insight is crucial considering the growing prevalence of female obesity in our modern society (Liang et al., 2009; Sarker et al., 2019; Gawlinska et al., 2021).

1.3.2 The model

Mouse models help to overcome some of the challenges faced in human studies, such as early drop-out, ethics and randomization (Brehm et al., 2010; Tuttle et al., 2018). Therefore, mouse models are often used in intergenerational studies (Rice et al., 2024). In such mouse studies, only a few address this specific maternal x offspring diet interaction, focusing on its effect on offspring health (Samuelsson et al., 2008; Keleher et al., 2018) and suggested that offspring born to obese mothers were more sensitive to the effects of an OB diet, compared to offspring born to healthy mothers. The OB born offspring showed exacerbated obesity and major insulin resistance, with a significant effect of the maternal x offspring diet interaction. Therefore, an increased sensitivity to an OB diet was suggested in these OB born offspring (Samuelsson et al., 2008; Keleher et al., 2018). However, these studies used inbred mice, while significant differences are reported between inbred and outbred mice strains that may impact their intergenerational disease susceptibility.

Inbreeding reduces population diversity and increases genetic drift, defined as random fluctuations in gene allele frequencies (Pekkala et al., 2014). As such, males of the inbred C57BL/6 strains are shown to have a defect in insulin secretion, which affects their overall insulin responsiveness (Clee and Attie, 2007). C57BL/6 mice strains were also found to carry mutations in the nicotinamide nucleotide transhydrogenase (Nnt) gene, which may predispose for glucose intolerance (Freeman et al., 2006) and result in mitochondrial redox abnormalities (Ronchi et al., 2013). Furthermore, Marei et al. (2020) noticed that female C57BL/6 mice carry a high rate of inborn oocyte mitochondrial ultrastructural alterations compared to Swiss mice, even when born to control lean mothers and fed a control diet. Feeding a HF/HS diet in C57BL/6 mice increases mtDNA copy numbers in oocytes, suggesting accumulation of damaged mitochondria due to lack of mitophagy, while it decreases mtDNA copy numbers in Swiss mice oocytes, suggesting that damaged mitochondria are removed (Marei et al., 2020; Nicholas et al., 2020). Therefore, the transmission of aberrant mitochondria through the female germline may differ between mouse strains.

Due to the differences in metabolic stress responses, inborn oocyte ultrastructural abnormalities and the accumulation of OB diet induced damaged oocyte mitochondria in inbred mice, there is a good reason to assume that the intergenerational carry-over of diet induced mitochondrial aberrations to the offspring tissues, including their impact on offspring metabolic health that is reported in C57BL/6 OB born offspring, might be strain specific. Hence, it is necessary to investigate the intergenerational transmission of metabolic diseases and the maternal x offspring diet interaction in outbred mice, thereby also increasing the pathophysiological extrapolation to humans.

1.3.3 Inborn or acquired effects

Even though maternal OB diet induced mitochondrial alterations are suggested to be transmitted through the female germline in inbred mice (Saben et al., 2016a), several mechanisms preclude the transfer of damaged mitochondria to the offspring, such as the mitochondrial self-repair mechanisms via fission and fusion as mentioned earlier, and the bottleneck phenomenon (Lee et al., 2012; Cox et al., 2021). Two

types of mitochondrial bottlenecks occur. The first mitochondrial bottleneck refers to the random segregation of mitochondria during early embryonic cell division, leading to a reduction in mitochondrial diversity per cell. During oogenesis, an enormous expansion of the mitochondrial population occurs. After fertilization, the oocyte contributes this large number of mitochondria to the zygote and this amount remains equal throughout the initial stages of early embryonic development. Then, as the zygote undergoes subsequent cell divisions, these mitochondria are distributed among the daughter cells, leading to a reduction in total mitochondria per cell. Over multiple rounds of mitotic cell division, this segregation results in a reduction in mitochondrial diversity within the developing embryo (Wilson et al., 2016; Zhang et al., 2018).

Around embryonic implantation, a second mitochondrial bottleneck occurs that limits the transmission of dysfunctional mitochondria to next generation's oocytes marked by a survival of only these germ cells containing healthy mitochondria (Bergstrom and Pritchard, 1998; Wai et al., 2008). Since key questions remain unanswered about how these series of events facilitate the maintenance of mitochondrial quality over generations, a computational model was developed using data involving derived mitochondrial mutations from previous human studies to predict the prevalence of mitochondrial mutations over generations. It was found that selection through pooling of high quality mitochondria via Balbiani bodies was a likely theory to prevent transmission of dysfunctional mitochondria through the germline (Colnaghi et al., 2021). Supporting this theory, in mice only 20 % of germ cells eventually become oocytes, implying indeed a natural selection. This natural selection can occur via so-called nurse cells. Around embryonic day 10-11, murine primordial germ cells undergo synchronous mitotic divisions and form germline cysts, connected by intercellular bridges. Per cysts, one germ cell receives organelles such as mitochondria from the surrounding cells (the nurse cells) that undergo programmed cell death after this organelle transmission (Lei and Spradling, 2016). This implies a natural selection of high quality germ cells, that will result in only healthy oocytes.

Throughout oocyte development, the number of mtDNA copies increases enormously (Van Blerkom, 2009), and the mitochondrial DNA requires multiple rounds of replications (Kirillova et al., 2021a) to regenerate the amount of mitochondria present in mature oocytes, increasing the chance of *de novo* mutations. Therefore it is likely that the mitochondrial defects present in offspring oocytes born to OB mothers (Saben et al., 2016a; Xhonneux et al., 2023) are caused by environmental stressors after the primordial germ cell establishment and are not transmitted through the germline.

Due to this gap in knowledge it remains unknown if offspring born to OB mothers carry mitochondrial abnormalities in their oocytes already at birth. Nevertheless, this information is crucial for optimal fertility management in daughters born to obese mothers. Mitochondria play a role in the activation of the primordial follicles (John et al., 2008) and are important for further follicle and oocyte development (Van Blerkom, 2009). Therefore, alterations in the primordial follicle pool already at birth may contribute to premature ovarian insufficiency (POI) and DOR.

1.3.4 Age and stage specific effects

While the effect of maternal OB diet consumption during lactation on offspring health and the risk of developing obesity are described (Ellsworth et al., 2018; 2020; Zhao et al., 2020), it is still unknown if the ovarian follicle reserve is also vulnerable at this stage. Exploring potential mitochondrial dysfunction at birth and at weaning can be interesting because an early malfunction of the primordial follicle pool may contribute to POI and DOR in daughters born to OB mothers. It is likely that, even if offspring from obese mothers are born with oocyte mitochondrial dysfunction or not, the effect of lactation can still aggravate any maternal OB diet effect at birth.

Folliculogenesis is a lengthy process which involves highly complex cytoplasmic changes in organelle structure and functions, including mitochondrial replication and gradual increase in mitochondrial bioenergetic activities (Telfer et al., 2023). Long et al. (2022) noticed that cell-cell interactions between granulosa cells were affected by maternal obesity via dysregulation in the granulosa cell differentiation and steroidogenesis with an increased follicular differentiation status. These alterations were also present in the mature oocytes (Long et al., 2022). Furthermore, obesity affects the anti-Müllerian hormone (AMH) production directly, impacting proper folliculogenesis (Bedenk et al., 2020). Therefore, if obesity affects folliculogenesis and granulosa cell differentiation, it is likely that the different stages of folliculogenesis may be affected by maternal OB diets, offspring OB diets and/or their interaction. Understanding the timings of mitochondrial alterations is pivotal since maintaining a healthy primordial follicle pool is essential for sustaining subsequent folliculogenesis and guaranteeing oocyte quality upon ovulation. **Therefore, determining the stage at which oocytes are affected is crucial to optimize reproductive management of obese patients by e.g. optimizing the duration or timing of the interventions during the preconception period. However, very little research addresses this topic.**

1.4 Concluding remarks

The intergenerational effects of maternal OB diet consumption imply a heavy burden on the health of future generations. Maternal OB diet consumption leads to oocyte mitochondrial dysfunction, severely reducing the quality of the oocyte. The exclusive matrilineal inheritance of mitochondria implies detrimental effects of maternal OB diet consumption on the corresponding embryo. Embryonic development in the oviductal and uterine environment of the OB mother may even developmentally reprogram the offspring, a process that continues postnatal during lactation, and may thereby increase the offspring's sensitivity to the same OB diet, leading to an exacerbated effect of an OB diet on the offspring's health and oocyte quality in particular. However, such modified dietary responses remain yet to be discovered. In addition, it is important to investigate when these effects occur in the offspring's life to design accurate preconception care strategies and halt the intergenerational cycles of maternal obesity. Furthermore, knowing when during adult folliculogenesis the maternal and offspring OB diet have a significant influence can help further develop strategies for improving the offspring's fertility.

1.5 References

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Chapter 2 Hypothesis and aims

Maternal obesogenic (OB) diet consumption leads to oocyte mitochondrial dysfunction and reduced oocyte quality that imply detrimental effects on the corresponding embryo. The hampered OB oviductal and uterine environment may reprogram the offspring, thereby increasing the offspring's sensitivity to an OB diet and affecting their metabolic health profile and the quality of the offspring's oocytes. However, this has never been thoroughly investigated. In addition, it remains unknown when during the offspring's life the effects of maternal OB diet consumption become present. Therefore, we hypothesized that **the metabolic health of adult offspring, and the quality of their preantral follicle oocytes and mature ovulated oocytes, are not only affected by direct exposure to an obesogenic (OB) diet, but also by the maternal dietary background. More importantly, we also hypothesized that the maternal OB diet influences the direct impact of the offspring's OB diet. In addition, we hypothesized that maternal OB diet induced oocyte mitochondrial alterations are inborn, and may further aggravate during lactation from an obese mother**. These hypotheses were tested in one experiment, using an outbred Swiss mouse model. Mothers were fed a control (C) or OB diet for 7 weeks, similar to previous experiments performed in our lab to induce obesity. Then, at the age of 10 weeks, the mothers were mated with the same control fed Swiss males in a cross-over design. During pregnancy and lactation, the mothers stayed on their allocated diet. This experiment was done in different replicates to facilitate animal handling and sample collection. In one replicate female offspring were sacrificed at birth, to collect offspring ovaries focusing on mitochondrial parameters in primordial follicle oocytes. In other replicates, samples from a few female offspring per litter were collected at weaning, focusing on weight and abdominal fat, and focusing on mitochondrial parameters in primordial follicle oocytes. The remaining sisters were equally weaned on either a C or an OB diet for 7 weeks, creating a 2 x 2 factorial design. Of these adult offspring, samples were collected at 10 weeks of age, resembling young adulthood with an optimal fertile age in mice, focusing on different parameters related to mitochondrial functions in oocytes and somatic cells.

More specifically, we aimed to:

1) investigate the effect of the maternal OB diet, the effect of the offspring's OB diet, and the interaction between the effect of both diets on the offspring's health at adult age. Effects on litter size and characteristics, offspring growth patterns, abdominal fat deposition, metabolic health and insulin sensitivity, as well as on mitochondrial structure and functions in oxidative skeletal muscle of adult offspring are evaluated and reported (Chapter 3).

2) investigate the effect of the maternal OB diet, the effect of the offspring's OB diet, and the interaction between the effect of both diets on the offspring's oocyte quality at adult age. Here, we evaluated oocyte lipid content and oocyte mitochondrial qualitative and quantitative measures, including mitochondrial bioenergetic activity, morphology and cell metabolism (Chapter 4).

3) investigate the effect of the maternal OB diet, the effect of the offspring's OB diet, and the interaction between the effect of both diets on the offspring's oocyte mitochondrial ultrastructure in primordial and preantral ovarian follicles at adult age. We used transmission electron microscopy to investigate mitochondrial morphology of oocytes of the primordial and early activated follicles (primary and secondary follicles) in ovarian sections of adult offspring (Chapter 5).

4) investigate the effect of a maternal OB diet on oocyte mitochondrial morphology, biogenesis and mitochondrial dynamics at birth and at weaning, thereby investigating if offspring from OB mothers are born with mitochondrial dysfunction or adaptational mechanisms, and if an additive effect of lactation is involved (Chapter 6).

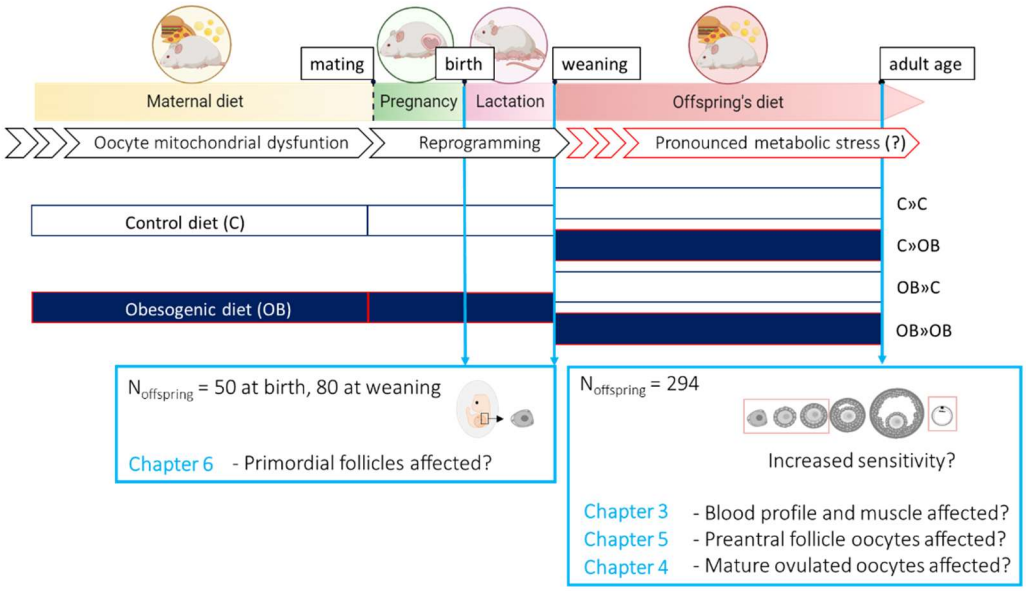

Figure 1. Schematic overview of the experimental design using an outbred Swiss mouse model. A 2 x 2 factorial design was created with control (C) or obesogenic (OB) fed offspring born to C or OB fed mothers. Offspring samples were collected at birth (offspring age less than 1h), weaning (offspring age 3 weeks) and at adulthood (offspring age 10 weeks). Adult offspring treatment groups are reported as MaternalDiet»OffspringDiet.

Chapter 3

The Interplay of Maternal and Offspring Obesogenic Diets: Impact on Offspring Metabolism and Muscle Mitochondria in an Outbred Mouse Model

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3.1 Abstract

Consumption of obesogenic (OB) diets increases the prevalence of maternal obesity worldwide, causing major psychological and social burdens in women. Obesity impacts not only the mother's health and fertility, but also elevates the risk of obesity and metabolic disorders in offspring. Family lifestyle is mostly persistent through generations, possibly contributing to the growing prevalence of obesity. We hypothesized that offspring metabolic health is dependent both on maternal and offspring diet and their interaction. We also hypothesized that the sensitivity of the offspring to the diet may be influenced by the match or mismatch between offspring and maternal diets. To test these hypotheses, outbred Swiss mice were fed a control (C, 10 % fat, 7 % sugar, n=14) or OB diet (60 % fat, 20 % sugar, n=15) for 7 weeks, then mated with same control males. Mice were maintained on the same corresponding diet during pregnancy and lactation, and the offspring were kept with their mothers until weaning. The study focused only on female offspring, which were equally distributed at weaning and fed C or OB diets for 7 weeks, resulting in 4 treatment groups: C-born offspring fed C or OB diets (C»C, C»OB) and OB-born offspring fed C or OB diets (OB»C, OB»OB). Adult offspring systemic blood profile (lipid and glucose metabolism) and muscle mitochondrial features were assessed. We confirmed that offspring OB diet majorly impacted offspring's health by impairing offspring's serum glucose and lipid profiles, associated with abnormal muscle mitochondrial ultrastructure. Contrarily, maternal OB diet was associated with increased expression of mitochondrial complex markers and mitochondrial morphology in offspring muscle, but no additive effects of (increased sensitivity to) an offspring OB diet were observed in pups born to obese mothers. In contrast, their metabolic profile appeared to be healthier than those born to lean mothers and fed an OB diet. These results are in line with the Thrifty Phenotype Hypothesis, suggesting that OB-born offspring are better adapted towards an environment with a high energy availability later in life. Thus, using a murine outbred model, we could not confirm that maternal obesogenic diets as such contribute to female familial obesity in the following generations

Keywords

DOHaD; Intergenerational diseases; Offspring health; Obesogenic diet; Maternal obesity; Thrifty Phenotype Hypothesis

3.2 Introduction

Sedentary lifestyle and consumption of Western-type diets (high-fat high-sugar (HF/HS) diets) are associated with an increased worldwide prevalence of obesity, type II diabetes and other metabolic disorders. According to Global Health Observatory (GHO) data, the prevalence of obesity in most countries is higher in women than men, with poorer prognosis, imposing a major psychological and social burden on women (Bentley-Lewis et al., 2007; Westerman and Kuhnt, 2022). On top, obesity in women is problematic as it does not only impact their own health and fertility, but also increases the risk of metabolic disorders and obesity in the next generations, affecting future mothers and eventually the female germline (Eriksson et al., 2014; Contreras et al., 2016; Saben et al., 2016).

Currently, up to 40 % of pregnancies in Western countries are to either overweight or obese mothers (Gaillard, 2015; Boutari and Mantzoros, 2022). Maternal BMI is positively associated with disease risk in adult offspring (Eriksson et al., 2014). This can be due to oocyte and embryo epigenetic alterations around conception and during pregnancy. However, whether such an increased risk is truly a direct consequence of maternal obesity is less clear, because the family lifestyle is mostly persistent, and maternal dietary preferences and lifestyle are likely to influence children's dietary choices (Gray et al., 2018; Pfledderer et al., 2021). Therefore, the increased risk of metabolic diseases in the offspring can be, at least in part, due to consuming an OB (OB) diet in early life. Children who develop obesity at young age are also more prone to the development of metabolic diseases later in life (Godfrey and Barker, 2000; Eriksson et al., 2014; Contreras et al., 2016; Stacy et al., 2019). In this context, the extent by which maternal obesity per se contributes to the continued propagation of the risk of obesity and metabolic disorders across generations, and its interaction with the offspring's response to a continued OB diet, are not clearly defined.

Multigenerational human studies are often confronted with difficulties linked with ethics, recruitment, randomization, equalization, drop-outs and follow-up. Diet-induced obese mouse models circumvent some of these challenges and enable studying the underlying pathogenesis in a highly controlled setting (Brehm et al., 2010; Tuttle et al., 2018). The majority of studies in this field use inbred mice (most commonly the C57BL/6 strain). However, inbreeding is known to increase genetic drift, which may confound responses to environmental and nutritional stress (Nicholson et al., 2010). The C57BL/6 strain carries mutations in the nicotinamide nucleotide transhydrogenase (Nnt) gene, which may predispose for glucose intolerance (Freeman et al., 2006) and result in mitochondrial redox abnormalities (Ronchi et al., 2013). We have tested and validated the use of the outbred Swiss mice to further increase the pathophysiological relevance to humans (Marei et al., 2020a; Smits et al., 2021; Smits et al., 2022). In these studies, we showed that Swiss females fed HF/HS diet exhibit obesity, hyperglycemia, hypercholesterolemia and reduced insulin sensitivity. While maternal obesity has been shown to impact offspring health in murine inbred adult offspring (Samuelsson et al., 2008), this has not been tested in outbred mice.

In mice, HF diet-induced hypercholesterolemia and dyslipidemia are known to induce lipotoxicity in muscle tissue. Skeletal muscles account for 60-70 % of the insulin-stimulated glucose uptake and are described as a primary determinant of metabolic disorders (Turpin et al., 2009; Montgomery et al., 2017; Mikovic and Lamon, 2018). Lipotoxicity results in oxidative stress and mitochondrial dysfunction, with a reduction in oxidative phosphorylation capacity. This is associated with impairment of insulin signal transduction pathways and reduced insulin sensitivity (Hauck and Bernlohr, 2016; Sergi et al., 2019a).

While mitochondrial dysfunction is considered as a key factor in the pathogenesis of metabolic diseases (Saris and Heymsfield, 2007; Sergi et al., 2019b), mitochondria are also essential in intergenerational programming and transmission of environmental effects towards next generations (Alhassen et al., 2021).

Maternal obesity is directly linked with mitochondrial alterations leading to placental dysfunction, modulating fetal growth and development (Sobrevia et al., 2020; Diniz et al., 2023), with gestational obesity directly affecting offspring muscle metabolism (Walter and Klaus, 2014; Ampong et al., 2022). Previous research in inbred C57BL/6 mice suggested that ultrastructural aberrations in oocyte mitochondria induced by a maternal OB diet are transferred to the subsequent embryos. Mitochondria are indeed exclusively maternally inherited. These aberrations were detected in oocytes and skeletal muscle tissues of offspring fed a standard chow diet but born to obese mothers (Saben et al., 2016). However, C57BL/6 mice have been found to already exhibit high rates of mitochondrial ultrastructural abnormalities in their oocytes even when fed a control diet when compared to outbred strains (Marei et al., 2020b), which highlights the importance of the mouse strain used. Furthermore, the additive effect of offspring diet on these maternally induced effects on the offspring has never been investigated.

While most studies focus on the sole effect of maternal obesity on offspring health, Barker's Thrifty phenotype hypothesis states that fetuses are programmed to adapt to the environment they expect to be born in (Barker, 1997a; Godfrey and Barker, 2000). The interaction between maternal and offspring dietary effects are therefore critical, yet underexplored. Similar links between early life adaptations and an individual's phenotype development have been described (Saitou and Yamaji, 2012; Bateson et al., 2014). Hence, some of the reported effects of maternal obesity on offspring health might be due to the mismatch created by weaning offspring born to obese mothers on a control diet, or vice versa. Understanding such interaction is important to develop tailored strategies to minimize risks of metabolic diseases. This fundamental insight is crucial considering the current actual society needs (Liang et al., 2009; Sarker et al., 2019; Gawlinska et al., 2021).

Therefore, we hypothesized that 1) offspring's metabolic health is not only affected by direct exposure to OB diet, but also by the maternal dietary background. More importantly, we also hypothesized that 2) offspring health is dependent on whether the maternal diet matches with the offspring diet. We aimed to test these hypotheses using an outbred Swiss mouse model in a 2 x 2 factorial design. Mothers were fed a Control (C) or OB diet from 6 weeks before pregnancy until end of lactation. After weaning, female offspring were fed either a C or OB diet. Effects on litter characteristics, offspring growth patterns, abdominal fat weight, blood profile, as well as on mitochondrial features in oxidative skeletal muscle of female adult offspring are evaluated and reported.

3.3 Materials and methods

3.3.1 Animal model and experimental design

This study was approved by the Ethical Committee for Animal Testing and performed accordingly (ECD 2018-05). In total 14 female Swiss F_0 mice were fed a control diet (C, 10 % fat, 7 % sugar, Sniff diets D12450J, containing 10 kJ % fat and 7 % sucrose (E157453-04)) and 15 female Swiss F_0 mice were fed an obesogenic diet (OB, E15741-34, 60 % fat (beef Tallow), 20 % sugar (fructose adjusted in the drinking water)) for 7 weeks. All females were mated (age 10 weeks) with the same Swiss males (n = 6) fed a standard chow diet, in a cross-over design. Two days after birth, litters were weighed, pups were counted and their sex was recorded. Then, while there is conflicting evidence pro (Knight et al., 1986; Konig et al., 1988) and against (Enes-Marques and Giusti-Paiva, 2018; Briffa et al., 2019; Xavier et al., 2019) litter size normalization, we decided to equalize litter size at 10 pups by only sacrificing male pups, allowing equal milk supply per pup and rule out associated consequent effects on offspring health. As reported below, the litter characteristics were not affected by diet. At 3 weeks, at least 2 female pups of 6 C and 7 OB mothers were weighed and sacrificed to measure the weight of the lower abdominal fat (Tan et al., 2018). All other female offspring from each litter were weighed and equally weaned on a C or OB diet for 7 weeks, creating a 2 x 2 factorial study design (Fig. 1) and resulting in 4 treatment groups named as MaternalDiet»OffspringDiet: 1) C»C, pups born to C mothers (C-born) and fed a C diet (C-fed pups), 2) C»OB, C-born and OB-fed pups, 3) OB»C, OB-born and C-fed pups and 4) OB»OB, OB-born and OB-fed pups. All pups were weighed weekly to record body weight trajectory (Figure. 2). During the trial, several steps of stratification and randomization were applied to minimize the effects of potential confounders. As such, litters were randomly but equally weaned onto a C or an OB diet to have equal number of sisters between different diet groups. Also, pups of at least 2 mothers were grouped per cage, with equal amount of total mice/cage, to avoid potential cage effects. Adult pups from 6 C and 7 OB mothers were used to perform an insulin tolerance test (ITT) at 9 weeks of age, after 6h of fasting. After overnight fasting at 10 weeks of age, offspring from 6 C and 7 OB mothers were sacrificed by decapitation, quickly performed by trained personnel using a sharp blade. Blood was collected for serum insulin, total cholesterol, triglycerides (TG) and alanine aminotransferase (ALT) analysis, together with the abdominal fat as described by Tan et al. (2018). Of these pups, one muscle (m. soleus) per pup per litter per group ($nF_0 = 6$) was immediately snap-frozen in liquid nitrogen for later analysis of the expression of mitochondrial complex markers using Western blotting. The other m. soleus ($nF_0 = 5$) was fixed in 2.5 % glutaraldehyde solution for ultrastructural analysis using transmission electron microscopy (TEM). Offspring born to the other 8 C and 8 OB mothers were decapitated at 10 weeks of age and blood was collected for non-esterified fatty acid analysis (NEFAs) (Tor et al., 2021). The criteria to choose which animal was killed for each outcome parameter were random within nests (Excel, function =RAND()) but practical between nests, to allow blocking for mother effect and father effect for all investigated outcome parameters.

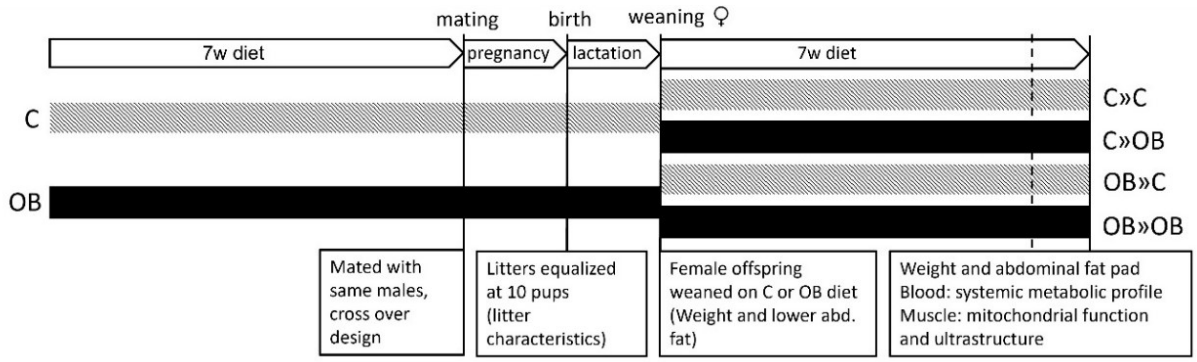

Figure 1. A schematic overview of the experimental design. Offspring were fed a control (C) or an obesogenic diet (OB) and were born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. The groups are named as MaternalDiet»OffspringDiet. This results in four experimental groups (C»C, C»OB, OB»C and OB»OB).

3.3.2 Assessment of the peripheral insulin response

All female offspring of 6 C and 7 OB mothers were intraperitoneally injected with 0.75 IU Insulin (Novo Nordisk, Denmark, Bagsværd, 0.15 IU/ml) per kg body weight after 6 h of fasting. Before the injection, the basal glycemia was measured using glucose meter and sticks from OneTouch Verio (LifeScan Belgium BV, Antwerp, Belgium) after a small tail cut. Post-injection, the glycemia was measured after 15, 30, 60, 90 and 120 minutes. The area under the curve (AUC) and the elimination rate of glucose during the first 30 minutes after insulin injection (ER30) were calculated as described by Smits et al. (2021). The proportional reduction in glycemia was calculated as the difference between peak and nadir, as well as the time needed from the start of the test to reach the glycaemic nadir.

3.3.3 Assessment of the systemic metabolic profile

The ultra-sensitive mouse insulin ELISA kit (CrystalChem, Zaandam, Netherlands) for low-range assays (0.1 – 6.4 ng/mL) was used on 15-16 offspring/group born to 6 C and 7 OB mothers for the determination of insulin concentrations after overnight fasting. After equilibration, 95 µl of sample diluent was mixed with 5 µl of sample in antibody-coated microplate modules and incubated for 2h at 4 °C. Samples were washed and 100 µl of anti-insulin enzyme was added to stop the reaction, then incubated for 30 minutes at room temperature (RT). Afterwards, 7 washing steps, followed by a 40 minute incubation of enzyme substrate solution (RT) were done and the absorbance was measured with TECAN Infinite M200pro microplate reader (TECAN Group Ltd., Männedorf, Switzerland). NEFAs (of 8 offspring/group born to 8 C and 8 OB mothers) were colorimetric and enzymatic determined (Randox Laboratories, CrumLin, United Kingdom) in serum (A.M.L., Antwerp, Belgium) with a semi-automatic RX monza analyzer for clinical chemistry assays (Randox Laboratories Ltd, Crumlin, United Kingdom; within CV 1-3.1 %, between CV 4.7 %). ALT, TG and cholesterol (of 10 offspring/group born to 6 C and 7 OB mothers) were measured enzymatic (A.M.L., Antwerp, Belgium) on an Abbott Architect c16000 (Abbott, Illinois, U.S.A; ALT within CV 2.34 %, between CV 5.5 %; triglycerides within CV 0.53 %, between CV 2 %; cholesterol within CV 0.37 %, between CV 2.5 %), after 12 h overnight fasting. The analytical methodology of TG measurement is based on the reaction sequence described by Fossati and Prencipe (1982) and by Mcgowan et al. (1983). The cholesterol detection is based on the formulation of Allain et al. (1974) and Roeschla.P et al. (1974). The catalyzing action of ALT, transfers the amino group from L-alanine to 2-oxoglutarate in the presence of pyridoxal-5'-phosphate, forming pyruvate and L-glutamate. Pyruvate in the presence of NADH and lactate dehydrogenase is reduced to L-lactate and in this reaction NADH is oxidized to NAD. The rate of decrease in absorbance at 340 nm was monitored as recommended by International Federation of Clinical Chemistry (IFCC).

3.3.4 Assessment of mitochondrial complex markers in muscle

Total OXPHOS Rodent WB Antibody Cocktail (Abcam, United Kingdom, Cambridge, ab110413) was used on muscle tissue of 6 offspring/group born to 6 C and 6 OB mothers, to analyse the expression of complex I, II, III, IV and V markers in offspring oxidative muscle tissue. Housekeeping protein Beta-actin Antibody (ACTB, Cell Signalling Technology®, United States, Massachusetts, 4967S) was included to correct for differences in sample load. Samples were lysed with RIPA buffer (300µl/sample, ThermoFisher Scientific™, Belgium, Dilbeek, 89900) combined with protease and phosphatase inhibitors (3µl/sample, ThermoFisher Scientific, 78425 and 1862495 respectively) followed by crushing (microtube homogeniser system, SP Bel-Art, Wayne, USA) and sonication (on ice, 6x10s, amplitude 50 %) steps. The samples were centrifuged (5min, 15000 rpm, 4°C), the supernatant was collected and heated (95°C) after adding Laemmli Buffer (1/1) (Bio-Rad Laboratories, Belgium, Temse, 1610737) and Beta-mercaptoethanol (5 %, Sigma Aldrich®, United States, Missouri, M3148). SDS-page was performed on mini PROTEAN pre-cast gels (Bio-rad, TGX Precast Protein Gels, 4561021) at 170 V and 0.5 A for 70 minutes. Afterwards, proteins were transferred on PVDF-membranes at 50 V and 0.1 A for 1 hour 35 minutes, then blocked for 2 hours in 0.1 % Skimmed milk (Applichem GmbH, Germany, Darmstadt, A0830) and overnight blotted with OXPHOS Rodent kit (dilution 1:500). Goat-anti-Mouse secondary antibody (dilution 1:2000, Agilent Dako Products, United States, California, P04448) HRP labelled and dissolved in 10 % bovine serum albumin (Sigma Aldrich®, S2002) solution containing 0.1 % of NaN_3 was used. In between different steps of blotting, the membrane was washed with TBST-Tween solution for 2 x 5 minutes, followed by 20 minutes of washing. To correct for differences in protein load, ACTB was blotted on the same membrane after stripping in 20ml6.25 mM TRIZMA-base (pH 6.7, Sigma-Aldrich, United States, Missouri, T1503) containing 175µl 2-mercaptoethanol (Sigma-Aldrich, M3148) on rocking shaker for 30-45min (RT). Protein bands were quantified with Chemidoc XRS+ System (Bio-Rad Laboratories).

3.3.5 Assessment of mitochondrial ultrastructure in muscle

M. soleus of 5 offspring/group born to 5 C and 5 OB mothers was fixed upon collection in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5 % glutaraldehyde solution), embedded in 2 % agarose blocks and washed in 0.1 M sodium cacodylate-7.5 % saccharose solution (pH 7.4). Then the blocks were incubated for 2 h in 1 % OsO4 solution and dehydrated in an ethanol gradient. Sections were cut ultrathin using EM-bed812, stained with lead citrate and examined with transmission electron microscope Tecnai G2 Spirit Bio TWIN microscope (Fei, Europe BV, Zaventem, Belgium) at 120 kV. Mitochondria were morphologically evaluated and classified by two researchers blind to the treatment group according to Hayden (2022). As morphological and functional differences occur between subsarcolemmal (SS) mitochondria and intermyofibrillar (IMF) mitochondria, these categories were investigated separately (Koves et al., 2005; Chomentowski et al., 2011). Mitochondria were classified as normal when they were homogenous without vacuoles as described by Hayden (2022). Mitochondria with abnormal morphology contained large vacuoles (one large vacuole as described by Cook et al. (2014)), small dispersed vacuolization (characterised by enlarged intercristal spaces) as described by Hayden (2022) and other abnormalities such as the presence of mitochondrial derived vesicle-like structures as described by He et al. (2020), abnormal inner membrane formation and mitochondria showing changed electron density.

3.3.6 Statistical analysis

Statistical analysis was done using IBM SPSS Statistics 28 (for Windows, Chicago, IL, USA). Data were confirmed to be homogenous in variance using Levene's Test, and follow normal distribution based on the residual QQ-plots. Therefore only parametric tests were used. Differences in maternal weight after 7 weeks of being fed their corresponding diet, and maternal diet effects at birth and at weaning were analyzed using Independent Sample T-test. Correlations between maternal weight and litter

characteristics were checked using Pearson's Correlations. Changes in maternal and offspring live body weight over time were compared using Repeated Measures ANOVA.

To test the effect of offspring diet, maternal diet as well as their interaction at the age of 10 weeks (hypothesis 1), Two-way ANOVA was used. In all models, the maternal diet and the offspring diet were classified as fixed factors. If two or more sisters were present in the same offspring treatment group, the mother ID was included as a random factor using linear mixed models. If the interaction was not significant, the interaction was omitted from the final model.

To estimate the effect of match/mismatch between the maternal diet and the offspring diet (hypothesis 2), numerical data (e.g. abdominal fat and live body weight, blood analysis, mitochondrial complex marker expression) were analyzed using One-way ANOVA (post-hoc LSD correction) to compare the means and differences in effect sizes of all treatment groups *vs.* C»C offspring, as the healthy reference group.

Categorical data (e.g. proportions of abnormal mitochondrial ultrastructure in muscle tissue) were analyzed using binary logistic regression, testing both hypotheses in the same manner as numerical data. Effect sizes were calculated using Cohen's d coefficient and are shown separately in Figure 7. Subjective evaluations (e.g. mitochondrial ultrastructure) were independently generated by two experienced researchers blind to the corresponding treatment groups and these data were merged after testing interrater reliability using interclass correlation coefficient, as described by Koo and Li (2016).

Differences with P-values ≤ 0.05 are reported as statistically significant, and 0.05 < P-values ≤ 0.1 are reported as tendencies. All main results are displayed in the manuscript and summarized in Supplementary file 1. To account for the multiple comparisons performed in this study, we also included the corresponding q-values in the supplementary file 1.

3.4 Results

3.4.1 Obesogenic diet effects on maternal weight and litter characteristics at birth

Before looking at the offspring health parameters, we analyzed the effects of the OB diet on the mother's weight and litter characteristics. Maternal OB diet increased the weight of the mothers at mating, but had no effect on litter size, litter weight or the sex ratio of the pups. The weight of the mother at the time of mating was also not correlated with litter size (r = 0.020). The original litter size was not correlated with adult offspring body weight (r = 0.974). Detailed information about the litter characteristics is included in Supplementary file 2.

3.4.2 Offspring live body weight and abdominal fat weight (weaning and post-weaning)

At weaning, maternal OB diet increased offspring body weight and abdominal fat weight (See detailed information in Supplementary file 3).

After weaning, offspring body weight trajectory was affected by time, offspring diet, and their interaction. Maternal OB diet significantly increased offspring body weight at weaning and at adulthood. The maternal OB diet did not interact with the effect of the offspring diet. Offspring adult body weight was increased in all treatment groups, compared to the reference group C»C.
Adult offspring abdominal fat weight was increased by offspring OB diet and tended to be reduced by a maternal OB diet. The interaction maternal x offspring diet was not significant. Adult offspring abdominal fat weight was increased in C»OB pups and in OB»OB pups, but tended to be reduced in OB»C pups, compared to C»C.

Results of offspring adult live body weight and abdominal fat weight are displayed in Figure 2.

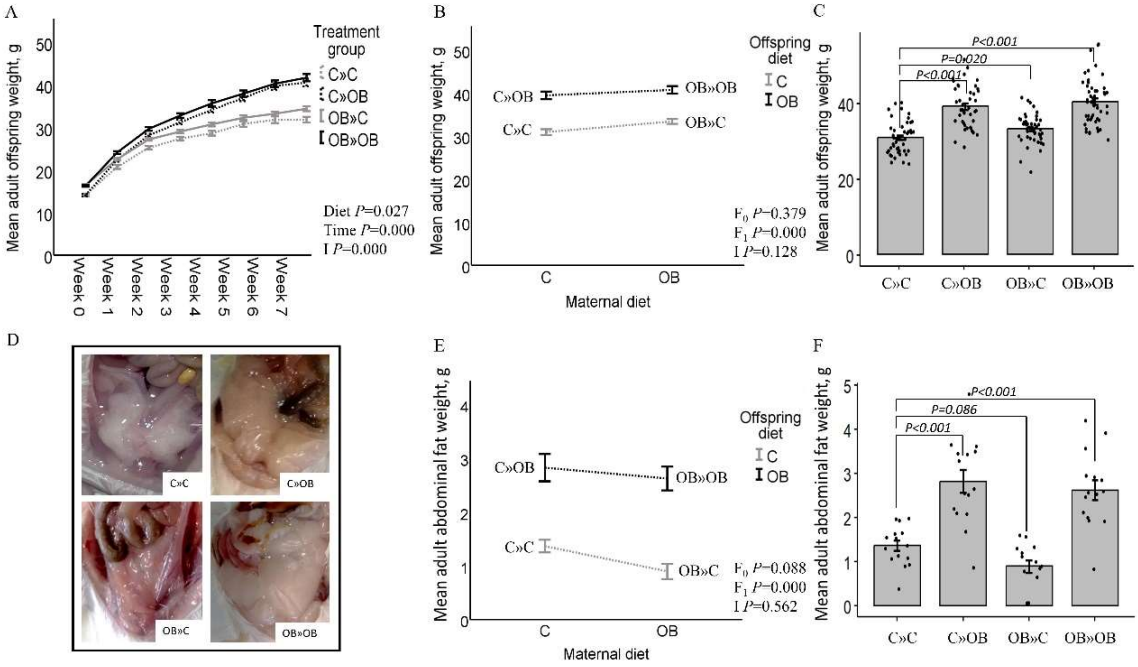

Figure 2. Maternal and offspring diet effects on adult offspring body weight and abdominal fat weight. Offspring growth curve (A), interaction plot (B, Two-way ANOVA) and bar charts with SE error bars and row data points (C, One-way ANOVA with C»C as reference group) of the mean body weight (g) of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as mean ± S.E.M and are derived from all offspring born to 6 C and 7 OB mothers. D. Offspring abdominal fat. Interaction plot (E) and bar charts with SE error bars and row data points (F) of the mean abdominal fat weight (g) of all treatment groups. Data are presented as mean ± S.E.M and are derived from all offspring born to 14 C and 15 OB mothers. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I = interaction). *P*-values of significant differences and tendencies between treatment groups are displayed on the graphs.

3.4.3 Offspring serum glucose and insulin

According to the Two-way ANOVA analyses, offspring OB diet significantly increased the AUC of the ITT, basal glucose and insulin levels, and decreased ER30 rates, with no significant effect of- or interaction with- maternal diet.

The effect of offspring OB diet in increasing ITT (AUC) and basal glycemia and decreasing ER30 rate was evident in both C»OB pups compared to C»C. The effect on basal insulin was not significant in the One-way ANOVA comparison for this group. In the OB»OB group, basal glycemia was also significantly increased compared to C»C with only a tendency of increased ITT (AUC), which could be due to the significantly higher basal insulin levels that was only detected in this group.
In contrast, while maternal diet had no significant effect, the dietary mismatch in the OB»C was associated with a tendency to reduce the AUC of ITT and the basal glycemia compared to the controls.

Results of offspring serum glucose profile are displayed in Figure 3.

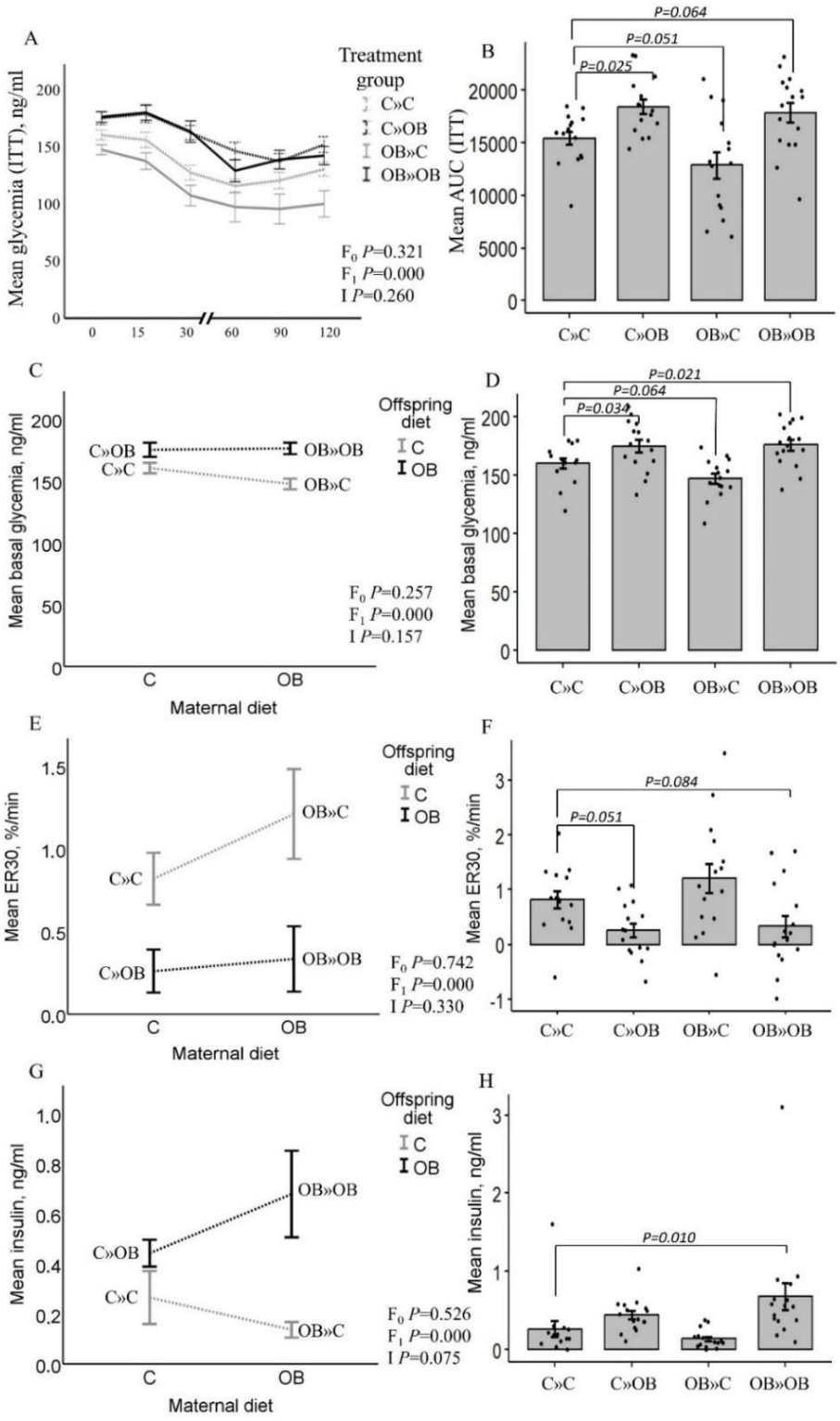

Figure 3. Maternal diet effects on offspring serum glucose profile and insulin concentrations. Graphs representing the mean glycemia (AUC (ITT), A, B), the basal glycemia (C and D, ng/ml), the elimination rate during the first 30 minutes after insulin injection (ER30, E and F, %/min) and serum insulin (G and H) of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as mean ± S.E.M and are derived from 15-16 offspring/group, born to 6 C and 7 OB mothers. Interaction plots show Two-way ANOVA, bar charts with SE error bars and row data points show One-way ANOVA comparisons with C»C as reference group. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I= interaction). *P*-values of significant differences and tendencies between treatment groups are displayed on the graph.

3.4.4 Offspring serum lipid profile

Offspring serum cholesterol was significantly increased by offspring OB diet, but tended to be reduced by maternal OB diet. The interaction was not significant. Serum cholesterol was significantly increased in C»OB offspring, but reduced in OB»C pups compared to C»C.

Offspring serum NEFA concentrations were only significantly increased by offspring diet with no effect or interaction of maternal diet. This increase was only significant in the C»OB pups, compared to C»C. Results of offspring serum cholesterol and NEFA levels are displayed in Figure 4.

Offspring serum ALT and TG were not affected by offspring or maternal diet nor their interaction. No differences were detected between groups (data are presented in Supplementary file 4).

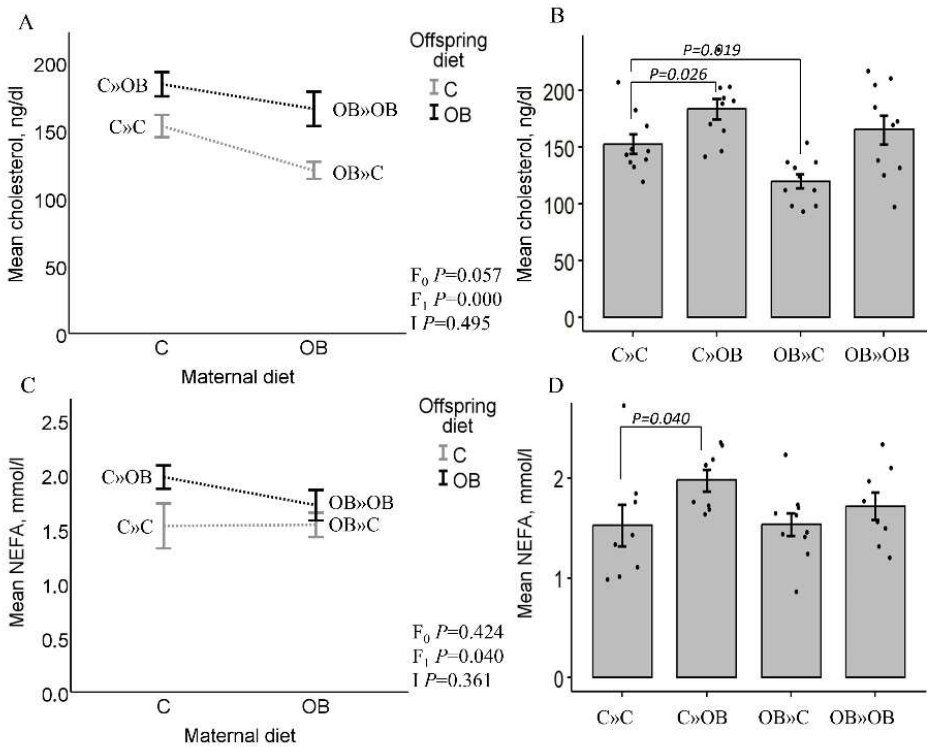

Figure 4. Maternal diet effects on offspring serum cholesterol and NEFA concentrations. Interaction plot (A) and bar chart (B) of mean serum cholesterol (ng/dl) from offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as mean ± S.E.M and are derived from 10 offspring/group, born to 6 C and 7 OB mothers. Interaction plot (C) and bar chart (D)) of the mean serum NEFA (mmol/l) of all treatment groups. Data are presented as mean ± S.E.M and are derived from 8 offspring/group born to 8 C and 8 OB mothers. Interaction plots show Two-way ANOVA analysis, bar charts with SE error bars and row data points show One-way ANOVA comparisons with C»C as reference group. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I= interaction). *P*-values of significant differences and tendencies between treatment groups are displayed on the graph.

3.4.5 Expression of mitochondrial complex markers in offspring m. soleus

In contrast with the systemic metabolic effects described above, significant maternal OB diet effects were detected at the muscle tissue level. Maternal diet increased the expression of mitochondrial complex markers III and V in m. soleus, with no effect of- or interaction with- offspring diet. Interestingly, the pairwise comparison of the One-way ANOVA test showed a strong additive effect of maternal and offspring diet on both complexes, since OB»OB was the only significantly different group compared to C»C. Diet mismatch did not affect complex marker expression. The expression of complex I, II and IV markers was not affected by any of the examined factors.

Results of mitochondrial complex markers III and V expression are shown in Figure 5. Results of mitochondrial complex markers I, II and IV and PVDF-membranes of all samples are displayed in Supplementary file 5.

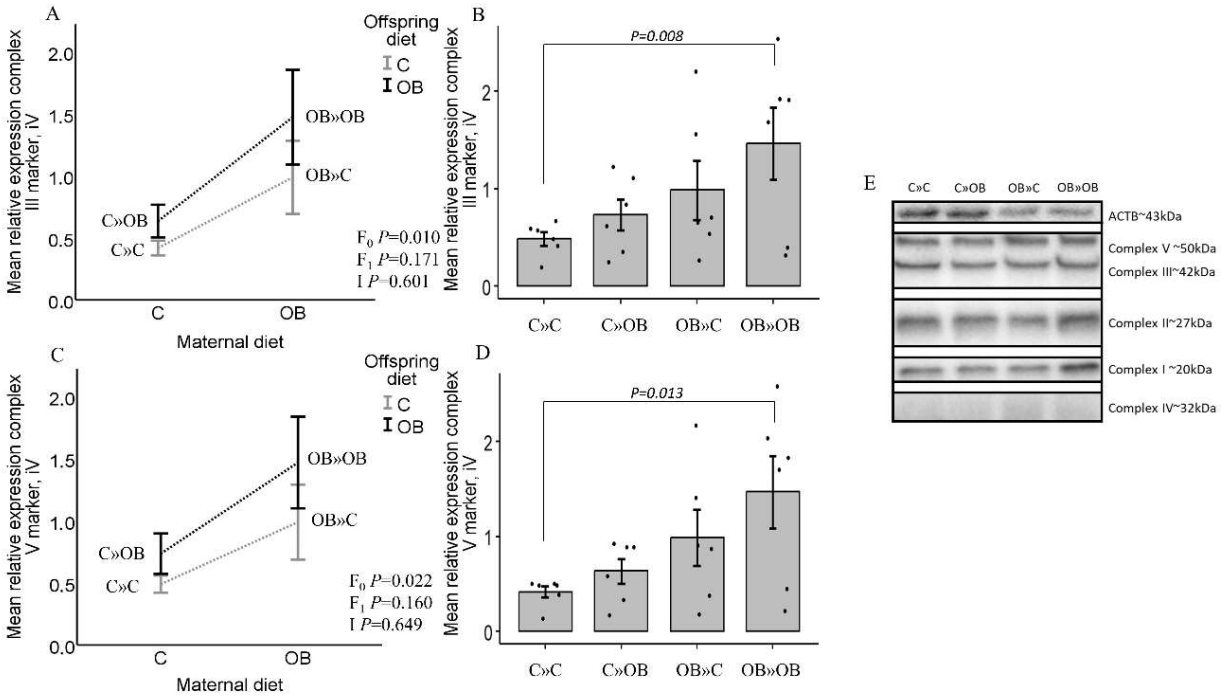

Figure 5. Maternal diet effects on offspring complex III and V marker expression. A. Graphs show the mean relative expression of complex III marker (A, B, iV) and complex V marker (C,D, iV) of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. All data are presented as mean ± S.E.M and are derived from 6 offspring/group born to 6 C and 6 OB mothers. Interaction plots show Two-way ANOVA analysis, bar charts with SE error bars and row data points show One-way ANOVA comparisons with C»C as reference group. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I= interaction). *P*-values of significant differences and tendencies between treatment groups are displayed on the graph. E. PVDF-Membrane of 3 replicates, showing complex I, II, III, IV (increased exposure time) and V, and housekeeping protein Beta-actin (ACTB).

3.4.6 Offspring mitochondrial ultrastructure in m. soleus

Maternal diet also significantly reduced the proportion of morphologically normal mitochondria and increased the proportion of small and large vacuolization in both SS and IMF mitochondria in m. soleus. Interestingly, this maternal diet effect was highly dependent on the offspring diet (significant interaction). Offspring diet effects were mainly detected in IMF mitochondria but less or not significant in SS mitochondria.

While the proportion of normal mitochondria was reduced in all treatment groups compared to C»C, it is notable that the lowest values were observed in the offspring born to obese mothers but fed a mismatched control diet (OB»C). This group exhibited significantly higher rates of both small and large vacuolization in SS and IMF mitochondrial. The dietary mismatch created in C»OB resulted in a different effect with only large but no small vacuolization. There was no additive effect of maternal and offspring diet effect noted (i.e. in OB»OB) since the rate of mitochondrial damage was comparable to other treatment groups. Results of offspring mitochondrial ultrastructure in m. soleus are displayed in Figure 6.

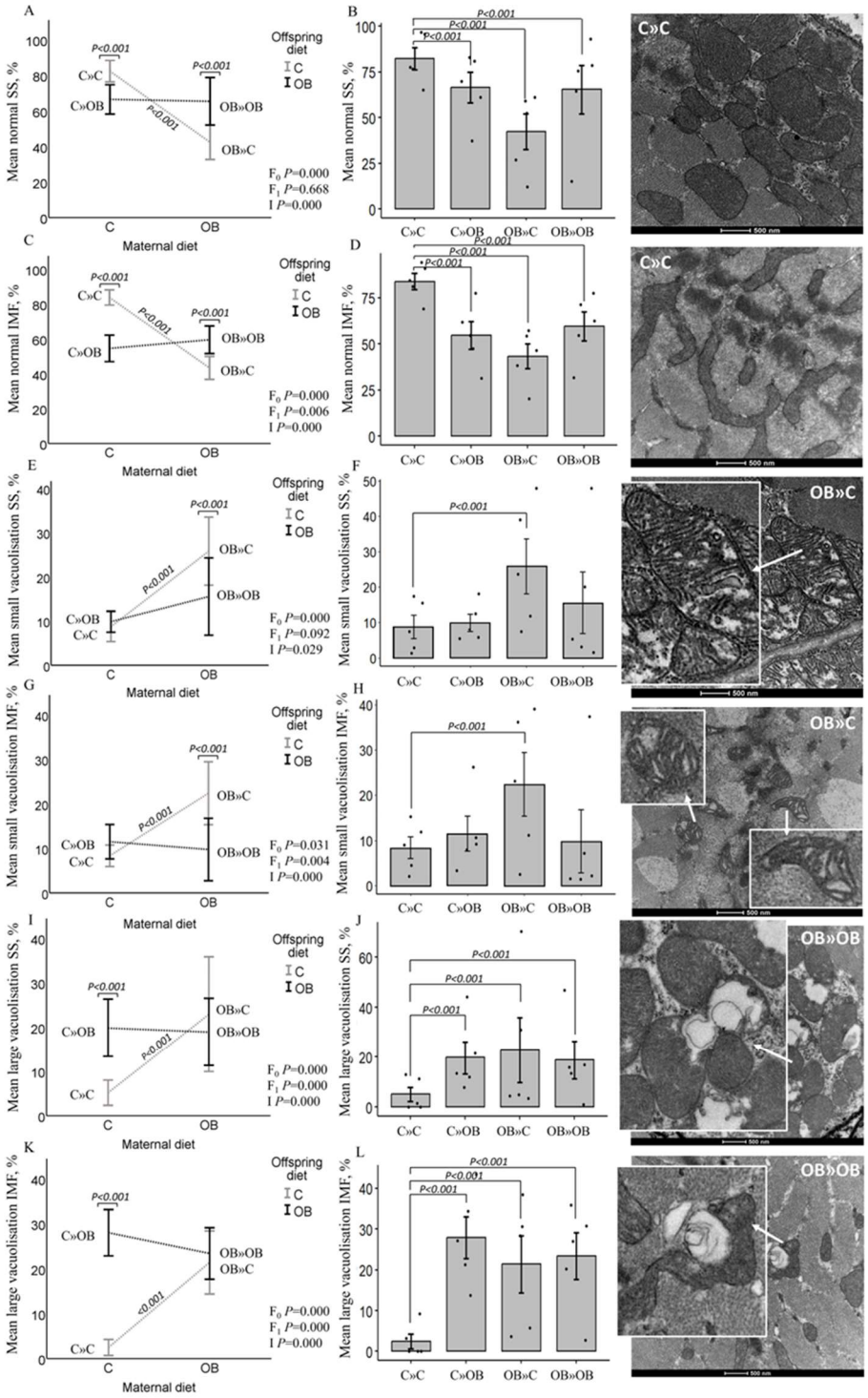

Figure 6. Maternal diet effects on offspring muscle SS and IMF mitochondrial ultrastructure. Mitochondrial ultrastructural morphology was classified and reported as homogenous SS (A, B) and IMF (C, D) mitochondria, small vacuolised SS (E, F) and IMF (G, H) and large vacuolised SS (I, J) and IMF (K, L) muscle mitochondria of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented using interactions plots and bar charts with SE error bars and row data points using binary logistic regression. Representative pictures for each mitochondrial phenotype are shown (right). All data are presented as mean ± S.E.M and are derived from 5 offspring/group born to 5 C and 5 OB mothers. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I= interaction). *P*-values on the interaction plots represent pairwise comparison performed due to the significant interaction. *P*-values of significant differences between treatment groups compared to C»C are displayed on the bar chart.

3.4.7 Effect sizes

Finally, we provide an overview of the effect sizes of the different treatment groups compared to C»C in all the examined outcome measures. Notably, the effect of OB»C (denoted by orange triangles) is always distinct and sometimes opposite (in abdominal fat weight, ITT (AUC), basal glycemia, ER30, insulin, and cholesterol) from the other two groups (C»OB and OB»OB) in which offspring diet effect is prominent. We also notice that the effect sizes of C»OB and OB»OB compared to C»C are mostly similar, showing no additive effects of maternal and offspring diets on metabolic parameters or muscle mitochondrial features.
Effect sizes are displayed in Figure 7.

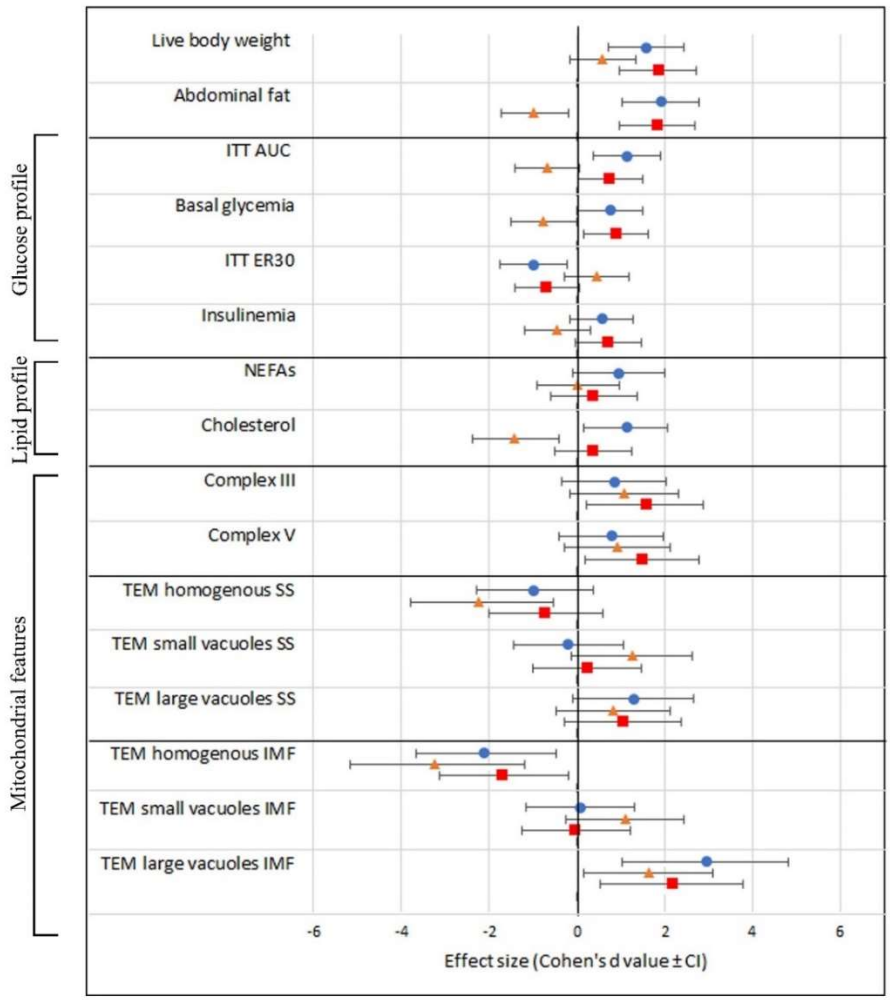

Figure 7. Schematic overview of all investigated outcome parameters with effect sizes estimated using Cohen's d. The black vertical line represents the reference group C»C. Effect sizes are shown as Cohen's d value ± CI and blue dot (equals C»OB), orange triangle (equals OB»C) and red square (equals OB»OB).

3.5 Discussion

We aimed to study the effect of maternal and offspring OB diets and their interaction on offspring health and metabolism using an outbred mouse model. On top, we aimed to examine if the dietary effects are influenced (attenuated or increased) by the match or mismatch between maternal and offspring's diet. This is the first study to perform such intergenerational analysis in a 2 x 2 factorial design using an outbred mouse model, which increases the pathophysiological relevance in a set-up that mimics the human situation of familial obesity. We chose to focus on female offspring health, since increased prevalence of obesity and metabolic diseases particularly in women across generations is alarming worldwide, with not only physical health but also significant psychological and social implications. In addition, while studying the same effects in male offspring in parallel can be of additive value, this would have doubled the data and significantly complicated the experimental design, data presentation, statistical comparisons and discussion in this report. Therefore, this was practically not feasible.

The results we have generated in C-born pups fed an OB diet (**C»OB *vs.* C»C**) can be compared with the results of other studies investigating the direct effect of an OB diet in F_0 female mice following standard breeding protocols. C»OB pups showed increased body weight and abdominal fat, and significantly increased basal blood glucose levels. An increased AUC of the ITT and a slower clearance of glucose after insulin injection (ER30) indicate a reduced glucose tolerance due to an impaired sensitivity to insulin. Systemic glycemia reflects the balance of liver gluconeogenesis (starving or pre-prandial) and glucose uptake in somatic tissue (Freeman et al., 2023). However, no liver functions were assessed in this study and only general liver damage was estimated through ALT measurements. On top, these pups also exhibited increased serum cholesterol and NEFA concentrations. Our data are in line with other studies reporting the direct response to OB diets in both inbred mice (male and female) (Wang and Liao, 2012; Vinué et al., 2018; Avtanski et al., 2019) and outbred mice (female) (Marei et al., 2020a; Smits et al., 2021; Smits et al., 2022). This validates the model used in the present study. Fasting cholesterol levels were relatively high in the control mice, but are in line with previous observations under the same experimental conditions (Marei et al., 2020).

Since mitochondrial dysfunction plays a key role in the development of reduced insulin sensitivity, our next step was to look at mitochondrial features in muscle tissue. Muscle tissue accounts for 60-70 % of the insulin-stimulated glucose uptake and is described as a primary determinant of metabolic disorders (Montgomery et al., 2017; Mikovic and Lamon, 2018; Sun et al., 2020; Smits et al., 2022). We noticed that the offspring OB diet-induced systemic alterations were indeed associated with mitochondrial abnormalities in muscle. While the expression of the mitochondrial complex markers in offspring muscle remained unaffected, offspring OB diet clearly altered offspring muscle mitochondrial morphology, with a significant increase in mitochondria with large vacuoles and a concomitant reduction in the percentage of normal mitochondria. These effects were different between the SS and IMF, since Two-way ANOVA analysis showed a significant reduction of normal mitochondria only in IMF and not in SS mitochondria in response to offspring diet. Koves et al. (2005) stated that SS and IMF mitochondria of gastrocnemius muscle react differently to environmental changes due to increased uncoupling protein 3 content in SS mitochondria, a protein important in fatty acid metabolism, redox regulation and ROS protection (Mailloux and Harper, 2011; Crescenzo et al., 2014). A human study performed by Chomentowski et al. (2011) showed that in m. vastus lateralis only IMF, and not SS, mitochondrial volume density was correlated with BMI. Also, IMF mitochondria were affected in patients with insulin resistance, whereas the SS mitochondria were not (Chomentowski et al., 2011). In our study, these differences were rather insubstantial.

Our main focus in this study was to investigate the maternal diet effects, and if the offspring diet effects were dependent on the maternal OB background. Maternal OB diet as a factor resulted in marked

significant effects on muscle mitochondrial morphology and expression of mitochondrial complex III and V markers. An interaction between the maternal and offspring diet effects was also significant (or tended to be significant) on basal insulin levels, and on muscle mitochondrial ultrastructural features. These results are in line with what has been previously described in studies using a similar experimental design (Saben et al., 2016; Dearden et al., 2020). However, in contrast, we were surprised that despite the major effects on muscle mitochondrial morphology, no maternal diet effects could be detected on adult offspring body weight, blood NEFA concentrations and glucose homeostasis in response to insulin. We could even detect a tendency to a reduced total blood cholesterol levels and abdominal fat in offspring born to OB mothers. This is all contradictory to what has been described in inbred mouse models (Samuelsson et al., 2008; Keleher et al., 2018). To further contemplate these results, and also to find out whether the sensitivity to the diet is influenced (attenuated or increased) by the match or mismatch between maternal and offspring's diet, it was necessary to study the differences and effect sizes in direct comparisons with the C»C mice as the most biologically healthy control group.

Focusing on the **OB»C** group, while these mice were slightly but significantly heavier than C»C, they had (or tended to have) a lower abdominal fat weight, lower serum cholesterol, reduced basal glycemia and reduced AUC of glycemia during the ITT, with normal ER30 and mean insulin concentrations. So an OB maternal background only seems to result in a higher sensitivity to insulin and a better metabolic blood profile in the offspring fed a control diet after weaning. This is interesting, since we expected the maternal OB diet to worsen the metabolic profile of the offspring (even when fed a C diet) instead of improving it as described by others. Our results contradict the findings of Samuelsson et al. (2008) who associated maternal OB diet with an increased weight gain, increased risk of hyperglycemia, hypertension, fatty liver disease and reduced insulin sensitivity both in male and female pups. The latter study used inbred C57BL/6 mice, highlighting again the possible influence of the genetic background and inbreeding. It is also surprising that the normal (or slightly improved) metabolic profile in OB»C occurs while a significant reduction in the proportion of morphologically normal mitochondria was found in the m. soleus muscles. The mitochondria in the OB»C mice were specifically characterized by a significant increase in small vacuolization, with larger intercristal spaces. Increased intercristal space may be indicative for intra organellar accumulation of fatty acids, since Ho et al. (2002) and Schrauwen et al. (2010) described that fatty acids can translocate into the mitochondrial matrix, bypassing acyl-Coenzyme A synthase and carnitine palmitoyl acyl transferase I, resulting in the accumulation of fatty acids inside the mitochondria. This feature is observed without occurrence of hyperlipidemia. Contrarily, maternal OB diet tended to improve offspring serum cholesterol in these mice. Such phenotype is similar to that described in the Athlete Paradox, where an increase in fat deposition in muscle tissue was an adaptive mechanism of muscles associated with a more efficient muscle energy production in highly trained athletes, since these lipid droplets were used as a direct energy source, obviously not as a consequence of hyperlipidemia (Goodpaster et al., 2001; Sergi et al., 2019b). However, since muscle cellular respiratory functions, metabolic intermediates, lipid content and lipid peroxidation levels were not analyzed in the present study, further investigations are required to tests these notions at a functional level. In addition, since we show that increased mitochondrial ultrastructure abnormalities due to maternal diet effects are not detrimental for offspring health up to 10 weeks of age, studies using mitochondrial ultrastructural changes in muscle tissue as a functional indication or explanation of altered individual metabolic health should take our results into considerations.

Last but not least, the metabolic profile and cellular characteristics of the **OB»OB** mice in the present model were also unexpected. Based on previous reports, we anticipated stronger synergetic effects due to increased sensitivity to the OB diet in pups born to OB mothers. Keleher et al. (2018) showed that female offspring born to OB mothers (in inbred SM/J mice) then fed an OB diet had exacerbated obesity with increased abdominal fat weight, compared to offspring born to lean mothers then fed an OB diet. Interestingly, in the present study using outbred Swiss mice, the abdominal fat weight of OB»OB was similar to C»OB. The effect sizes induced by the OB»OB exposure on most of the investigated blood

parameters were very similar, if not smaller, compared to the effects of C»OB compared with C»C(namely on abdominal fat, ITT(AUC), basal glycemia, ER30, NEFAs and cholesterol). In contrast with C»OB, the numerical increase in serum cholesterol and NEFA in OB»OB were not significant compared to C»C. Therefore, the matching OB»OB dietary exposure appears to result in a more favorable metabolic profile than the C»OB group. In addition to the strain differences, other factors may explain these contrasting results. For example, in the study of Keleher et al. (2018) pups were cross-fostered to lean mothers immediately after birth. In a study cross-fostering C-born pups to obese mothers during lactation, Monks et al. (2018) suggested that nourishing from a mother consuming an OB diet may protect the offspring from the OB diet-induced effects later in life. Since we did not use cross-fostering here, trying to mimic the natural situation in human the best way possible, this may partially explain the differences.

Another interesting observation was that while there was a tendency of an maternal x offspring diet interaction (with $P = 0.075$) on blood insulin levels, elevated concentrations of insulin were only significant in the OB»OB mice compared to C»C. While this may indicate a more severe reduction in insulin sensitivity, effects on basal glycemia were similar to that in OB»C, and changes in glucose levels during the ITT (AUC) were even better (only a tendency compared to C»C). Thus, our results could not confirm a synergetic or aggravating effect of maternal and offspring OB diets on offspring metabolic health parameters.

In addition, while maternal diet had a significant effect on muscle mitochondrial complex III and V marker expression, this increase was again only significant in the OB»OB group, and not in the OB»C group, compared to C»C. This is also opposite to what we expected because only a reduced expression of muscle mitochondrial complex I, II, IV and V markers was associated with insulin resistance (Lee et al., 2021). Mitochondrial complexes are an assembly of multiple polypeptide chains, arranged via functional intermediates and multiple chaperones (Vercellino and Sazanov, 2022). The electron transport system, including complex III, as well as complex V (ATP synthase) assembly and functions are key elements in energy provision and cellular functions (Nakamoto et al., 2008; Nolfi-Donegan et al., 2020). The increased expression of complex III and V markers may be adaptive mechanisms to compensate for mitochondrial damage and to improve mitochondrial energy production efficiency. We believe that these alterations at the cellular level are indicative for adaptation of offspring towards an energy-rich environment. However, mitochondrial proteins can be regulated by post-translational modifications and super complex assembly. Therefore, functional tests are still required to confirm these notions. Complex V assembly is also associated with changes in mitochondrial ultrastructure (Jonckheere et al., 2012). Abnormal complex V dimerization (due to depleting specific subunits) may hamper normal cristae formation and results in onion-like structures (Paumard et al., 2002) similar to the features observed here in the mitochondria with large vacuoles, which were increased both due to maternal and offspring diet. Mitochondria with large vacuoles containing loose inner membranes are also suggested by Cook et al. (2014) to be indicative for mitophagy, a temporary process by which damaged mitochondria are removed. Increased mitophagy is described as an adaptive response to improve insulin sensitivity (Ning et al., 2022). Future studies should include outcome parameters focusing on mitophagy. While the effect on the proportions of these mitochondria was similar in OB»OB compared to C»OB *vs.* C»C, the magnitude (effect size) was smaller, and numerically more normal mitochondria were detected. Also, the small vacuolization that was associated with the dietary mismatch in the OB»C group was not present in the OB»OB group. Mitochondrial shape, size and cristae formation always adapt in response to changed energy requirements (Kondadi et al., 2019). Therefore, considering the metabolic profile, associated with changes in mitochondrial morphology and complex III and V marker expression, OB»OB Swiss mice appear to perform better compared to C»OB.

The suggested changes in the OB»OB group may be adaptive, and in line with the hypothesis of Holt (2023), stating that the environmental developmental plasticity allows species to quickly adapt to changes over following generations. Bateson et al. (2014) also indicated with the Predictive Adaptive Response hypothesis that early life signals, predominantly stemming from the maternal environment, significantly

influence an individual's phenotype development, aiming at a better adaptation to similar environmental conditions later in life. The Dutch famine Cohort study revealed that increased prevalence of obesity was seen in offspring conceived during the Dutch famine of 1944-1945, showing that perturbations established during gestation may contribute to the development of offspring obesity in later life (Ravelli et al., 1999) and highlighting the importance of matching maternal and offspring diets. Vice versa, we believe that our data imply that the OB-born offspring are adapted to an energy-rich environment and thus ultimately support Barker's theory of adaptational mechanisms towards changed nutrient availability, and the Thrifty Phenotype Hypothesis (Barker, 1997b).

We have previously shown that Swiss mice are responsive to the HF/HS diet leading to an increase in weight gain, including hypercholesterolemia, and altered serum glucose (GTT) and insulin tolerance (ITT) after 7 weeks of feeding (Smits et al., 2021). In the present study, to avoid maternal stress before or during pregnancy that may interfere with the study results on offspring's metabolic profile (Enes-Marques and Giusti-Paiva, 2018; Sheng et al., 2023), we decided not to assess insulin sensitivity during gestation. Only maternal weight gain was recorded, and shown to be significantly increased in OB mothers after 7 weeks, compared to C-fed mothers. Offspring were sacrificed at 10 weeks of age, while often older mice are used in studies focusing on insulin sensitivity and metabolic health (Fraulob et al., 2010; Ravichandran et al., 2019; Lee et al., 2021). Therefore, it is possible that offspring aging, coinciding with age-related mitochondrial dysfunction, may still affect or aggravate the mitochondrial features and the metabolic profile later in life. This may lead to more distinct differences between the treatment groups investigate in our study. Also, in-depth intergenerational research may be needed to investigate mitochondrial functions in muscle tissue of OB-born offspring, to dissect the cause of the mitochondrial adaptation. Finally, since sex-differences may occur in the development of metabolic disorders (Tramunt et al., 2020), comparison between males and females can be of additive value in future intergenerational research to better understand the intergenerational impact on disease susceptibility.

In conclusion, this study is the first to investigate the effect of maternal and offspring OB diets and their interaction on offspring metabolic health using an outbred Swiss mouse model. While maternal diet was associated with abnormalities in muscle mitochondrial morphology, effects of the maternal OB background on the metabolic profile were very limited. On the contrary, offspring born to obese mothers and fed a normal diet seemed to have an improved metabolic health during early adulthood. No additive effects (increased sensitivity) to an obese offspring diet were observed in pups born to obese mothers. In contrast, the metabolic profile appeared to be better than those born to lean mothers and fed OB diet. These results are in line with the Thrifty Phenotype Hypothesis, suggesting that OB-born offspring are better adapted towards an environment with a high energy availability later in life. These results differ from previous reports using inbred mice, highlighting the importance of the model when designing and interpreting intergenerational studies. We have shown that, using a murine outbred model, maternal OB diets as such do not embark upon female familial obesity in the following generations.

Acknowledgements

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3.6 Supplementary file 1

3.6.1 Post-weaning data

Table S1.1. Post-weaning data of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are shown as mean ± S.E.M. Significant differences between treatment groups and C»C are shown by different letters (a, b). Tendencies are reported as dollar signs ($).

	C»C	C»OB	OB»C	OB»OB
Body weight (g)	24.38±1.03^{a}	39.37±0.86^{b}	33.48±0.56^{b}	40.61±0.86^{b}
Abd. Fat weight (g)	1.37±0.12^{a}	2.83±0.25^{b}	0.90±0.0.14$^{b\$}$	2.63±0.22^{b}
Cholesterol (ng/dl)	152.9±8.3^{a}	183.8±9.0^{b}	120.2±6.3^{b}	165.6±12.8^{a}
NEFA (mmol/l)	1.53±0.21^{a}	1.98±0.11^{b}	1.54±0.11^{a}	1.72±0.14^{a}
AUC (ITT)	15451.5±644.0^{a}	18435±702.3^{b}	12870±1238.9$^{b\$}$	17861.3±919.7$^{b\$}$
Basal glycemia (ng/ml)	160.3±4.3^{a}	175±5.6^{b}	147.5±4.3$^{b\$}$	176.1±4.7^{b}
ER30 (%/min)	0.8±0.2^{a}	0.3±0.1^{b}	1.2±0.3^{a}	0.3±0.2$^{b\$}$
Insulin (ng/ml)	0.2±0.1^{a}	0.5±0.1^{a}	0.1±0.0^{a}	0.7±0.2^{b}
C III (iV)	0.42±0.06^{a}	0.64±0.13^{a}	0.99±0.29^{a}	1.47±0.38^{b}
C V (iV)	0.49±0.07^{a}	0.74±0.16^{a}	0.99±0.30^{a}	1.47±0.37^{b}
Normal SS (%)	83.32±6.07^{a}	66.49±8.24^{b}	42.35±9.70^{b}	65.37±13.35^{b}
Normal IMF (%)	83.80±4.37^{a}	54.57±7.05^{b}	44.45±6.61^{b}	59.53±7.92^{b}
Small vac. SS (%)	8.71±3.36^{a}	9.85±2.39^{a}	25.81±7.70^{b}	15.53±8.75^{b}
Small vac. IMF (%)	8.33±2.41^{a}	11.50±3.86^{a}	22.39±7.07^{b}	9.74±7.02^{a}
Large vac. SS (%)	5.15±2.85^{a}	19.83±6.49^{b}	22.90±12.97^{b}	18.91±7.59^{b}
Large vac. IMF (%)	2.47±1.79^{a}	27.99±5.14^{b}	21.69±7.02^{b}	23.38±5.73^{b}

Table S1.2. Corresponding q-value of post-weaning data of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are shown as mean ± S.E.M. Significant differences between treatment groups and C»C are shown by different letters (a, b). Tendencies are reported as dollar signs ($).

Parameter	Comparison	***P*-value**	**q-value**	Parameter	Comparison	***P*-value**	**q-value**
Body weight	C»C - C»OB	0.001	0.048	C III	C»C - C»OB	0.549	0.561
Body weight	C»C - OB»C	0.020	0.042	C III	C»C - OB»C	0.125	0.167
Body weight	C»C - OB»OB	0.001	0.024	C III	C»C - OB»OB	0.008	0.020
Abd. fat	C»C - C»OB	0.001	0.016	C V	C»C - C»OB	0.502	0.524
Abd. fat	C»C - OB»C	0.086	0.118	C V	C»C - OB»C	0.180	0.216
Abd. fat	C»C - OB»OB	0.001	0.012	C V	C»C - OB»OB	0.013	0.030
AUC (ITT)	C»C- C»OB	0.025	0.046	Normal SS	C»C - C»OB	0.001	0.010
AUC (ITT)	C»C - OB»C	0.074	0.108	Normal SS	C»C - OB»C	0.001	0.008
AUC (ITT)	C»C - OB»OB	0.021	0.042	Normal SS	C»C - OB»OB	0.001	0.007
B glycemia	C»C - C»OB	0.034	0.058	Normal IMF	C»C - C»OB	0.001	0.006
B glycemia	C»C - OB»C	0.064	0.096	Normal IMF	C»C - OB»C	0.001	0.005
B glycemia	C»C - OB»OB	0.021	0.040	Normal IMF	C»C - OB»OB	0.001	0.005
ER30	C»C - C»OB	0.051	0.079	S vac SS	C»C - C»OB	0.314	0.359
ER30	C»C - OB»C	0.168	0.218	S vac SS	C»C - OB»C	0.001	0.004
ER30	C»C - OB»OB	0.084	0.119	S vac SS	C»C - OB»OB	0.174	0.220
Insulinemia	C»C - C»OB	0.256	0.300	S vac IMF	C»C - C»OB	0.038	0.063
Insulinemia	C»C - OB»C	0.415	0.443	S vac IMF	C»C - OB»C	0.001	0.004
Insulinemia	C»C - OB»OB	0.010	0.024	S vac IMF	C»C - OB»OB	0.178	0.219
Cholesterol	C»C - C»OB	0.026	0.046	L vac SS	C»C - C»OB	0.001	0.004
Cholesterol	C»C - OB»C	0.019	0.041	L vac SS	C»C - OB»C	0.001	0.003
Cholesterol	C»C - OB»OB	0.345	0.385	L vac SS	C»C - OB»OB	0.001	0.003
NEFA	C»C - C»OB	0.040	0.064	L vac IMF	C»C - C»OB	0.001	0.003
NEFA	C»C - OB»C	0.958	0.958	L vac IMF	C»C - OB»C	0.001	0.003
NEFA	C»C - OB»OB	0.368	0.401	L vac IMF	C»C - OB»OB	0.001	0.003

B glycemia = basal glycemia, Abd. Fat = abdominal fat weight, S vac = small vacuolization, L vac = large vacuolization.

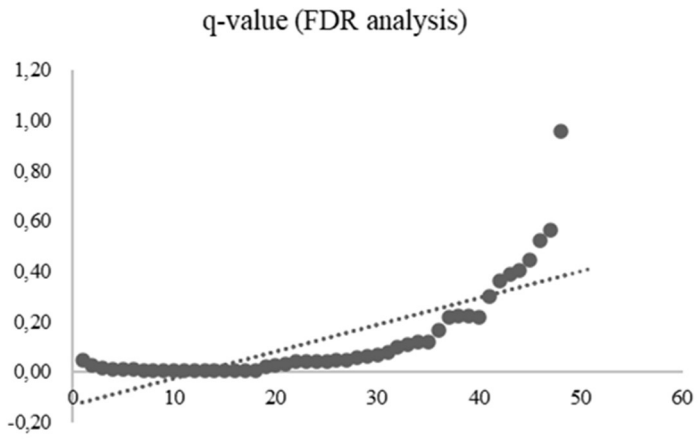


Figure S1.1 Q-values of post-weaning data of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design.

3.7 Supplementary file 2

3.7.1 Maternal live body weight

During the trial, mothers were weighed weekly. Maternal live body weight significantly differed between C-fed mothers (32.97 ± 1.24, g) and OB-fed mothers (41.15 ± 1.69, g) after being fed their corresponding diet for 7 weeks (*P*<0.001). The maternal growth curve is shown in figure S2.1, including the corresponding *P*-values for each timepoint.

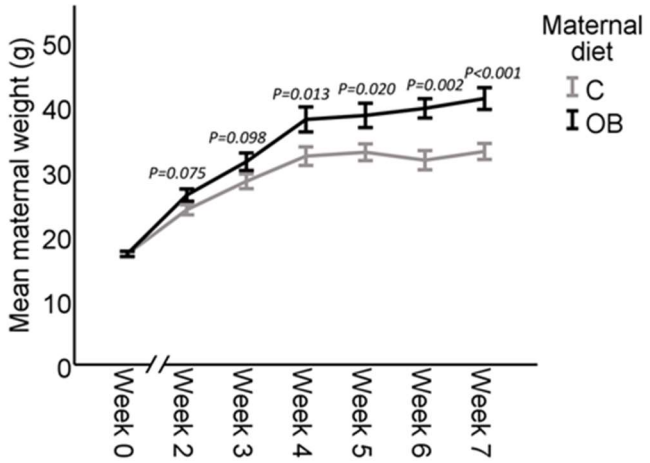


Figure S2.1. Maternal growth curve of mothers fed a C or an OB diet for 7 weeks. Data are shown as mean ± S.E.M. and are derived from 14 C-fed and 15 OB-fed mothers. *P*-values of significant differences and tendencies are displayed on the graph.

3.7.2 Litter characteristics.

Table S2.1. Litter characteristics of C- and OB- mothers, and their correlation with the maternal live body weight at mating. Data are shown as mean ± S.E.M. The corresponding *P*- and r- values of the correlations are stated.

	Litter size	Litter weight (g)	Pup weight (g)	Female pups (%)	Male pups (%)
C mothers	14.6 ± 0.8	33.0 ± 0.9	2.4 ± 0.5	52.3 ± 3.5	60.9 ± 10.5
OB mothers	14.2 ± 0.4	31.7 ± 0.9	2.2 ± 0.1	51.5 ± 3.2	49.1 ± 2.1
P-value	0.904	0.943	0.58	0.842	0.335
r-value	0.020	0.012	-0.093	0.034	-0.161

The original litter size was not correlated with adult offspring body weight (r = -0.109, *P*=0.974).

3.8 Supplementary file 3

3.8.1 Offspring body weight and abdominal fat weight at weaning

Table S3.1. Pre-weaning data of offspring body weight and abdominal fat weight of offspring born to C- and OB-mothers. Data are shown as mean ± S.E.M. Significant differences are shown by different letters (a, b). Tendencies are reported as dollar signs ($).

	C-born pups	OB-born pups
Body weight (g)	14.02 ± 0.67[a]	16.14 ± 0.15[b]
Weight abdominal fat (g)	0.06 ± 0.01[a]	0.12 ± 0.01[b]

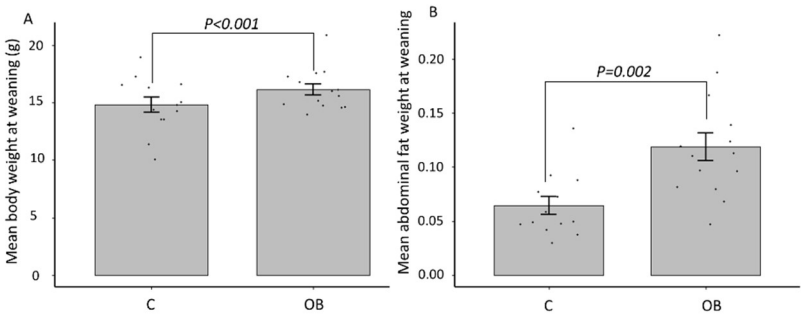


Figure S3.1. Maternal diet effect on offspring live body weight and abdominal fat weight at weaning. Bar chart with SE and row data points of mean offspring live body weight (A, g) and offspring abdominal fat weight (B, g) at weaning. Data are presented as mean ± S.E.M and are derived from at least 2 offspring born to 6 C and 7 OB mothers. Corresponding *P*-values are displayed on the graph.

3.8.2 Offspring body weight trajectory after weaning

After weaning, offspring body weight trajectory was affected by time (P = <0.001), offspring diet (P = 0.027), and their interaction (P < 0.001). Maternal OB diet significantly increased offspring body weight at week 1 (P < 0.001), wk2 (P = 0.001) , wk3 (P = 0.006) and wk4 (P = 0.083) post-weaning, but this effect disappeared from 5wk onwards. The effect of the offspring OB diet was already significant at wk1 post-weaning and was further significant until sample collection at wk7 (10 weeks of age). During this growth trajectory, the maternal OB diet never interacted with the effect of the offspring diet.

3.9 Supplementary file 4

3.9.1 Offspring serum alanine aminotransferase (ALT) and triglycerides (TG)

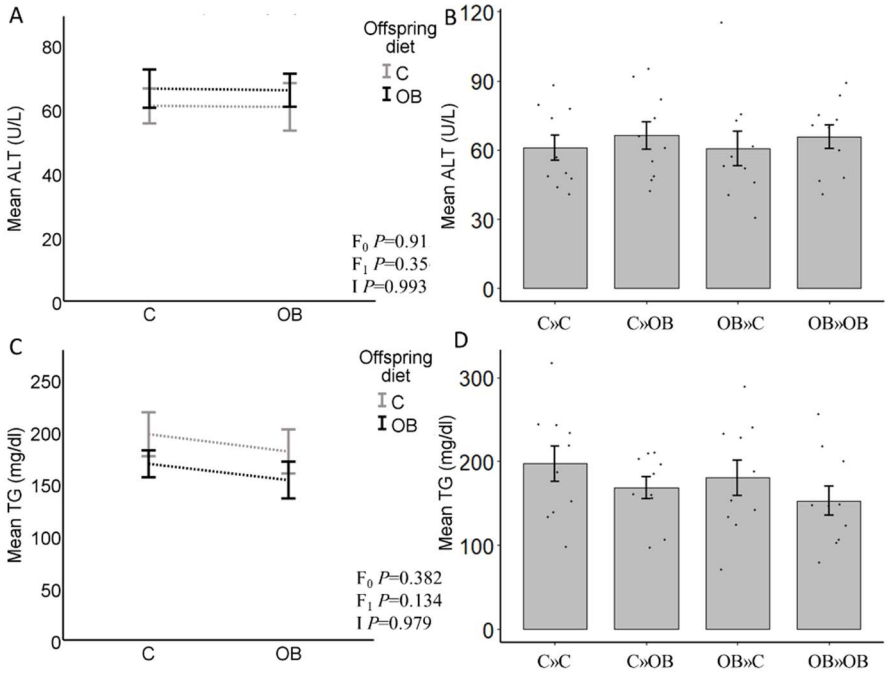


Figure S4.1. Maternal diet effects on offspring serum ALT and TG concentrations. Interaction plots (A,C) and bar chart (B, D) of mean serum ALT (U/L) and TG (mg/dl) from offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as mean ± S.E.M and are derived from 10 offspring/group, born to 6 C and 7 OB mothers. Interaction plots show Two-way ANOVA analysis, bar charts with SE error bars and row data points show One-way ANOVA comparisons with C»C as reference group. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I= interaction).

3.10 Supplementary file 5

3.10.1 Offspring muscle mitochondrial complex I, II and IV marker expression

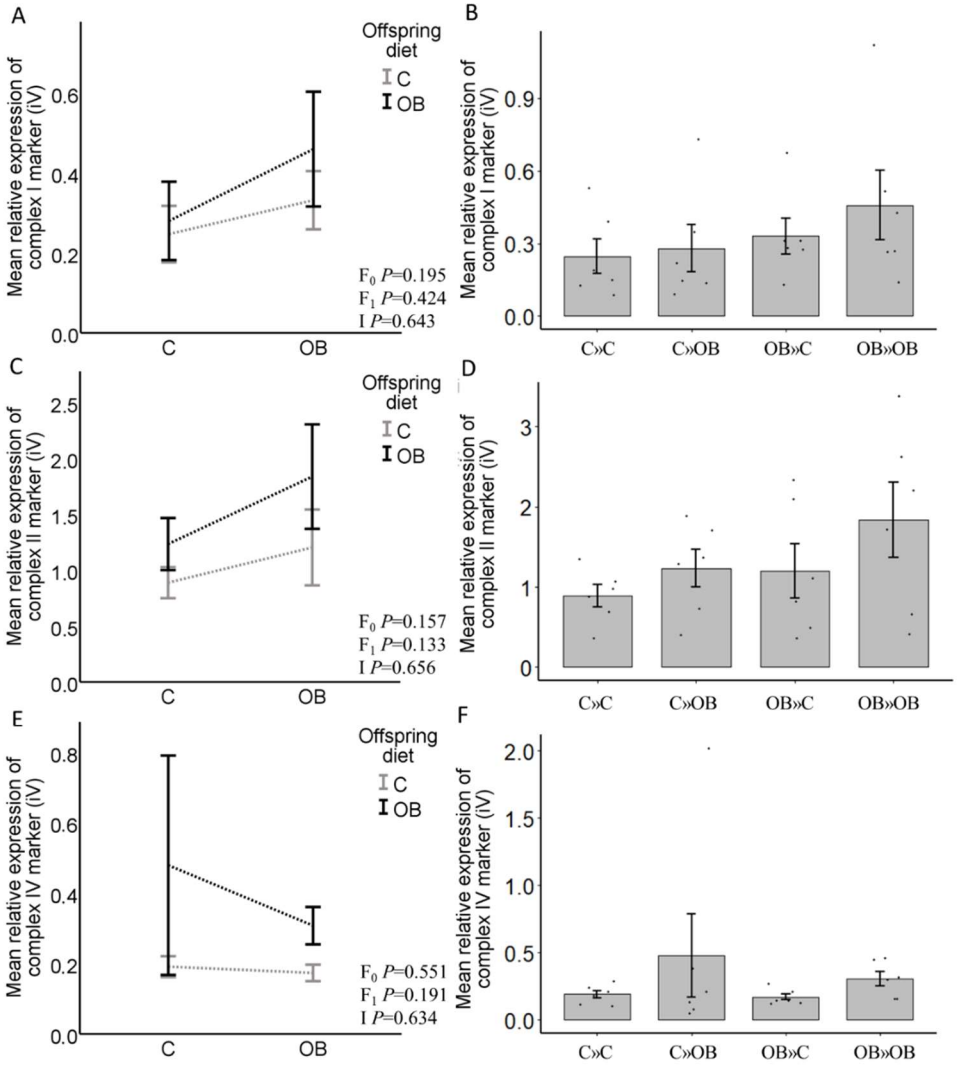


Figure S5.1. Maternal diet effects on offspring complex I, II and IV marker expression (muscle). A. Graphs show the mean relative expression of complex I marker (A, B, iV), complex II marker (C,D, iV) and complex IV marker (E,F) of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. All data are presented as mean ± S.E.M and are derived from 6 offspring/group born to 6 C and 6 OB mothers. Interaction plots show Two-way ANOVA analysis, bar charts with SE error bars and row data points show One-way ANOVA comparisons with C»C as reference group. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I= interaction).

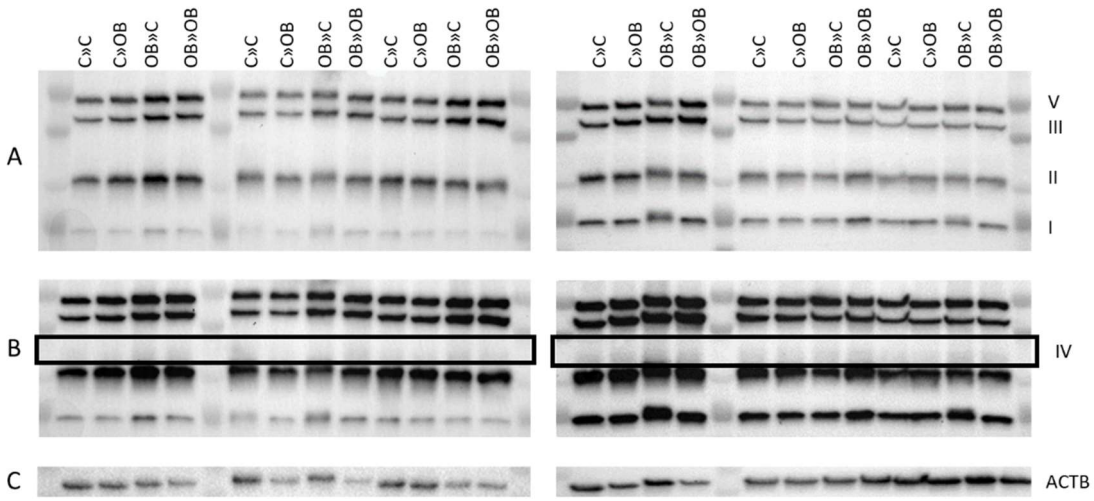
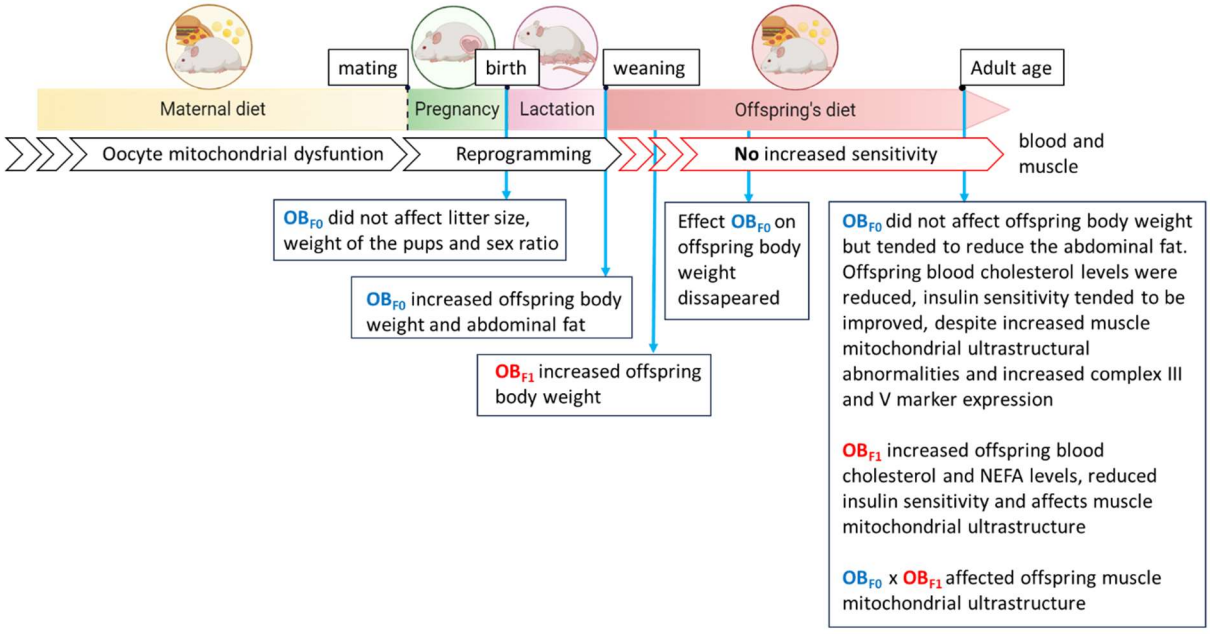


Figure S5.2. PVDF-Membrane of all replicates, showing complex I, II, III, IV (increased exposure time) and V, and housekeeping protein Beta-actin (ACTB). A. PVDF-membrane for measuring mitochondrial complex I, II, III and V expression. B. PVDF-membrane prolonged exposure (290s) for measuring complex IV expression. C. PVDF-membrane for measuring Beta-actin (ACTB) as housekeeping protein after stripping, to correct for differences in sample load.

3.11 Key message

The direct effect of the offspring's OB diet was confirmed to perturb the adult offspring's metabolic profile in outbred Swiss mice. Considering the relatively young offspring age (10 weeks) that we have investigated, the maternal OB diet did not hamper the offspring's metabolic profile, or increased the offspring's sensitivity to an OB diet. Maternal OB diet consumption even improved the offspring's metabolic profile, despite the changes present in the offspring muscle mitochondria.

3.12 Graphical summary



Schematic overview of the main effect of the maternal OB diet (OBF_0), the offspring OB diet (OBF_1), and the maternal x offspring diet interaction (OBF_0 x OBF_1) on offspring health at birth (age = less than 1h), weaning (age 3 weeks) and adulthood (age 10 weeks). Results of the pairwise comparisons are not included in this overview.

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Chapter 4

The Impact of a Maternal and Offspring Obesogenic diet on Daughter's Oocyte Mitochondrial Ultrastructure and Bioenergetic Responses. Insights from an Outbred Mouse Model

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4.1 Abstract

Obesity affects oocyte mitochondrial functions and reduces oocyte quality and fertility. Obesity may also increase the risk of metabolic disorders in the offspring. Children are likely to follow their parents lifestyle and diet, which also contributes to the increased prevelance of obesity across generations. We hypothesise that the impact of obesogenic (OB) diet and obesity on oocyte mitochondrial functions is different in offspring born to obese mothers compared to those born to healthy mothers. To test this hypothesis, we fed a control (C, 10 % fat, 7 % sugar) or an OB diet (60 % fat, 20 % sugar) to female mice (for 7 weeks (w)) and then to their female offspring (for 7w after weaning) in a 2 x 2 factorial design (C»C, n = 35, C»OB, n = 35, OB»C n = 49 and OB»OB, n = 50). Unlike many other studies, we used an outbred Swiss mouse model to increase the human pathophysiological relevance. Offspring were sacrificed at 10w and their oocytes were collected. Offspring OB diet increased oocyte lipid droplet content, mitochondrial activity and reactive oxygen species (ROS) levels, altered mitochondrial ultrastructure and reduced oocyte pyruvate consumption. Mitochondrial DNA copy numbers and lactate production remained unaffected. Mitochondrial ultrastructure was the only factor where a significant interaction between maternal and offspring diet effect was detected. The maternal OB background resulted in a small but significant increase in offspring's oocyte mitochondrial ultrastructural abnormalities without altering mitochondrial inner membrane potential, active mitochondrial distribution, mitochondrial DNA copy numbers, or ROS production. This was associated with reduced mitochondrial complex III and V expression and reduced pyruvate consumption which may be compensatory mechanisms to control mitochondrial inner membrane potential and ROS levels. Therefore, in this Swiss outbred model, while offspring OB diet had the largest functional impact on oocyte mitochondrial features, the mitochondrial changes due to the maternal background appear to be adaptive and compensatory rather than dysfunctional.

Key words

DOHaD; Intergenerational diseases; Oocyte quality; High-fat diet; Maternal obesity; Mitochondrial quality

4.2 Introduction

Obesity, linked to a sedentary lifestyle and an unbalanced diet, is a globally increasing problem leading to disappointing fertility. Obesity represents a major health issue associated with cardiovascular diseases, metabolic syndrome, insulin resistance and fatty liver disease among other metabolic disorders (Keller and Lemberg, 2003; Blondeau et al., 2011; Cardozo et al., 2011; Jialal et al., 2014; Johns et al., 2015; Broughton and Moley, 2017; Liu et al., 2021; Gonzalez et al., 2022). Almost 60 % of women in Europe are overweight or obese. Furthermore, a steep increase is also seen in the proportion of children suffering from the same condition. About 30 % of pregnant women suffer from obesity, which is known to increase the risk of offspring health issues (Armitage et al., 2008; Gahagan et al., 2012; Gaillard, 2015) such as metabolic syndrome, obesity and diabetes (Armitage et al., 2008; Bansal and Simmons, 2018). This could be replicated in mouse models fed an obesogenic (OB) diet during pregnancy while offspring were kept on a standard diet after weaning (Samuelsson et al., 2008; Igosheva et al., 2010; Jungheim et al., 2010; Keleher et al., 2018; Andreas et al., 2019; McMurray et al., 2019). While there is an increasing number of studies that describe an impact of maternal obesity on offspring health, the consequences for offspring fertility remain largely underexplored.

A key factor in the pathogenesis of subfertility linked to metabolic health disorders is a directly affected oocyte (Robker, 2008; Cardozo et al., 2011; Antunes et al., 2014; Leary et al., 2015). Obesity reduces oocyte quality mainly by inducing lipotoxicity. Lipotoxicity is defined as lipid accumulation in non-adipose tissue (Engin, 2017), causing inflammatory responses and oxidative stress, resulting in deleterious effects such as mitochondrial dysfunction (Wu et al., 2010; Van Hoeck et al., 2013; Leroy et al., 2022). Obesity alters the lipid composition of the ovarian follicular fluid, the microenvironment in which oocyte growth and maturation takes place. This is associated with intracellular accumulation of reactive oxygen species, altered mitochondrial membrane potential, lower mitochondrial mass and less available ATP, affecting not only cellular bioenergetics in the oocyte but also in the subsequent embryo, since mitochondria are exclusively maternally inherited. Mitophagy was shown not to be activated in oocytes in response to mitochondrial dysfunction, suggesting that dysfunctional mitochondria may be transmitted to the embryo (Boudoures et al., 2017c). This will not only lead to subfertility in the affected patient, but can also lead to mitochondrial aberrations transmitted to the offspring, as claimed by Saben et al. (2016) in inbred C57BL/6 mice. More mitochondrial ultrastructural abnormalities were detected in oocytes and muscles of adult offspring born to diet-induced obese C57BL/6 mothers, suggesting a transmission of aberrant mitochondria through the female germline (Saben et al., 2016). On the other hand, other studies show evidence of clearance of damaged mitochondria in the embryos during the peri-implantation period. At this stage, the so called bottleneck phenomenon only allows embryonic cells with healthy mitochondria to survive, which is suggested to minimize the transfer of defective mitochondria to the offspring (Lee et al., 2012; Cox et al., 2021). Of course, it can also be assumed that de novo mitochondrial dysfunction may take place in the offspring during prenatal and/or early postnatal development (during lactation) under OB conditions.

In either cases, transmission or appearance of aberrant mitochondria in the early embryo and eventually the offspring, may directly influence metabolism and growth, and may render this next generation more sensitive or vulnerable to lipotoxic conditions upon consuming an OB diet. However, it may also induce many indirect effects on the developing embryo or the offspring, through alterations in epigenetic programming (Desmet et al., 2016). Oocyte maturation under lipotoxic conditions changes mitochondrial functions and DNA methylation in the resulting preimplantation embryo (Meulders et al., 2022; Taschereau et al., 2023). This can result in epigenetic modifications in imprinted and developmentally important genes, affecting implantation, placentation, fetal development and compromising postnatal health (Dearden and Ozanne, 2015). On top, children born to obese mothers are more likely to develop the same dietary preference, which may lead to an increased preference to an OB diet with a higher risk of developing metabolic disorders and other complications such as infertility (Keleher et al., 2018;

Pfledderer et al., 2021). However, the additive effect of the interaction between maternal and offspring OB conditions on the offspring's oocyte quality, to the best of our knowledge, has not been studied yet.

Therefore we hypothesized that the impact of an OB diet on oocyte quality and mitochondrial function is influenced by the offspring's maternal metabolic background. We aimed to study the influence of a maternal OB background on the effect of an offspring OB diet on oocyte quality and mitochondrial functions. For this an outbred mouse model was used in a two-by-two factorial design. We investigated the effect of 1) an offspring OB diet and/or 2) a maternal OB background and 3) the interaction between offspring and maternal diet, on offspring oocyte mitochondrial features. Our analyses were focused on oocyte lipid content and on oocyte mitochondrial qualitative and quantitative measures, including mitochondrial bioenergetic activity, morphology and cell metabolism.

4.3 Materials and methods

4.3.1 Animal model and Experimental design

This study was approved by the Ethical Committee for Animal Testing (ECD 2018-05). All animal procedures were performed in accordance with the relevant guidelines and regulations. We worked with outbred mice because inbred control fed C57BL/6 mice exhibit already a high rate of mitochondrial ultrastructural abnormalities in their oocytes (Marei et al., 2020) which may create a bias if used to answer the research questions listed above. A total of 27 female Swiss mice (F_0) and a total of 169 of their female offspring were used in this study. The mothers were either fed a control diet ("C", n=11, Sniff diets D12450J, containing 10 kJ % fat and 7 % sucrose (E157453-04),) or an OB diet (n=15, (E15741-34), 60kJ % fat (beef Tallow), 20 % fructose adjusted in the drinking water) for 7 weeks starting at 3 weeks of age. Mothers were weekly weighed for 7 weeks of being fed the corresponding diet. Detailed information on maternal weight is included in supplementary file 1. All females were mated with Swiss males (n=4) fed a standard chow diet, in a cross over design (i.e. each male is used in both groups). Litter sizes were equalized at 10 pups, to correct for differences in nutrient availability from postnatal day 5 to 22 to equalize establishment of maternal imprints. Detailed information on litter size is included in supplementary file 1. Female F_1 offspring from each litter were equally divided at weaning on a C or OB diet creating a 2 x 2 factorial study design, ultimately resulting in 4 treatment groups: 1) C»C, n=35 offspring born to control mothers and fed a control diet, 2) C»OB, n=35 offspring born to control mothers, and fed an OB diet 3) OB»C, n=49 offspring born to obese mothers and fed a control diet 4) OB»OB, n=50 offspring born to obese mothers and fed an OB diet. Offspring were weaned at 3 weeks and were fed their corresponding diet for 7 weeks afterwards. Adult offspring were weighed weekly and sacrificed by decapitation at 10 weeks of age after intraperitoneal injection of 10IU PMSG (Pregnant Mare Serum Gonadotropin, Folligon 1000UI, MSD Intervet, Boxmeer, The Netherlands) and 10IU hCG (human choriogonadotropin, Pregnyl 5000IE, MSD Intervet), respectively. Decapitation was quickly performed by trained personnel (FELASA C) using a sharp blade. Blood was collected for total serum cholesterol, enzymatically analyzed (A.M.L., Antwerp, Belgium) on an Abbott Architect c16000 (Abbott, Illinois, U.S.A) as described by (Allain et al., 1974) and (Roeschla.P et al., 1974). Cumulus-oocyte complexes (COCs) were collected directly from the oviduct, i.e. after in vivo maturation and ovulation, immediately after euthanasia as described by Marei et al. (2020). One COC from each offspring was fixed in 2.5 % glutaraldehyde solution for transmission electron microscopy (TEM). The remaining COCs were denuded of cumulus cells using stripper tips fitted on EZ-grip (Origio) in droplets of L15 medium (Thermofisher Scientific), supplemented with 50IU/ml Penicillin G Sodium salt, 0.3mg/ml hyaluronidase and 10 % Fetal Bovine Serum. Oocytes were denuded and used directly or after fixation (as described in the methods sections) to determine the lipid droplet content (LDC), the mitochondrial inner membrane potential (MMP), the distribution of the active mitochondria and the reactive oxygen species (ROS) accumulation. The mitochondrial DNA copy number was assessed using qPCR and the oxidative phosphorylation (OXPHOS) was estimated by assessing electron transport chain (ETC) complex markers. The lactate production and pyruvate consumption of the oocytes was analyzed to estimate the cellular metabolic activity.

4.3.2 Assessment of oocyte lipid droplet content

Lipid droplet content (LDC) was assessed using BODIPY® staining and confocal microscopy. Offspring from 7-8 C mothers and 6-8 OB mothers were used for the analysis of the oocyte LDC. At least 5 oocytes per offspring (n = 7-8 offspring per group) were fixed in paraformaldehyde 4 % and stored in PBP containing 1 mg/mL PVP (PBS-PVP) at 4 °C for a maximum of 1 month. Oocytes were permeabilized in PBS containing saponin (0.1 %w/v) and glycine 0.1M solution, then washed and incubated in 20µg/ml BODIPY (BODIPY® 496/503, Thermo Fisher Scientific, Belgium) in PBS for 1h. The presence of a polar body was included in the analysis. After each step, oocytes were washed twice in PBS-PVP. Oocytes were mounted in glass-

bottom dishes in droplets of PBS-PVP and immediately examined using confocal microscope (Nikon Eclipse Ti-E inverted micro-cope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Zaventem, Belgium)). High resolution images were acquired with 488 nm diode lasers, excitation 493nm and 503 nm emission. Z-stack projections of 40µm with 1µm steps were taken for each oocytes. The volume of lipid droplets was measured using Volocity® software (Quorum Technologies Inc, Puslinch, Canada). To exclude background noise, objects smaller than 0.5 μm^3 were excluded from the analysis. Finally, lipid volume per oocyte was calculated based on the oocyte diameter.

4.3.3 Assessment of oocyte mitochondrial DNA copy number

DNA extracts from pools of at least 15 oocytes per litter from 11 C and 13 OB mothers were used to determine the absolute amount of mitochondrial DNA qPCR of the mitochondrial gene ND4. After simultaneous purification of genomic and total RNA using AllPrep® DNA/RNA MicroKit (#80284, Qiagen) and CYBR Green (SYBR Green supermix #172-5270, Bio-Rad) was mixed with 20pmol/µl forward and reverse primers in nuclease-free water and sample DNA. The absolute amount of mitochondrial DNA was measured using Avogadro's constant as "Number of copies=(ng*[$6.022*10^{23}$])/(length*[$1*10^{9}$]*650)" in a standard curve with known concentration and copies of ND4 in Bio-Rad CFX Manager 3.1.

4.3.4 Assessment of mitochondrial inner membrane potential, reactive oxygen species accumulation and distribution of active mitochondria

One offspring of each of the 6-8 C and 6-8 OB mothers was used to analyze the MMP, the active mitochondrial distribution and accumulation of reactive oxygen species (ROS), using specific staining and confocal microscopy. Only mature oocytes, with a polar body, were used for this assessment. Six to eight oocytes per offspring were incubated for 30 minutes at 6 % CO_2 37°C in L15 medium containing 5µg/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Invitrogen) and 2.5mM CellROX™ Deep Red Reagent (Thermo Fisher Scientific, Belgium) as described by Komatsu et al. (2014) and De Biasi et al. (2015) directly after collection of the oocytes. i.e. without a fixation step. Afterwards, oocytes were washed and transferred to equilibrated L15 medium droplets under mineral oil on 35 mm glass-bottom dishes. Images were taken using LeicaSP8 laser scanning confocal microscope (Leica Microsystems, Machelen, Belgium) in a humid chamber at 37°C. The uptake of JC-1 and formation of J-aggregates in the mitochondria is MMP dependent and hence used to estimate mitochondrial bioenergetic activity. Images from three confocal planes from each oocyte: the equatorial plane (at largest diameter), the peri-cortical plane (closest to the objective lens), and plane in-between) were acquired at excitation/emission 488/525 nm (monomers, mitochondria with low MMP or a reduced activity; green), 561/590 nm (formation of J-aggregates with high MMP, active mitochondria; yellow) and 644/665 nm (to detect CellROX or accumulation of ROS; red). The mean grey scale intensity of the MMP and ROS was afterwards quantified using ImageJ software. Active mitochondria are reported as mean grey scale intensity of the J-aggregates. Inactive mitochondria are reported as the mean grey scale intensity of the JC-1 monomers. The presence and thickness of the active mitochondrial ring in the peri-cortical region was measured using ImageJ and used to categorize the regional distribution of active mitochondria as peri-cortical or diffuse. Mitochondria showing no activity were categorized as uncoupled and mitochondrial aggregates were classified as aberrant clustering.

4.3.5 Assessment of mitochondrial ultrastructure in oocytes

One COC per offspring from 8 C and 8 OB mothers was fixed upon collection in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5 % glutaraldehyde solution, embedded in 2 % agarose blocks and washed in 0.1 M sodium cacodylate-7.5 % saccharose solution (pH 7.4). Then the blocks were incubated for 2 h in 1 % OsO4 solution and dehydrated in an ethanol gradient. Sections were cut ultrathin using EM-bed812, stained with lead citrate and examined with transmission electron microscope Tecnai G2 Spirit Bio TWIN microscope (Fei, Europe BV, Zaventem, Belgium) at 120 kV. For each oocyte, 10-20 random images were

acquired at 16500x magnification. Mitochondria were morphologically evaluated and classified blindly according to (Marei et al., 2020).

4.3.6 Assessment of mitochondrial electron transport chain complexes

Pools of 45-80 oocytes of 2-5 offspring from 3-4 C and OB mothers were used to analyse the expression of the ETC complex markers for Western blotting (WB) after collection. Different complex markers of the ETC were analysed (from 2-5 offspring per mother), using total OXPHOS Rodent WB Antibody Cocktail (Abcam, ab110413) and GAPDH Antibody (Thermo Fisher Scientific™, US, PA1-16777) as a housekeeping protein for relative quantification. After lysis of denuded oocytes in lysis buffer containing Trizma® hydrochloride solution (Sigma-Aldrich, US, T3038, 63.5mM), Glycerol (Sigma-Aldrich, G7893, 10 % v/v), sodium dodecyl sulphate (Sigma-Aldrich, L5750, 4 % w/v) and protease (Thermo Fisher Scientific™, 78425)-phosphatase (Thermo Fisher Scientific™,78420) inhibitor at pH 6.8, followed by freeze-thaw cycles, SDS-page electrophoresis was performed on mini PROTEAN pre-cast gels (Bio-rad, TGX Precast Protein Gels, 4561021) at 170V and 0.5A for 70 minutes. Proteins were transferred on PVDF-membranes at 50V and 0.1A for 1 hour 35 minutes and blocked for 2 hours in 0.1 % Skimmed milk (Applichem, A0830). After overnight blotting with OXPHOS Rodent kit (dilution 1:500), Goat-anti-Mouse biotinylated antibody (dilution 1:2000, Dako E0433) was used as secondary antibody, followed by Streptavidine-HRP (dilution 1:2000, Dako P0397) blotting. As a last step, LumiGLO™ (Cell Signalling, 7003S) was utilized and images were acquired and analysed with Chemidoc XRS+ Gel Documentation System (BioRad, Belgium). Antibodies were dissolved in 10 % bovine serum albumin (Sigma, S2002) solution containing 0.1 % of NaN_3. In between different blotting steps, the membrane was washed with TBST-Tween solution for 2x5 minutes, followed by 20 minutes of washing. Negative controls, without the primary antibody, were included for validation.

4.3.7 Assessment of oocyte lactate production and pyruvate consumption

Six to 8 freshly collected mature oocytes of 1 offspring coming from 8 C and 8 OB mothers were immediately incubated for 4 hours in 7µl droplets of L15 lactate-free 5 % CO_2 and 5 % O_2 equilibrated medium containing 0.5mM pyruvate under mineral oil. The fluorometric assay is based on the generation or consumption of the reduced pyridine nucleotides, NADH and NADPH, in coupled enzymatic reactions. The nucleotides fluoresce when excited at 340 nm, whereas the oxidized forms, NAD^+ and $NADP^+$, do not. The fluorometric conversion of NADH to NAD^+ (or vice versa) was measured with TECAN Infinite 200Pro (TECAN Group Ltd., Männedorf, Switzerland) after adding lactate dehydrogenase (Roche, 10127876001 5mg/ml, 1,100U/mg), with or without adding hydrazine (Sigma, H3376) (Gardner D.K. and Lane M., 2013).

4.3.8 Statistical analysis

Statistical analysis was done using IBM SPSS Statistics 28 (for Windows, Chicago, IL, USA). Numerical data (e.g. LDC, MMP, ROS, OXPHOS, lactate production and pyruvate consumption) were checked for equality of variance (Levene's Test) and normality of distribution (residual QQ-plots). The effect of the maternal diet, the offspring diet and their interactions were investigated using Two-way ANOVA. If the interaction was not significant, the interaction was omitted from the model. Comparisons of the two offspring dietary groups within each maternal group, and vice versa, were done using Independent Samples T-tests (summarized in supplementary file 2). In all models, the maternal dietary groups and the offspring dietary groups were used as fixed factors, while the mother ID was used as a random factor if two or more sisters were present in the same offspring treatment group (e.g. offspring weight). Categorical data (e.g. proportions of different distribution patterns of the active mitochondria and ultrastructural classifications) were analyzed using general linear mixed models (binary logistic regression). Pairwise comparisons within each offspring or maternal group were done using Chi-square analysis or Fisher's Exact analysis when the expected frequency was below five (e.g. mitochondrial clustering and uncoupling (supplementary file 2)). The differences in mean weight data recorded weekly throughout the experiment were analyzed using repeated measures ANOVA, and Two-way ANOVA measurements were performed

to study the effect of maternal diet and offspring diet on offspring mean weight at each timepoint. Correlations between offspring body weight and oocyte outcome parameters were done when no oocyte pooling between offspring and litters was needed to acquire sufficient sample size (i.e. this was done for JC-1 and CellROX™ data, metabolic assay measurements and ultrastructural abnormalities only).

4.4 Results

In all results sections below, the results of main effects using Two-way ANOVA or binary logistic regression are reported first to show the effects of maternal diet, offspring diet and their interaction. This is followed by analyzing maternal effects in pairwise comparison within each offspring diet group, or vice versa. A summary of the main effects and pairwise comparisons of all outcome parameters are shown in table S2 of the supplementary file 2. Results of the maternal diet effects on mother weight and litter size are implemented in supplementary file 1.

4.4.1 Offspring live body weight and total cholesterol

The body weight trajectory was significantly affected by diet, time, and the interaction between both factors (P<0.05, Fig. 1).
The effect of the offspring OB diet on offspring body weight was significant already after one week of dietary exposure and was further significant until sample collection at 10 weeks of age (P<0.05, Table 1). Maternal OB diet significantly increased offspring body weight at week 1 and week 2 post-weaning, but disappeared from week 3 onwards (P>0.05, Table 1). An interaction between maternal and offspring diet effects was only present after being fed the corresponding diet for 7 weeks (P<0.05, Table 1). Offspring body weight was not correlated with litter size (r = 0.015, P<0.015).

When splitting the offspring based on their maternal metabolic background, body weights of both C-born and OB-born offspring were increased when fed an OB diet compared to the C-fed mice in the same subgroup. Interestingly, although the two-way ANOVA showed no maternal diet effect, only control offspring from OB mothers were significantly heavier compared to control offspring born to control mothers. A significantly higher weight could be detected in offspring born to OB mothers only when the offspring were fed a control diet (32.92 ± 0.5 g in OB»C vs 30.25 ± 0.61 g in C»C, P<0.05,), but not in offspring fed an OB diet (40.90 ± 1.04 g in C»OB vs 40.52 ± 0.73 g in OB»0B, P>0.05).

Offspring diet caused a significant increase in offspring total serum cholesterol (C»C 112.9 ± 8.5 vs C»OB 180.0 ± 5.4 and OB»C 126.9 ± 68.2 vs OB»OB 193.0 ± 10.0, P<0.05). Maternal diet and the interaction maternal x offspring diet showed no significant differences (P>0.05). Pairwise comparison between offspring or maternal diet groups showed no significant differences (P>0.05). Offspring total serum cholesterol was positively correlated with offspring body weight (r = 0.786, P<0.05).

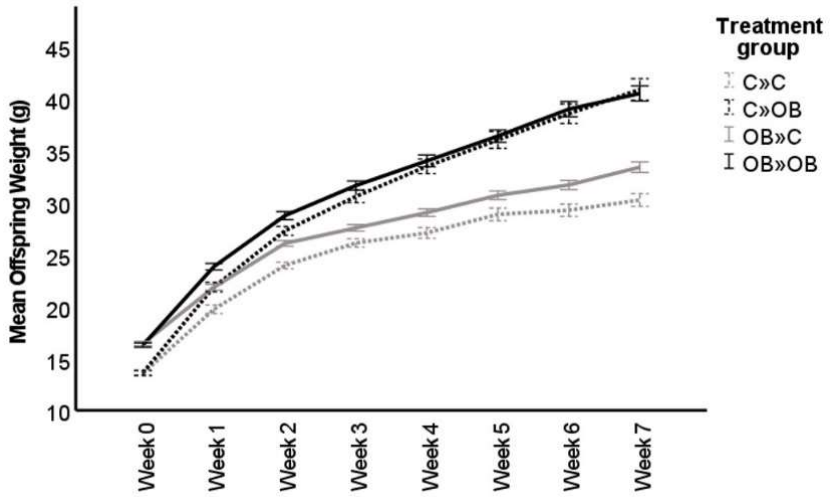


Figure 1. Maternal and offspring diet effects on offspring weight trajectory. The graph shows the live body weight trajectory (g) in offspring fed a control (C) or an obesogenic diet (OB) and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. The groups are named as MaternalDiet»OffspringDiet. Data are presented as mean ± S.E.M. and are derived from 11 litters of C mothers and 15 litters of OB mothers.

Table 1: Offspring weekly weights of C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as mean ± S.E.M. and are derived from 40-41 pups of 11 C mothers and 56-57 pups from 15 OB mothers. *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I= interaction).

	Week 0 (weaning, g)	Week 1 (g)	Week 2 (g)	Week 3 (g)	Week 4 (g)	Week 5 (g)	Week 6 (g)	Week 7 (g)
C»C	13.55 ± 0.24	19.74 ± 0.41	23.96 ± 0.32	26.12 ± 0.40	27.09 ± 0.51	28.87 ± 0.61	29.27 ± 0.59	30.25 ± 0.61
C»OB	13.62 ± 0.24	21.88 ± 0.46	27.28 ± 0.43	30.60 ± 0.60	33.50 ± 0.70	36.04 ± 0.81	38.57 ± 0.95	40.90 ± 1.04
OB»C	16.43 ± 0.20	21.85 ± 0.28	26.08 ± 0.27	27.57 ± 0.32	29.04 ± 0.36	30.71 ± 0.41	31.70 ± 0.44	32.92 ± 0.50
OB»OB	16.28 ± 0.21	23.84 ± 0.31	28.75 ± 0.39	31.65 ± 0.44	34.02 ± 0.57	36.41 ± 0.60	39.01 ± 0.73	40.52 ± 0.73
F_0 *P*	0.000	0.034	0.006	0.110	0.174	0.286	0.198	0.332
F_1 *P*	0.518	0.000	0.000	0.000	0.000	0.000	0.000	0.000
I *P*	0.691	0.744	0.328	0.727	0.163	0.194	0.135	0.025

4.4.2 Offspring oocyte lipid droplet content

The LDC was significantly higher in oocytes collected from offspring fed an OB diet (P<0.05) regardless of their maternal background. The LDC was significantly increased in C»OB compared to C»C (7.2 ± 0.3 vs 5.7 ± 0.3, x10^2 µm^3) and in OB»OB vs OB»C (6.8 ± 0.3 vs 5.5 ± 0.3, x10^2 µm^3, Fig. 2). Maternal diet had no effect, and there was no interaction between the maternal and offspring diet effects (P>0.05). The presence of a polar body did not influence the LDC of the oocytes and did not influence the effect of diet on LDC (separate data not shown).

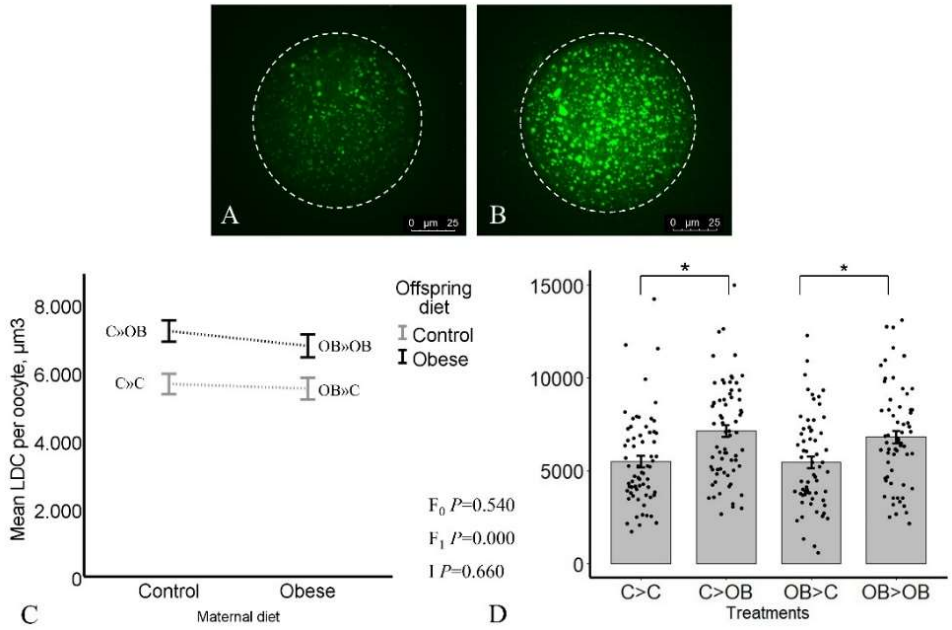


Figure 2. Maternal and offspring diet effects on offspring oocyte lipid droplet content (LDC). Representative confocal projections of oocytes stained with BODIPY® showing lipid droplets (green) from the C»C (A) and the C»OB (B) groups. Graphs showing LDC (µm3/oocyte) in oocytes collected from offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design using an interaction plot (C) and a dot plot (D). Data are presented as mean ± S.E.M. and are derived from 5-10 oocytes per litter per group, from 6-7 litters or mothers. P-values of the main effects are stated (F0 = maternal diet effect, F1 = offspring diet effect, I= interaction). Significant differences are indicated with an asterisk (*), tendencies with a dollar sign ($). Pairwise comparisons within treatment groups are presented in supplementary file 2.

4.4.3 Offspring oocyte mitochondrial DNA copy number

Offspring oocyte mitochondrial DNA copy number was not affected by offspring diet, nor by maternal diet (C»C, 87.7 ± 8.9; C»OB, 89.0 ± 6.2; OB»C, 75.7 ± 4.3; OB»OB, 86.7 ± 11.1, x10^3 copies/oocyte, *P*>0.05, Fig. 3). Pairwise comparisons within maternal diet groups or offspring diet groups showed no differences (*P*>0.05).

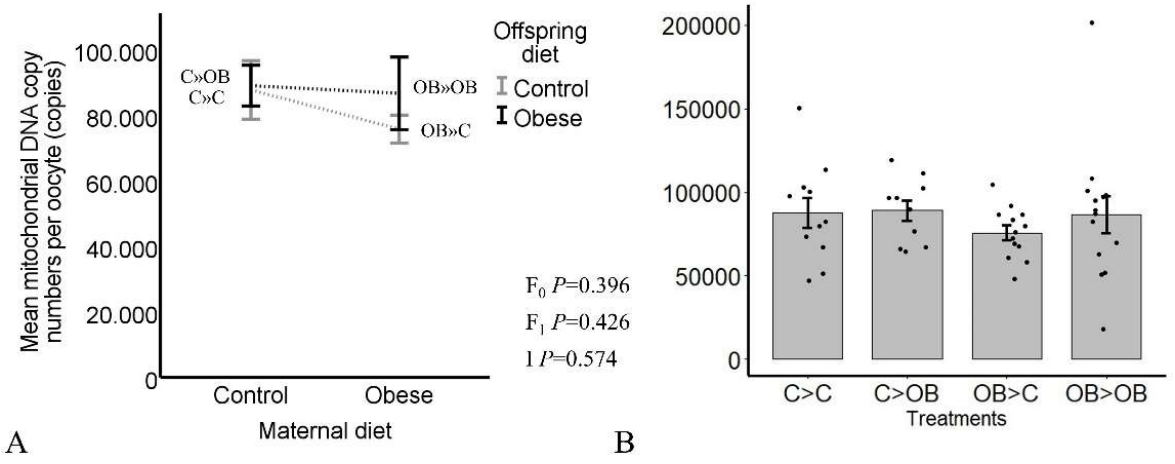


Figure 3. Maternal and offspring diet effects on offspring oocyte mitochondrial DNA copy number. Graphs representing mean mitochondrial DNA copy number (n copies/oocyte) in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design, using an interaction plot (A) and a dot plot (B). Data are presented as mean ± S.E.M. and are derived from pools of at least 15 oocytes per group per litter, from 11-13 litters each. *P*-values of the main effects are stated (F0 = maternal diet effect, F1 = offspring diet effect, I = interaction). Significant differences are indicated with an asterisk (*), tendencies with a dollar sign ($). Pairwise comparisons within treatment groups are presented in supplementary file 2.

4.4.4 Offspring oocyte MMP, distribution of active mitochondria and ROS

Offspring oocyte MMP was only altered by offspring diet (*P*<0.05). Maternal diet effect and maternal x offspring diet interaction were not significant (*P*>0.05, Fig. 4). In the pairwise comparisons within C mothers, C»OB mice had a significantly higher oocyte MMP compared to C»C (55.7 ± 2.9 vs 46.9 ± 2.3,x10^3 grey scale intensity of J-aggregates, *P*<0.05), while this increase was only a tendency in OB-born offspring (OB»OB, 56.3 ± 2.7 vs OB»C, 52.9 ± 3.0, x10^3 *P*<0.1). In comparisons within each offspring diet group, maternal diet tended to affect MMP in oocytes from offspring fed a control diet (comparing OB»C versus C»C (*P*<0.1)), whereas no differences occurred between OB»OB and C»OB (*P*>0.05). Oocyte MMP was positively correlated with offspring body weight (r = 0.402, *P*<0.05).

Oocyte ROS levels (grey scale intensity) were only affected by offspring diet (*P*<0.05). Maternal diet effect was not significant (*P*>0.05, Fig. 5). Despite no significant interaction, the increase in oocyte ROS levels due to offspring diet was only significant in C-born offspring (C»OB, 31.9 ± 2.6 vs C»C 24.2 ± 2.0 x10^3, *P*<0.05) and this was only a tendency (*P*<0.1) in OB-born offspring (OB»OB, 30.6 ± 3 vs OB»C, 25.3 ± 2.4, x10^3). The ratios of ROS concentrations to active mitochondria (ROS : J-aggregates) were not affected by offspring nor maternal diet (separate data are not shown). Oocyte ROS accumulation was not correlated with offspring body weight (r = 0.061, *P*>0.05).

The distribution of active mitochondria was neither affected by the offspring diet nor the maternal diet (*P*>0.05). Nonetheless, a small fraction (6-8 %) of the oocytes of OB-born offspring showed either

clustering of highly active mitochondria (Fig. 6) or, on the other extreme, very low mitochondrial activity (uncoupling). This was associated with high intra-oocyte ROS accumulation. The proportions of oocytes exhibiting these defects were 6.25 ± 4.72 % in OB»C and 8.71 ± 4.60 % in OB»OB compared to 0 ± 0.0 % in both C»C and C»OB (*P*<0.05). The distribution of active mitochondria was not correlated with offspring body weight (r = -0.047, *P*>0.05).

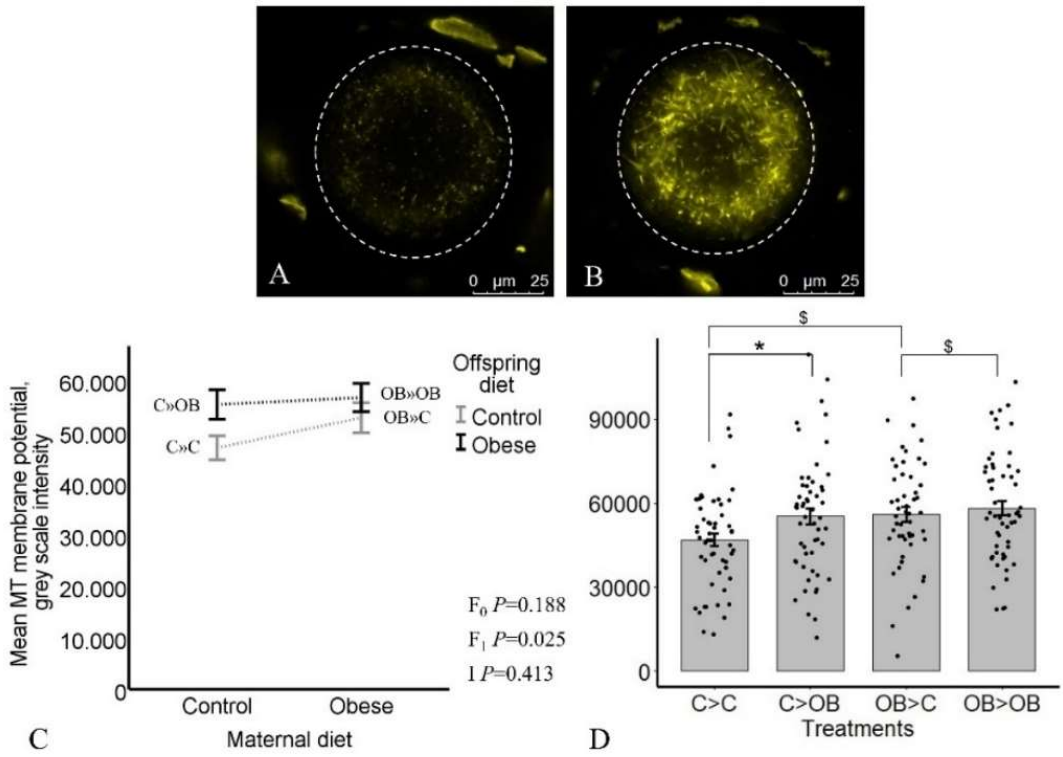


Figure 4. Maternal and offspring diet effects on offspring oocyte mitochondrial membrane potential (MMP). Representative confocal projections of oocytes stained with JC-1 (J-aggregates, yellow) from the C»C (A) and the C»OB (B) groups. Graphs representing mean MMP ((grey scale intensity)/oocyte) in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design, using an interaction plot (C) and a dot plot (D). Data are presented as mean ± S.E.M. and are derived from 8 oocytes per offspring per group per litter, from 8 litters each. *P*-values of the main effects are stated (F0 = maternal diet effect, F1 = offspring diet effect, I = interaction). Significant differences are indicated with an asterisk (*), tendencies with a dollar sign ($). Pairwise comparisons within treatment groups are presented in supplementary file 2.

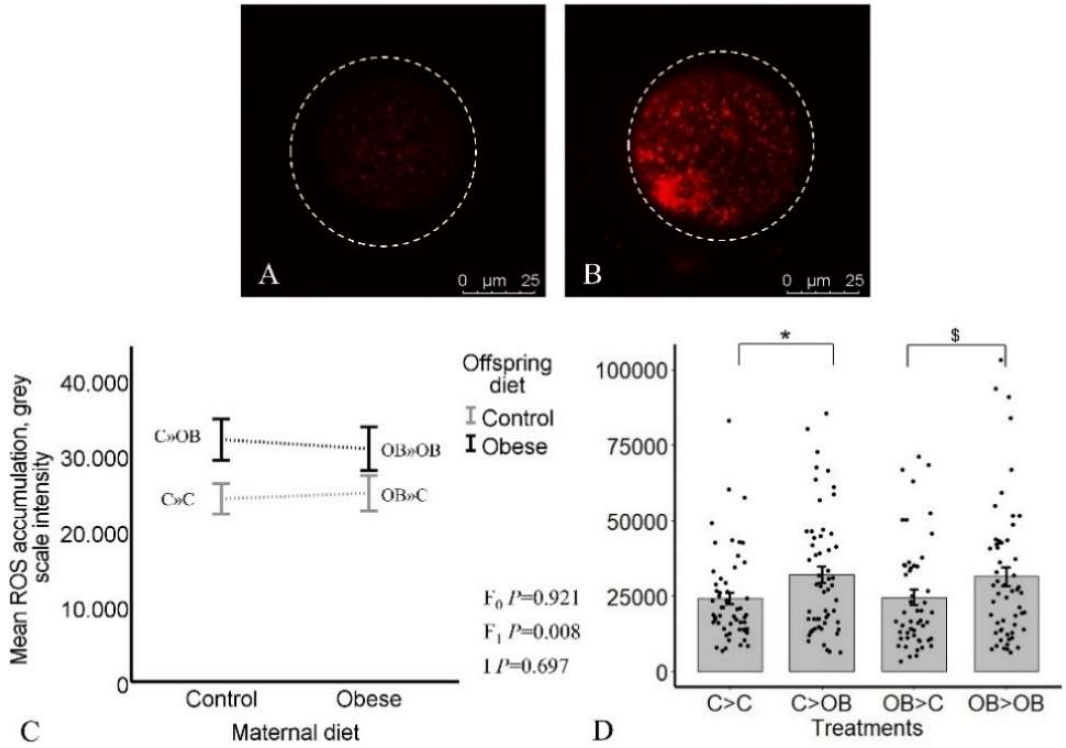


Figure 5. Maternal and offspring diet effects on offspring intraoocyte reactive oxygen species (ROS) accumulation. Representative confocal projections of oocytes stained with CellROXTM Deep Red from the C»C (A) and the C»OB (B) groups. Graphs showing intraoocyte ROS accumulation ((grey scale intensity)/oocyte) in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design, using an interaction plot (C) and a dot plot (D). Data are presented as mean ± S.E.M. and are derived from 8 oocytes per offspring per group, from 8 litters or mothers. *P*-values of the main effects are stated (F0 = maternal diet effect, F1 = offspring diet effect, I = interaction). Significant differences are indicated with an asterisk (*), tendencies with a dollar sign ($). Pairwise comparisons within treatment groups are presented in supplementary file 2.

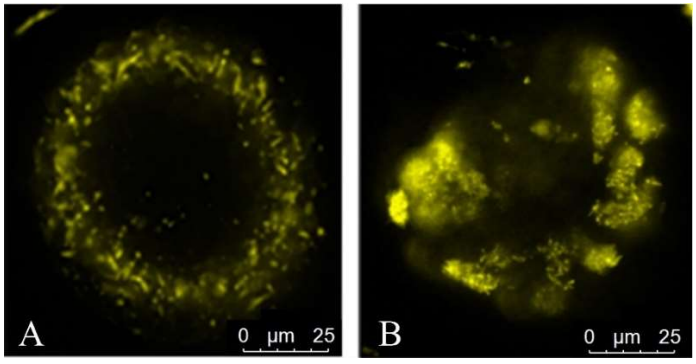


Figure 6. Peri-cortical distribution (A) of active mitochondria in an oocyte from the C»C group and mitochondrial clustering (B) in an oocyte from the OB»OB group.

4.4.5 Offspring oocyte mitochondrial ultrastructure

Oocyte mitochondrial ultrastructure was classified as normal when they were spherical and homogenous, with or without regular vacuoles. Mitochondria were classified as abnormal if they showed irregular vacuolated with or without loose membranous structures, dumbbell, elongated, or rose petal shape, electron dense foci or signs of degeneration (as described in the supplementary material in Marei et al., 2020).

The proportions of oocyte abnormal mitochondria in total were significantly increased both by offspring and maternal OB diet ($P<0.05$). In addition, maternal x offspring diet interaction was also significant ($P<0.05$, Fig. 7). This is because the increase in response to offspring OB diet was only significant in C-born offspring (32.11 ± 1.6 in C»OB vs 25.7 ± 2.2 in C»C, $P<0.05$), but was not significant in OB-born mice (37.3 ± 2.6 in OB»OB vs 35.5 ± 4.48 in OB»C, $P>0.05$). Maternal OB diet tended to increase the proportion of abnormal mitochondria within both offspring groups (i.e. in OB»C vs C»C and in OB»OB vs C»OB ($P<0.1$). The percentage of oocyte abnormal mitochondria was positively correlated with offspring body weight ($r = 0.613$, $P<0.05$).

When looking at the different categories of abnormal mitochondrial ultrastructure separately, we noticed that the proportions of dumbbell shaped mitochondria and degenerative mitochondria were increased by offspring diet ($P<0.05$) whereas the proportions of elongated mitochondria, mitochondria with electron dense foci and degenerative mitochondria were increased by the maternal OB background ($P<0.05$). The maternal effect on the proportion of mitochondria with electron dense foci was dependent on offspring diet (interaction $P<0.05$).

Detailed information about the different categories of aberrant mitochondrial ultrastructure with the corresponding *P*-values is shown in Table 2. The separate effects of maternal and offspring diet in the pairwise comparisons are shown in supplementary file 2.

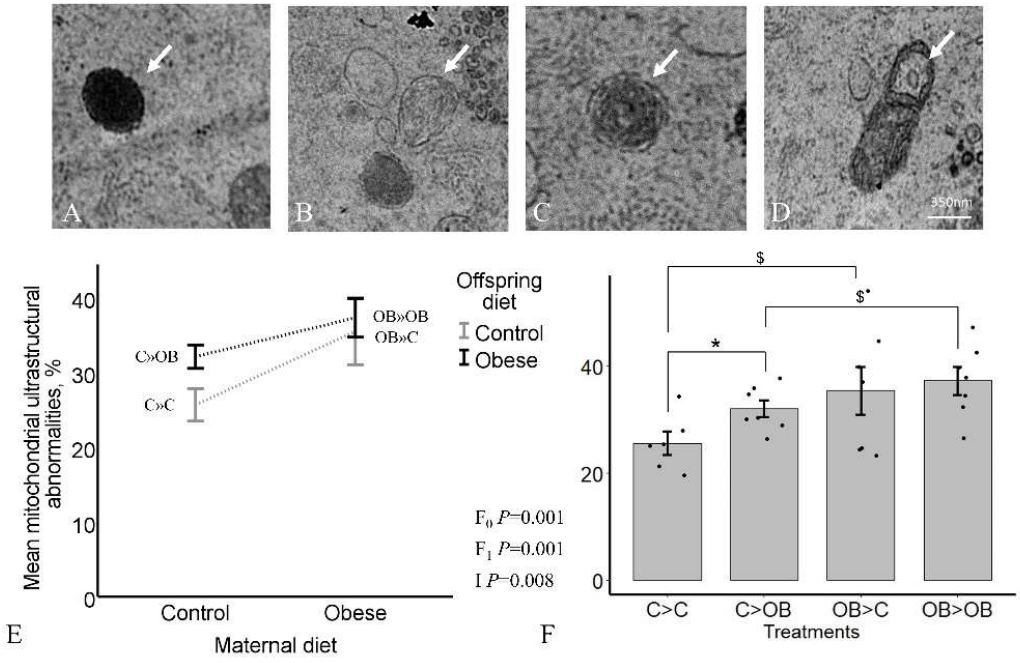


Figure 7. Maternal and offspring diet effects on offspring oocyte mitochondrial ultrastructure. Representative TEM images of oocytes showing electron dense foci (A), degenerated (B), rose-petal (C) and elongated mitochondria with loose inner membrane (D) are shown. The graphs represent mitochondrial ultrastructural abnormalities in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design, using an interaction plot (E) and a dot plot (F). Data are presented as mean proportions ± S.E.M. and are derived from 1 oocyte per offspring per group, from 8 litters or mothers. *P*-values of the main effects are stated (F0 = maternal diet effect, F1 = offspring diet effect, I = interaction). Significant differences are indicated with an asterisk (*), tendencies with a dollar sign ($). Pairwise comparisons within treatment groups are presented in supplementary file 2.

Table 2: Mitochondrial (MT) abnormal ultrastructure in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design, categorized in elongated, rose petal shaped, e-dense and dumbbell shaped mitochondria, together with mitochondria showing lose inner membranes and mitochondrial degeneration. Data are presented as mean proportions ±S.E.M. and are derived from 1 oocyte per offspring per group, from 8 litters or mothers. *P*-values of the binary logistic regression are stated (F0 = maternal diet effect, F1 = offspring diet effect, I = interaction). Pairwise comparisons within treatment groups are presented in supplementary file 2.

Group	Total abnormal MT	Elongated MT	Rose Petal shaped MT	MT with E-dense foci	Dumpbell shaped MT	MT degeneration	MT with loose inner membranes
C»C	25.6±2.16	2.0±0.96	5.5±1.10	2.1±0.59	1.9±0.70	7.3±0.92	5.9±1.27
C»OB	32.1±1.57	2.8±0.84	5.5±1.04	4.0±0.91	3.0±0.93	9.2±1.5	6.6±0.95
OB»C	35.5±4.48	3.4±0.71	6.1±1.46	5.2±1.83	3.3±0.90	10.9±2.27	5.7±2.00
OB»OB	37.3±2.58	3.3±0.74	4.8±0.86	3.7±0.79	4.0±1.65	13.8±1.99	6.0±1.02
F_0 *P*-value	0.001	0.005	0.715	0.042	0.115	0.000	0.674
F_1 *P*-value	0.001	0.391	0.597	0.717	0.042	0.004	0.684
I *P*-value	0.008	0.627	0.078	0.005	0.135	0.672	0.586

4.4.6 Expression of ETC complex markers in offspring oocytes

Complex I was not detected. Offspring diet did not influence the expression of any of the OXPHOS complexes in the offspring oocytes (*P*>0.05). Maternal diet significantly reduced the expression of complex III and complex V marker, regardless of the offspring diet (*P*<0.05, Fig. 8). Complex II and IV were not affected by a maternal OB diet and maternal x offspring diet interaction was not significant (*P*>0.05).

Pairwise comparison within maternal diet groups showed no differences in expression of ETC markers caused by the offspring diet. In pairwise comparisons within each offspring diet group, no differences were seen in the expression of complex III marker (in C»C, 0.25 ± 0.13 compared to OB»C, 0.06 ± 0.03; and in C»OB, 0.21 ± 0.03 compared to OB»OB 0.09 ± 0.02, Integral volume (iV) (*P*>0.05)) but the expression of complex V marker was significantly higher in C»C (0.07 ± 0.01, iV) compared to OB»C (0.03 ± 0.01, iV, *P*<0.05). It did not differ in C»OB (0.07 ± 0.02, iV) compared to OB»OB (0.04 ± 0.00, iV, *P*>0.05).

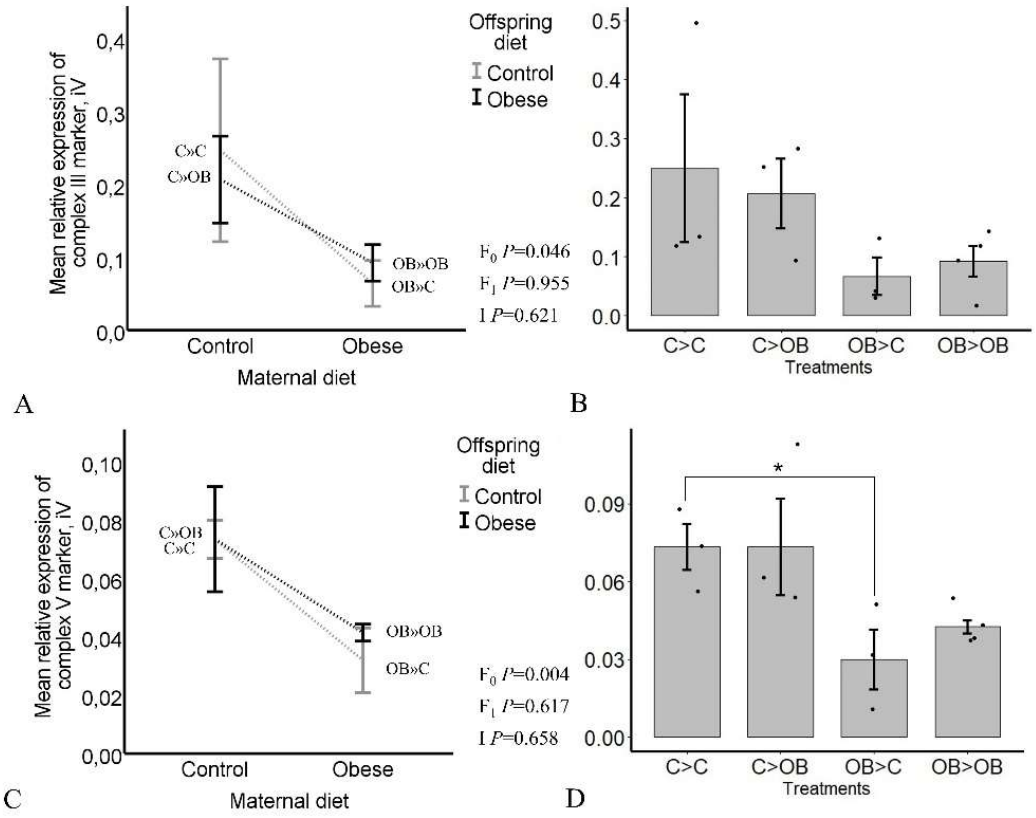


Figure 8. Maternal and offspring diet effects on the expression of the ETC complexes III and V markers in oocytes. Graphs showing expression of ETC complexes III markers in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design, using an interaction plot (A) and a dot plot (B). Graphs showing expression of ETC complexes V markers in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design, using an interaction plot (D) and a dot plot (E). Data are presented as mean ± S.E.M. and are derived from pools of 40-80 oocytes per group, from 3-4 C and OB mothers. P-values of the main effects are stated (F0 = maternal diet effect, F1 = offspring diet effect, I = interaction). Significant differences are indicated with an asterisk (*), tendencies with a dollar sign ($). Pairwise comparisons within treatment groups are presented in supplementary file 2.

4.4.7 Offspring oocyte metabolic activity

Oocyte pyruvate consumption was significantly reduced both by offspring and by maternal OB diets (*P*<0.05). The maternal x offspring diet interaction was not significant (Fig. 9, *P*>0.05). In pairwise comparison within each maternal diet group, OB-fed offspring had a reduced pyruvate consumption when born to OB mothers, (OB»OB, 57 ± 3.5 vs OB»C, 65 ± 3.0, pmol/oocyte/h, *P*<0.05) whereas the pyruvate consumption only tended to differ in offspring born to C mothers (C»OB, 64 ± 4.5 vs C»C, 73 ± 3.2, pmol/oocyte/h, *P*<0.1). The pyruvate consumption tended to be reduced in OB»C compared to C»C. No differences were seen between OB»OB and C»OB. The oocyte pyruvate consumption was negatively correlated with offspring body weight (r = -0.449, *P*<0.05).

Oocyte lactate production was not affected by offspring diet and tended to be reduced by a maternal metabolic background (C»C, 1.02 ± 0.23; C»OB, 1.29 ± 0.61; OB»C, 0.55 ± 0.32; OB»OB, 0.35 ± 0.34, pmol/oocyte/h, *P*>0.1). The maternal x offspring diet interaction was not significant (Fig. 9, *P*>0.05). Oocyte lactate production was not different between groups in pairwise comparisons within both

maternal and offspring groups. Oocyte lactate production was not correlated with offspring body weight (r = -0.067, *P*<0.05).

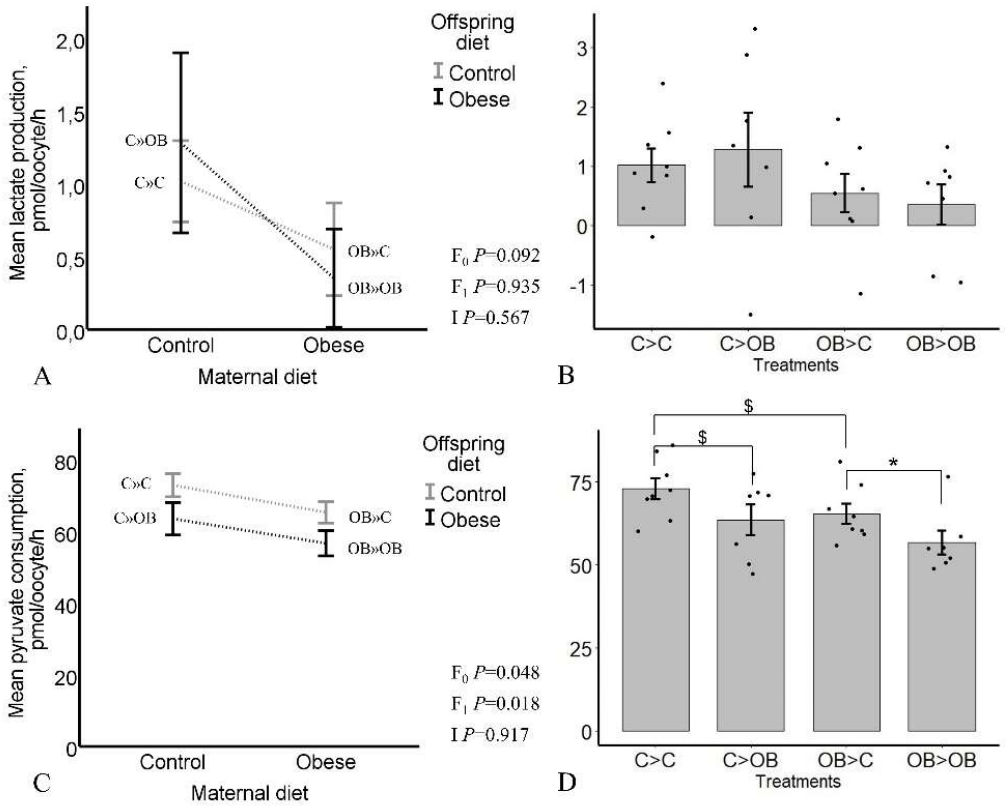


Figure 9. Maternal and offspring diet effects on offspring oocyte lactate production and pyruvate consumption. Graphs representing lactate production in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design using an interaction plot (A) and a dot plot (B). Graphs representing pyruvate consumption in oocytes collected from C- or OB-fed offspring and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design using an interaction plot (C) and a dot plot (D). Data are presented as mean ± S.E.M. and are derived from 6-8 oocytes per offspring per group, from 8 litters or mothers. P-values of the main effects are stated (F0 = maternal diet effect, F1 = offspring diet effect, I = interaction). Significant differences are indicated with an asterisk (*), tendencies with a dollar sign ($). Pairwise comparisons within treatment groups are presented in supplementary file 2.

4.5 Discussion

The aim of this study was to investigate the influence of a maternal OB background on offspring oocyte quality, when the offspring is fed either a control or an OB diet. A validated and robust outbred Swiss mouse model was used to increase the pathophysiological relevance and translatability to the human situation. We have previously shown that Swiss mice are sensitive to an OB diet, develop hypercholesterolemia, obesity, and insulin resistance, reflecting in a reduced oocyte quality with mitochondrial functional and morphological alterations (Marei et al., 2020; Smits et al., 2021; Moorkens et al., 2022; Smits et al., 2022a). We focused on several complementary oocyte mitochondrial qualitative and quantitative parameters. We found that the majority of the assessed mitochondrial functions in oocytes were significantly affected by the offspring OB diet. However, the extent of this impact, at least for some outcome parameters, seemed to depend on the metabolic background of the mother. The mitochondrial changes in offspring oocytes associated with a maternal OB diet only were significant but relatively limited.

Most studies investigating the effect of diet-induced obesity on oocyte quality and fertility do not consider the potential interacting effects of the maternal metabolic background. In human, obese daughters are often born to obese mothers, making such interaction a very important factor to consider. The data generated in the present study in offspring born to control mothers are comparable with other studies using mice born to control healthy mothers, following standard breeding protocols. In these mice, we found that feeding a high fat and high sugar diet (in C»OB) significantly increased live body weight trajectory, total serum cholesterol, as well as oocyte LDC, MMP, ROS and mitochondrial ultrastructural abnormalities, compared to the control group (C»C). This is in line with previous findings from our laboratory using the same outbred Swiss model (Marei et al., 2020; Smits et al., 2021; Smits et al., 2022a), and also in line with other studies where the inbred C57BL/6 mouse model was used (Igosheva et al., 2010; Boots et al., 2016). On top, we showed that the offspring diet effect was already present at 1 week post-weaning and was influenced by the maternal metabolic background at week 7 post-weaning. In our study, offspring's oocyte pyruvate consumption was reduced, and the mitochondrial activity and percentage of mitochondrial ultrastructural abnormalities were increased when offspring gained more weight. Consumption of an OB diet and development of obesity are known to alter the biochemical composition of the blood and follicular fluid (Leroy et al., 2022), leading to increased lipid accumulation in oocytes, which creates a nutrient overload and metabolic dysregulation resulting in lipotoxicity, oxidative stress, and mitochondrial dysfunction (Boudoures et al., 2017a; Zhao et al., 2017; Bradley and Swann, 2019; Marei et al., 2020; Smits et al., 2021; Leroy et al., 2022). We could also detect a tendency towards a reduced oocyte pyruvate consumption in C»OB compared with C»C mice. These results are thus in agreement with the expected increase in fatty acid beta-oxidation, and may explain, at least in part, the increased MMP and ROS accumulation in the affected oocytes.

While the direct impact of OB diet was confirmed in our experimental model, our main focus was to determine if the maternal OB background might influence the offspring oocyte quality and its mitochondrial functions and how such maternal impact may affect the effects of the offspring OB diet on oocyte mitochondrial function. For this, a 2 x 2 factorial design was used. The most prominent effect of maternal diet observed here was on offspring oocyte mitochondrial morphology. Maternal OB diet significantly increased the proportions of total mitochondrial ultrastructural abnormalities in offspring oocytes, and more specifically on the proportion of mitochondria classified as degenerated, elongated or electron dense. These abnormalities were limited to only 5-10 % of the mitochondria in total. Similar results have been previously reported by a few studies using C57BL/6 mice (Saben et al., 2016; Boudoures et al., 2017a), where oocyte mitochondria were found to be significantly larger and less round in offspring born to obese mothers compared to those born to lean mothers (Saben et al., 2016). The authors did not report the exact proportion of the abnormal mitochondria in the affected oocytes. Mitochondrial

morphology can be linked to its function. Increased mitochondrial elongation may be a consequence of altered fusion and fission machinery due to high levels of cellular stress (Runkel et al., 2014) and may imply alterations in energy metabolism pathways (Boudoures et al., 2016; Saben et al., 2016). Increased mitochondrial electron density is linked with altered glucose and oxygen metabolism (Grindler and Moley, 2013). In our study, we detected both features being more prevalent in oocytes from OB-born offspring, together with a reduction in pyruvate consumption, suggesting maternally induced alterations in offspring oocyte energy efficiency mechanisms. However, the proportions of elongated or electron dense mitochondria were relatively low (about 2 %) and only increased by 1-2 % in response to maternal and/or offspring OB diet, suggesting that the overall impact may be rather limited. The interaction between maternal diet and offspring diet effects on mitochondrial ultrastructural morphology was also significant, meaning that the effect of an offspring OB diet on offspring mitochondrial morphology is dependent on the maternal metabolic background.

It is important to keep in mind that the increased oocyte mitochondrial ultrastructural abnormalities in response to maternal and/or offspring OB diet does not necessarily mean that other mitochondrial or cellular metabolic functions are affected. Oocytes contain around hundred thousand mitochondria and display several adaptive mechanisms that may be used to compensate for energy deficit or overload (Runkel et al., 2014; Marei and Leroy, 2022). Previous studies from our laboratory have shown that the percentage of mitochondria with abnormal morphology in control Swiss oocytes is around 20 % (Smits et al., 2022a). An increase of 5-10 % of mitochondrial ultrastructural abnormalities detected here may have no, or limited, functional impact. For a more comprehensive assessment, we investigated several other mitochondrial functions and indeed we found that MMP and intracellular levels of ROS were not affected by the maternal diet effects despite the observed effects on mitochondrial morphology. Assessment of MMP is commonly used as an indicator of oocyte mitochondrial activity (Blerkom et al., 2003). The MMP increases during the final stage of maturation in murine oocytes and is linked to an increase in oxidative phosphorylation and ATP production (Blerkom et al., 2003; Al-Zubaidi et al., 2019) and correlates with preimplantation development (Komatsu et al., 2014). Increased ROS concentrations in oocytes is also frequently used in other studies to indicate oxidative stress. Increased MMP and ROS in oocytes have both been linked with reduced oocyte quality, reduced developmental competence (Marei et al., 2019) and lower pregnancy rates in diet-induced obese mice (Smits et al., 2021). Interestingly, also in C57BL/6 mice, maternal diet-induced obesity did not influence ATP production in the oocytes of the offspring despite the associated increase in mitochondrial ultrastructural abnormalities (Boudoures et al., 2017b). Offspring in the later study were fed a normal chow diet. Here we also found no maternal effects on oocyte MMP and ROS even in offspring fed an OB diet, showing that the maternal metabolic background did not increase the sensitivity of the oocyte to a high fat and high sugar diet. It is also interesting to mention that among the different classes of abnormal oocyte mitochondria in offspring from OB mothers, degenerated mitochondria were the most prevalent. This might be a sign of clearance of damaged mitochondria rather than retaining the defective ones. We suggest that, as oocytes do not have the capacity to efficiently remove damaged mitochondria (Boudoures et al., 2017c), they eventually degenerate.

It was also important in the present study to examine the effects on mitochondrial DNA copy numbers. The results were highly variable within each treatment group and we could not detect any significant effect of maternal or offspring diet. There was a numerical decrease in mitochondrial DNA copy numbers in C»OB oocytes compared to C»C, which is in line with our previous report (Marei et al., 2020). Our data are in contrast with the data reported using the inbred C57BL/6 mice where maternal obesity resulted in a significant reduction in mitochondrial DNA copy numbers in the offspring's oocytes (Saben et al., 2016). Mitochondrial DNA copy numbers are tightly regulated by the rate of mitochondrial biogenesis and replication, as well as the rate of mitophagy by which damaged mitochondria might be removed. Mitochondrial biogenesis is active in primordial germ cells and during early stages of folliculogenesis (St John, 2019). High levels of cellular metabolic stress can influence mitochondrial regulation of fusion and fission and alter mitochondrial DNA copy numbers (Fukunaga, 2021). Therefore, if the maternal metabolic

effects on oocyte mitochondrial functions would persist during prenatal and postnatal development, or if mitochondrial dysfunction would be transmitted through the female germline as suggested in inbred mice, we would expect an impact on mitochondrial DNA copy numbers and mitochondrial bioenergetic functions in the offspring oocytes. In the present study using outbred Swiss mice, we were unable to detected such impact.

Oocytes normally contain a heterogenous population of active and inactive mitochondria (Van Blerkom, 2011). Active mitochondria move towards the peri-cortical area of the oocyte during the final phase of oocyte maturation to support the cortical granule reaction to prevent polyspermy and provide energy for polar body extrusion (Kirillova et al., 2021). A peri-cortical distribution of active mitochondria also guarantees an equal division of the mitochondria among the blastomeres after cleavage and is therefore of the utmost importance for optimal embryo development (Liu et al., 2003; Van Blerkom and Davis, 2006; 2007; Van Blerkom, 2008). In our study, the mitochondrial distribution was not affected by offspring and maternal diets. We did however observe aberrant mitochondrial uncoupling and clustering in a few oocytes only in offspring born to obese mothers. Mitochondrial clustering may imply alterations in oocyte energy production due to overexpression of mitochondrial fusion proteins (Babayev and Seli, 2015). Igosheva et al. (2010) and Hou et al. (2016) reported clusters of mitochondria in some oocytes of obese mice being indicative of metabolic or functional damage, involving a greater need for energy production to preserve oocyte viability and competence. Nevertheless, since these features were only limited to 6-8 % of the oocytes, with a normal MMP and active mitochondrial distribution in all the rest, the potential impact may also be limited. In addition, not only clustering but also mitochondrial lactate and pyruvate serve as a redox buffer that equilibrates the NADH/NAD^+ ratio across the cell. Lactate acts, amongst other functions, as an important redox carrier. Its production is increased in anaerobic respiration when glucose is converted into lactic acid, being energetically less efficient than oxidative phosphorylation (Lagarde et al., 2021). We could not detect differences in offspring oocyte lactate production between treatment groups. However, both offspring and maternal OB diet reduced the consumption of pyruvate, the principle energy substrate of the oocyte during final maturation (Richani et al., 2021) with the lowest pyruvate consumption seen in OB-born OB fed offspring. Oocytes with reduced pyruvate may be more sensitive towards increased ROS production, as a reduced pyruvate consumption may lead to reduced glutathione levels in oocytes (Funahashi et al., 2008). We could indeed detect dietary induced increased ROS accumulation in the oocyte, but only due to offspring OB diet and not linked with the mother's OB background. The reduced pyruvate consumption together with the numerical reduction in lactate production may suggest a change in the metabolic preference towards β-oxidation for energy provision. However, no differences could be detected in oocyte LDC, MMP or ROS to support this notion.

Finally, this study is the first to investigate the potential effect of maternal and offspring OB diet on the expression of protein complexes of the electron transport chain in oocytes. Complex I was not detected, which is line with very recent observations reported by Rodriguez-Nuevo et al. (2022), who illustrated that suppressing complex I in oocytes is an intrinsic mechanism to maintain a ROS-free mitochondrial metabolism. In the present study, this appears not to be changed by maternal or offspring diet. Also no differences in expression of complex II and IV markers were detected. Complex I and II oxidize NADH and $FADH_2$ respectively, transferring the resulting electrons to ubiquinol, which carries electrons to complex III to be subsequently used for ATP production (Rutter et al., 2010). In the absence of complex I, complex II activity becomes crucial for the function of the ETC and for energy production but it was not influenced by diets. We also found that the expression of the complex III and V markers were significantly reduced by maternal OB diet. However, in the pairwise comparisons, this was only confirmed for complex V and only in offspring fed a control diet. There was a tendency for a lower complex III expression in OB fed offspring born to OB mothers. Complex III uses energy released in downhill electron transfers to pump more protons across the inner mitochondrial membrane (Chandel, 2010). The resulting proton gradient (MMP) is then used to produce ATP by the fifth complex, also known as ATP-synthase (Lenaz et al., 2006; Zhao et al., 2019; Maclean et al., 2022). Reactive oxygen species are produced as a by-product of the ETC

(Brand, 2010). Protective mechanisms by reducing expression of ROS generating ETC complexes have been reported by others in somatic tissue (Gleason et al., 2011; Le Vasseur et al., 2021; Groen et al., 2022). Therefore, the slightly reduced expression of complex III in OB»OB oocytes may be a beneficial compensatory mechanism for the oocytes to control ROS production. Since MMP was not affected, no consequence for energy production is expected. Lower complex V expression in OB»C oocytes compared to C»C is very interesting and may be secondary to metabolic adaptations also leading to reduced pyruvate consumption and suggesting a lower metabolic activity in these oocytes. However, these adaptations were not sufficient to impact MMP.

It is also important to highlight that, except for specific mitochondrial ultrastructural features, there was no interaction between the maternal diet and offspring diet effects. Following the systematic 2 x 2 factorial design of the data, we did not directly compare OB»OB oocyte quality measures with those of the C»C. Nevertheless, we can clearly see from the figures that the highest degree of alteration or change in most outcome parameters were due to the combined effect of maternal and offspring OB diets. The specific maternal contribution of these differences in some parameters are evident but not significant. In our study, oocytes were collected of 10 weeks old offspring. It is possible that treatment effects will become more pronounced when offspring age increases, especially since aging is linked with mitochondrial damage (Sun et al., 2016). Therefore the functional impact of the maternal OB background on offspring oocyte mitochondria may still become significant over time. Also, our experimental design tried to mimic the societal situation in which daughters may be raised in the same OB environment after weaning. Therefore, the present study does not allow to determine the specific window of impact during which the maternal diet could have resulted in reduced quality of the daughter's oocyte. However, our data do suggest that the pool of dormant oocytes in the ovary of the fetus or the newly born pup is already sensitive to an adverse metabolic condition leading to mitochondrial defects in the offspring oocytes at adult age, even if the post-weaning diet was perfectly healthy. Similar long-term carry over effects on oocyte mitochondrial features were reported also in our previous study in obese mice submitted to diet normalization for 4 weeks (Smits et al., 2022b). In our study, litter sizes were reduced to allow equal milk supply per pup. However, litter size is linked with offspring body weight and the expression of each phenotype as such may be relevant to fully understand maternal-offspring nutrient interactions in developmental studies. Also, artificial reduction of litter size itself can be considered as a confounding factor, as it may affect maternal care behavior and influence offspring health (Enes-Marques and Giusti-Paiva, 2018; Briffa et al., 2019; Xavier et al., 2019). Nevertheless, our data show that litter size at birth was similar between C- and OB-fed mothers and hence did not act as a confounding factor in this study. Finally, maternal obese diets during pregnancy and lactation may redirect metabolic programming resulting in an altered offspring metabolism (Wells, 2014; Gawlinska et al., 2021; Furse et al., 2022). This may indirectly affect oocyte quality in the offspring.

This study is the first to use an outbred model to investigate the effect of both maternal and offspring OB diets and their interaction on offspring oocyte mitochondrial ultrastructure and function. We show evidence that especially the offspring diet had the most obvious impact on mitochondrial features and thus, the most obvious impact on oocyte quality. However, the extent of this impact was often dependent on the maternal metabolic background with the most prominent effects seen in oocytes from OB offspring born to OB mothers. The maternal OB background on its own appears to have a limited but significant impact on the offspring's oocyte mitochondrial morphology but not on bioenergetic functions, on mitochondrial DNA copy numbers, mitochondrial activity (estimated by MMP), distribution of active mitochondria, as well as on ROS levels. These results strongly suggest that aberrant mitochondria in oocytes (induced by OB diet) are not transferred through the female germline to the next generation. The majority of abnormal oocyte mitochondria in OB-born offspring were degenerated suggesting that, at least in this outbred model, defective mitochondria can be removed and replaced without compromising mitochondrial DNA copy numbers. Reduced oocyte pyruvate consumption and lower expression of the ETC complex V marker without a significant change in MMP, ROS and lactate production indicates that

these adaptations may have no, or limited, functional consequences. These results are in contrast with previous reports using inbred models and highlight marked differences in responses between inbred and outbred mice strains. Therefore, extrapolation of data from inbred mouse models, particularly related to oocyte mitochondrial functions and intergenerational effects of metabolic stress, should be done with caution. Pathophysiological relevant outbred mouse models should be used in further studies to investigate the potential effects on somatic cell functions in the offspring born to obese mothers, the developmental competence of their oocytes and potential effects on the second generation.

4.6 Supplementary file 1

4.6.1 Maternal weight

Maternal OB diet significantly increased the weight of the mother at mating (weight at week 7, *P*<0.05). The maternal OB diet significantly increased live body weight from week 3 onwards. Overall body weight trajectory of the mothers (using repeated measures ANOVA) was affected by diet, time and the interaction between both factors (*P*<0.05). The weight of the mother at mating was not correlated with litter size (r = 0.32, *P*>0.05). More detailed information is shown in Figure S1 and table S1. Litter size was not correlated with offspring body weight (r = 0.015, *P*>0.05).

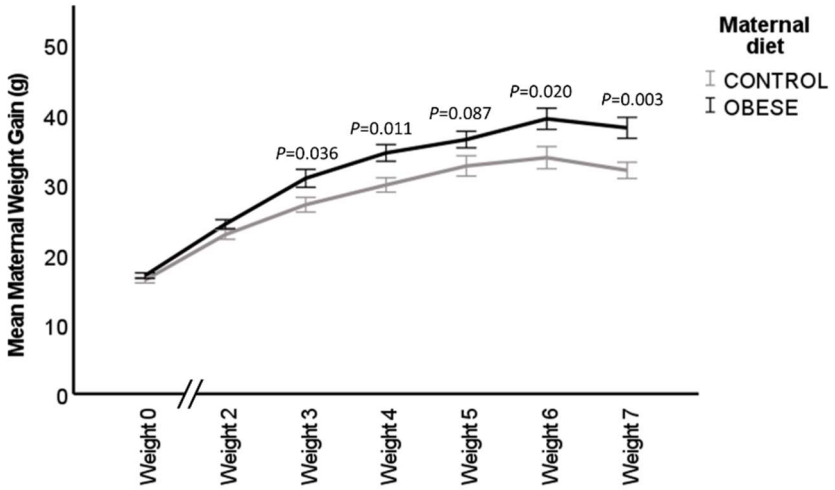


Figure S1. Maternal diet effect on maternal weight gain trajectory. The graph shows maternal weight at the start of the trial (Weight 0, weaning of the mothers) until mating (Weight 7) in mothers fed a control (C) or an obesogenic diet (OB). Data are presented as mean ± S.E.M. and are derived from 11 C mothers and 15 OB mothers. Significant differences per timepoint (Independent Samples T-test) are indicated with an asterisk (*), tendencies with a dollar sign ($).

4.6.2 Litter characteristics

Table S1. Litter characteristics of C-fed or OB-fed mothers and the corresponding correlation with the weight of the mother at the time of mating (7w). Data are represented as mean ± S.E.M. and are derived from 11C and 15 OB mothers.

	Litter size	Litter weight	Pup weight at birth
CONTROL n = 11	14.45 ± 0.61	30.26 ± 1.35	2.22 ± 0.20
OBESE n = 15	14.26 ± 0.41	30.15 ± 0.93	2.14 ± 0.07
r weight F_0	0.32	0.119	-0.059
P-value	0.876	0.562	0.775

4.7 Supplementary file 2

4.7.1 Pairwise comparisons

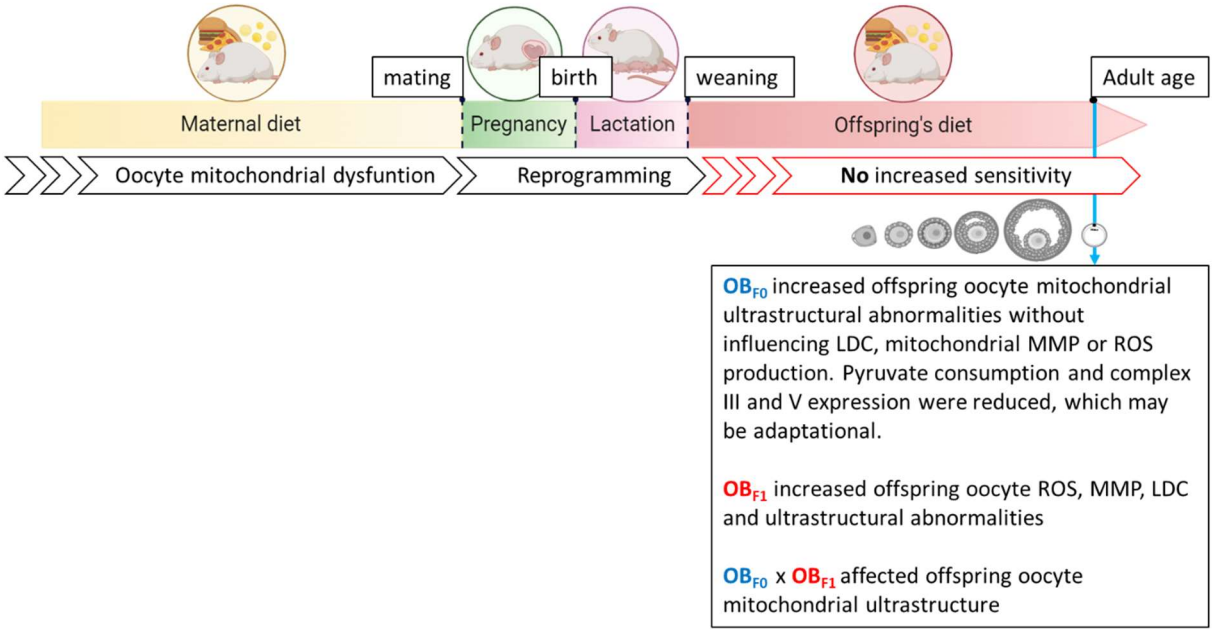
Table S2: Overview of all investigated outcome parameters with main effects and pairwise comparisons within treatment groups. Significant differences are indicated by an asterisk (*), while tendencies are indicated by a dollar sign ($). NS, not-significant; ND, not-detected. Arrows show the direction of change in each comparison.

	Main effects			In C fed offspring only	In OB fed offspring only	In offspring from C fed mothers only	In offspring from OB fed mothers only
Parameter	Maternal diet effect	Offspring diet effect	Inter-action	Maternal diet effect	Maternal diet effect	Offspring diet effect	Offspring diet effect
				OB»C vs C»C	OB»OB vs C»OB	C»OB vs C»C	OB»OB vs OB»C
Weight (7wk)	NS	*↑	*	*↑	NS	*↑	*↑
LDC	NS	*↑	NS	NS	NS	*↑	*↑
mtDNA copy number	NS	NS	NS	NS	NS	NS	NS
MMP	NS	*↑	NS	$↑	NS	*↑	$↑
ROS	NS	*↑	NS	NS	NS	*↑	$↑
Distribution	NS	NS	NS	NS	NS	NS	NS
Ultrastr. (Total)	*↑	*↑	*	$↑	$↑	*↑	NS
Dumbbell	NS	*↑	NS	NS	NS	NS	NS
Degenerative	*↑	*↑	NS	NS	*↑	*↑	NS
Elongated	*↑	NS	NS	NS	NS	NS	NS
E-dense	*↑	NS	*	$↑	NS	$↑	NS
Rose-petal	NS	NS	$	NS	NS	NS	NS
Loose inner membrane	NS	NS	NS	NS	NS	NS	NS
ETC CI	ND	ND	ND	ND	ND	ND	ND
ETC CII	NS	NS	NS	NS	NS	NS	NS
ETC CIII	*↓	NS	NS	NS	NS	NS	NS
ETC CIV	NS	NS	NS	NS	NS	NS	NS
ETC CV	*↓	NS	NS	*↓	NS	NS	NS
Pyruvate	*↓	*↓	NS	$↓	NS	$↓	*↓
Lactate	$↓	NS	NS	NS	NS	NS	NS

4.8 Key message

The direct effect of the offspring's OB diet was confirmed to hamper the adult offspring's mature oocyte quality in outbred Swiss mice. However, up to the relatively young offspring age that we have investigated, maternal OB diet consumption did not lead to any apparent effect on offspring's oocyte quality, and did not interact with the impact of the offspring's diet on the oocytes.

4.9 Graphical summary



Schematic overview of the main effect of the maternal OB diet (OBF_0), the offspring OB diet (OBF_1), and the maternal x offspring diet interaction (OBF_0 x OBF_1) on offspring mature oocyte quality at adulthood (age 10 weeks). Results of the pairwise comparisons are not included in this overview.

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Chapter 5

The impact of Offspring and Maternal Obesogenic diets on Adult Offspring Oocyte Mitochondrial Morphology in Primordial and Preantral Follicles. Insights from an Outbred Mouse Model

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5.1 Abstract

Diet-induced obesity reduces oocyte quality mainly by impacting oocyte mitochondrial functions. Moreover, maternal obesity is associated with mitochondrial dysfunction in oocytes of their adult offspring. However, these effects were reported only in fully grown oocytes, mainly in the form of abnormal mitochondrial ultrastructure. It is unknown if obesogenic (OB) diets or maternal obesity already impact the primordial and preantral follicles. Considering the long duration and dynamics of folliculogenesis, determining the stage at which oocytes are affected and the extent of the damage is crucial for optimal reproductive management of obese patients and their daughters. Potential interaction between maternal and offspring diet effects are also not described, yet pivotal in our contemporary society. Therefore, here we examined the impact of OB diets on oocyte mitochondrial ultrastructure in primordial and activated preantral follicles in offspring from diet-induced obese or lean mothers. We used an outbred Swiss mouse model to increase the pathophysiological relevance to humans. Female mice were fed control or OB diets for 7 weeks, then mated with control males. Their female offspring were fed control or OB diets after weaning for 7 weeks (2 x 2 factorial design). Adult offspring ovarian sections were examined using transmission electron microscopy. We characterized and classified unique features of oocyte mitochondrial ultrastructure in the preantral follicles. An increase in mitochondrial matrix density was the most predominant change during follicle activation in secondary follicles, a feature that is linked with a higher mitochondrial activity. Maternal obesity increased mitochondrial density already in the primordial follicles suggesting an earlier increase in bioenergetic capacity. Maternal obesity did not induce aberrant ultrastructure (abnormalities and defects) in primordial or preantral follicles. In contrast, offspring OB diet increased mitochondrial abnormalities in the primordial follicles. Further investigation of the consequences of these changes on oocyte metabolic regulation and stress levels during folliculogenesis is needed.

5.2 Introduction

The growing global prevalence of obesity has raised significant concerns regarding its detrimental consequences on health and fertility, and its effects across generations. Obese women have an increased risk of subfertility, involving endocrine imbalance, ovulatory malfunction, polycystic ovarian disorders, and reduced oocyte quality (Cena et al., 2020; Marinelli et al., 2022). Moreover, children born to obese mothers have increased risk of developing metabolic syndrome, including obesity and diabetes (Godfrey et al., 2017), and are thus more prone to the development of metabolic diseases later in life (Godfrey and Barker, 2000; Eriksson et al., 2014; Contreras et al., 2016; Stacy et al., 2019). A few studies in diet-induced obese mouse models suggested that maternal obesity increases the risk of reproductive disorders in the offspring as well (Cheong et al., 2014; Ramadan et al., 2023; Wei et al., 2023). Since children mostly follow the same lifestyle and dietary habits of their parents (Pfledderer et al., 2021), some of the reported effects of maternal obesity on offspring health and fertility can be, at least in part, due to direct effects of consuming obesogenic (OB) diets after ablactation. Dissecting these effects and investigating the potential interaction between offspring's OB diet and the maternal metabolic background became crucial in our contemporary society, in which most obese woman are born to obese mothers (Liang et al., 2009; Sarker et al., 2019; Gawlinska et al., 2021).

Reduced oocyte quality in diet-induced obesity has been described in several studies and is linked with low maturation and fertilization rates, reduced embryo developmental competence and low pregnancy success rates after ART treatments (Metwally et al., 2007; Grindler and Moley, 2013b). This was usually attributed to biochemical alterations in the follicular fluid after antrum formation (Carrell et al., 2001; Robker et al., 2009; Valckx et al., 2014b). We and others have shown that mitochondrial dysfunction plays a key role in the pathogenesis of reduced oocyte quality. Consumption of OB diets was shown to alter the oocyte mitochondrial ultrastructural morphology, for example by increasing mitochondrial vacuolization and altering inner membrane organization, or by inducing mitochondrial elongation (Koripella et al., 2020; Marei et al., 2020; Trebichalská et al., 2021). This was associated with concurrent alterations in mitochondrial inner membrane potential, reduced ATP production and increased mitochondrial reactive oxygen species (ROS) production (Xu et al., 2015; Hou et al., 2016; Xhonneux et al., 2023). Importantly, most of these studies examined the effect of obesity on oocyte quality in fully grown oocytes either in the preovulatory follicles or after ovulation (Purcell and Moley, 2011). However, it is not known if the dormant primordial pool and early preantral follicular stages are also affected. The primordial follicle pool is established around birth (Telfer et al. 2023). After primordial follicle activation, they first develop to preantral primary follicles. Then, primary follicles develop into preantral secondary follicles, and eventually into antral (tertiary) follicles, to become the dominant follicle ready for ovulation (Telfer et al. 2023). Folliculogenesis is a lengthy process which involves highly complex cytoplasmic changes in organelle structure and functions, including mitochondrial replication and gradual increase in mitochondrial bioenergetic activities (Adhikari et al., 2022). This is crucial to meet the increasing metabolic demand to support cytoplasmic and nuclear maturation in the oocyte and grant oocyte developmental competence (Telfer et al., 2023). Determining the stage at which oocytes are affected is crucial to optimize reproductive management of obese patients by e.g. optimizing the duration or timing of the interventions during the preconception period.

There are several indications that suggest that preantral follicles are vulnerable to metabolic stress. This is most clearly described in the dairy cow model. High yielding dairy cows suffering from severe negative energy balance during the early post-partum period and during heat stress show elevated concentrations of lipotoxic fatty acids and reactive oxygen species in the blood and follicular fluid, which induces oocyte mitochondrial dysfunction and reduced oocyte quality, similar to those described in obese women (Shehab-El-Deen et al., 2010; Valckx et al., 2014a; Leroy et al., 2022). Nevertheless, it was noticed that the reduced oocyte quality persists for a few months after the restoration of energy balance and alleviation of stress (Carvalho et al., 2014). According to the Britt's hypothesis (1992), this suggests that early

preantral stages of follicular development are also affected. Recent data could provide strong evidence that is in line with this notion (Marei et al., 2022). Similarly, studies from our laboratory recently indicated that an increased rate of mitochondrial ultrastructural abnormalities is still detectable in diet-induced obese mice after 4-6 weeks of diet normalization or caloric restriction, implying that the dormant follicle pool may indeed be affected (Smits et al., 2021b). Nevertheless, the nature and extent by which oocyte mitochondria can be affected by obesogenic diets in primordial and preantral follicles has never been investigated.

In addition, maternal obesity is also associated with mitochondrial dysfunction in oocytes of their offspring, mainly in the form of abnormal mitochondrial ultrastructure (Saben et al., 2016). Again, this was only described in fully grown oocytes from preovulatory follicles in the adult offspring, while the occurrence of these defects in the primordial pool was not examined. This fundamental knowledge, and the potential interaction with the direct effects of the offspring's diet can be useful to develop more efficient strategies to prevent and treat subfertility in females born to obese mothers.

Therefore, in this study we hypothesized that both maternal and offspring OB diet consumption can affect the oocyte mitochondrial morphology in the primordial and early preantral ovarian follicles in the offspring. We also hypothesized that the effects of the offspring diet may depend on the maternal dietary background. To test these hypotheses, we used a diet-induced obese mouse model in which mothers and their female offspring were fed a control or high-fat high sugar diet in a 2 x 2 factorial design. This design enabled us to address the interaction between the effect of the maternal and offspring OB diet, mimicking the same pattern of female familial obesity in our society. Outbred Swiss mice were used to increase the pathophysiological relevance to the human physiology, as validated and described in our previous studies (Smits et al., 2021a; Smits et al., 2022). While examining mitochondrial parameters in oocytes of preantral follicles can be challenging, we used transmission electron microscopy to investigate mitochondrial morphology, which also enables precise identification of the corresponding stage of follicular development. Oocytes of the primordial and early activated follicles (primary and secondary follicles) were examined in ovarian sections of adult offspring. Since studies describing mitochondrial morphology in early developing follicles are lacking, it was necessary to first provide a comprehensive characterization and classification of the normal mitochondrial ultrastructure in oocytes of primordial and early activated follicles of the control adult offspring.

5.3 Materials and Methods

5.3.1 Experimental design

This study was approved by the Ethical Committee for Animal Testing and performed in accordance with the ARRIVE guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Antwerp (Protocol Number: ECD 2018-05). All efforts were made to minimize animal suffering. Female Swiss mice (age 3 weeks) were fed a C diet (10 % fat, 7 % sugar, Sniff diets D12450J, containing 10 % fat and 7 % sugar (E157453-04), n = 6) or an OB diet (Sniff diets E15741-34, 60 % fat (beef Tallow), 20 % sugar, n = 6) for 7 weeks, then mated with the same Swiss males (n = 2) fed a standard chow diet in a cross-over design. Their weight gain was recorded weekly during the trial. All mice stayed on their corresponding diet during pregnancy and lactation. Litter sizes were recorded and equalized to 10 pups to avoid unequal nutrient supply between different litters (Knight et al., 1986; Konig et al., 1988). Female offspring were randomly and equally weaned onto either a C or an OB diet at 3 weeks of age, creating a 2 x 2 factorial design and resulting in four treatment groups named as MaternalDiet»OffspringDiet: 1) C»C: C-born C-fed pups 2) C»OB: C-born OB-fed pups 3) OB»C: OB-born C-fed pups and 4) OB»OB: OB-born OB-fed pups (Figure 1). They were fed their corresponding diet for 7 weeks after weaning and were weighed weekly. At 10 weeks, the estrus cycles of the mice were synchronized using the Whitten-effect 12 h before euthanasia and sample collection (Moorkens et al., 2022). Each pup was euthanized by cervical dislocation. One ovary per offspring per mother (n = 6 per group) was collected and immediately fixed in sodium cacodylate-buffered glutaraldehyde solution. Random ultrathin ovarian sections were examined for mitochondrial ultrastructure using TEM. All primordial, primary, and secondary follicles in each section were examined. To compare the oocyte mitochondrial morphology in preantral follicles with the already well described mitochondrial morphology of mature (ovulated) oocytes, few ovulated oocytes were collected of adult C»C offspring, according to the protocol described by Xhonneux et al. (2023).

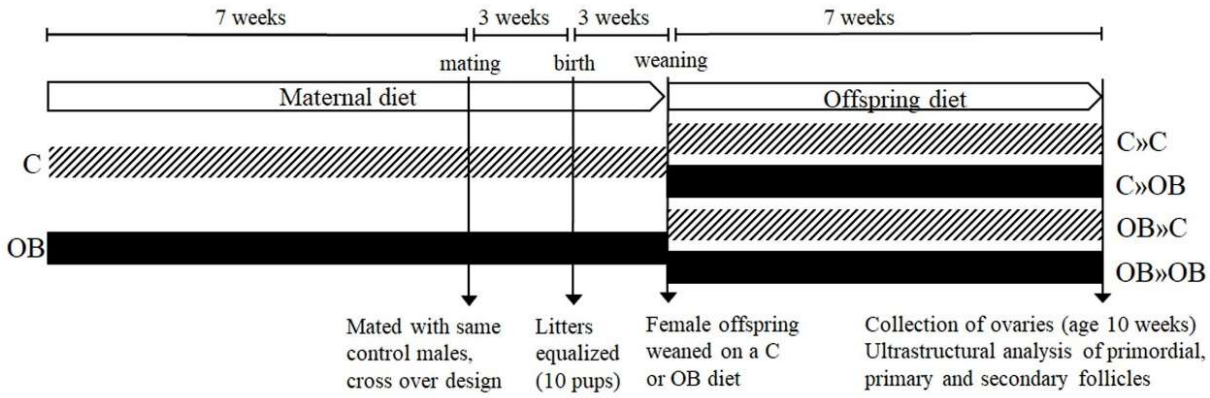

Figure 1. Schematic 2 x 2 factorial design of the study. Offspring were fed a control (C) or an obesogenic diet (OB) and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Offspring treatment groups are named as MaternalDiet»OffspringDiet.

5.3.2 Sample collection and preparation

Immediately post-collection, ovaries were fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5 % glutaraldehyde solution with 0.05 % $CaCl_2.2H_20$ at 4 °C. The fixative was removed, and the tissue was rinsed in 0.1 M sodium cacodylate-buffered (pH 7.4) 0.05 % $CaCl_2.2H_2O$, 7.5 % sucrose solution (RT). Post-fixation tissue was incubated (2 h, RT) in 1 % osmium tetroxide (OsO_4) in a 0.033 M veronal acetate buffer containing 4 % sucrose. The tissue was rinsed with 0.05 M veronal acetate buffer (pH 7.4) containing 6 % sucrose RT after OsO_4 removal. Afterwards, 1 % tannic acid staining was performed in veronal acetate 6

% sucrose (1 h, RT) and the samples were washed thoroughly with veronal acetate 6 % sucrose, followed by dehydration using an ethanol gradient starting from 50 % to 100 % ethanol. Dehydration was continued with propylene oxide for 30 minutes. To impregnate the samples, the tissue was treated overnight with a propylene oxide/EMBed 812 resin (EMBed-812 Kit, E14120) mixture (1:3) without accelerator (DMP-30), while on a rotary shaker (RT). Subsequently, the sample was treated with EMBed 812 resin mixture (without accelerator) for 2 x 2 h at 37 °C, followed by EMBed 812 resin mixture with accelerator (1 h, 37 °C). Before the sample was embedded, it was transferred into a gelatine capsule or into a flat embedding mold, then covered with EMBed 812 resin mixture containing accelerator to allow polymerization (36 h, 65 °C). Afterwards, ultrathin sections (50 nm thick) were cut using Ultramicrotome (Leica EM UC7) with diamond knife (ultra 45°, Diatome), then stained with lead citrate lead citrate, rinsed with 0.05 M sodium hydroxide and CO_2-free ultrapure water and examined with TEM Tecnai G2 Spirit Bio TWIN microscope (Fei, Europe BV, Zaventem, Belgium) at 120 kV. All primordial, primary, and secondary follicles present in one or two random section(s) midway through the ovary (with both ovarian medulla and cortex present) were assessed. Non-overlapping pictures were taken of all mitochondria present in the oocytes of these follicles. All mitochondria per oocyte section were morphologically evaluated and classified by two independent experienced assessors blind to the treatment groups.

5.3.3 Classification of the follicles

Follicles were classified as 1) Primordial, when the oocyte is surrounded by a single layer of flattened (pre-) granulosa cells, 2) Primary, surrounded by one layer of cuboidal granulosa cells, 3) Secondary, surrounded by two to four layers of granulosa cells without antrum (Aerts and Bols, 2010) (Figure 2). Primordial follicles are also referred to as dormant follicles. After follicle activation occurs, the primary and secondary follicles are also referred to as activated or growing follicles. Follicles were excluded from the analysis when signs of follicular atresia were evident, such as oocyte fragmentation, granulosa cell pyknosis, degeneration, and damaged cell membrane, as described by Yu et al. (2004) and Regan et al. (2018) (see example of atretic follicles in S1 Figure 1).

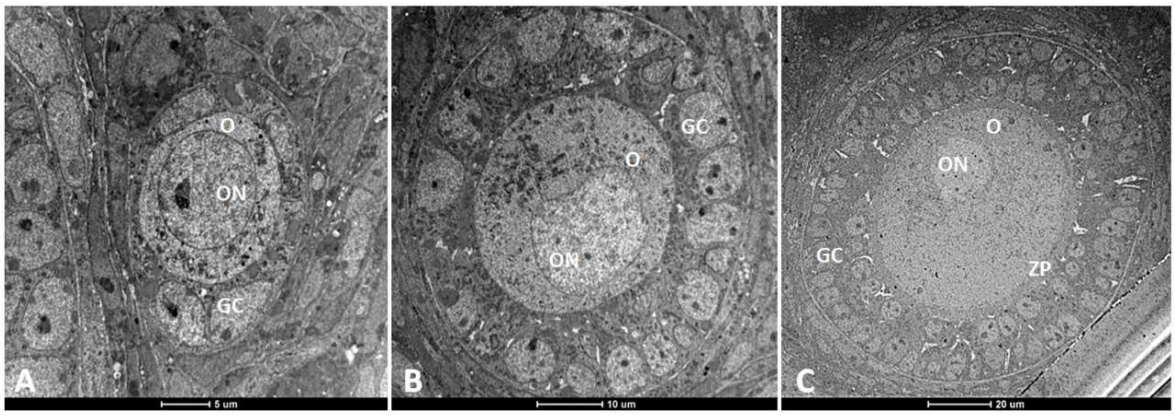

Figure 2. Transmission electron microscopic overview of assessed follicles (C»C) in this study. **A**. Primordial follicle **B**. Primary follicle **C**. Secondary follicle (O = oocyte; ON = oocyte nucleus; ZP = zona pellucida; GC = granulosa cells).

5.3.4 Classification of oocyte mitochondria within follicles

Since studies investigating mitochondrial morphology in the preantral follicles of adult mice are lacking, we have first characterized and classified different oocyte mitochondria according to their ultrastructure. This was done using the preantral follicle oocytes of the C»C pups as a reference. The mitochondrial morphology was classified based on matrix density (dense or light), shape (spherical or non-spherical),

the presence of cristae (not present, sparse or abundant), and the presence of a vacuole. Differences in mitochondrial density, shape, or presence of cristae are not considered abnormalities at these preantral follicle developmental stages, and can rather imply acquisition of bioenergetic function (Schatten et al., 2014; Udagawa et al., 2014; Yildirim and Seli, 2024). In addition, other specific ultrastructural features which clearly illustrate an abnormality or defect in the oocyte mitochondrial were classified separately, such as mitochondria showing loose inner membranes, broken or protruded outer membranes, rose-petal shaped mitochondria, enlarged mitochondria (>1µm), and mitochondria having electron dense foci. These forms were collectively classified here as abnormal mitochondria, as described in mature oocytes by Marei et al. (2020) and Xhonneux et al. (2023).

5.3.5 Statistical analysis

Statistical analysis was done using IBM SPSS Statistics 29 (for Windows, Chicago, IL, USA). Data were checked for equality of variance (Levene's Test) and normality of distribution (residual QQ-plots). Differences in weight of the mothers at mating (after 7 weeks of being fed their corresponding diet) were analyzed using Mann-Whitney-U, since the data were not homogenous in variance. Effect of the maternal diet on litter sizes and differences in offspring weight at birth and at weaning were investigated using Independent Sample T-test. Changes in mean live body weight (recorded weekly) of the mothers and their offspring were compared using Repeated Measures ANOVA. The maternal and offspring diet effect and their interaction on offspring weight at adulthood (age 10 weeks) was tested using Two-way ANOVA. If the interaction was not significant, it was omitted from the model. Differences in the proportions of different classes of mitochondrial ultrastructure between follicular stages (from primordial to secondary follicular stage) within the control offspring (C»C) were examined using generalized linear mixed models (binary logistic regression) with follicle stage as factor. Correction for nesting of the data collected from multiple follicles within the same offspring ovary was always implemented in the statistical analysis, blocking for differences in number of follicles per offspring. To estimate the effect of the maternal and offspring diets and their interplay on the mitochondrial morphology (proportion of different mitochondrial ultrastructural classes) in oocytes of preantral follicles generalized linear mixed models (binary logistic regression, with diet as factor) was used. If the interaction was not significant, it was omitted from the model. Differences with *P*-values ≤ 0.05 are reported as statistically significant, and 0.05 < *P*-values ≤ 0.1 are reported as tendencies. Numerical data are presented as mean ± S.E., while categorical data are presented as proportion percentage ± S.E. An overview of the number of mitochondria assessed in each follicular stage in each group is presented in S1 Table 1-3. All proportions are summarized in S2 Table 1-3.

5.4 Results and Discussion

5.4.1 Maternal and Offspring body weight

The OB mothers weighed significantly more at mating (after 7 weeks on diet) compared to the controls (C: 35.89 ± 1.71 *vs.* OB: 42.53 ± 3.23, g, *P* = 0.020), but this did not impact the litter sizes (C: 15 ± 0.82 *vs.* OB: 13 ± 0.86, pups/litter, *P* = 0.122). Therefore, litter size equalization was similar in all groups. The maternal growth curve is shown in Figure 3A.

The maternal OB diet did not increase offspring weight at birth (C: 2.40 ± 0.01, OB: 2.37 ± 0.12, g, *P* = 0.650), at weaning (C: 15.36 ± 0.40; OB: 16.27 ± 0.46, g, *P* = 0.150), or at adulthood (at sample collection, after 7 weeks on diet, C»C: 35.60 ± 1.95 *vs.* C»OB: 43.34 ± 2.15, and OB»C 31.57 ± 1.99 *vs.* OB»OB 40.62 ± 1.38, g, *P* = 0.113).

Offspring weight at adulthood was increased by offspring OB diet (*P* < 0.001), which was not dependent on the maternal dietary background (interaction *P* = 0.861). The offspring growth curve is shown in Figure 3B.

Our previous studies have shown that despite no obvious effects on offspring weight, maternal obesity in Swiss mice resulted in significant detrimental effects on mitochondrial ultrastructure in the muscles (Xhonneux et al., 2024) and fully grown oocytes (Xhonneux et al., 2023) of the offspring. Here we further investigate the impact on mitochondrial morphology in the preantral stages of folliculogenesis.

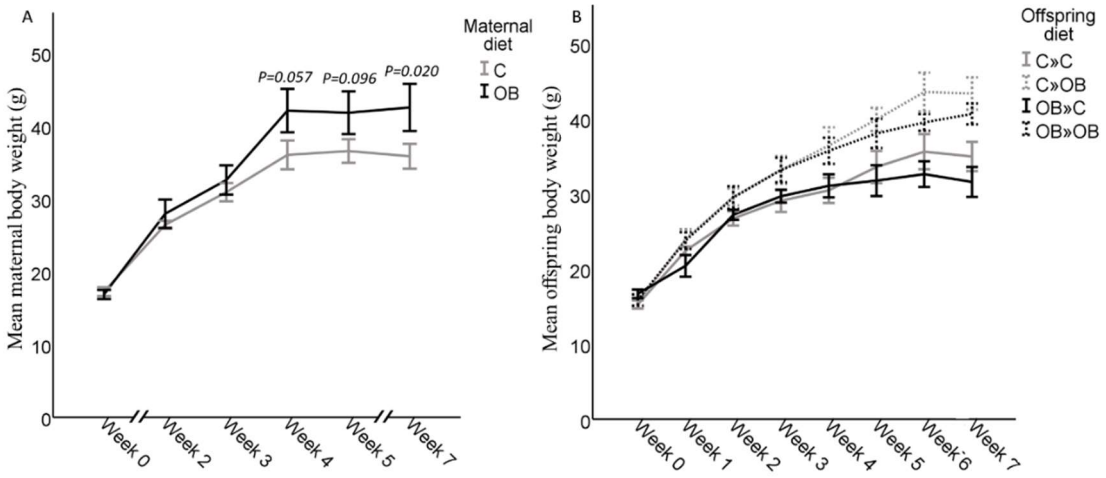

Figure 3. Growth curve of the mothers (A) fed a C or OB diet, and their offspring after weaning (B) in the different treatment groups where C or OB-born offspring were fed a C or an OB diet, in a 2 x 2 factorial design. Data are presented as mean ± S.E.M. and are derived from 6 mothers fed a C or OB diet and one offspring per mother (n = 6 offspring/group).

5.4.2 Mitochondrial ultrastructure within oocytes of primordial, primary and secondary follicles in C-born C-fed pups (C»C)

Since studies involving mitochondrial morphology in young developing follicles are lacking, it was necessary to first analyze and compare oocyte mitochondrial morphology during early folliculogenesis in primordial, primary and secondary follicles of adult control offspring (C»C), before dietary effects were investigated. The mitochondrial classifications detected in C»C, and used throughout the study are described in Table 1.

Unlike the mitochondrial ultrastructure in mature oocytes (after ovulation) reported in other studies and displayed here in Figure 4, the majority of the oocyte mitochondria within primordial follicles were characterized by low electron density and a translucent or light matrix, with distinct but sparse cristae present. After follicle activation, a proportion of oocyte mitochondria became electron dense, without distinct cristae (Figure 4).

Table 1. Different mitochondrial classifications recorded in oocytes within primordial, primary, and secondary follicles in ovaries of C»C pups.

Mitochondrial class	Description of the mitochondria
Spherical:	
Full Cristae (FCr)	Abundant distinct cristae and a translucent/light matrix
Sparse Cristae (SCr)	A few distinct cristae and a light matrix
No Cristae (NCr)	Mitochondria without cristae and a light matrix
Dense (D)	An electron dense matrix without distinct cristae
Dense-vacuolated (DV)	An electron dense matrix with a vacuole and no distinct cristae
Non-spherical:	Pear, Elongated and Dumbbell-shaped mitochondria
Abnormal	Mitochondria showing loose inner membranes, broken or protruded outer membranes, rose-petal shaped mitochondria, enlarged mitochondria, and mitochondria having electron dense foci

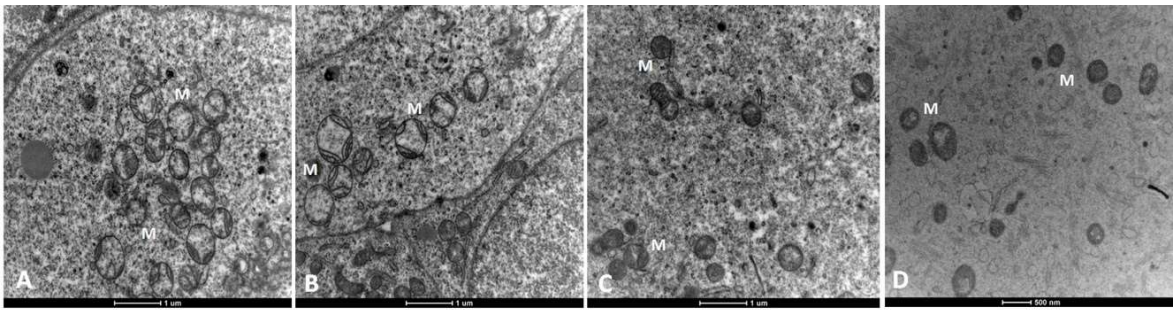

Figure 4. Oocyte mitochondria (M) in a primordial (**A**), primary (**B**) and secondary (**C**) follicle. **D.** Oocyte mitochondria in a mature ovulated oocyte.

The majority of the mitochondria in the oocytes of the **primordial ovarian follicles** of adult control (C»C) mice were spherical with light electron density containing full (FCr, 16.8 ± 5.9 %), sparce (SCr, 55 ± 5.3 %) or no cristae (NCr, 3.8 ± 1.3 %) (Figure 5). Electron dense mitochondria were not abundant (1.4 ± 0.8 % D and 4.7 ± 1.6 % DV). Only 5.4 ± 1.1 % of the mitochondria were non-spherical, and 13.4 ± 1.6 % of the mitochondria showed ultrastructural abnormalities (listed in Table 1).

Oocytes of the primordial follicles remain arrested at the diplotene stage of the first meiotic division from shortly before birth and until the follicle is activated. They form a limited stock of dormant primordial follicle pool which is crucial for fertility and reproductive longevity after puberty (Lussier et al., 1987;

Gougeon, 2010; Clarke, 2017). Odor and Blandau (1969) studied oocyte mitochondrial morphology in murine primordial follicles from prenatal day 18 until postnatal day 3. In this study, the oocyte mitochondrial morphology in the dormant primordial follicle pool was described to be translucent, with sparse cristae (Odor and Blandau, 1969). This specific mitochondrial morphology is also similar to the predominant feature that we observed here in oocytes of primordial follicles at adulthood (age 10 weeks) and suggests that the mitochondrial morphology in these follicles does not change with advancing age.

Cinco et al. (2016) suggested, while studying *ex-vivo* neonatal and prepuberal murine ovaries, that oocytes within primordial follicles exhibit elevated free/bound auto-fluorescent NADH ratios, often associated with a reduced cellular respiration (Kolenc and Quinn, 2019). Therefore, the presence of large proportions of mitochondria with low electron density and only few cristae, as detected in our study, may imply an immature oxidative phosphorylation capacity. Oocytes at this stage of development are arrested and do not undergo cytoplasmic or nuclear changes. Thus, these oocytes are not in need of high-energy provision. Throughout this dormant stage they even uphold an adapted mitochondrial metabolism, characterized by low ROS levels through the inhibition of complex I, hereby ensuring minimal cellular stress levels and long-lasting viability (Rodríguez-Nuevo et al., 2022).

In **primary follicles** of C»C, we found that the proportions of different classes of oocyte mitochondrial morphology were very similar to those at the primordial stage; with spherical, low electron dense mitochondria with full (FCr, 11.9 ± 2.8 %) or sparce cristae (SCr, 57.9 ± 4.4 %) being most abundant (Figure 5).

In contrast, oocytes of the **secondary follicles** exhibited drastic changes in mitochondrial morphology compared to earlier stages. In these oocytes, SCr mitochondria were still the most predominant (30.6 ± 3.1 %), but their presence was reduced by over 25 %, along with an increase in the proportions of high electron dense mitochondria (around 18 % increase in total, 6.6 ± 1.3 % D and 22 ± 2.4 % DV) compared to oocytes of earlier stages (primordial and primary) (Figure 5).

Follicle activation initiates oocyte growth and volume expansion (Telfer et al., 2023), accompanied by increased metabolic activity, energy production and concomitant mitochondrial biogenesis (Adhikari et al., 2022). During this process, the oocyte's metabolism gradually shifts from a mainly glycolytic metabolism to a combination of glycolytic and aerobic metabolism, involving increased oxidative phosphorylation (Cinco et al., 2016) and substantial changes in mitochondrial dynamics (Adhikari et al., 2022). As follicles mature to the secondary stage, the oocyte starts to rely on highly glycolytic cumulus cells as a source of pyruvate, while continuing to be active in oxidative phosphorylation (Cinco et al., 2016; Warzych and Lipinska, 2020). Since oxidative phosphorylation involves electrons being transported over the inner mitochondrial membrane to produce ATP (Tang et al., 2020), we would expect an increase in the amount of cristae, and thus an increase in the proportion of FCr mitochondria. However, only dense mitochondria (D and DV) were significantly increased in secondary follicle oocytes, as in this stage the subtle increase in the proportion of D and DV mitochondria seen in the primary follicles became apparent. Interestingly, these dense mitochondria look similar to the majority of the mitochondria in mature oocytes (Van Blerkom, 2009; Saben et al., 2016; Marei et al., 2020), where the matrix is also electron dense. The mitochondrial matrix contains essential components such as DNA, ribosomes, enzymes and co-factors needed for the citric acid cycle and oxidative phosphorylation (Argan et al., 1983; Haggie and Verkman, 2002). In mature oocytes, while all mitochondria exhibit electron dense matrix and no or very few distinct cristae, these oocytes exhibit a high mitochondrial membrane potential, and detectable O_2-linked ATP production as shown using Seahorse analysis (Van Blerkom and Davis, 2006; Muller et al., 2019). Thus, the increase in mitochondrial matrix density detected in secondary follicle oocytes may be linked with an increased mitochondrial activity (Trebichalská et al., 2021). However, high electron density of the mitochondrial matrix may also obscure the distinction of cristae.

A small but significant 1 % reduction in the proportion of elongated mitochondria was seen in the oocytes as they transit from the primordial to the secondary follicle stage (1.1 ± 0.4 % in oocytes of secondary follicles vs 2.8 ± 0.9 % and 2.5 ± 0.6 % in oocytes of primordial and primary follicles respectively). However, it is only after the blastocyst stage (5-8 days after fertilization) that the mitochondria of the embryo will gradually transform from immature spherical to elongated mitochondria with clear cristae, as found in somatic cells (Sathananthan and Trounson, 2000). In mature oocytes, elongated mitochondria are described as abnormal and indicative of a disturbed fusion/fission machinery (Runkel et al., 2014), which may imply alterations in energy metabolism (Boudoures et al., 2016; Saben et al., 2016). Nevertheless, it remains questionable if a decrease of only 1 %, as seen in our results, can be linked with changes in mitochondrial biogenesis or bioenergetic functions.

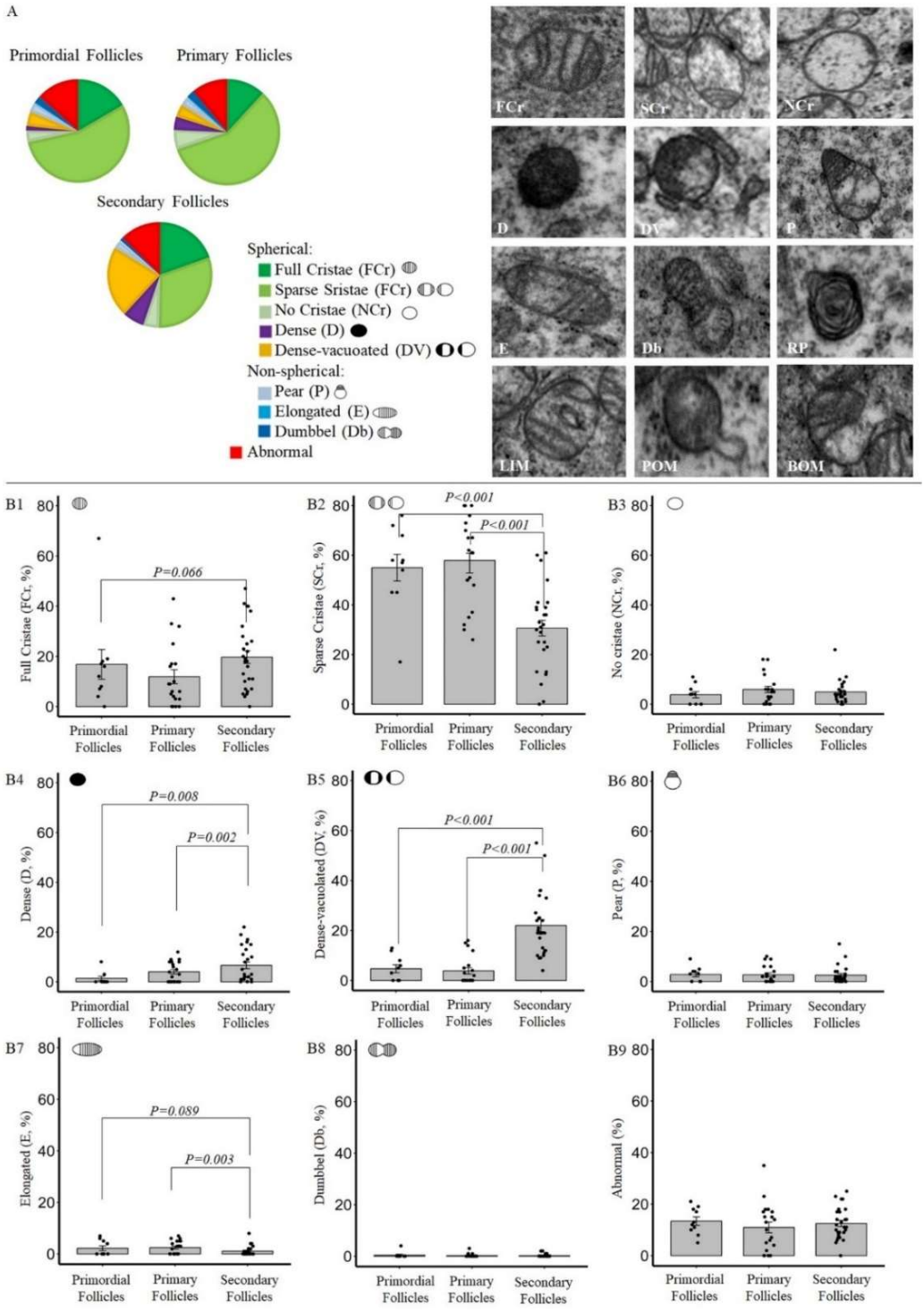

Figure 5. A. Description of the oocyte mitochondrial ultrastructure (Pie-charts) in primordial, primary and secondary follicles, and corresponding TEM. Pictures of different mitochondrial subcategories (FCr = Full cristae, SCr = Sparse cristae, NCr = No Cristae, D = Dense, DV = Dense-vacuolated, P = pear-shaped, E = elongated, Db = dumbbell-shaped, RP = rose-petal shaped, LIM = loose inner membrane, BOM = broken outer membrane, POM = protruded outer membrane) in control (C»C) ovaries. **B**. Changes in the proportion of each morphological class between different stages of early follicle development in control (C»C) ovaries. Data are presented as proportion percentages ± S.E. and are derived from 10 primordial, 20 primary and 27 secondary follicles of 6 C»C mice. *P*-values of the significant differences and tendencies are displayed on the graphs.

5.4.3 The effect of the maternal and offspring diets on oocyte mitochondrial morphology in offspring preantral ovarian follicles

Following the initial examination of oocyte mitochondrial morphology in preantral follicles of healthy adult mice (C»C), we proceeded to explore the impact of maternal and offspring OB diets and their interaction.

5.4.3.1 *The effect of the maternal diet on the oocyte mitochondrial morphology of the primordial follicles*

In primordial follicles, maternal OB diet resulted in a significant reduction (by about 18%) of the proportion of mitochondria with low electron-density (particularly with SCr) (C»C: 55.0 ± 5.3 % ; C»OB: 40.9 ± 4.4 %; OB»C: 27.2 ± 4.1 %, OB»OB: 32.2 ± 4.0 %), associated with a corresponding increase (by up to 10%) in dense mitochondria with vacuoles (DV, C»C: 4.7 ± 1.6 % ; C»OB: 11.2 ± 1.7 %; OB»C 14.8 ± 1.9 %; OB»OB 14.1 ± 1.9 %) and without vacuoles (D, C»C: 1.4 ± 0.8 %; C»OB: 2.9 ± 1.3 %; OB»C 5.6 ± 1.3 %; OB»OB 5.3± 0.8 %, Figure 6).

Such maternal diet induced change in mitochondrial electron density in the primordial follicle oocytes is similar to that observed after follicle activation at the secondary stage in control (C»C) oocytes, and was previously associated with an increased cellular respiration. This may imply an earlier onset of oxidative phosphorylation, or activation of the mitochondria in the primordial follicles of the offspring born to obese mothers.

The maternal OB diet also slightly increased the proportion of elongated mitochondria with 4 - 5 % (C»C: 2.2 ± 0.9 %; C»OB: 3.3 ± 1.3 %; OB»C: 7.9 ± 1.8 %; OB»OB: 6.2 ± 1.0 %). Mitochondrial elongation is associated with changes in energy efficiency and may even indicate alterations in mitochondrial dynamics and replication, as described by others (Boudoures et al., 2016; Saben et al., 2016; Singh et al., 2021).

Interestingly, only the presence of cristae, differences in mitochondrial density or shape were affected by a maternal OB diet, and may imply rather functional adaptations instead of dysfunction (Yildirim and Seli, 2024). The proportions of mitochondrial abnormalities, directly associated with mitochondrial dysfunction (Marei et al., 2020) remained unaffected by a maternal OB diet.

It is suggested that mitochondrial dysfunction in oocytes of offspring born to obese mothers may originate from the maternal aberrant oocyte mitochondria that are transferred to the embryo at conception, due to the inability of oocytes to activate mitophagy (Saben et al., 2016; Boudoures et al., 2017). However, these studies were performed in inbred C57BL/6 mice, while it is known that C57BL/6 mice already show an increased rate of inborn oocyte mitochondrial ultrastructural abnormalities (compared to Swiss mice of the same age, fed the same control diet), which may affect the mitochondrial transmission to the next generations (Marei et al., 2020). On top, several mechanisms preclude such transfer of mitochondrial damage to the offspring, e.g. the bottleneck phenomenon and mitochondrial self-repair (Lee et al., 2012; Cox et al., 2021). Multiple rounds of mitochondrial segregation and replication during the formation of the primordial follicle pool even increase the chance of inducing *de-novo* mutations (Lei and Spradling, 2016; Yamashiro et al., 2020), making this germline transmission of mitochondrial dysfunction rather unlikely. Presumably, the maternal OB diet induced alterations that we detected in the oocytes of the offspring's primordial follicle pool, may occur due to the direct effects of the maternal OB diet on the dividing primordial germ cells during pregnancy, drastically impacting the early formation of the primordial follicle pool, or even affect the offspring's oocyte mitochondria post-gestational during lactation. Currently, we are trying to make similar assessments to investigate if these primordial follicle pool oocytes are already affected at birth.

5.4.3.2 *The effect of the offspring diet on the oocyte mitochondrial morphology of the primordial follicles*

In contrast with maternal diet effects, offspring OB diet significantly increased the proportion of abnormal mitochondria in the primordial follicle oocytes by around 10 % (C»C: 13.4 ± 1.6 % ; C»OB: 22.4 ± 2.9 %; OB»C: 15.5 ± 1.9 %; OB»OB: 23.1 ± 2.5 %). Offspring diet also had a significant effect on the proportions of SCr mitochondria (C»C: 55.0 ± 5.3 %; C»OB: 40.9 ± 4.4 %; OB»C: 27.2 ± 4.3; OB»OB: 32.2 ± 4.0 %) and a tendency to increase the proportion of dense mitochondria. However these effects were only obvious in offspring born to mothers fed a control diet, and may be masked by maternal obesity. Nevertheless, the interaction between offspring and maternal diet effects on SCr and D mitochondria was never significant (Figure 6).

The mitochondrial abnormalities detected in our study involved mitochondria with protruded or ruptured outer membrane, which may suggest mitochondrial bursting. Mitochondrial bursting indicates exacerbated levels of oxidative stress, ultimately leading to complete mitochondrial dysfunction (Eliassen et al., 2006; Sesso et al., 2012). Also, loose inner membrane structures inside an enlarged intercristal space were detected, previously described as an abnormal mitochondrial feature in mature oocytes (Marei et al., 2020; Xhonneux et al., 2023). Mitochondria with electron dense foci and rose-petal shaped mitochondria were also classified here as abnormal. Others associated the presence of electron dense foci with altered glucose and oxygen metabolism (Grindler and Moley, 2013a), and rose-petal shaped mitochondria are linked with affected ATP-synthase (Paumard et al., 2002; Arselin et al., 2003) which is a major component of the fifth mitochondrial complex, needed to produce energy (Cogliati et al., 2013). Our results thus indicate that offspring OB diet is detrimental for oocyte quality in the primordial follicles. Most direct effects of an OB diet on fully grown (matured or ovulated) oocytes are attributed to biochemical changes in the composition of the follicular fluid, such as increased lipid concentrations and pro-inflammatory cytokines (Carrell et al., 2001; Robker et al., 2009; Valckx et al., 2014b). With our results, we show for the first time that these effects may already be induced even before the formation of the antrum.

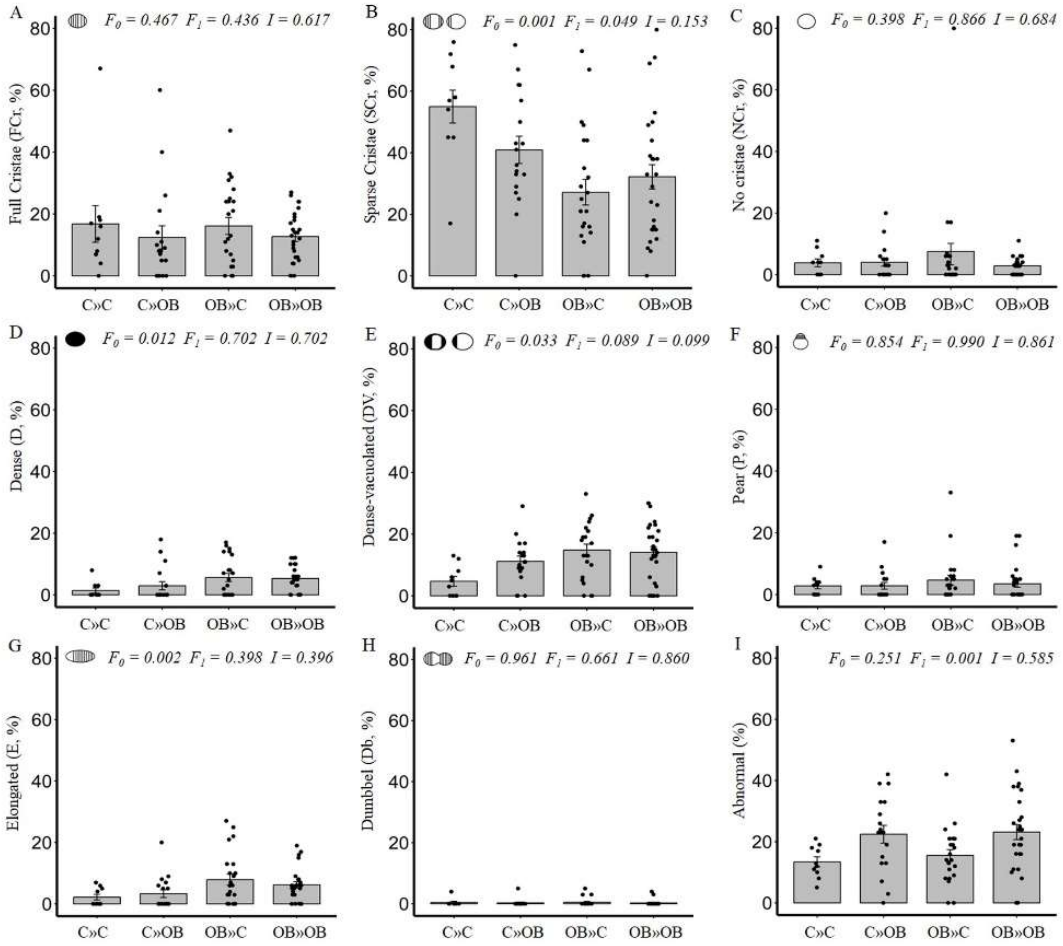

Figure 6. The effect of maternal and offspring OB diets and their interaction on mitochondrial ultrastructure in oocytes of primordial follicles within ovaries of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as percentage ± S.E. and are derived from primordial follicles of 6 mice per treatment group (C»C n = 10, C»OB n = 18, OB»C n = 23, OB»OB n = 27 follicles). *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I = effect of the interaction).

5.4.3.3 *Effects of maternal and offspring diets on mitochondria in primary and secondary follicles*

We could not detect any effects of the maternal OB diet on oocyte mitochondrial ultrastructure in the primary and secondary follicles (Figure 7 and Figure 8 respectively). This could be due to changes in the oocyte mitochondrial morphology that already occur when the primordial follicles are activated (as described above), which may mask the detection of maternal diet effects. However, while follicle atresia was not assessed in this study, the absence of dietary effects in these late stages of preantral follicular development may also imply that only the follicles with the best oocyte mitochondria are able to reach the secondary stage. Nevertheless, further research is required to understand these notions at a functional level.

Similarly, we could not detect any effect of the offspring OB diet on mitochondrial density or shape in the primary and secondary oocytes (Figure 7 and Figure 8 respectively). However, the effects on the proportions of abnormal mitochondria in primary oocytes were almost identical to those observed in the

primordial follicles (C»C: 11.0 ± 2.1 %; C»OB: 20.5 ± 3.5 %; OB»C: 14.1 ± 2.7 %; OB»OB: 20.4 ± 2.1 %), and indicative of mitochondrial damage.

A parallel study done at our lab, using the same mouse model and experimental design but focusing on the dietary impact on fully grown mature oocytes (collected after ovulation), clearly showed that the direct effect of the offspring's OB diet on mitochondrial ultrastructure in these mature ovulated oocytes was influenced by the maternal metabolic background (Xhonneux et al., 2023). Therefore, the dietary effects on the mitochondrial ultrastructure detected here in the offspring's primordial follicles may have long-term repercussions throughout subsequent folliculogenesis.

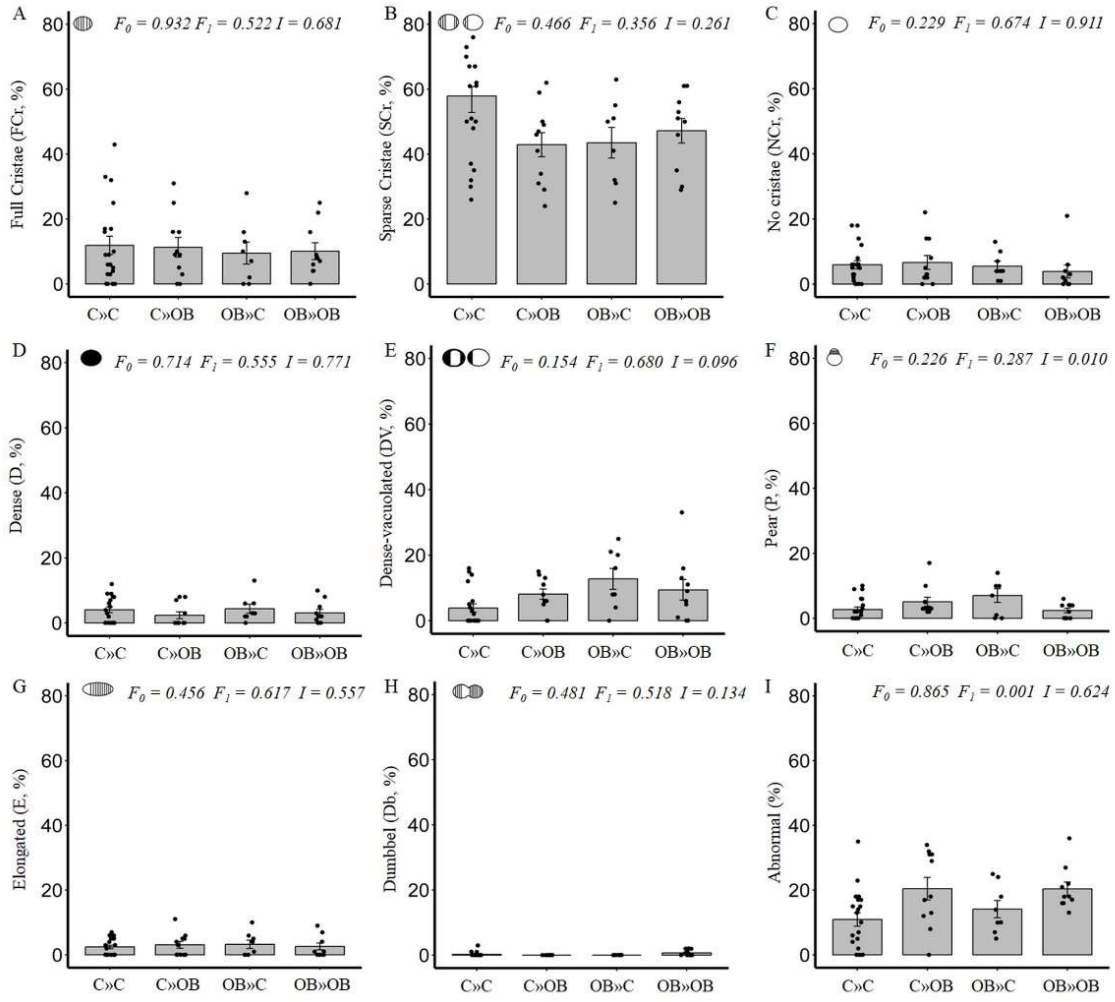

Figure 7. The effect of maternal and offspring OB diets and their interaction on mitochondrial ultrastructure in oocytes of primary follicles within ovaries of offspring fed a C or an OB diet and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as percentage ± S.E. and are derived from primary follicles of 6 mice per treatment group (C»C n = 20, C»OB n = 11, OB»C n = 8, OB»OB n = 10 follicles). *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I = effect of the interaction).

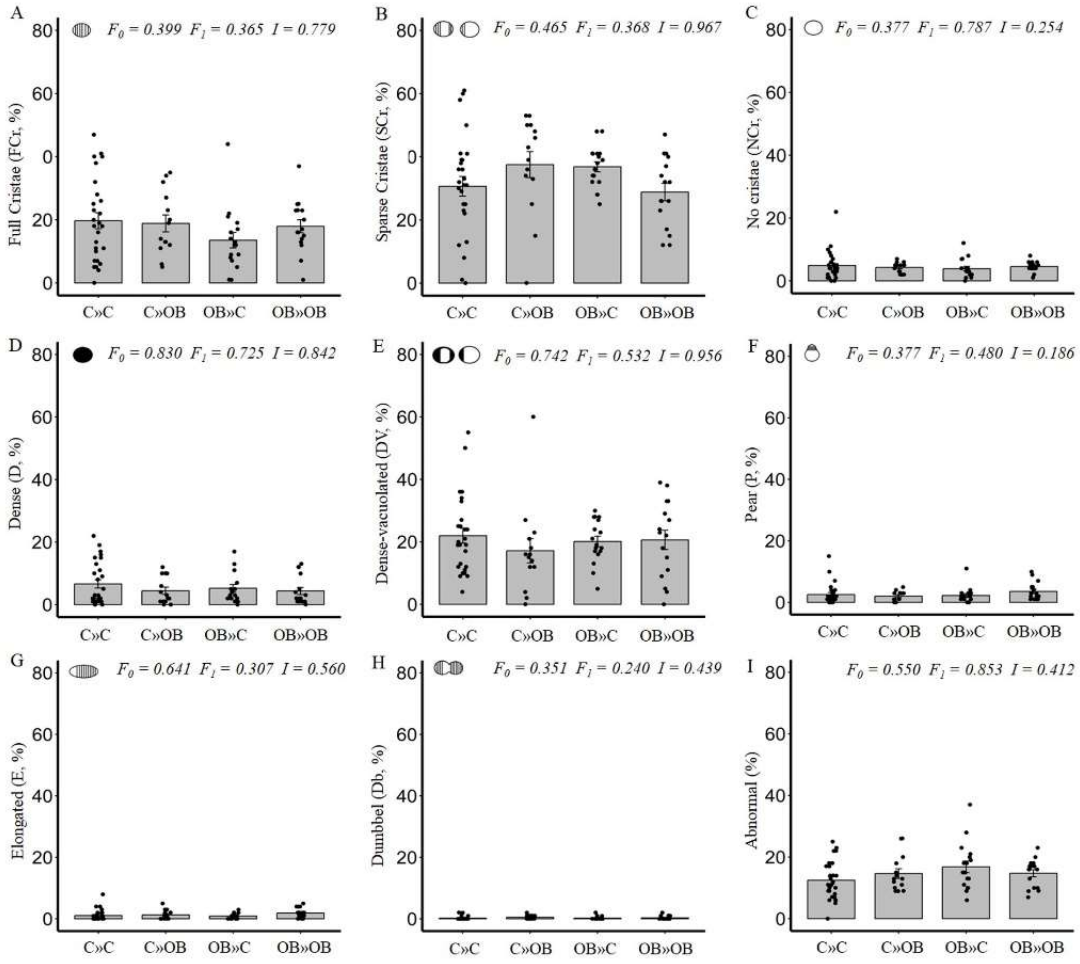

Figure 8. The effect of maternal and offspring OB diets and their interaction on mitochondrial ultrastructure in oocytes of secondary follicles within ovaries of offspring fed a control (C) or an obesogenic diet (OB) and born to mothers that were either fed a C or OB diet, in a 2 x 2 factorial design. Data are presented as percentage ± S.E. and are derived from secondary follicles of 6 mice per treatment group (C»C n = 27, C»OB n = 14, OB»C n = 17, OB»OB n = 16 follicles). *P*-values of the main effects are stated (F_0 = maternal diet effect, F_1 = offspring diet effect, I = effect of the interaction).

5.5 Conclusions

In conclusion, this study characterized and described the oocyte mitochondrial morphology in primordial and preantral follicles. We showed that the matrix density of primordial follicle oocyte mitochondria increases after follicle activation, thereby becoming similar to the mitochondrial morphology of the well described mature (ovulated) oocytes. On top, the effect of both maternal and offspring OB diets and their interaction on oocyte mitochondria in primordial and early activated follicles was investigated using a pathophysiological relevant outbred mouse model in a 2 x 2 factorial design, thereby mimicking the human situation of female familial obesity. We are the first to show that the oocyte mitochondria of the dormant primordial follicle pool are already changing under the influence of both maternal and offspring OB diets, which may impact the quality of the oocyte during subsequent stages of folliculogenesis. While our study remains descriptive, focusing only on the mitochondrial morphology without functional analysis, it was clear that the maternal OB diet increased the mitochondrial density already in the primordial follicles, suggesting an earlier increase in bioenergetic capacity that may be adaptive in nature, rather than dysfunctional. Interestingly, maternal obesity did not induce abberant oocyte mitochondrial ultrastructure (abnormalities and defects) in preantral follicles of their offspring, and did not influence the direct effect of an offspring OB diet. In contrast, only offspring OB diet increased the oocyte mitochondrial abnormalities in the primordial follicles, indicative for mitochondrial damage. While further functional investigations are still required, the unique and novel insights provided here at these very early stages strongly suggests that preantral folliculogenesis is a crucial window of sensitivity of the oocyte for the effects of maternal and offspring OB diets, which may require attention when developing or optimizing reproductive managing protocols for obese patients.

Acknowledgements

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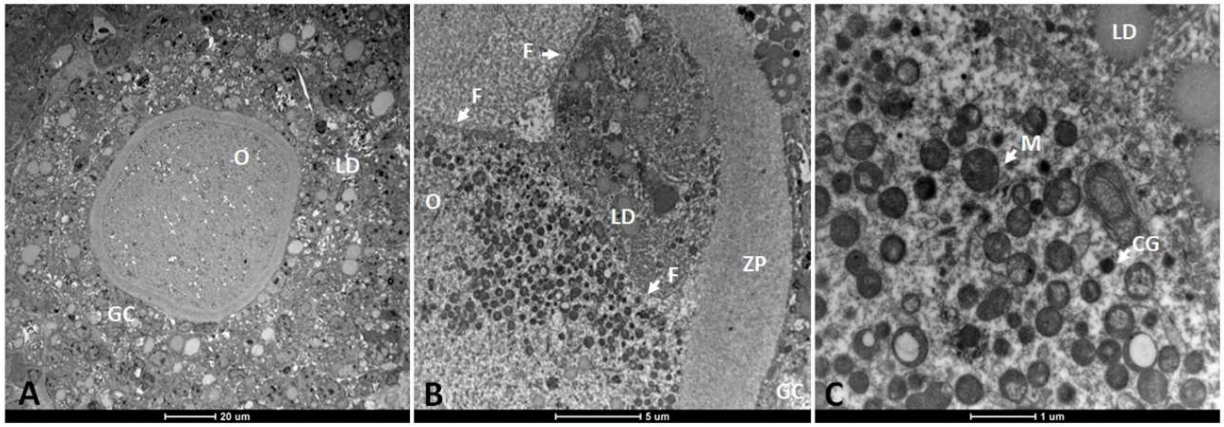
5.6 Supplementary information



S1 Figure 1. TEM overview of apoptotic follicles. A. overview of an apoptotic follicle; B. fragmentation of the oocyte within the apoptotic follicle; C. close-up image of mitochondria in the oocyte within an apoptotic follicle (O = oocyte; M = mitochondria within the oocyte cytoplasm; ZP = zona pellucida; GC = granulosa cells, F = oocyte fragmentation, CG = cortical granule, LD = lipid droplet). Whereas the mitochondria in oocytes of non-atretic follicles were more or less equally dispersed throughout the cytoplasm, the mitochondria in oocytes of apoptotic follicles were not. Only in these follicles, signs of mitochondrial clustering were detected with cortical granules present in the peri-cortical area of the oocyte. Lipid droplets were seen in the oocyte and in the surrounding granulosa cells. The oocyte itself was irregularly shaped, showing signs of fragmentation.

S1 Table 1. Amount of mitochondria studied in primordial follicles in all treatment groups.

	Mean number of oocyte mitochondria per follicle ± S.E.M	Maximum number of mitochondria per follicle	Minimum number of mitochondria per follicle
C»C	24.1 ± 3.2	38	6
C»OB	20.4 ± 2.5	39	3
OB»C	24.3 ± 2.8	49	1
OB»OB	23.8 ±1.8	49	11

Data are derived from all primordial follicles of 6 mice per treatment group (C»C n = 10, C»OB n = 18, OB»C n = 23, OB»OB n = 27 follicles).

S1 Table 2. Amount of mitochondria studied in primary follicles in all treatment groups.

	Mean number of oocyte mitochondria per follicle ± S.E.M	Maximum number of mitochondria per follicle	Minimum number of mitochondria per follicle
C»C	40.2 ± 4.9	91	11
C»OB	36.5 ± 3.6	63	19
OB»C	49.9 ± 11.4	97	8
OB»OB	61.3 ± 8.4	99	15

Data are derived from all primary follicles of 6 mice per treatment group (C»C n = 20, C»OB n = 11, OB»C n = 8, OB»OB n = 10 follicles).

S1 Table 3. Amount of mitochondria studied in secondary follicles in all treatment groups.

	Mean number of oocyte mitochondria per follicle ± S.E.M	Maximum number of mitochondria per follicle	Minimum number of mitochondria per follicle
C»C	91.2 ± 6.7	157	31
C»OB	125.6 ± 9.6	202	60
OB»C	117.2 ± 11.3	175	26
OB»OB	136.7 ± 9.6	199	65

Data are derived from all secondary follicles of 6 mice per treatment group (C»C n = 27, C»OB n = 14, OB»C n = 17, OB»OB n = 16 follicles).

S2 Table 1: Different mitochondrial phenotypes of primordial follicles per treatment group primordial.

	FCr	SCR	NCr	D	DV	P	E	DB	abnormal
C»C	0.168 ± 0.059	0.550 ± 0.053	0.038 ± 0.013	0.014 ± 0.008	0.047 ± 0.016	0.028 ± 0.009	0.022 ± 0.009	0.004 ± 0.004	0.134 ± 0.016
C»OB	0.124 ± 0.037	0.409 ± 0.044	0.040 ± 0.013	0.029 ± 0.013	0.112 ± 0.017	0.028 ± 0.011	0.033 ± 0.013	0.003 ± 0.003	0.224 ± 0.029
OB»C	0.161 ± 0.028	0.272 ± 0.041	0.075 ± 0.043	0.056 ± 0.013	0.148 ± 0.019	0.047 ± 0.016	0.079 ± 0.018	0.05 ± 0.003	0.155 ± 0.019
OB»OB	0.127 ± 0.016	0.322 ± 0.040	0.029 ± 0.006	0.053 ± 0.008	0.141 ± 0.019	0.035 ± 0.011	0.062 ± 0.010	0.003 ± 0.002	0.231 ± 0.025

The groups are named as MaternalDiet»OffspringDiet. Mitochondria showing loose inner membranes, broken or protruded outer membranes, rose-petal shaped mitochondria, enlarged mitochondria (>1µm), and electron dense mitochondria as described by Marei et al. (2020), are classified as abnormal. Data are presented as proportion ± S.E. and are derived from all primordial follicles of 6 mice per treatment group (C»C n = 10, C»OB n = 18, OB»C n = 23, OB»OB n = 27 follicles).

S2 Table 2: Different mitochondrial phenotypes of primary follicles per treatment group.

	FCr	SCr	NCr	D	DV	P	E	DB	abnormal
C»C	0.119 ± 0.028	0.579 ± 0.044	0.060 ± 0.013	0.041 ± 0.009	0.039 ± 0.013	0.028 ± 0.009	0.025 ± 0.006	0.003 ± 0.002	0.110 ± 0.021
C»OB	0.113 ± 0.030	0.429 ± 0.037	0.066 ± 0.021	0.024 ± 0.011	0.081 ± 0.016	0.051 ± 0.014	0.031 ± 0.011	0.000 ± 0.000	0.205 ± 0.035
OB»C	0.095 ± 0.034	0.435 ± 0.047	0.055 ± 0.015	0.044 ± 0.014	0.128 ± 0.032	0.070 ± 0.021	0.033 ± 0.013	0.000 ± 0.000	0.141 ± 0.027
OB»OB	0.101 ± 0.026	0.472 ± 0.038	0.039 ± 0.020	0.031 ± 0.011	0.094 ± 0.032	0.042 ± 0.007	0.026 ± 0.010	0.007 ± 0.003	0.204 ± 0.021

Data are presented as proportion ± S.E. and are derived from all primary follicles of 6 mice per treatment group (C»C n = 20, C»OB n = 11, OB»C n = 8, OB»OB n = 10 follicles).

S2 Table 3: Different mitochondrial phenotypes of secondary follicles per treatment group.

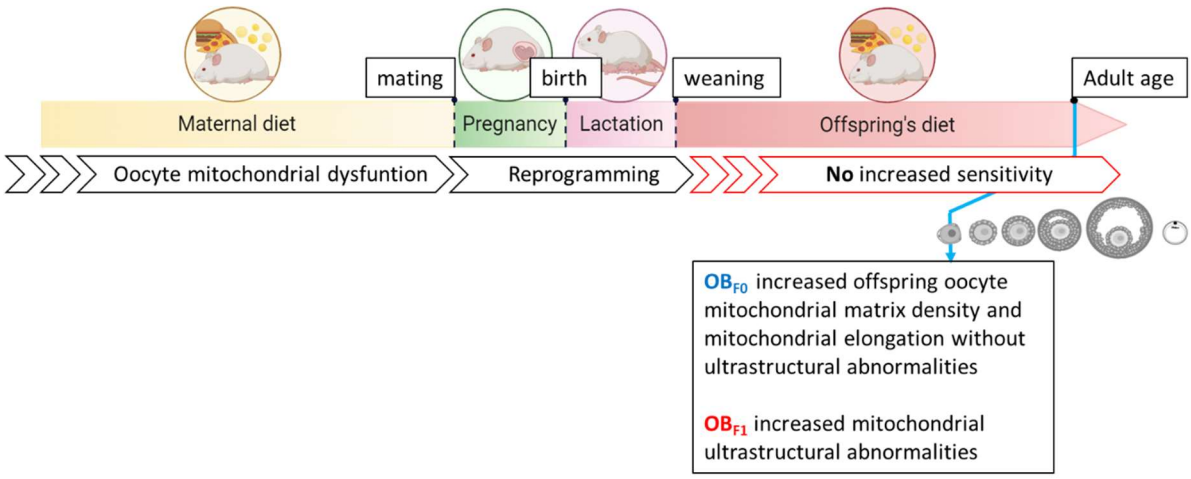
	FCr	SCr	NCr	D	DV	P	E	DB	abnormal
C»C	0.197 ± 0.025	0.306 ± 0.031	0.049 ± 0.009	0.066 ± 0.013	0.220 ± 0.024	0.025 ± 0.007	0.011 ± 0.004	0.002 ± 0.001	0.125 ± 0.012
C»OB	0.189 ± 0.027	0.375 ± 0.041	0.043 ± 0.004	0.044 ± 0.012	0.171 ± 0.039	0.020 ± 0.004	0.013 ± 0.004	0.005 ± 0.002	0.146 ± 0.016
OB»C	0.135 ± 0.024	0.368 ± 0.016	0.039 ± 0.007	0.052 ± 0.012	0.201 ± 0.017	0.022 ± 0.006	0.009 ± 0.002	0.002 ± 0.001	0.168 ± 0.019
OB»OB	0.179 ± 0.02	0.288 ± 0.028	0.046 ± 0.004	0.044 ± 0.012	0.206 ± 0.031	0.036 ± 0.007	0.019 ± 0.004	0.003 ± 0.002	0.148 ± 0.011

Data are presented as proportion ± S.E. and are derived from all secondary follicles of 6 mice per treatment group (C»C n = 27, C»OB n = 14, OB»C n = 17, OB»OB n = 16 follicles).

5.8 Key message

We are the first to describe that the direct effect of the offspring's OB diet perturbed the adult offspring's primordial follicle oocyte mitochondrial quality by inducing ultrastructural damage in outbred Swiss mice. However, maternal OB diet consumption did not induce ultrastructural damage in their offspring's primordial follicle oocytes (assessed at 10 weeks of age), and did not interact with the offspring diet effects on the primordial follicle oocytes.

5.9 Graphical summary



Schematic overview of the main effect of the maternal OB diet (OBF_0), the offspring OB diet (OBF_1), and the maternal x offspring diet interaction (OBF_0 x OBF_1) on offspring primordial follicle oocyte mitochondrial ultrastructure at adulthood (age 10 weeks).

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Chapter 6

The Impact of Maternal Diet-Induced Obesity on Offspring Primordial Oocyte Mitochondria at Birth and at Weaning

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6.1 Abstract

Maternal diet-induced obesity (DIO) may affect adult offspring oocyte quality due to mitochondrial dysfunction. We investigated if offspring of DIO mothers exhibit mitochondrial abnormalities in their primordial follicle oocytes already at birth, and if (further) alterations can be detected at weaning. Female Swiss mice were fed a control or obesogenic (OB) diet for 7 weeks before mating and throughout pregnancy and lactation. Offspring primordial follicle oocytes in ovarian sections were examined at birth for mitochondrial ultrastructural abnormalities via transmission electron microscopy. Key markers of cell stress (Hsp70), mitochondrial biogenesis (PGC-1α), mtDNA replication (TFAM), fusion (Mfn2, OPA1), and fission (Drp-1) were examined using immunofluorescence and confocal microscopy at birth and weaning. Maternal DIO did not induce mitochondrial ultrastructural abnormalities in offspring primordial follicle oocytes at birth, nor altered Hsp70 or PGC-1α, suggesting that cellular homeostasis and mitochondrial biogenesis were unaffected. In contrast, maternal DIO reduced TFAM expression at both timepoints, which may impact mtDNA replication/stability. Drp1 and OPA1 expression were altered at birth, but without major ultrastructural changes, suggesting regulatory alterations. In contrast, at weaning, PGC-1α expression was significantly increased, indicating additional postnatal effects on primordial follicle oocytes mitochondrial biogenesis. Mfn2 and OPA1 expression also increased during this period with a persistently low TFAM. In conclusion, the previously reported oocyte mitochondrial abnormalities in adult offspring from DIO mothers are not inborn. More significant changes in primordial follicles linked to maternal DIO are detectable post-lactation. The impact of these alterations on the maintenance of the follicular reserve and oocyte quality later in life requires further investigation.

6.2 Introduction

The link between maternal obesity and reduced offspring metabolic health is well established (Contreras et al., 2016). Offspring born to obese women have higher risks of developing obesity, diabetes, coronary heart disease, stroke, and cancer (Eriksson et al., 2014). There is increasing evidence that maternal obesity can also impact offspring fertility (Cinzori and Strakovsky, 2022). Maternal obesity was linked with offspring genital defects such as elongated anogenital distance in girls (Kloboves et al., 2022), hypospadias, cryptorchidism and compromised germ cell maturation in boys (Waller et al., 2007; Blomberg and Källén, 2010; Arendt et al., 2017), as well as precocious onset of menarche, adrenarche and puberty (Cinzori and Strakovsky, 2022). These anomalies and alterations are usually attributed to placental dysfunctions, oxidative stress and hormonal imbalance during pregnancy, which leads to disruption of the gonadal development and fetal programming of the hypothalamus-pituitary-gonadal axis (Grumbach, 2005). In contrast, the effects of maternal obesity on the female germline, more specifically on the quality of the oocytes forming the ovarian reserve of the next generation, are less characterized.

A study in a diet-induced obese (DIO) mice showed that obesogenic (OB) diet consumption during conception and pregnancy lead to compromised adult offspring GV oocyte quality, presented as increased mitochondrial ultrastructural abnormalities (larger, less round mitochondria with irregular vacuoles enclosing lamellar membranes), reduced OPA1 expression and reduced mtDNA copy numbers, suggesting altered mitochondrial fusion/fission and biogenesis (Saben et al., 2016). Similarly, mitochondrial mass and bioenergetic activity were reduced in ovulated MII oocytes of adult offspring born to obese mice (Andreas et al., 2019). However, the primordial follicle pool was never tested. Aiken et al. (2016a) noticed that whole ovaries of adult offspring born to obese mice exhibit altered gene expression of mtDNA replication (TFAM) and mitochondrial antioxidant markers (e.g. SOD), without determining the cell type or follicular stage in which these alterations occur. Recently, we showed that oocytes of adult offspring born to obese mothers have global hypermethylation of their nuclear DNA (Meulders et al., 2024), which controls mitochondrial biogenesis and replication (Kelly et al., 2012). We also examined if primordial follicle oocytes of adult offspring born to obese mothers carry mitochondrial ultrastructural alterations, and found no increased proportions of mitochondria with irregular vacuoles, but observed a premature increase in mitochondrial density, indicating changes in bioenergetic capacity (Xhonneux et al., 2024b). Notably, adult offspring born to DIO mothers may suffer from altered metabolic health as described above. Therefore, the effects on offspring oocytes may occur *de novo* during post-weaning development, after puberty, and/or during late stages of folliculogenesis. It is yet to be determined whether primordial follicle oocytes are already affected by maternal DIO at birth.

Since mitochondria are exclusively maternally inherited, oocyte mitochondrial dysfunction due to DIO (in F0) can significantly impact pre- and post-implantation embryo development (Van Blerkom, 2011; Grindler and Moley, 2013). Studies using *in vivo* and *in vitro* exposure models have shown that oocytes matured under an obesogenic (lipotoxic) environment exhibit mitochondrial alterations that disturb mitochondrial ion transport and electron transport complex protein assembly, leading to ultrastructural abnormalities and cristae malformation (Emelyanova et al., 2016; Vincent et al., 2016a; Hayden, 2022). When such defective mitochondria are transferred to the embryo, mitophagy is not activated (Boudoures et al., 2017) and the mitochondrial dysfunction and cellular stress levels persist (Saben et al., 2016; Marei et al., 2019). Subsequently, this alters the concentrations of ATP and metabolic byproducts that derive epigenetic programming during early embryo development, with potential long-lasting effects on embryonic cell proliferation, differentiation and metabolic functions after hatching and implantation (Desmet et al., 2020). In addition, such mitochondrial alterations may also be induced by maternal DIO during pregnancy due to a direct impact on placental functions (Higa and Jawerbaum, 2013).

On the other hand, several mechanisms take place in the female germline during early embryonic stages to limit the transfer of dysfunctional mitochondria and mtDNA mutations to the next generation. In the initial stages, continuous cell division with inactive mitochondrial biogenesis significantly reduces the number of mitochondria per cell (bottleneck) (Cao et al., 2007), allowing selection of cells with sound mitochondria to survive and proliferate further. At a later stage, healthy mitochondria are donated and pooled from sister cyst germ cells to the surviving oocyte pool that will later form the ovarian reserve (Lei and Spradling, 2016). The establishment of the primordial follicle reserve also involves several mitochondrial changes during the initial stages of oogenesis, including mitochondrial replication, biogenesis and a fine balance of mitochondrial fission and fusion processes, aiming to reach the mtDNA set-point, and the well-characterized immature fragmented form of mitochondria in arrested oocytes (Motta et al., 2000; Chiaratti, 2021; Telfer et al., 2023). While these processes are tightly regulated, it is not known if they could be altered by maternal obesity during pregnancy. Examining mitochondrial biogenesis and replication of primordial follicle oocytes at birth should reveal whether previously described defects in adult offspring oocytes are inborn, and whether the process of primordial follicle formation is affected by maternal DIO. However, this has not been described yet.

Finally, the impact of the early postnatal period on offspring ovarian development, particularly when nursing from an obese mother, should not be undermined. The Developmental Origins of Health and Disease (DOHaD) attributes offspring health disorders to the developmental programming that takes place during the first 1000 days of life (in humans), which corresponds to pregnancy and lactation (Wrottesley et al., 2016; Cumerlato et al., 2023). Obesity is known to change the milk composition, creating a nutritional imbalance for the offspring during the first postnatal weeks (Bravi et al., 2016). The potential additional impact of that on the primordial follicles has not been described.

Therefore, the aim of this study was to examine the primordial follicle oocytes in ovarian sections collected at birth and at weaning from offspring born to DIO and control mothers. We focused our analysis on key mitochondrial markers of biogenesis, replication, fusion/fission using immunofluorescence staining. In addition, we also examined the offspring ovarian sections using transmission electron microscopy at birth to check if the oocyte mitochondrial ultrastructural abnormalities previously detected in adult offspring from DIO mothers (Saben et al., 2016) are inborn. We used an outbred Swiss mouse model - previously validated in our laboratory (Smits et al., 2022) - to increase the relevance to the human pathophysiology.

6.3 Materials and methods

Chemicals are purchased from Sigma Sigma-Aldrich (Missouri, US) and Life Technologies – ThermoFisher (Bleiswijk, The Netherlands) unless stated otherwise.

6.3.1 Experimental design

This study was approved by the Ethical Committee for Animal Testing (ECD 2018-05). All animal procedures were performed in accordance with the relevant guidelines and regulations.

Female Swiss mice (age 3 weeks) were fed either a Control diet ("CTRL", Sniff diets D12450J, containing 10 kJ % fat and 7 % sucrose, n = 10), or an OB diet (Sniff Diets E15741-34, 60 kJ % fat, in addition to 20 % fructose added to the drinking water, n = 10) for 7 weeks. Then, the mothers were weighed and mated with the same chow-fed control Swiss males (n = 2) in 2 replicates using a cross-over design. The dams stayed on their corresponding diet during pregnancy and during lactation, which commonly happens in pregnant women. Pregnancies were closely monitored. At birth, within 1 h post-partum (TP1, Figure 1), and at weaning (TP2, offspring age 3 weeks), ovaries of one female pup per mother (5 mothers per TP/group) were collected and washed in PBS, after recording the offspring's weight. One ovary per offspring was used for ultrastructural analysis using TEM at birth. The other ovary was used for immunofluorescence staining and confocal microscopy both at birth and at weaning, as described below.

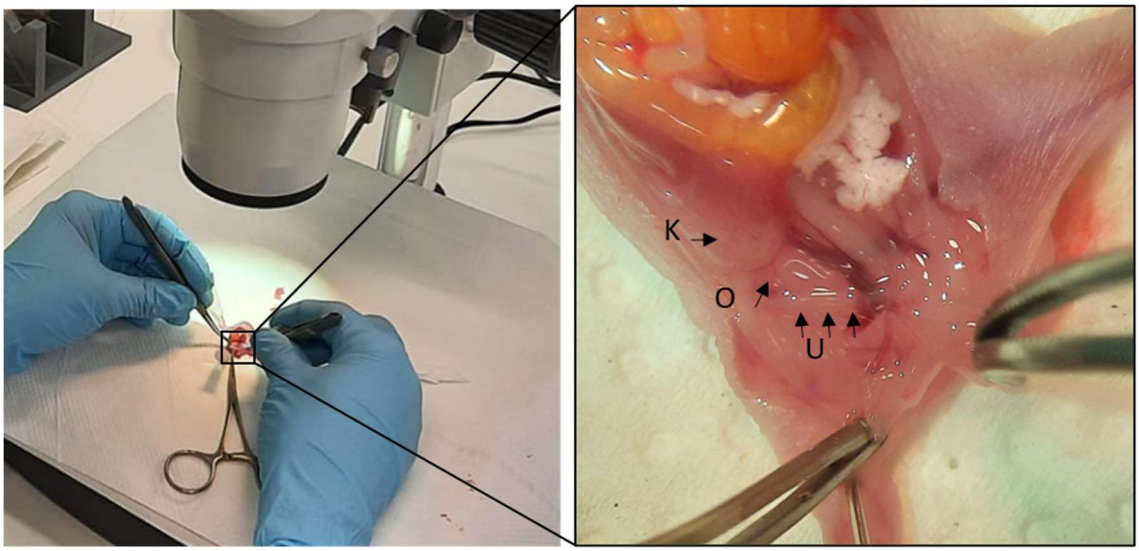

Figure 1. Collection of ovaries of newborn pups within 1h after birth. K= kidney, O = ovary, U = uterine horn.

6.3.2 Ovarian sectioning and immunofluorescence (IF) to assess cellular stress and mitochondrial markers

One TP1 and one TP2 ovary from offspring from the same mother were embedded and oriented together in the same agar drop and processed further to facilitate finding and sectioning the small ovaries. The agar drops containing the ovaries were processed following the standard histological procedures and embedded in paraffine and stored at RT until serial sectioning on super frost slides. Next, sections were dewaxed in xylol (3 x 5 min) and rehydrated in ethanol (100 % - 50 %) series. Antigen retrieval was done in 10 mM sodium citrate buffer (pH 6) for 10 minutes at 100 °C. Sections were permeabilized using DPBS

containing 1 % (v/v) Triton X-100 and 0.05 % Tween-20 solution for 20 - 40 min, then rinsed with DPBS containing 0.05 % Tween-20 and incubated in blocking buffer: DPBS containing 4 % BSA and 10 % Normal Goat Serum (NGS) for 30 minutes in a humidified chamber. The sections were washed with primary antibody buffer: PBS with 1 % NGS, and incubated with primary antibodies of markers of cellular stress (Hsp70), mitochondrial replication (TFAM), biogenesis (PGC-1α), fission (Drp1), and fusion (Mfn2 and OPA1). One random section per ovary was used for each marker. Since TP1 and TP2 ovaries were on the same slides, this reduced the technical variation between the timepoints. Negative controls were included and incubated with primary antibody buffer containing an equivalent concentration of normal rabbit IgGs. Next, secondary Cy™3 AffiniPure™ antibody (1:500, Jackson ImmunoResearch Europe, Ltd., Newmarket, UK) was applied for 1 h (RT) in a humidified chamber after washing (3 x 5 min) with PBS 0,05 % Tween-20. Following the incubation, the sections were washed with PBS 0,05 % Tween-20 and counterstained with Hoechst (10 µg/ml, Invitrogen, Carlsbad, CA, USA), mounted with DAPCO (Carl Roth, Karlsruhe, Germany) and covered with cover-slips.

6.3.2.1 *Identification and localization of primordial follicles*

At birth, folliculogenesis is not active and only primordial follicles are present in the ovary. At weaning (age 3 weeks), mice approach puberty and different stages of follicular growth can be observed. In this study, we focused on the primordial follicle pool at both timepoints.

Prior to investigation, ovaries were mapped and all primordial follicles in the examined TP1 and TP2 sections were identified. Primordial follicles, characterized by clear round nuclei surrounded by one layer of flattened granulosa cell nuclei (Telfer et al., 2023), were localized and mapped on the Hoechst-stained serial sections. These maps were used to find and evaluate various mitochondrial markers in the same regions of interest, contributing to the accuracy and specificity of interpreting the results. To further confirm our follicle identification, we did immunostaining using DEAD-box helicase 4 DEAD-box helicase 4 (DDX4, a protein predominantly expressed in germ cells (A. R. White et al., 2009), 1µg/ml, ON at 4 °C, Abcam) which specifically labels the oocytes (Figure 2).

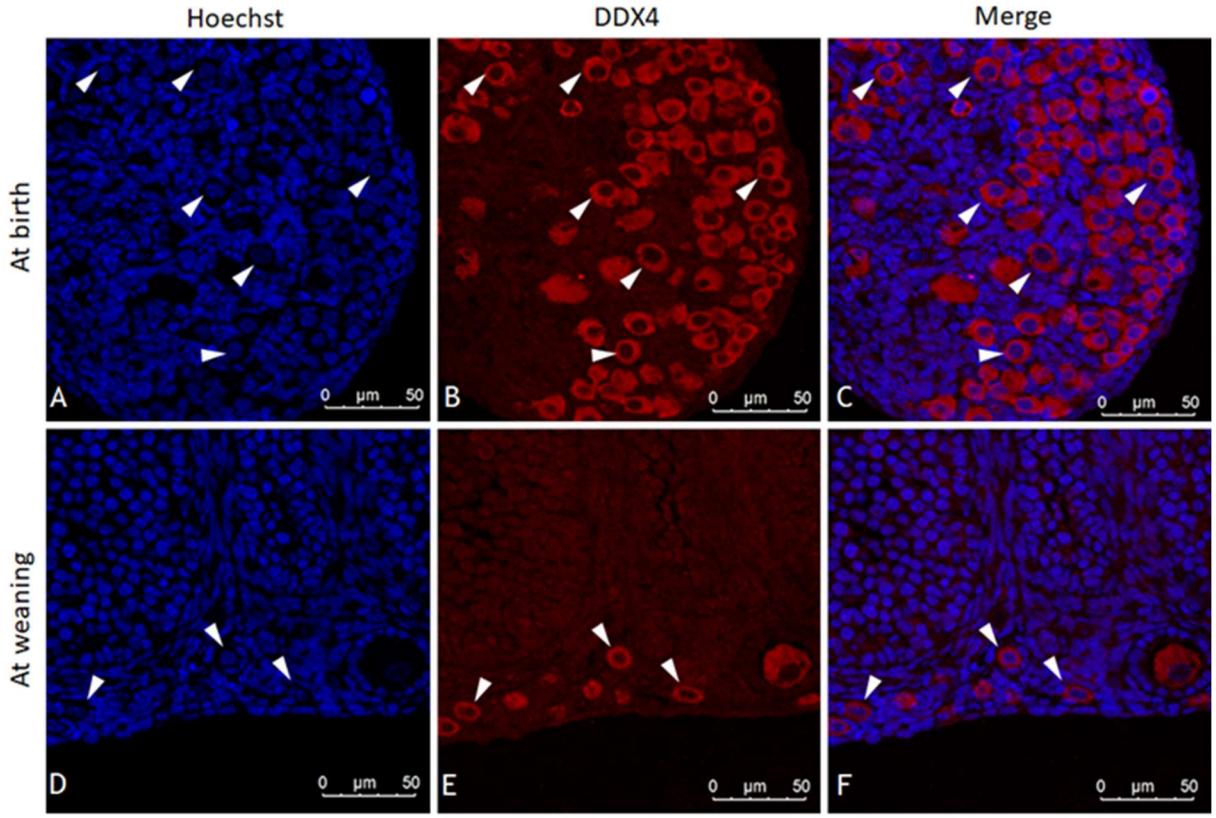

Figure 2: Identification of primordial follicles in murine ovaries using Hoechst and DDX4 immunofluorescence (**IF**) staining at birth (**A, B, C**) and at weaning (**D, E, F**). Primordial follicles were identified using their characteristic round nuclei of the enclosed oocytes, surrounded by a few flattened nuclei of the surrounding granulosa cells (see representative examples indicated by arrowheads). This was further confirmed using the DDX4 immunostaining and used to map ovarian sections for subsequent imaging of other markers on the adjacent serial sections. Follicles with incomplete or abnormal morphology were not used in the analysis.

6.3.2.2 *Image quantification*

Quantification of the images was done using ImageJ® software. Two random regions of interest in the cytoplasm of the oocyte within each primordial follicle were chosen, and the grey scale intensity in these regions was measured in three z-positions where the oocyte nucleus was at the biggest in diameter. When the examined marker was expressed in the nucleus (namely for PGC-1α and OPA1), an additional region of interest was chosen that comprised the nucleus. The average grey scale intensity in the cytoplasm and in the nucleus was then calculated per follicle and used further in the analysis. Two examples are shown in Figure 3.

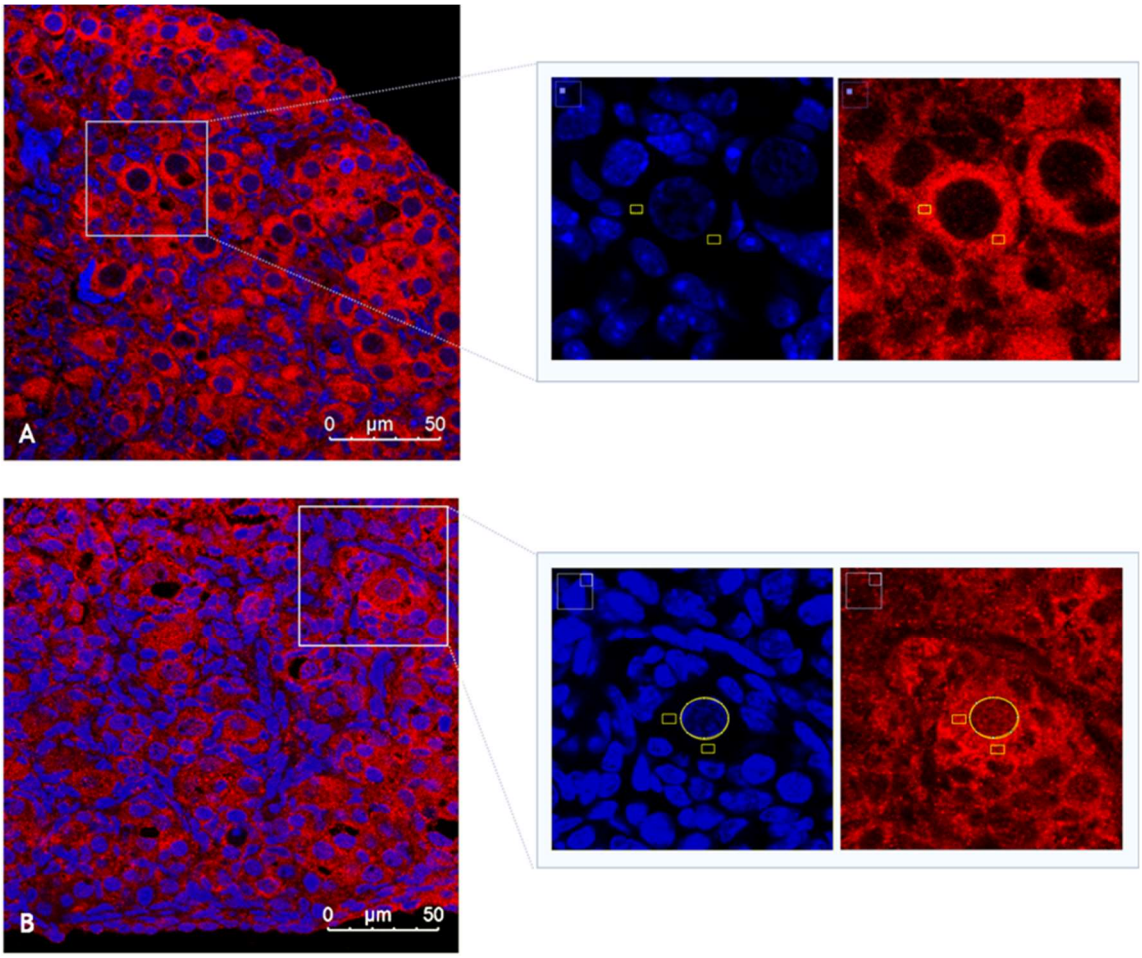

Figure 3. Regions of interest after ovarian mapping for Drp1 (A, only present in the cytoplasm) and PGC-1α (B, present in nucleus and cytoplasm). Two regions of interest were selected on the Hoechst stained images in the cytoplasm of the oocyte within the follicle on three different z-positions, presented as squares. When marker expression was present in the nucleus, an additional region of interest covering the nucleus was selected, presented as circle.

6.3.3 Assessment of oocyte mitochondrial ultrastructure

One ovary from one female offspring per mother was collected and fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5 % glutaraldehyde solution, embedded in 2 % agarose blocks and washed in 0.1 M sodium cacodylate-7.5 % saccharose solution (pH 7.4). Then, the blocks were incubated for 2 h in 1 % OsO_4 solution and dehydrated in an ethanol gradient. Sections were cut ultrathin using EM-bed812, stained with lead citrate and examined with TEM. Primordial follicles were identified (6 - 10 per section) and 5 non-overlapping images were acquired of one random section of each enclosed oocyte, at 16500 x magnification using a transmission electron microscope (Tecnai G2 Spirit Bio TWIN; Fei, Europe BV, Zaventem, Belgium) at 120 kV. The ultrastructure of all mitochondria in the examined offspring primordial follicle oocytes was determined and categorized based on shape (spherical or non-spherical = elongated or dumbbell-shaped), the presence of a vacuole, abnormal membrane structures, clustering (multiple mitochondria attached to each other by an electron dense spot) and the presence of electron dense foci by two independent researchers blind to the corresponding treatment group. Proportions of each category were recorded and analyzed. Since the primordial follicle oocyte mitochondrial ultrastructure at birth was found to be different from that reported at adulthood, additional images are presented in the Supplementary File 1 for comparison.

6.3.4 Statistical analysis

Statistical analysis was done using IBM SPSS Statistics 29 (for Windows, Chicago, IL, USA). Graphs were created using R (ggplot2). Categorical data (ultrastructural analysis) were analyzed using generalized linear mixed models. For numerical data (body weight, IF), data were checked for equality of variance using Levene's test and normality of distribution (residual QQ plots). The weight of the mother and the offspring used in this study was examined using independent student t-tests. Litter size was investigated with Mann-Whitney U-test, as the assumptions for parametric testing were not met. The main effects of the maternal diet (CTRL/OB), time point (TP1/TP2), and their interaction were examined using Two-way ANOVA. When there was an expression in both cytoplasm and nucleus (PGC-1α and OPA1), a separate analysis was performed for each site. If the interaction between the effect of the maternal diet and time was significant, the data were split for further analysis using an independent student t-test to study the effect of maternal diet and time separately, or Mann-Whitney U test if assumptions for parametric testing were not valid. When the interaction was not significant, the interaction term was omitted from the model. Significance level was set at $P < 0.05$.

6.4 Results

6.4.1 Maternal and offspring weight

Maternal DIO significantly increased the weight of the mothers at the time of mating (Figure 4A, *P* = 0.025), but did not affect the litter size (*P* = 0.752) or the weight of the pups at birth (*P* = 0.998, Figure 4B). In addition, maternal DIO diet did not affect the weight of the pups at weaning (*P* = 0.271).

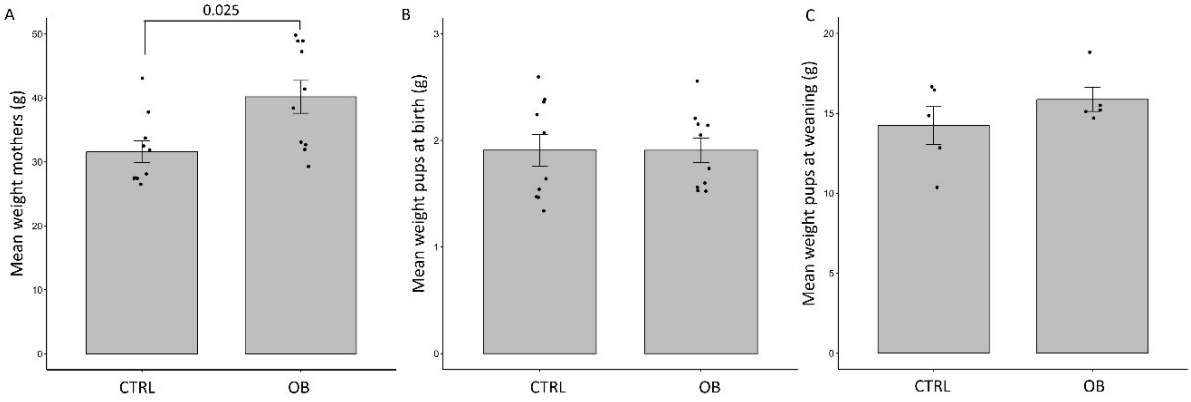

Figure 4. The Effect of the obesogenic diet (OB) on the live body weight of the mothers compared to control (CTRL)-fed mothers, and the effects on the weight of the offspring. **A.** Mothers weight at mating. **B.** Weight of the offspring at birth (including the offspring of both replicates, n = 10 per group). **C.** Weight of the offspring at weaning (n = 5 per group). Data are presented as mean ± S.E.M. and are derived from 10 CRTL and 10 OB mothers and from one offspring of each mother per timepoint.

6.4.2 Immunofluorescence (IF) staining patterns of the target markers in the primordial follicles

All target markers could be detected using IF staining both at birth and at weaning, and the pattern of the staining in the primordial follicle oocytes was distinctly target-specific (Figure 5). All markers were strictly expressed only in the cytoplasm, except PGC-1α and OPA1 which were also detected in the nucleus. The expression of HSP70 and Drp1 were specifically high in the primordial follicle oocytes compared to the surrounding somatic cells, and were homogenous across the cytoplasm.

The expression pattern of TFAM, Mfn2 and OPA1 followed a punctate pattern of expression throughout the cytoplasm, which matches with their known co-localization with the mitochondria.

The expression level of Mfn2 was the lowest of all markers but still considered positive when compared to the negative controls which showed no Cy3 signal. A higher level of expression of Mfn2 was noticed in the surrounding somatic cells, suggesting that the low expression level is specific to the oocytes.

Surprisingly, while OPA1 is known to be expressed only in the mitochondria (Zou et al., 2021), a strong OPA1 IF labelling was detected here in the nuclei of the primordial follicle oocytes. The surrounding nuclei of the somatic cells were negative, showing that this nuclear expression appears to be specific to the oocytes. Nuclear OPA1 expression has never been previously reported.

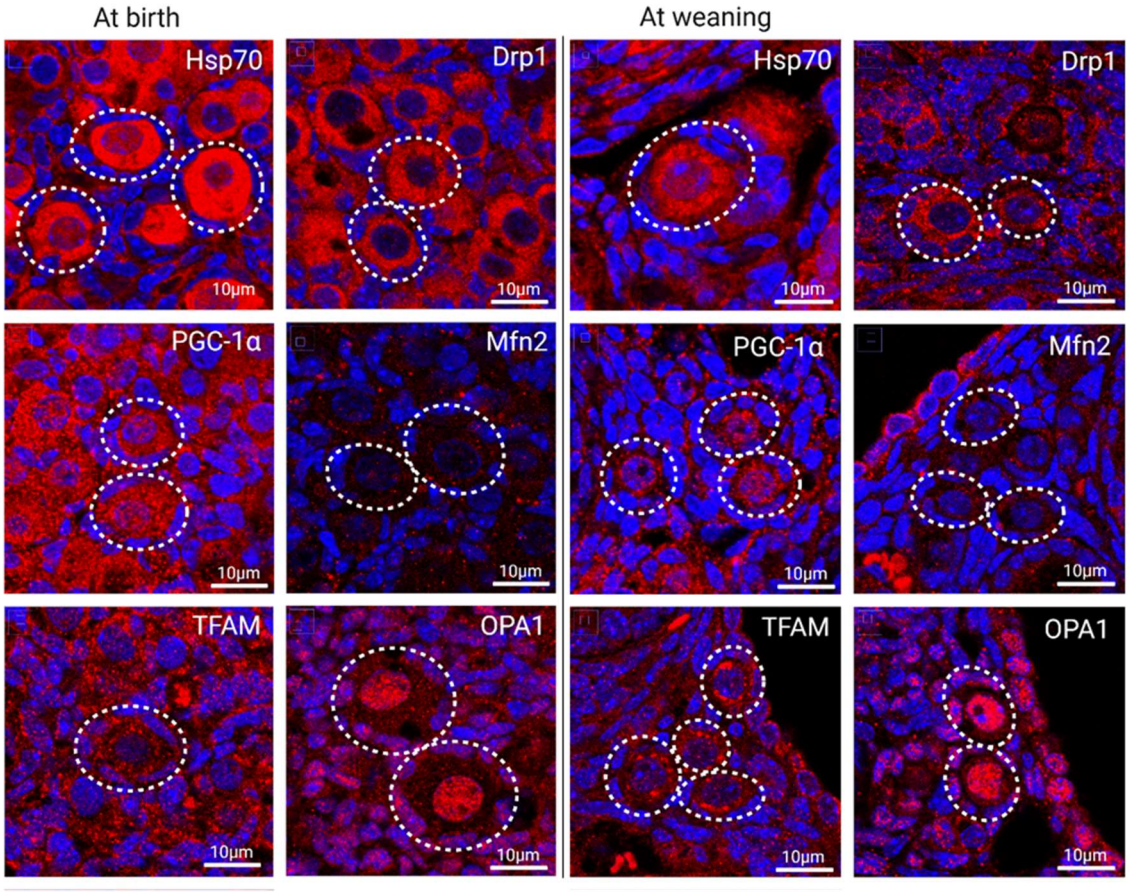

Figure 5: Representative confocal images of primordial follicles at birth and at weaning showing distinct patterns of expression of each target marker. The ovarian sections were immunolabeled with marker specific primary antibodies and Cy3 labelled secondary antibodies (red) as well as Hoechst counterstaining of the nuclei (blue). A few primordial follicle are highlighted in circles.

6.4.3 Effect of maternal diet-induced obesity on cell stress and mitochondrial markers in the primordial follicle oocytes at birth and at weaning

At birth, maternal DIO did not affect the expression of Hsp70 ($P = 0.087$), cytoplasmic PGC-1α ($P = 0.095$), nuclear PGC-1α ($P = 0.627$)) nor Mfn2 ($P = 0.135$) in offspring primordial follicle oocytes. However, the expression of TFAM ($P < 0.001$), Drp1 ($P < 0.001$) and cytoplasmic OPA1 ($P < 0.001$) was reduced by maternal DIO, while the expression of nuclear OPA1 was increased ($P < 0.001$), compared to the primordial oocytes in offspring born to control mothers.

At weaning, maternal DIO did not affect the expression of Hsp70 ($P = 0.087$) and Drp1 ($P = 0.725$) in offspring primordial follicle oocytes. However, the expression of TFAM ($P < 0.001$) was still reduced by maternal DIO, and the expression of both cytoplasmic PGC-1α ($P = 0.040$) and nuclear PGC-1α ($P = 0.046$), as well as Mfn2 ($P = 0.006$), cytoplasmic OPA1 ($P < 0.001$) and nuclear OPA1 ($P < 0.001$)) was significantly increased, compared to the primordial follicle oocytes in offspring born to control mothers.

We also noticed significant differences between the timepoints. When comparing the expression levels at weaning to those at birth we detected a reduced expression of Hsp70, cytoplasmic PGC-1α, Drp1, cytoplasmic OPA1 and nuclear OPA1 (all at $P < 0.001$), and increased expression of nuclear PGC-1α ($P = 0.004$), TFAM ($P = 0.042$) and Mfn2 ($P < 0.001$). The interaction between the effect of time and treatment

was significant for cytoplasmic PGC-1α ($P = 0.008$), nuclear PGC-1α ($P = 0.029$), Drp1 ($P = 0.002$), Mfn2 ($P < 0.001$) and cytoplasmic OPA1 ($P < 0.001$), but not for Hsp70 ($P = 0.236$), TFAM ($P = 0.482$) and nuclear OPA1 ($P = 0.897$).

The main effects of time and treatment and their interactions of all investigated markers are displayed on the interaction plots of Figure 6 and 7, including the corresponding *P*-values. The effects of treatment at birth and at weaning on all investigated markers are presented in the bar charts of Figure 6 and 7, including corresponding significant *P*-values ($P < 0.05$).

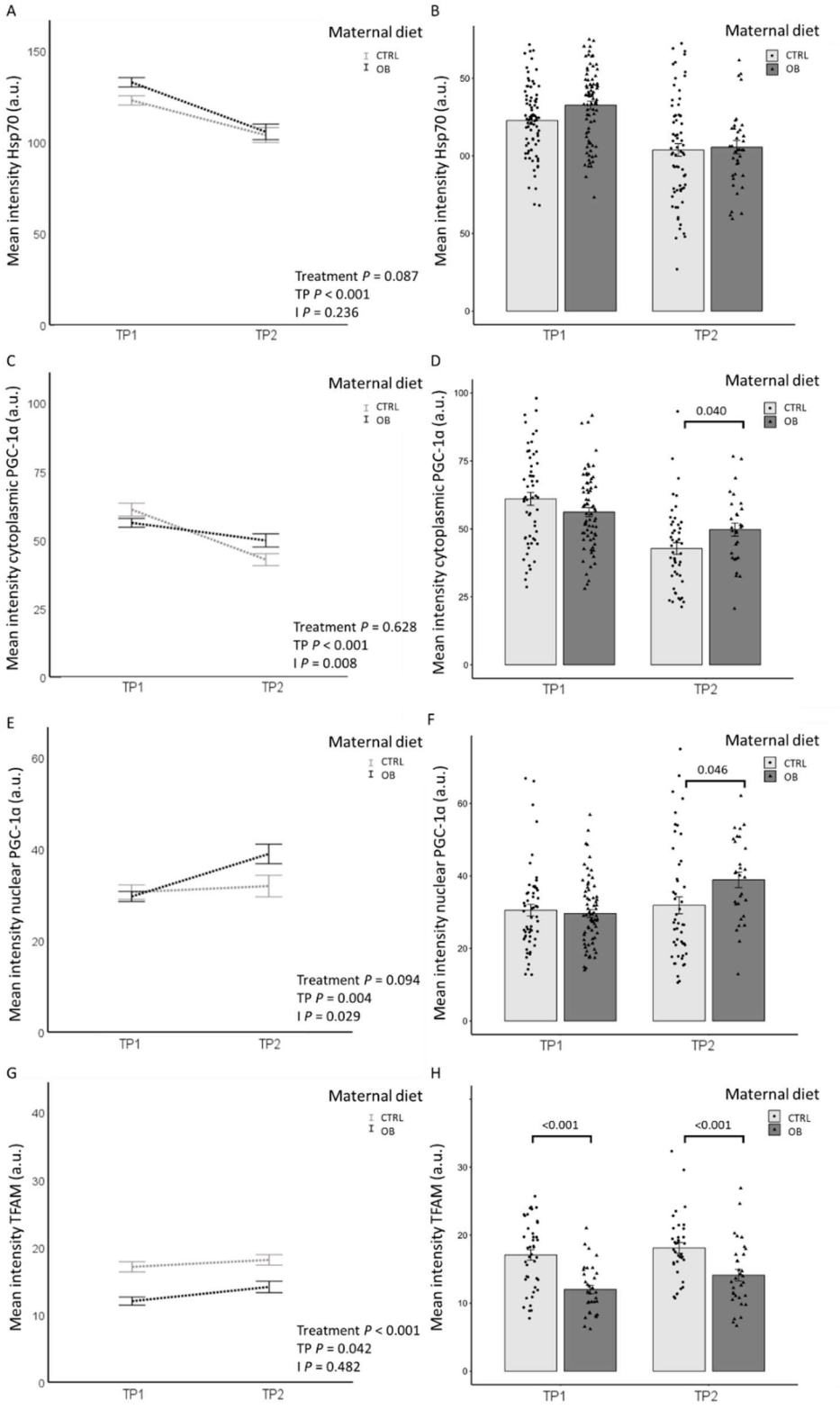

Figure 6. The effect of maternal obesogenic (OB) diet consumption on the expression of markers related to cellular stress (Hsp70); mitochondria biogenesis (PGC-1α), and mtDNA replication (TFAM) in offspring primordial follicle oocytes assessed at birth (TP1) and at weaning (TP2). Interaction plots (left) represent the main effects of time and treatment and their interaction. Bar charts with data points display the effect of treatment split per timepoint. Data are presented as mean ± S.E.M. and are derived from 81, 55 and 43 control (CTRL) and 82, 71 and 34 OB offspring primordial follicle oocytes at birth and 69, 48 and 37 CTRL and 39, 29 and 32 OB offspring primordial follicle oocytes at weaning for Hsp70, PGC-1α and TFAM analysis respectively.

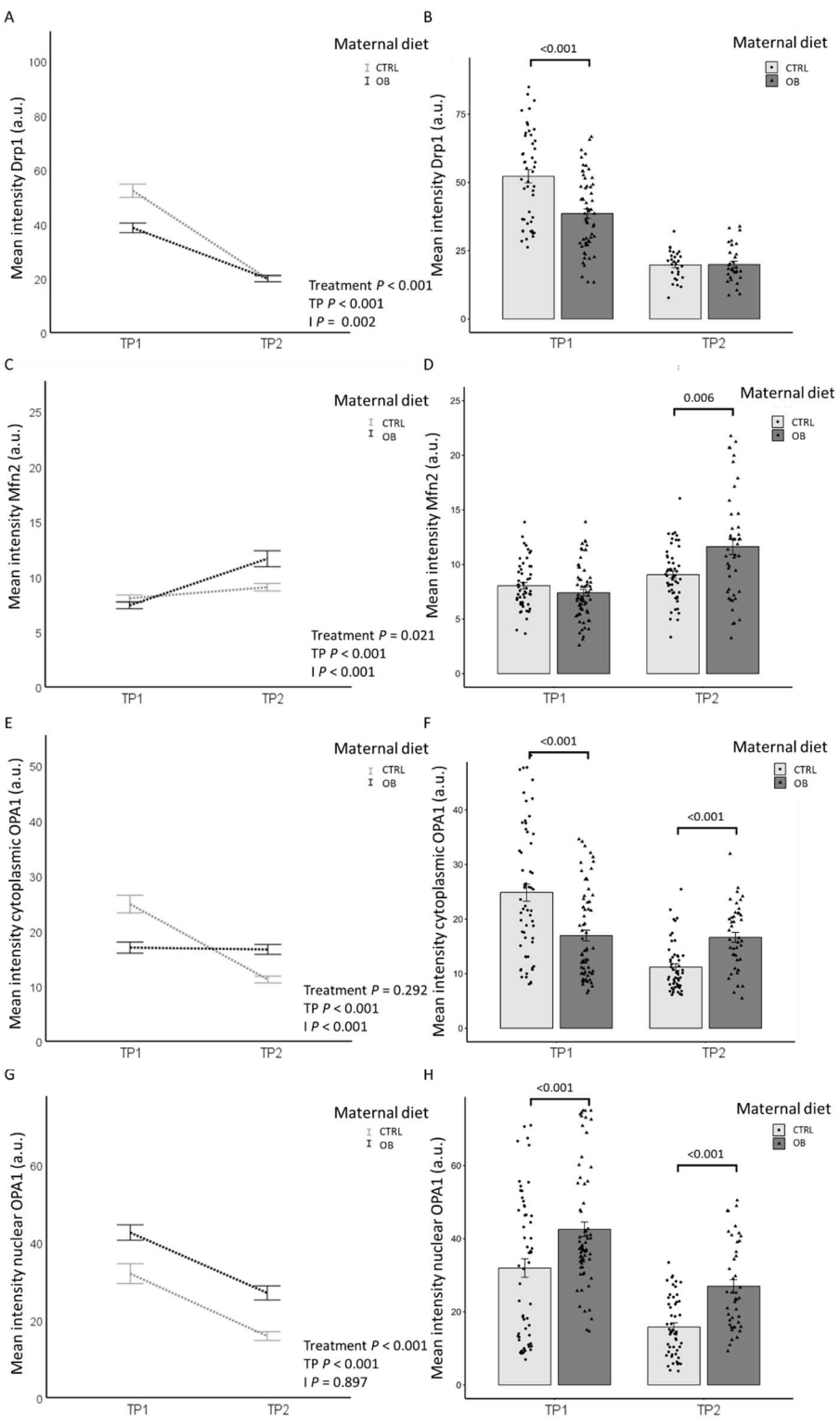

Figure 7: The effect of maternal obesogenic (OB) diet consumption on the expression of markers related to mitochondrial fusion (Mfn2, OPA1) and fission (Drp1) in offspring primordial follicle oocytes assessed at birth (TP1) and at weaning (TP2). Interaction plots represent the main effects of time and treatment and their interaction. Bar charts with data points display the effect of treatment split per time point. Data are presented as mean ± S.E.M. and are derived from 47, 53 and 58 CTRL and 29, 53 and 52 OB offspring primordial follicle oocytes at birth and 57, 68 and 67 CTRL and 31, 45 and 43 OB offspring primordial follicle oocytes at weaning for Drp1, Mfn2 and OPA1 analysis respectively.

6.4.4 Mitochondrial ultrastructure at birth

Maternal DIO reduced the proportion of mitochondria classified as spherical with 2.7 % (*P* = 0.020), and reduced the proportion of mitochondria with electron dense foci with 12.2 % (*P* = 0.035) in primordial follicle oocytes of their offspring at birth. Other mitochondrial ultrastructural features such as elongation, vacuolization, clustering or the presence of loose inner membranes (LIM) or broken outer membranes (BOM) (which are described as abnormal features in other studies (Marei et al., 2020; Xhonneux et al., 2023a) were not altered by maternal DIO (Figure 8, Table 1).

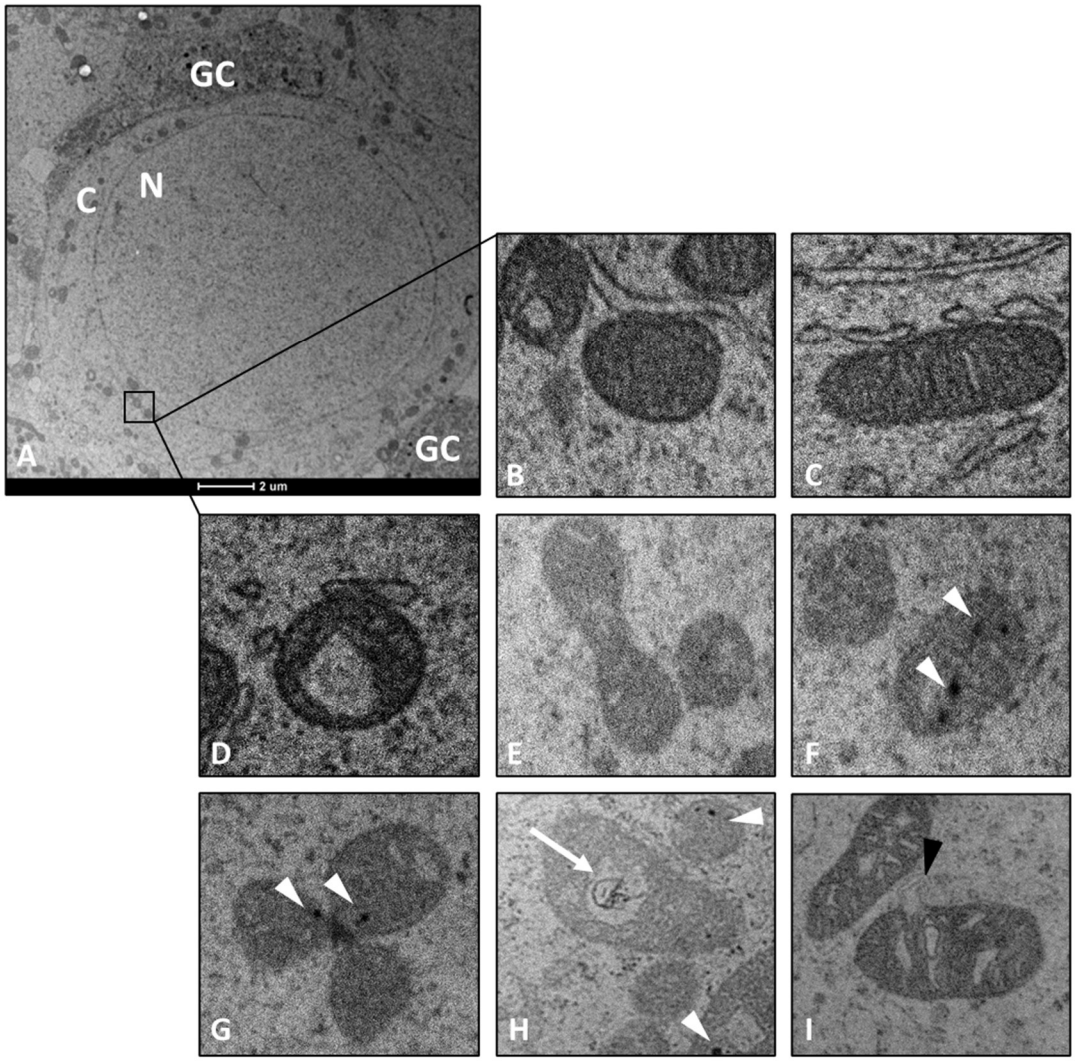

Figure 8: Different classes of mitochondrial ultrastructure in offspring primordial follicle oocytes at birth. **A.** A primordial follicle with the oocyte (N = oocyte nucleus, C = oocyte cytoplasm) and its surrounding flattened granulosa cells (GC). Oocyte mitochondria were classified as **B.** homogenous and spherical, **C.** elongated, **D.** spherical and vacuolated, E. dumbbell shaped, **F.** spherical with electron dense foci (arrowheads), **G.** clustered with electron dense foci, **H.** containing loose inner membranes (arrow), or **I.** broken outer membranes (black arrowhead).

Table 1**:** The proportions of different classes of oocyte mitochondrial ultrastructure in primordial follicles of offspring born to control and OB mothers at birth.

Diet	S	E	Db	H	V	ED	C	LIM	BOM
CTRL n = 1587	88.2±1.0	10.5±0.9	1.1±0.3	58.1±2.6	38.7±2.5	34.5±2.2	8.7±0.9	2.9±0.6	1.0±0.5
OB n = 1820	85.6±1.1	12.5±1.1	1.8±0.4	59.2±2.5	36.5±2.4	22.7±1.7	6.5±0.7	2.5±0.5	0.7±0.3
***P* - values**	0.020	0.061	0.268	0.894	0.818	0.035	0.194	0.607	0.742

Data are presented as proportion percentage ± S.E. and are derived from 6 - 10 follicles investigated on one midway section in one ovary per offspring per mother, and 5 mothers per group. S = spherical, E = elongated, Db = dumbbell, H = homogenous, V = vacuolated, ED = electron dense structures, C = clustered, LIM = loose inner membrane, BOM = broken outer membrane.

6.5 Discussion

A few studies have demonstrated the impact of maternal DIO on adult offspring oocyte quality, particularly focusing on mitochondrial abnormalities. However, it remains unclear if these reported changes are inborn, or occur early postnatal. In this study, oocyte mitochondria of the offspring primordial follicles were examined at birth and at weaning, to investigate if maternal DIO induces inborn alterations in mitochondrial ultrastructure, biogenesis, replication and fusion/fission dynamics, and if further aggravation or *de novo* effects can be detected at weaning after nursing from a DIO mother.

After consumption of the OB diet for 7 weeks, the weight of the OB mothers at the time of mating (10 weeks) was significantly increased and was on average > 15 % higher than the weight of the controls, illustrating the induction of maternal DIO. Maternal DIO did not affect litter size or offspring weight at birth, confirming previous results of a parallel study using the same outbred Swiss mouse model (Xhonneux et al., 2023; Xhonneux et al., 2024a). The numerical increase in offspring's weight at weaning is also in line with our previous reports (Xhonneux et al., 2023; Xhonneux et al., 2024a) but did not reach the statistical power here to be significant. Nevertheless, as further discussed hereafter, several alterations were detected in the primordial follicles of offspring born to OB mothers at weaning despite the limited effect on offspring's weight.

The expression of Hsp70 in the primordial follicle oocytes tended to increase, but was not significantly affected by maternal DIO, neither at birth nor at weaning. Hsp70 is a key molecule involved in cellular homeostasis during cell cycle progression, protecting chromatin structure and regulating transcription activity while serving as a first line of defense against oxidative stress (Stamperna et al., 2021). Rapid alteration in Hsp70 levels has been reported in young growing oocytes in response to stressors such as heat stress (Lánská et al., 2006), indicating its role in controlling oxidative damage in oocytes. We noticed that the expression of Hsp70 was significantly higher at birth compared to at weaning, which may be attributed to the highly active dynamics during development of the primordial follicle pool in the pre- and perinatal period (Wear et al., 2016). Throughout oocyte development, oogonia cluster in germ cell nests, thereby increasing the quality of organelles and nutrients required for later development of the primary oocyte. During transition from these nests into primordial follicles (nest breakdown), the dynamics of cellular and organellar remodulations markedly increase to assist proliferation, transcription and translation processes during primordial follicle formation (Witkin et al., 2017; Pfeiffer et al., 2018), which requires regulation of protein folding to maintain homeostasis (Pepling, 2006; Tingen et al., 2009; Wear et al., 2016). This may explain the relatively high Hsp70 levels in the primordial follicle oocytes at birth, compared to at weaning. Since the expression level of Hsp70 was not significantly affected by DIO neither at birth nor at weaning, this suggests that the above mentioned cellular processes were not altered by maternal DIO, and shows no signs of persistent cellular stress in the female germline, and no additive direct impact of nursing from an obese mother on cellular stress levels in the arrested ovarian follicles. The changes in some of the other examined mitochondrial markers discussed hereafter are therefore not (oxidative) stress-induced.

Focusing on mitochondrial biogenesis, we found that PGC-1α was also not altered by maternal DIO in the offspring primordial follicle oocytes at birth. As mentioned above, mitochondrial biogenesis plays an important role during primordial germ cell proliferation and the initial steps of oogenesis during the last phase of pregnancy (Pepling, 2006; Tingen et al., 2009; Wear et al., 2016). PGC-1α also regulates pathways involved in oxidative phosphorylation, ROS detoxification and lipid metabolism (Abu Shelbayeh et al., 2023). It appears that these processes were not altered in the primordial pool by the exposure to maternal DIO throughout pregnancy. Importantly, the subcellular localization of PGC-1α is tightly regulated, and can shift between nucleus and cytoplasm based on cellular signals and modifications (Anderson et al., 2008). Cytoplasmic PGC-1α expression in the primordial follicle oocytes was relatively higher at birth compared to at weaning, which is in line with its role during ovarian prenatal development as described above. The reduced cytoplasmic PGC-1α expression at weaning in control offspring is crucial to maintain

the immature state of the oocyte mitochondria and the reduced metabolic activity in the arrested oocytes (Abu Shelbayeh et al., 2023). Here we reported for the first time that nursing from an OB mother may increase the PGC-1α expression both in the cytoplasm and in the nucleus in the primordial follicle oocytes (as shown at weaning). Increased mitochondrial biogenesis and oxidative metabolism at this stage may potentially increase mitochondrial bioenergetic capacity, promote oocyte ROS accumulation and result in a gradual deterioration of oocyte quality. On the other hand, PGC-1α is also involved in stimulating the expression of antioxidant enzymes, such as GPX and SOD (Ramalho-Santos et al., 2009; Abu Shelbayeh et al., 2023), and thus, the PGC-1α upregulation reported here may also play protective roles. Other studies have shown that PGC-1α and some closely related factors (e.g. SIRT1 and FOXO3) are involved in primordial follicle activation to support the metabolic demands of the growing oocytes (Anderson et al., 2008; Cinco et al., 2016; Panes et al., 2020). The increase in nuclear PGC-1α expression at weaning in the DIO-born offspring may indicate that their primordial follicles can be prone to premature activation or possess higher bioenergetic capacities after nursing from an OB mother. This is in line with our previous study, where a premature increase in mitochondrial matrix was observed in the primordial follicle oocyte of adult offspring born to DIO mice, indicating an increased bioenergetic capacity (Xhonneux et al., 2024b). This may have long-term consequences on the offspring ovarian reserve, and may increase follicular depletion rate and impact reproductive lifespan. While we have not observed any differences in ovulation and oocyte retrieval following hormonal ovarian stimulation in adult Swiss offspring (at 10 weeks of age) born to DIO mothers, these notions may require further investigations, particularly after further advancement of age. Other reports from C57BL/6J (inbred) mice have shown a decreased ovarian reserve and increased oxidative stress in ovaries of offspring born to DIO mothers at 12 weeks of age regardless of the offspring diet (Aiken et al., 2016). Based on our results, PGC-1α might be used as a target to reduce acceleration in follicle depletion, particularly during early postnatal development. Further studies are needed to test this hypothesis.

Next, focusing on mtDNA replication, we found that maternal DIO reduced the expression of TFAM in offspring primordial follicle oocytes at birth. This may suggest a reduced mtDNA transcription and replication during the final stages of the formation of primordial follicle oocytes (or earlier) in OB pregnancies compared to the controls (Antelman et al., 2008a). Since TFAM activation is one of the downstream effects of PGC-1α activation (Abu Shelbayeh et al., 2023b), these results may seem to be contradictory because PGC-1α was not affected by maternal DIO at birth. Nevertheless, TFAM also binds nonspecifically on mtDNA, promoting mitochondrial chromosome stabilization. A few studies have confirmed that mtDNA replication and biogenesis seem to be independent and separately regulated in somatic cells (Koh et al., 2021; Karamanlidis et al., 2010). Our results show that a similar independent expression of these factors appear to be also true in the oocytes. It is also important to note that our study only examines protein levels, not RNA transcripts. The observed decrease in TFAM, but not in PGC-1α, may be due to a regulatory reduction in TFAM translation to control mtDNA copy numbers. Note that although TFAM expression was persistently low in the examined follicles at weaning, we have previously shown that mtDNA copy numbers in adult offspring oocytes were not altered by maternal DIO (Xhonneux et al., 2023). Such regulatory capacity may differ between inbred and outbred mice strains, as maternal DIO resulted in decreased mtDNA copy numbers in oocytes of adult C57BL/6 offspring born to obese mothers (Saben et al., 2016).

It was also important to examine the oocyte mitochondrial fusion/fission dynamics in the primordial follicles formed under OB and control conditions. At birth, we noticed a reduced expression of Drp1 (fission) in pups from DIO mothers without changes in Mfn2 (fusion). In addition, the expression of the fusion factor OPA1 was reduced in the cytoplasm and increased in the nucleus. Drp1 controls the final stage of mitochondrial fission by separating the membrane stalk connecting two daughter mitochondria that require their own copy of mtDNA. This process is facilitated by TFAM (Zou et al., 2021). Therefore, the downregulation in both Drp1 and TFAM is consistent and confirmatory. The reduction in OPA1 may compensate for the reduction in Drp1 to maintain a normal fusion/fission balance. Alterations in OPA1 may also result in abnormal inner membrane fusion and cristae remodeling (Zhao et al., 2015; Liu et al., 2016). Nevertheless, we did not find any signs of altered fission or fusion in the TEM analysis as discussed

further below, suggesting that the inborn changes in Drp1 and OPA1 expression at birth are regulatory or compensatory to maintain normal mitochondrial features. The increase in nuclear OPA1 expression due to maternal DIO is interesting but difficult to interpret because such nuclear expression has not been previously characterized (Zou et al., 2021).

At weaning, Drp1 levels were not altered by maternal DIO, supporting the notion that the earlier alterations in Drp1 at birth are rather settlements of regulatory changes that took place during the prenatal stage. Interestingly, mitochondrial fusion markers Mfn2 and OPA1 were both increased at weaning in DIO-born offspring. This might be PGC-1α dependent (Chitra and Boopathy, 2014). OPA1 can facilitate triggering of apoptosis by facilitating the cytochrome c release from the mitochondria (Del Dotto et al., 2017; Zou et al., 2021), showing that these follicles may be prone to increased rates of follicular atresia, which can affect the maintenance of the ovarian reserve. Again, this is in line with the decreased ovarian reserve reported in the ovaries of adult OB born offspring (Aiken et al., 2016b). However, further research is required to investigate the functional and long-term consequences of the alterations reported here.

It is important to highlight that mothers in the present study remained on their allocated diet during pregnancy and lactation, with no cross-fostering of the offspring. While we see a more pronounced effect on the examined markers at weaning compared to that at birth, our study is not designed to dissect the effects of maternal DIO induced during lactation from those induced during prenatal development. The reported alterations are also likely to be cumulative. We opted to avoid the stress associated with cross-fostering and the nutritional-metabolic mismatch, which can induce acute effects and create bias in intergenerational studies such as the present one (Matthews et al., 2011; Sasaki et al., 2013). Our approach also mimics the human biological situation.

In addition to monitoring the above mentioned markers, this study also primarily aimed to assess the impact of maternal DIO on the offspring oocyte mitochondrial morphology in the primordial follicles at birth. Previous research using both inbred and outbred mice showed that mitochondrial ultrastructural abnormalities were present in mature oocytes of their adult offspring, and suggested a transmission of aberrant mitochondria through the female germline (Saben et al., 2016; Xhonneux et al., 2023). Whether these aberrations are inborn was never tested. Here, we found only a small reduction in the proportion of spherical mitochondria and mitochondria having electron dense foci in OB newborns. No aberrant or abnormal mitochondrial morphology such as loose inner membranes and broken outer membranes were induced by maternal DIO, showing that the previously reported abnormalities in oocytes of adult offspring are not inborn and may have developed *de novo* after birth. The mitochondrial selection mechanisms that take place during embryonic and fetal development such as the bottleneck phenomenon and the organelle exchange in the primordial germ nests (as mentioned in the introduction) can justify these findings.

In addition, TEM imaging also enabled us to examine if the altered expression of fusion and fission markers at birth (as discussed above) are reflected in the mitochondrial morphology. While the reduction in Drp1 expression is expected to increase fusion, and thus increase mitochondrial elongation and/or mitochondrial clustering, this was not evident in the TEM results. Drp1 reduction was previously associated with mitochondrial elongation in mature (ovulated) oocytes due to a reduced mitochondrial division (Smirnova et al., 2001), eventually leading to an aggregation of malformed clustered mitochondria (Udagawa et al., 2014). We could not make such association here at birth.

Maternal DIO also reduced the proportion of mitochondria having electron dense foci. Electron dense foci in mammalian somatic cell mitochondria are reported to contain granules of calcium phosphate (Chalmers and Nicholls, 2003). The mitochondrial calcium-intake depends on the membrane potential (Chalmers et al., 2003; Wolf et al., 2017), and increased calcium phosphate granules in somatic cells are associated with calcium overload and mitochondrial dysfunction (Strubbe-Rivera et al., 2021). In our study, maternal DIO reduced the presence of these electron dense foci, and may support the reduced TFAM expression at birth as TFAM regulates the mitochondrial membrane potential, thereby playing a role in the

mitochondrial calcium flux via calcium/calmodulin-dependent protein kinase β (CaMKKβ) (Koh et al., 2019). In addition, TFAM coordinates nuclear and ER signaling that can affect the mitochondrial calcium flux in response to metabolic challenges (Koh et al., 2021). Increased electron dense foci in the mitochondria of somatic cells are associated with calcium overload and mitochondrial dysfunction (Strubbe-Rivera et al., 2021), but in our study maternal DIO actually reduced these electron dense foci and may therefore be seen as an improvement rather than a deterioration of mitochondrial function.

6.6 Conclusions

Studying the impact of maternal DIO on offspring primordial follicle oocytes during prenatal and early postnatal development is crucial for gaining insights into the mechanisms that may impact offspring fertility on the long-term. With our data presented here, we are the first to show that the oocyte mitochondrial ultrastructural abnormalities reported in adult offspring from DIO mothers are not inborn. We also show that offspring born to DIO mothers do not exhibit signs of altered cellular homeostasis, and do not exhibit inborn alterations in mitochondrial biogenesis and other PGC-1α-dependent metabolic pathways in oocytes of primordial follicles at birth. Other changes in Drp1 and OPA1 (and probably also TFAM) expression at birth may be regulatory and were not associated with any morphological changes in the mitochondria. The alterations detected at weaning were more pronounced and may indicate increased mitochondrial biogenesis and bioenergetic capacity, and may make the follicles prone to atresia or further (mitochondrial) damage later in life. Further research is required to determine the functional implications of these changes on oocyte quality and metabolic activity during subsequent stages of folliculogenesis in offspring born to DIO mothers.

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6.7 Supplementary file 1

While oocyte mitochondria of adult primordial follicles are mainly translucent (Figure S1.B), their morphology at birth (Figure S1.A) resembles the dense morphology of mature ovulated oocyte mitochondria (Figure S1.D) that starts to appear in secondary follicles (Figure S1.C).

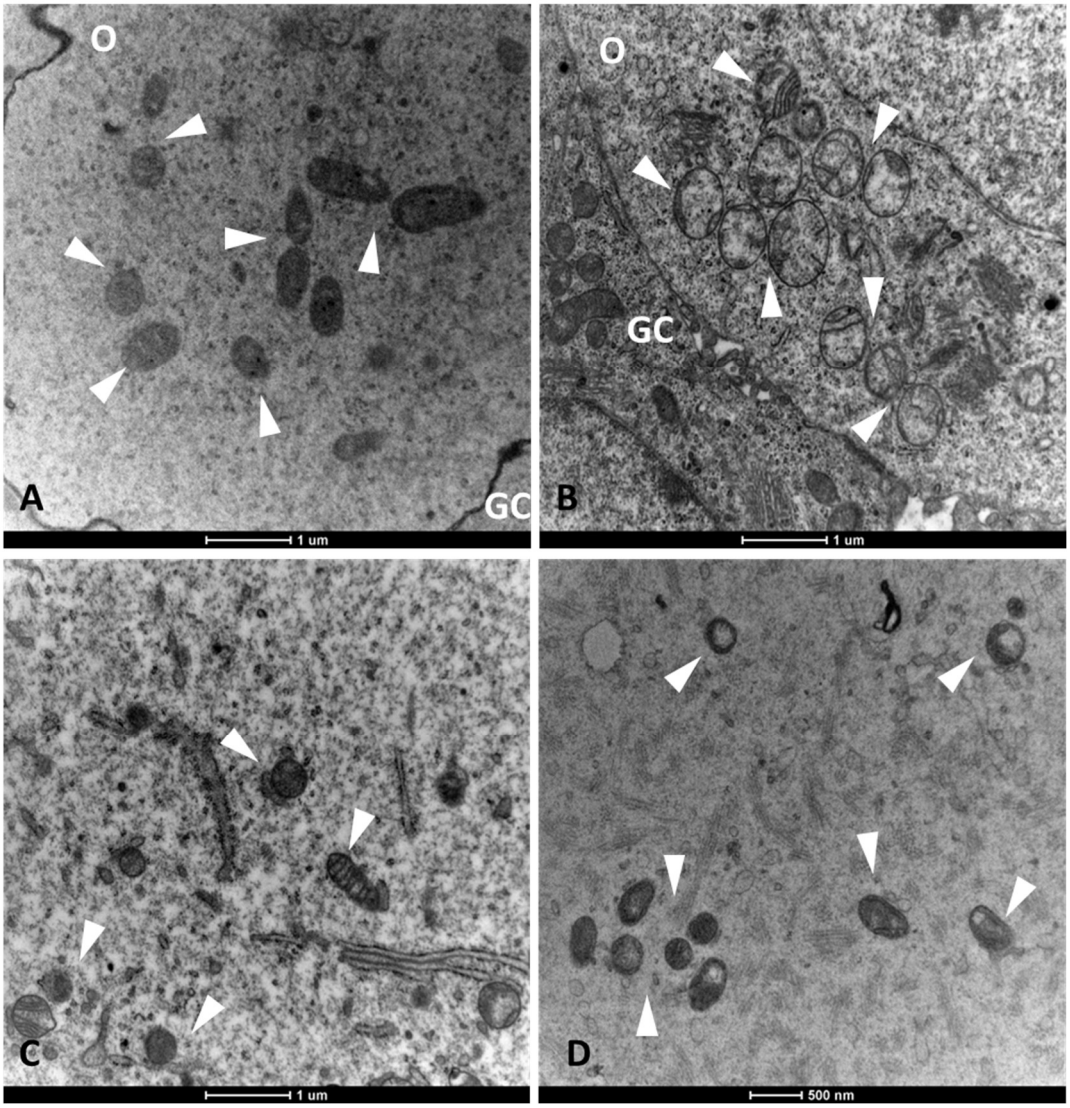
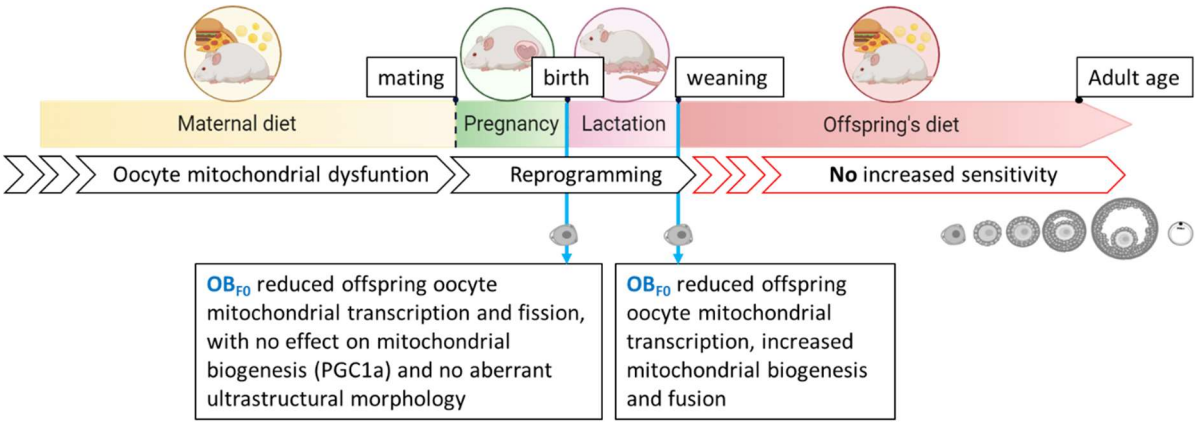


Figure S1. Mitochondria (white arrows) within a primordial follicle at birth (A) in oocytes of offspring born to C mothers. Oocyte mitochondria within a primordial follicle at adulthood (age mice 10 weeks, B), a secondary follicle at adulthood (C) and a mature (ovulated) oocyte at adulthood (D) of offspring born to C mothers and fed a C diet after weaning. O = oocyte, GC = granulosa cell.

6.8 Key message

Maternal OB diet did not increase cellular stress levels in the primordial follicle oocytes but reduced TFAM expression both at birth and at weaning, which may affect or regulate mtDNA replication. More prominent effects of the maternal OB diet were detected in the offspring primordial follicle oocytes after lactation, specifically impacting PGC-1α expression and markers of fusion and fission. The functional implications of these changes on oocyte quality during subsequent development are yet to be determined.

6.9 Graphical summary



Schematic overview of the main effect of the maternal OB diet (OBF_0), the offspring OB diet (OBF_1), and the maternal x offspring diet interaction (OBF_0 x OBF_1) on offspring oocyte (mitochondrial) quality at birth (age = less than 1h) and at weaning (age 3 weeks).

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Chapter 7 General Discussion and Conclusions

7.1 Overview of the obtained results

Obesity is associated with subfertility, due to (amongst others) a reduced oocyte quality and mitochondrial dysfunction. This will also affect their offspring (Baptiste-Roberts et al., 2006; Chandrasekaran and Neal-Perry, 2017; Bucher et al., 2021). In addition, the compromised oocytes of obese patients and subsequent embryo's will further develop in a pre- and postnatal OB environment (*in utero* and during lactation respectively), inducing offspring epigenetic (re)programming (Peral-Sanchez et al., 2022), that may change the offspring's sensitivity or vulnerability to an offspring OB diet (Bateson et al., 2014). In addition, family lifestyle persists through generations and significantly influences the dietary choices of their children (Pfledderer et al., 2021). Therefore, daughters born to obese mothers may continue the unhealthy lifestyle with unbalanced diets high in fat and sugar (Lopez-Gonzalez et al., 2020) that may cumulatively impact the offspring's health and fertility. The response to preconception care interventions (PCCI) and fertility treatments such as artificial reproduction techniques (ART) is largely dependent on the woman's health. Often, such treatments show disappointing results for obese patients, making it challenging to provide sustainable advice, univocal guidelines and treatments for patients with obesity (Johnson, 2016; Gonzalez et al., 2022). However, it is possible that the response to such PCCI and fertility treatments may depend on the maternal metabolic background. Consequently, the effect of the interaction between the maternal diet and the offspring's diet on the health and fertility of the daughters is extremely important in our contemporary society where most obese women are born to obese mothers (WHO, 2022). However, most research only addresses the sole effect of maternal OB diets on offspring health or fertility, not taking the offspring's current diet into account. Therefore it is possible that the reported effects of maternal obesity in daughters born to OB mothers are induced by the direct effect of the daughter's own diet by creating a maternal x offspring dietary match or mismatch (Bateson et al., 2014). In addition, previous studies mostly focus on the adult offspring and on the mature oocytes. There are no studies defining the dysfunction that might be present in the oocytes of offspring born to OB mothers at birth and early life, examining if the primordial pool is already affected, or examining the stage of follicular development at which most of the defects start to appear. Nevertheless, this information is crucial to provide fundamental basis for a better reproductive management.

In this study, we aimed to study the effect of the offspring OB diet, the effect of the maternal OB and the maternal x offspring diet interaction on the metabolic health of offspring and the quality of their preantral follicle oocytes and mature ovulated oocytes. In addition, we aimed to study if maternal OB diet induced alterations are inborn, and further aggravate during lactation from obese mothers. An outbred Swiss mouse model was used, to investigate the pathological consequences of obesity in highly controlled settings while increasing the pathophysiological relevance to humans (Brehm et al., 2010; Perlman, 2016) and avoiding possible strain-specific bias related to inbreeding (Freeman et al., 2006; Marei et al., 2020). The 2 x 2 factorial design used here in our studies enabled to closely study the direct effects of the offspring OB diet, the maternal OB diet and the maternal x offspring diet interaction. Such set-up using an outbred mouse model is unique.

Conducting the experiments described here was practically challenging. In total 801 mice were handled over a 6 months period. 294 female offspring born to 50 mothers were used for sample collection at offspring adult age, 50 were used for offspring sample collection at weaning and 80 female pups were culled at birth. Monitoring, handling, tacking, and investigating such high number of animals requires a perfect planning, to prevent mistakes and factors that may eventually cause inconsistencies and invalid results by inducing bias. Several factors were taken into consideration while designing the experiments to

eliminate or minimize the effect of potential confounders. For example, mating was done using the same Swiss males (6 in total) in a cross-over design to block for paternal effects (Anuradha et al., 2024). At weaning, sisters were equally divided between offspring diet groups, to block for any effect related to the mother herself, instead of the corresponding diet. Also, litters were equally divided between different cages, to avoid cage effects. All mice were weighed weekly, performed by the same person at the same time and in the same calm and stress-free circumstances. The experimental set-up of this study was intense, but allowed a unique setting where all mice were kept under exactly the same environmental conditions.

While our experimental design was not empowered to investigate different windows of exposure separately (pre/post-conception, *in utero* and early post-natal), the prolonged exposure of offspring to the effects of a maternal OB diet throughout oocyte development, maturation, embryonic and fetal *in utero* development and further post-natal offspring development during lactation, made our design highly biologically relevant to the human situation. Control and OB-born offspring were weaned on C and OB diets and the direct effect of the offspring's OB diet, the maternal OB diet and the maternal x offspring diet interaction was investigated on offspring health and oocyte quality. In addition, by collecting samples at different timepoints, namely at birth (within 1h after birth), at weaning (offspring age 3 weeks) and at adulthood (offspring age 10 weeks), we were able to examine if the mitochondrial alterations at adulthood were inborn, or developed or aggravated during lactation.

The following questions will be answered in this discussion, based on our obtained results:

- How does the maternal OB diet influence adult offspring health and oocyte (mitochondrial) quality and their responses to the offspring diet?

- Are the effects of the maternal OB diet on oocyte mitochondrial features inborn or determined during early postnatal development (during lactation)?

Graphical summaries of the obtained results are presented below. Figure 1 displays the most important effects of the offspring OB diet, the maternal OB diet and the maternal x offspring diet interaction on offspring health, while Figure 2 covers the most important effects on offspring oocyte quality at birth, weaning and adulthood.

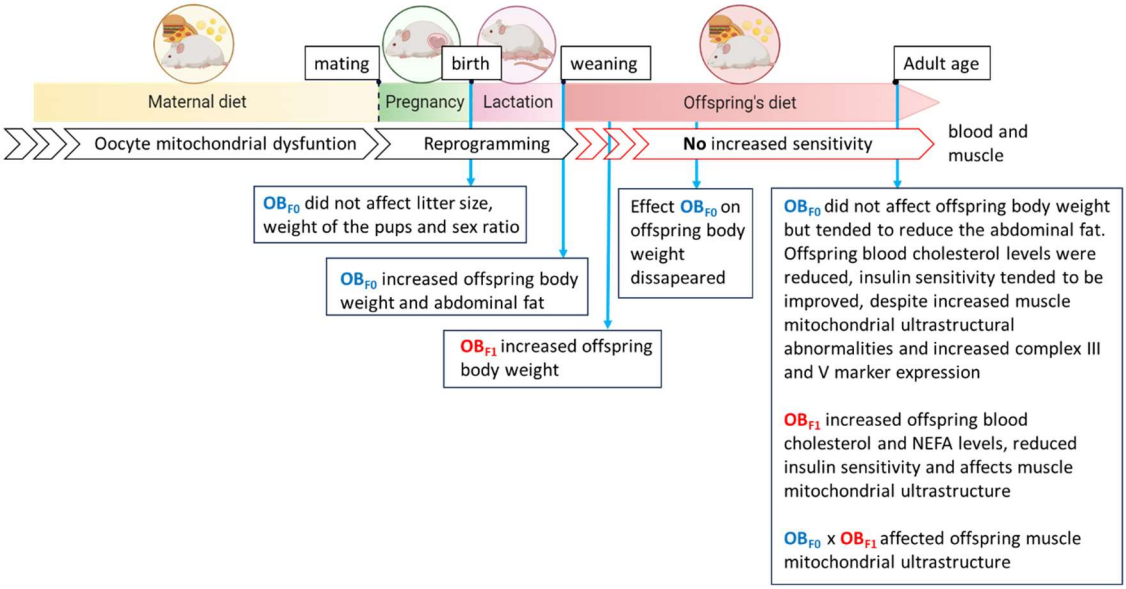

Figure 1. Schematic overview of the main effects of the maternal OB diet (OB_{F0}), the offspring OB diet (OB_{F1}), and the maternal x offspring diet interaction (OB_{F0} x OB_{F1}) on offspring health at birth (age = less than 1h), weaning (age 3 weeks) and adulthood (age 10 weeks). Results of the pairwise comparisons are not included in this overview.

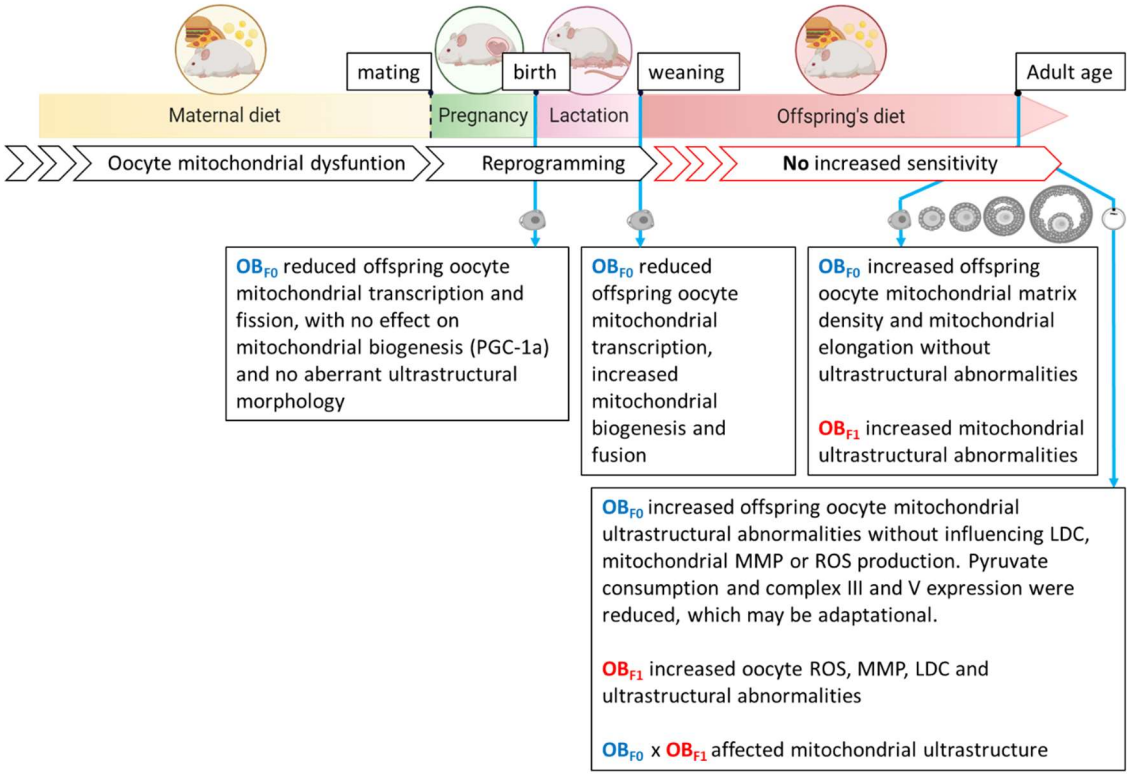

Figure 2. Schematic overview of the main effects of the maternal OB diet (OB_{F0}), the offspring OB diet (OB_{F1}), and the maternal x offspring diet interaction (OB_{F0} x OB_{F1}) on offspring oocyte (mitochondrial) quality at birth (age = less than 1h), weaning (age 3 weeks) and adulthood (age 10 weeks). Results of the pairwise comparisons are not included in this overview.

7.1.1 How does the maternal obesogenic diet influence adult offspring health and oocyte (mitochondrial) quality and their responses to the offspring diet?

7.1.1.1 *Maternal obesogenic diet consumption did not alter litter size, weight and sex ratio*

During this PhD study, 50 pregnant mothers gave birth to 749 pups in total and all litter characteristics were recorded. In our study, litter size and litter weight were not affected by the maternal OB diet. Our results are in line with the studies of Rosenfeld et al. (2003) and Meulders et al. (2024), feeding outbred Swiss mothers a HF or HF/HS diet before and during pregnancy, and during lactation. Although results are inconsistent in rodents, research has demonstrated that in inbred C57BL/6 mice fed a HF diet before and during pregnancy, the litter size was significantly reduced (Buffington et al., 2016), suggesting that the dietary effects on some fertility related outcome parameters may be influenced by the used mouse model.

The offspring sex ratio was not affected in our study, which contradicts the results of Rosenfeld et al. (2003) using the same outbred Swiss mice fed a HF diet, and contradicts the sex allocation theory of Trivers and Willard (1973), stating that females with increasing body condition score (BCS) would produce more sons than daughters (Rosenfeld et al., 2003). This theory states that healthy, strong males are likely to have more offspring than similar healthy females. If a mother is in good condition reflecting high nutrient availability, producing males can be advantageous, while in poorer environmental conditions associated with a lower maternal BCS, producing females may be favorable (Trivers and Willard, 1973; Chinn et al., 2023). However in mice, the diet composition as such seems to play a major role in this phenomenon, rather than the maternal BCS (Vanengelen et al., 1995). Diets high in fat but low in carbohydrates increase male pups, whereas diets low in fat but high in sugar increase female pups (Rosenfeld et al., 2003; Rosenfeld and Roberts, 2004). We fed a diet with both high fat and high sugar content to our mothers, without affecting the offspring's sex ratio. Nevertheless, the influence of the maternal diet composition as such on the sex ratio of offspring illustrates that maternal diet can have profound effects on reproductive outcomes beyond the maternal BCS. However, the mechanism behind this specific phenomenon remain to be elucidated (Rosenfeld et al., 2003; Rosenfeld and Roberts, 2004).

7.1.1.2 *Maternal obesogenic diet consumption tended to improve offspring insulin sensitivity and blood cholesterol levels despite significant alterations in mitochondrial ultrastructural morphology in the muscles. No aggravation of offspring obesogenic diet effects were detected, except in OXPHOS complex markers*

While the maternal OB diet did not affect the weight of the pups at birth, overall, maternal OB diet increased the offspring body weight at weaning (Chapter 3). A significantly high body weight due to the maternal OB diet as a factor was still persistent for up-to 4-5 weeks post-weaning, but was absent at adulthood (Chapter 3 and 4). This (partially) contradicts with findings using inbred mice strains where OB-born offspring (born to mothers fed a HF/HS diet 5-6 weeks before pregnancy, during pregnancy, and during lactation) significantly gained more weight at adulthood, compared to C-born offspring. This increased weight gain was present even when the offspring were fed a standard chow diet after weaning, creating a dietary mismatch between the maternal and offspring diet (Samuelsson et al., 2008b; Zieba et al., 2019). Even though pairwise comparisons revealed that the body weight of adult OB»C offspring was increased compared to C»C in our study, the maternal OB diet tended to reduce the weight of the abdominal fat in these offspring, while the maternal OB diet increased the offspring's adiposity in the study of Samuelsson et al. (2008a) using inbred C57BL/6 mice.

Offspring OB diet increased offspring weight already after 1 week post-weaning, with a continuous effect until adulthood (Chapter 3 and Chapter 4). In addition, the offspring's OB diet clearly affected the health

of the offspring by reducing the overall insulin sensitivity, inducing hypercholesterolemia and increasing the blood NEFA levels in C»OB (Chapter 3). Despite the fact that adult offspring weight appeared not to be affected by the maternal OB diet as a factor, it was still necessary to examine the effect of the maternal OB diet on the offspring's metabolic profile and muscle mitochondrial features. The maternal x offspring diet was also investigated, together with the effect of a dietary mismatch between both diets following the theory of Holt (2023), stating that mainly the mismatch between maternal and offspring environments may affect the offspring's phenotype (Bateson et al., 2014; Holt, 2023).

In adult offspring, the metabolic profile was not hampered by a maternal OB diet as such. In fact, OB-born offspring fed a matching OB diet (OB»OB) even had an improved systemic metabolic profile compared to C-born offspring fed a mismatching OB diet (C»OB). The most distinct metabolic profile (but with the lowest abdominal fat, basal glycemia and AUC of the ITT) was detected in OB-born offspring fed a mismatching C diet after weaning (OB»C, Chapter 3). Our results contradict the findings of Samuelsson et al. (2008a) using inbred mice, who associated maternal OB diet with an increased risk of hyperglycemia, hypertension, fatty liver disease and reduced insulin sensitivity both in male and female OB-born offspring fed a C diet, while we noticed the opposite in OB»C. Keleher et al. (2018) showed that female OB»OB offspring had exacerbated obesity with increased abdominal fat weight in OB»OB compared to C»OB. However, our results were again contradictory, as the weight and abdominal fat between OB»OB and C»OB in our study was equal (Chapter 3). While the pups in our study remained with their biological mother, Keleher et al. (2018) cross-fostered OB-born pups to control mothers at birth, thereby missing the important lactational window that can influence the offspring's development. In addition, the effect of stress due to the cross-fostering itself can significant impact the offspring's phenotype (Matthews et al., 2011; Sasaki et al., 2013). Both studies of Samuelsson et al. (2008) and Keleher et. al (2018) used inbred mouse strains (C57BL/6 and SM/J respectively). Epigenetic responses are genome dependent (Turner, 2009), and may partially explain the different dietary responses between mouse strains. Nevertheless, in our study using outbred Swiss mice, a maternal OB diet did not exacerbate the effect of an offspring OB diet on the offspring's metabolic profile, but even induced improvements.

Despite no concomitant impact on metabolic health, the significant effect of the maternal OB diet on the offspring's muscle mitochondrial morphology and ETC complex III and V marker expression cannot be overlooked. These mitochondrial alterations in the muscle tissues are in line with the observations of Saben et al. (2016b) using inbred mice. In this study conducted by Saben et al. (2016b), the mitochondrial alterations detected in chow diet-fed offspring born to HF/HS fed mothers (OB»C) were associated with a reduced insulin sensitivity. However, the muscle mitochondrial alterations detected in our study were not reflected in the metabolic profile of the affected offspring. Moreover, the response to insulin of OB»C offspring even seemed to be improved. Despite the maternal OB diet induced improvements of the offspring's metabolic profile, offspring born to OB mothers were significantly heavier at weaning, with increased abdominal fat weight compared to offspring born to C mothers, which may suggest that compensatory mechanisms may have occurred over time. We know that lactation to OB mothers is suggested to protect the offspring from the effects of an OB diet later in the offspring's life (Monks et al., 2018), and as these cellular changes in the muscle tissue were not associated with impairment of the offspring's metabolic profile but even coincided with some improvements, we suggest that the muscle mitochondrial alterations are signs that maternal OB diet consumption during lactation may prepare or reprogram the offspring for a nutrient availability that is equal to that of the mother. This is in line with Barker's Thrifty Phenotype Hypothesis, stating that associations between infant growth and development result from effects of the maternal nutrition in early life (Hales and Barker, 1992). In our mouse model, these associations do not necessarily have a bad outcome.

With our data we highlight important differences between used mouse models and experimental designs that need to be taken into account when interpreting data from intergenerational studies. Mismatches between maternal and offspring diets had the biggest impact on the offspring's health, whereas the

maternal OB diet as a factor did not hamper the metabolic profile of the offspring. The maternal OB diet only influenced the offspring muscle mitochondrial morphology, indicated by a significant maternal x offspring diet interaction.

7.1.1.3 *Maternal obesogenic diet consumption alters mature oocyte mitochondrial ultrastructure in adult offspring without influencing the mitochondrial inner membrane potential, ROS production, together with a compensatory reduction in pyruvate consumption and complex III and V expression*

As expected, offspring OB diet increased the offspring's oocyte lipid droplet content (LDC), ROS accumulation, membrane potential (MMP) and reduced the oocyte's pyruvate consumption. In addition, the mitochondrial morphology was altered in mature oocytes of offspring fed an OB diet (Chapter 4). In our study, oocyte developmental competence was not investigated, while this is an important outcome parameter to assess the oocyte's ability to produce an embryo. However, increased LDC, alterations in the MMP, ROS accumulation and mitochondrial ultrastructural abnormalities in mature oocytes are associated with reduced developmental competence after fertilization (Marei et al., 2019; Smits et al., 2022). Therefore, the outcome parameters addressed in our study estimate the quality of the oocyte, even though 'quality' as such involves a broad concept.

While offspring OB diet impeded offspring oocyte quality by increasing, amongst others, the oxidative stress, the maternal OB diet as such did not (Chapter 4). This can be explained by the absence of the maternal OB diet effect on the offspring's blood metabolic profile. Again, our findings partially contradict with reports from studies using inbred mice. For example, abnormal oocyte mitochondrial morphology and reduced mtDNA copy number was detected in oocytes of adult C57BL/6 mice born to OB mothers, together with OPA1 depletion and reduced intracellular citrate and phosphocreatine (Saben et al., 2016b). No proportions of the ultrastructural abnormalities were displayed in this latter study of Saben et al. (2016b), so direct comparison in magnitude of effect is not possible. Nevertheless, despite the mitochondrial morphological alterations associated with a maternal OB diet in our study, this increase was only 5 -10 % of the total number of mitochondria. Such increase is rather limited, knowing that oocytes of C-fed Swiss mice with a normal maternal background already show 20 % of mitochondrial ultrastructural abnormalities (Smits et. al. 2021). In addition, only a 20 % increase in mitochondrial matrix density with reduced cristae coincides with hampered oocyte quality, indicated by a 44 % decrease in mtDNA copy number and a 35 % decrease in ATP production (Simsek-Duran et al., 2013; Muhammad et al., 2021). Interestingly, in the study of Saben et al. (2016b), the overall oocyte ATP production was not affected while the mtDNA copy number was reduced, so the remaining mitochondria were producing more energy to reach the same level of ATP (Saben et al., 2016b). This reduction in mtDNA copy number in adult OB-born offspring was not detected in our study and may be due to strain specific differences in response to the indirect effect of maternal OB diet consumption between C57BL/6 and Swiss mice. A different, yet opposite, direct effect of an OB diet was noticed by Marei et al. (2020), as OB diet consumption resulted in increased mtDNA copy numbers in oocytes of C57BL/6 mice, while it reduced the mtDNA copy numbers in oocytes of Swiss mice (Marei et al., 2020). Such strain-related differences in response to dietary changes may also have an impact on the mitochondrial germline transmission to the next generation.

Since ROS accumulation, lipid droplet content (LDC), mitochondrial inner membrane potential (MMP) and also the lactate production remained unaffected by maternal OB diet consumption, we can assume that the OB-born offspring's oocytes do not suffer from increased oxidative stress levels. Mitochondrial aggregation was induced by a maternal OB diet, but only in a small fraction of oocytes and the mitochondrial aggregation did not coincide with changes in the MMP, similar to the findings of Lee et al. (2024). Lee et al. (2024) injected murine mature ovulated oocytes with adaptor protein Trak2, thereby

inducing mitochondrial aggregation, and noticed only a slight delay in meiotic resumption while the spindle organization was not impaired and no evidence of aneuploidy could be detected. In addition, the MMP remained unaffected in the study of Lee et al. (2024) and it was concluded that mitochondrial aggregation in mature oocytes does not lead to a reduced oocyte quality (Lee et al., 2024). The maternal OB diet induced reduction in pyruvate consumption and ETC III and V marker expression detected in our study even suggests that oocytes of OB-born offspring may have a different mitochondrial and metabolic profile. Pyruvate fuels the mitochondrial ETC by conversion into acetyl-CoA, generating NADH and $FADH_2$ via the TCA cycle for electron provision (Luo et al., 2015). The reduced pyruvate consumption together with the numerical reduction in lactate production may suggest a change in the metabolic preference towards the β-oxidation for energy provision, and may imply that the oocytes of OB-born offspring prefer the fatty acids supplied by oocyte lipid droplets as an energy source over the pyruvate that was supplemented in the media. The mitochondrial β-oxidation is essential for murine oocyte developmental competence (Dunning et al., 2010), and a preference of metabolizing fatty acids may spare glucose for anti-oxidant generation such as glutathione via the pentose-phosphate pathway (PPP), to cope with increasing oxidative stress. This pathway operates parallel to glycolysis and produces ribose 5-phosphate, a precursor for nucleotide synthesis, and NADPH, a substrate utilized for lipogenesis and glutathione regeneration by glutathione reductase (Downs et al., 1997; Sutton-McDowall et al., 2010). However, no signs of maternal OB diet induced dyslipidemia or hyperlipidemia were detected and no corresponding increased oocyte LDC was present. This implies that the mitochondrial activity and corresponding energy production may therefore be more efficient as it remains equal with less fuels, or with fuels different than the ones assessed in our study. Due to the lack of oocyte phosphofructokinase activity, the capacity of the oocyte to utilize glucose is limited (Da Broi et al., 2018; Turathum et al., 2021). Even though pyruvate is described as a primary energy source for mature oocytes (Johnson et al., 2007a; Liu et al., 2009), it may be that in oocytes of OB-born offspring the ability to utilize glucose is increased. Similar patterns are seen in the blood of OB»C offspring, as the most aberrant, but also the most optimal systemic glucose profile was noted in this treatment group (Chapter 3). However, no oocyte glucose consumption was measured in this study because of its limited glucose usage at this stage, so these thoughts require further investigation.

Maternal OB diet consumption also coincided with a small but significant 1 % increase in oocyte mitochondrial elongation, without the increased cellular stress (ROS) that is reported by others (Boudoures et al., 2016; Saben et al., 2016b). In murine fibroblast cells, fused mitochondria (associated with mitochondrial elongation) are reported to distribute fatty acids more efficiently, thereby ensuring optimal energy production via OXPHOS (Rambold et al., 2015). Although we did not investigate mitochondrial fusion as such at offspring adult age, these subtle changes in oocyte mitochondrial shape may be a sign of adaptation towards high energy provision. Even though somatic mitochondria are different than oocyte mitochondria (Chiaratti et al., 2018) and the maternal OB diet induced changes in oocyte mitochondrial shape were limited, we believe that oocytes of OB-born offspring may have different metabolic needs during maturation. Such metabolic reprogramming may occur already in the dormant primordial follicle pool during pregnancy or during lactation, especially since follicle activation, antrum formation and oocyte maturation of C-fed offspring but born to OB mothers occurs under normal conditions.

A clear and significant interaction was again only present for mitochondrial ultrastructure in mature oocytes of adult offspring. It seems that the mitochondrial ultrastructure is one of the first outcome parameters to be affected and influenced by a maternal OB background, and highlights the mitochondrial flexibility to adjust to different situations.

7.1.1.4 *Maternal obesogenic diet consumption induces a premature increase in mitochondrial matrix density in the adult offspring primordial follicle oocytes without causing ultrastructural abnormalities like those directly induced by the offspring obesogenic diet*

Following these adaptive changes in mature ovulated oocytes of OB-born offspring, we wanted to know if these changes are already present in the dormant primordial follicle pool. Reduced oocyte quality persists even after the restoration of energy balance and alleviation of stress, implying that primordial follicles can be affected by maternal stressors (Carvalho et al., 2014; Smits et al., 2021). While primordial follicles are generally considered to be in a resting state, the oocytes within are transcriptionally active, regulating their own development and activation (McLaughlin and McIver, 2009; Ernst et al., 2017), a process where mitochondria play a key role (John et al., 2008). Consequently, they may also be sensitive to the effects of a maternal OB diet, potentially influencing their survival and potential activation. Moreover, if mitochondrial aberrations are transmitted through the female germ cells as suggested by others (Saben et al., 2016b), we anticipate mitochondrial ultrastructural abnormalities in the primordial follicle oocytes similar to those found after oocyte maturation.

Up until now, it remained unknown whether or not the mitochondria within primordial follicles are affected by the direct effect of an OB diet, especially since these follicles are referred to as 'dormant' despite their intrinsic capacity to initiate follicle activation (John et al., 2008). We are the first to show that the mitochondria within these dormant follicle oocytes are indeed affected by an OB diet, as clear signs of mitochondrial damage were present already in the primordial ovarian follicle pool of adult offspring fed an OB diet (Chapter 5). Offspring OB diet coincided with mitochondrial dysfunction, as it was noted that the mitochondrial outer membrane was often protruded or ruptured, indicative for mitochondrial bursting (Sesso et al., 2012). Furthermore, the direct effect of an offspring OB diet increased the proportion of rose-petal shaped mitochondria and mitochondria with loose inner membranes. These ultrastructural changes are associated with mitochondrial dysfunction in mature oocytes (Marei et al., 2020). Since mitochondria are involved in the primordial follicle activation via forkhead proteins such as FOXO3 (John et al., 2008), it is possible that mitochondrial dysfunction already in the primordial follicle pool may lead to premature ovarian insufficiency (POI). This is in line with the findings of Skaznik-Wikiel et al. (2016), where the direct effect of an OB diet was associated with a reduced number of primordial follicles, and coincided with increased pro-inflammatory cytokines and macrophage infiltration in ovaries of adult mice fed a HF/HS diet for 10 and 32 weeks (Skaznik-Wikiel et al., 2016). Also in human studies an increased risk of POI was noticed in obese women, and was strongly linked with lower anti-Mullerian hormone (AMH) levels (Shelling and Nasef, 2023), needed for inhibiting premature follicle growth and maintaining ovarian reserve (Chumsri et al., 2024). In our study, the direct effect of an offspring OB diet on the primordial follicle oocyte mitochondria was clearly associated with a reduced quality of the corresponding ovulated mature oocytes, indicated by increased ROS accumulation, LDC, MMP, abnormal mitochondrial morphology and a reduced pyruvate consumption. Therefore, the direct effect of an offspring OB diet on primordial follicle oocytes clearly has a detrimental impact on offspring fertility.

While offspring OB diet caused major effects on the mitochondria within primordial follicle oocytes, a maternal OB diet as such did not increase any mitochondrial ultrastructural abnormalities in the primordial follicle oocytes at adulthood. Nevertheless, a few indirect changes in mitochondrial ultrastructure were attributed to the maternal OB diet, such as mitochondrial elongation and an increase in mitochondrial matrix density that normally only appears after follicle activation (Chapter 5). The increased mitochondrial matrix density may indicate a higher mitochondrial activity at an earlier state, and may explain the slightly increased rate of maternal OB diet induced mitochondrial abnormalities at the end of the folliculogenesis detected in mature ovulated oocytes (Chapter 4). Since mitochondrial activity is crucial for follicle activation, also changes in oocyte mitochondrial features suggestive for an earlier onset of OXPHOS may lead to increased ROS accumulation after follicle activation, or may influence

the process of follicle activation itself. A diminished ovarian reserve (DOR) has been described by Aiken et al. (2016) in OB-born C57BL/6 offspring ovaries, associated with an upregulation of markers related to lipid peroxidation and oxidative stress. However, in contradiction, the changes in mitochondrial shape and matrix density in primordial follicle oocytes detected in our study due to a maternal OB diet were not associated with increased ROS accumulation, LDC, MMP indicative for increased oxidative stress in the corresponding mature ovulated oocytes (Chapter 4). Moreover, the maternal OB diet did not impact the amount of ovulated oocytes of offspring at the time of collection. Therefore, the rather subtle changes induced by maternal OB diet seem not to be sufficient to induce increased oxidative stress during subsequent stages of development, or influence the process of follicle activation leading to an affected amount of ovulated oocytes. In addition, the effect of the offspring OB diet on oocyte mitochondrial ultrastructure in preantral follicles was not dependent on the maternal OB background.

7.1.2 Are the effects of maternal obesogenic diet consumption on oocyte mitochondrial features inborn or determined during early postnatal development (during lactation)?

7.1.2.1 *Maternal obesogenic diet consumption induces small adaptational changes in offspring primordial follicle oocytes at birth, but the mitochondrial ultrastructural abnormalities induced by an obesogenic diet are not inborn*

Our results show that the primordial follicle pool at adulthood is mainly affected by the direct effect of an offspring's OB diet. In addition, small changes in mitochondrial shape and matrix density were noticed due to the indirect effect of the maternal OB diet. Next, we examined if such changes were also present in the dormant follicle pool at weaning, or even earlier, at birth, due to the direct effect of a maternal OB diet.

Up until now, it remained unknown if OB-born offspring carry mitochondrial abnormalities in their oocytes already at birth that may have long-lasting effects on the quality of the oocyte within. Even though a maternal OB diet did not increase offspring weight at birth and did not hamper mature ovulated oocytes, ovaries of offspring born to either OB- or C-fed mothers were collected within 1 h after parturition for morphological and functional examination of the primordial follicle oocyte mitochondria. This enabled us to detect if the indirect effects of a maternal OB diet on primordial follicles at offspring adult age were equal to the direct effects of a maternal OB diet at birth or at weaning.

Similar to our findings at adulthood, a maternal OB diet did not increase the mitochondrial ultrastructural abnormalities in offspring primordial follicle oocytes at birth. Nevertheless, the oocyte mitochondria classified as spherical and having electron dense foci were reduced with 3 % and 12 % respectively. These morphological changes coincided with a reduction in TFAM, Drp1 and cytoplasmic OPA1 marker expression, while the expression of PGC-1α, Mfn2 and Hsp70 markers remained unaffected (Chapter 6). Interestingly, we noticed that the oocyte mitochondrial morphology at birth clearly resembles the mitochondrial morphology in mature ovulated oocytes of adult offspring, regardless of any diet effect (Figure 3). The oocyte mitochondrial matrix at birth was dense (Chapter 6), similar to the mitochondrial matrix density present in mature oocytes (Chapter 4), while the mitochondrial matrix of primordial follicle oocytes of adult offspring was mainly translucent with most mitochondria having only few cristae being present (Chapter 5). Even though no functional outcome parameters were assessed in the offspring's primordial follicle oocytes at adult age, we suggested a link between the increased mitochondrial density and the mitochondrial activity. Therefore, it is possible that the mitochondrial activity in oocytes at birth may resemble the mitochondrial activity in mature oocytes at adulthood. At birth, this mitochondrial energy is needed for final primordial follicle formation and oocyte germinal vesicle breakdown (Wear et al., 2016), while the mitochondria in mature ovulated oocytes support the final stages of maturation and prepare for fertilization (Robker et al., 2018). Even though the actual size of the mitochondria within the oocytes was never measured, it seemed that mitochondria within mature oocytes are a little bit smaller

than the mitochondria within primordial follicle oocytes. However, actual measurements of mitochondrial size is needed to support this observation.

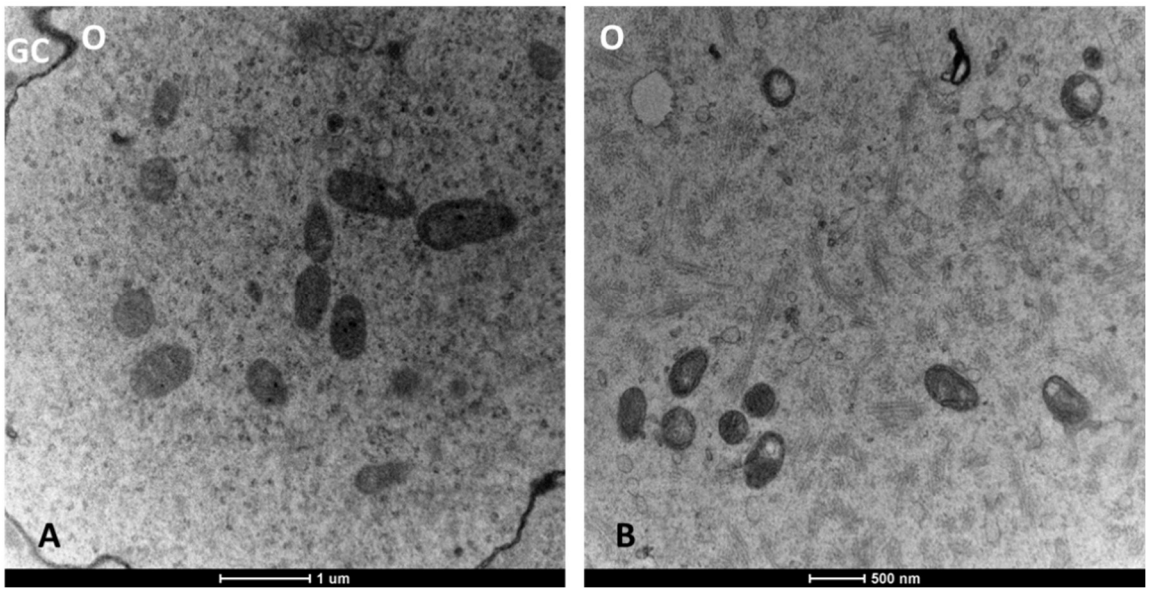

Figure 3: Mitochondria having a dense mitochondrial matrix within primordial follicle oocytes at birth (A). Mitochondria having a dense mitochondrial matrix within mature ovulated oocytes at adulthood (B). O = oocyte, GC = granulosa cell.

No inborn alterations in mitochondrial biogenesis due to a maternal OB diet are present in primordial follicle oocytes of their offspring, but the reduced TFAM expression may suggest a downregulation in mtDNA replication and transcription, independent of PGC-1α (Antelman et al., 2008). It is only recently discovered in a study using ICR mice that maternal obesity affects offspring primordial germ cells after the mitochondrial bottleneck (Tang et al., 2024b). In this latter study, mice were fed a HF diet and female embryonic gonads were collected at embryonic day 13.5 and 18.5. It was noted that maternal obesity disrupts the chromosomal synapsis and homologous recombination during fetal oogenesis, and induces global hypermethylation of genomic DNA in fetal oocytes (Tang et al., 2024b). However, as mentioned previously, several mechanisms can still preclude germ cell transmission of mitochondrial damage (Tingen et al., 2009; O'Connell and Pepling, 2021). We believe that the reduced mitochondrial replication and transcription detected in our study at birth may indicate a protective mechanism to prohibit the persistence of damaged mitochondria through the female germline. At adulthood, no differences in oocyte mtDNA copy number were present between offspring born to C or OB mothers (Chapter 4). Therefore we suggest that compensatory mechanisms may occur to eventually reach the required amount of mitochondria needed for maintaining the quality of mature oocytes at adulthood.

Also the Drp1 and cytosolic OPA1 marker expression were reduced in primordial follicle oocytes of OB newborns. In mature oocytes, depletion of Drp1 leads to an aggregation of malformed fused mitochondria (Udagawa et al., 2014; Liu et al.,2016). Such mitochondrial aggregation or clustering was slightly but significantly present in a few mature ovulated oocytes of adult OB-born offspring (Chapter 4), but the mitochondrial clustering at birth was not affected by maternal OB diet consumption, despite the reduced Drp1 expression (Chapter 6). Nevertheless, the reduced Drp1 expression in OB newborns at birth was linked with a reduced proportion of spherical mitochondria and a tendency towards and increased proportion of elongated mitochondria. In addition, the reduced cytosolic OPA1 expression can also contribute to the less spherical oocyte mitochondrial morphology of OB newborns by playing a crucial role in fusion and membrane remodeling (von der Malsburg et al., 2023). At birth, this was associated

with reduced electron dense foci in oocyte mitochondria within primordial follicles of newborns (Chapter 6). In mature oocytes, the same mitochondrial elongation was present but this coincided with an increase in mitochondria having electron dense foci (Chapter 4) suggestive for changes in the mature oocyte's glucose and oxygen metabolism (Grindler and Moley, 2013), and partially contradicting the findings at birth (Chapter 6). This may indicate that the oocyte's membrane remodeling is not persistent during oocyte development. Oocyte mitochondrial elongation was present in primordial follicles of OB newborns, in primordial follicle oocytes and in mature oocytes of adult OB-born offspring. Therefore, offspring oocyte mitochondrial elongation (albeit limited) can be seen as a consistent factor induced by maternal OB diet consumption. Hsp70, involved in the molecular defense in case of increased oxidative stress (Jovaisaite et al., 2014; Seli et al., 2019), was not increased in primordial follicle oocytes of OB newborns, thus the primordial follicle oocytes may not experience significant disruptions in cellular homeostasis due to maternal obesity. This is confirmed by the absence of maternal OB diet induced mitochondrial abnormalities and oxidative stress in oocytes of OB-born offspring, in their primordial follicle oocytes and mature oocytes respectively.

The direct effect of the offspring OB diet on the offspring primordial follicle oocyte mitochondrial morphology at adulthood is different than the direct effect of the maternal OB diet on offspring primordial follicle oocyte mitochondrial morphology at birth. Therefore, the oocyte mitochondrial abnormalities induced by an OB diet are not transmitted to the following generations. In addition, our results imply that the maternal OB diet reduced mitochondrial transcription during the first postnatal days may play a role in this phenomenon.

A schematic overview of all different categories of oocyte mitochondrial ultrastructure present at birth and at adulthood in Swiss mice, regardless of any diet, is presented in Figure 4, the Mini Atlas. All mitochondrial classifications noticed in our study at birth and at adulthood (primordial follicle oocytes and mature oocytes) are shown. Per timepoint or developmental phase, the mitochondrial morphology that is expected to be normal or abnormal is indicated based on findings by us (Marei et al., 2020) and others (Odor and Blandau, 1969; Van Blerkom, 2009; Yildirim and Seli, 2024).

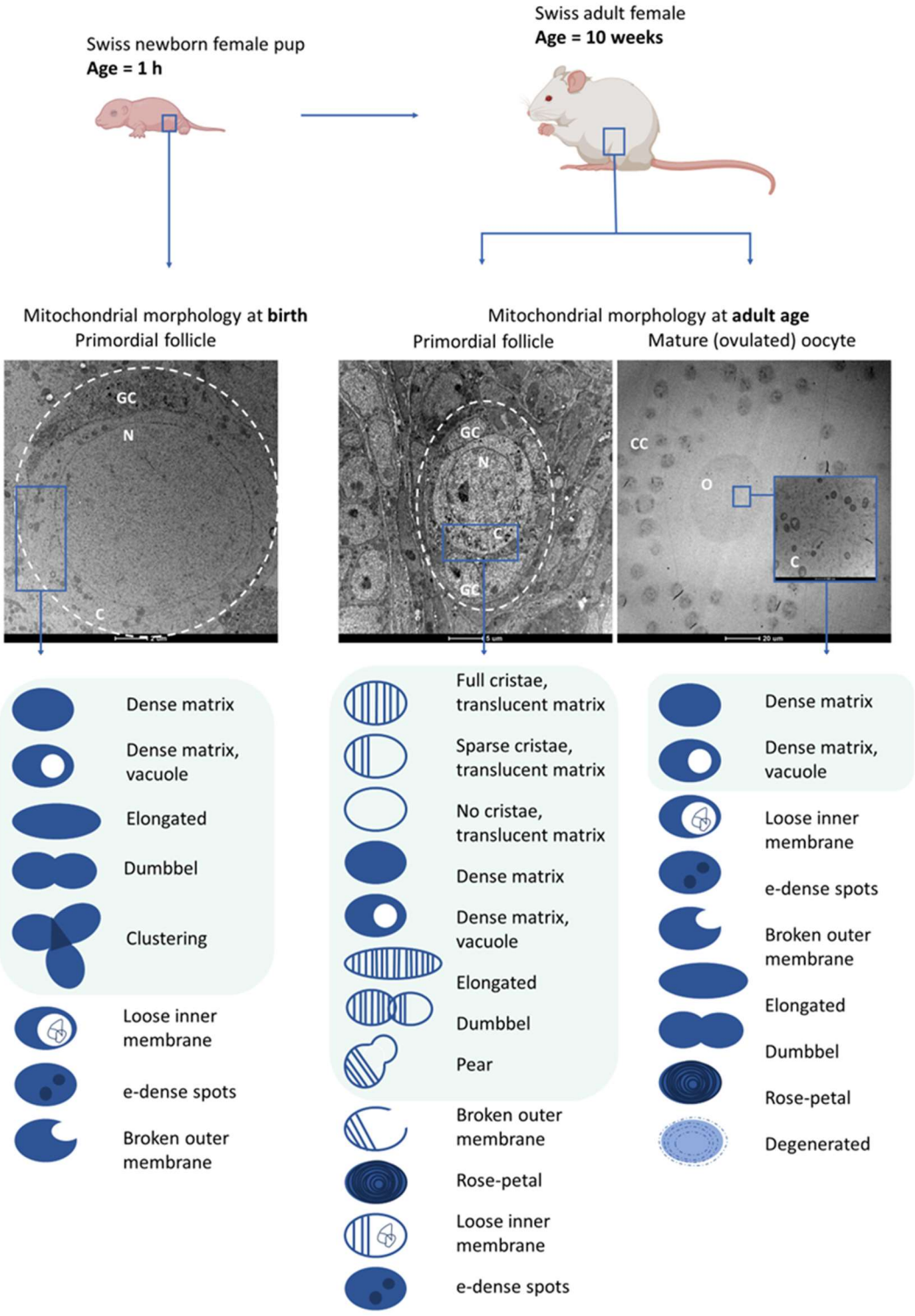

Figure 4. The mini atlas represents the mitochondrial morphology in oocyte mitochondria of primordial follicles of Swiss mice at birth (age less than 1 h) and at adulthood (age 10 weeks). In addition, the mitochondrial morphology is presented in mature oocytes of adult Swiss mice (age 10 weeks) after hormonal stimulation as described in Chapter 4 of this thesis. The green(ich) background represent the mitochondrial morphology that is suggested to be normal in each stage, whereas the others are suggested to be abnormal and indicative of mitochondrial dysfunction. The color blue in the drawings stands in for the electron density as detected under the transmission electron microscope. Dark blue mitochondria represent mitochondria with a dense matrix, whereas white mitochondria represent mitochondria with a translucent matrix, with detectable individual cristae. O= oocyte, N = nucleus, GC = granulosa cell.

7.1.2.2 *Maternal obesogenic diet consumption induces adaptational changes in offspring primordial follicle oocytes mostly at weaning*

The lactational window is a crucial window of exposure for metabolic reprogramming (Bagnell et al., 2005; Bartol et al., 2008; Bartol et al., 2017), and a maternal OB diet impacted the weight of the offspring in our study (Chapter 3). Therefore, the lactational window was implemented in our study examining the effect of a maternal OB diet on primordial follicle oocytes at weaning.

During the lactational period only in C-born offspring, fission protein expression was reduced, while fusion protein expression was increased (Chapter 6), which implies a natural elongation of the mitochondria with changes in cristae formation due to the reduced OPA1 expression (Zou et al., 2021; von der Malsburg et al., 2023). However, unfortunately, technical problems with tissue processing and sample dehydration for TEM prevented ultrastructural analysis of oocyte mitochondria within primordial follicles at weaning, so these suggestions of mitochondrial elongation from birth until weaning cannot be confirmed. Nevertheless, a few good TEM images could be taken at weaning, and it was clear that the overall mitochondrial morphology of primordial follicle oocytes at weaning did not show immediate signs of elongation, but it clearly resembled the mitochondrial morphology of primordial follicle oocytes at adulthood (a mitochondrial translucent matrix with few cristae, Figure 5). Although we were unable to include these few images in our statistical analysis, the transition from dense mitochondria at birth to translucent mitochondria at weaning coincided with a decreased expression of OPA1 and PGC-1α. This natural reduction of OPA1 expression at weaning was linked with only a few cristae being present with a translucent mitochondrial matrix, and the PGC-1α reduction seemed to be associated with a reduced mitochondrial density. However, good and representative TEM images at weaning are needed to confirm this interesting observation.

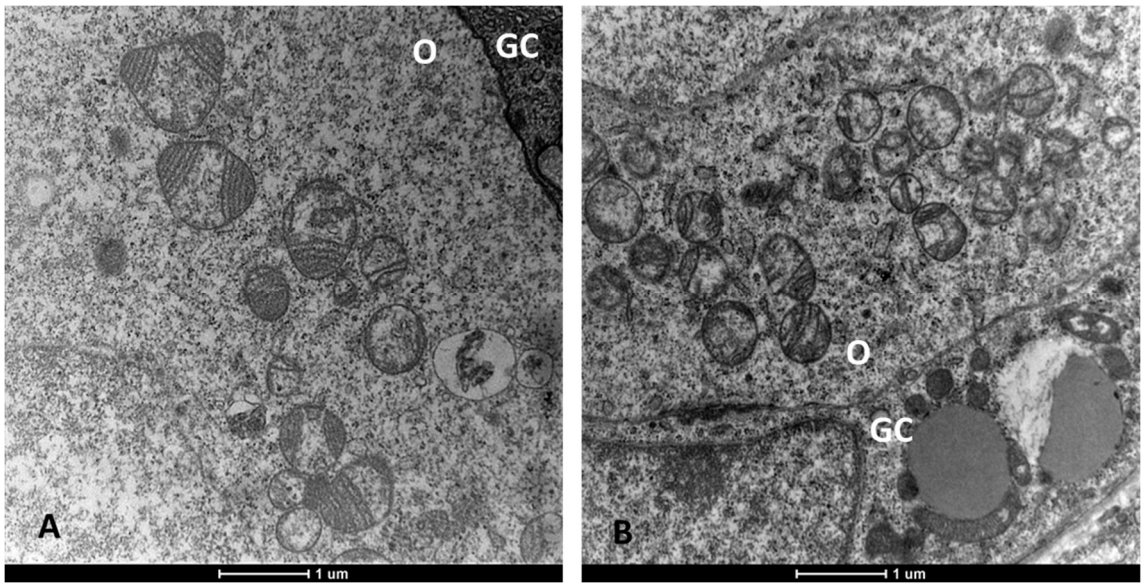

Figure 5. Mitochondrial with a translucent mitochondrial matrix and the presence of clear cristae within primordial follicle oocytes at weaning (A) and at adulthood (B). O = oocyte, GC = granulosa cell.

Maternal OB diet consumption coincided with reduced TFAM, but increased PGC-1α, OPA1 and Mfn2 marker expression in offspring primordial follicle oocytes at weaning. In addition, the weight of the offspring was also increased at weaning due to the maternal OB conditions, while it remained unaffected at birth (Chapter 3). No cross-fostering was performed in our study to investigate the effect of the

lactational window as such, and thus, no OB-born pups were nursed by C fed mothers. This means that our results only highlight a certain additive effect of lactation to an OB mother. It is possible that nursing by OB mothers only is enough to induce effects on the offspring's weight, or on their oocyte mitochondria at weaning. Nevertheless, our results support the findings of others highlighting the importance of the sensitive lactational window (Monks et al., 2018), albeit partially, as the effects of a maternal OB diet at weaning were increased compared to the maternal OB diet effects at birth. At offspring adult age, no signs of increased oxidative stress were noticed in offspring oocytes due to the maternal OB diet. Therefore we believe that the mitochondrial changes detected at birth, but mainly at weaning, are signs of adaptation and metabolic reprogramming of OB-born offspring to an environment with high energy availability. This reasoning is supported by the comparison of mature ovulated oocytes of C»OB with OB»OB. Oocytes of both groups suffer from the direct effect of an OB diet, and the investigated outcome parameters show similar results. However, OB»OB had the lowest pyruvate consumption and ETC marker V expression based on the measured effect sizes as shown in Chapter 4, and only degenerative mitochondria were significantly increased in OB»OB compared to C»OB when doing the pairwise comparisons within offspring diet groups. This may indicate that oocytes of OB»OB have an increased removal of damaged mitochondria. Since the mtDNA copy number between both groups remained equal, an increased biogenesis in OB»OB is expected, compared to C»OB that may ameliorate oocyte quality (Reynier et al., 2001). Such increased biogenesis (PGC-1α) was clearly detected in oocytes of OB-born offspring at weaning. Perhaps, the reduced TFAM indicates a lowered transcription and translation of mitochondria preventing transmission of mitochondrial dysfunction to the offspring, while the increased biogenesis at weaning implies more generation of mitochondria from the preexisting ones that have passed the selection process at birth.

The increased PGC-1α expression at weaning (but not at birth), and the increased mitochondrial density detected in primordial follicle oocytes in OB-born offspring at adulthood, may also indicate that the primordial follicles of OB-born offspring may transition to the primary stage earlier (Cinco et al., 2016), compared to primordial follicles of offspring born to C mothers. Eventually, an increased follicle activation may lead to a reduced follicle reserve, as the amount of dormant follicles is fixed around birth (Telfer et al., 2023). These thoughts may be in line with findings in C57BL/6 mice where DOR was detected in ovaries of offspring born to obese mothers (Aiken et al., 2016). However, further research involving the follicular depletion rate is needed and implementing the follicle count on all time points is therefore recommended. Nevertheless, again, the expression of Hsp70 was not increased at birth or at weaning, no increased oxidative stress was noticed in ovulated oocytes of OB-born offspring at adulthood and the amount of ovulated oocytes collected after super stimulation was equal between groups. This suggests that the offspring oocytes may not experience significant disruptions in cellular homeostasis due to a maternal OB diet at birth, at weaning or at adulthood.

While mature ovulated oocytes of adult OB»C offspring did not show major bad features compared to the other treatment groups, the results of the metabolic profile analysis of adult OB-born offspring hint towards the Thrifty Phenotype Hypothesis (Hales and Barker, 2001) and the Predictive Adaptive Response theory (Bateson et al., 2014) as mentioned earlier. Switching the *in utero* and post-natal adapted OB-born offspring suddenly to a mismatched C diet after weaning, has the highest impact in the offspring's metabolic profile, but without hampering the quality of the corresponding oocytes.

7.2 Practical implications of our findings – fundamental insights for sustainable advice

The results described here in our study using outbred Swiss mice differ from the findings that are previously reported by others using inbred mice strains. In inbred mice, the maternal OB diet caused exacerbated obesity in their offspring, and inbred offspring born to OB mothers even showed signs of an

increased sensitivity to an offspring OB diet after cross-fostering of the OB-born offspring to C mothers. In contradiction, our study using outbred Swiss mice provides new fundamental insights by showing that offspring born to OB mothers, remaining with their biological mother during lactation (like humans), have no perturbed metabolic health or increased oxidative stress in their mature oocytes. Moreover, even improvements are detected in the metabolic profile of adult offspring born to OB mothers. Even though no direct comparisons between inbred and outbred mice strains were implemented in this study, we provide evidence that it is of utmost importance to take the used mouse strain and experimental design into account when interpreting data obtained from intergenerational studies. However, despite the absence of a hampered adult offspring metabolic profile and the absence of oxidative stress or mitochondrial damage in offspring oocytes at birth, weaning, or adulthood due to maternal OB diet consumption, some subtle but consistent changes were detected in both muscle and oocyte mitochondria of offspring born to OB mothers that may be important to further obtain fundamental insights for sustainable advice.

Some studies link DOHaD and the expected increased risk of metabolic diseases in offspring born to obese mothers to mitochondrial dysfunction (Fukunaga, 2021; de Lima et al., 2023). In the present study we did not examine if the offspring from OB mothers were born with mitochondrial defects in their somatic cells (muscles), but these defects were predominant at adulthood and were associated with small improvements of the offspring's metabolic blood profile. Even though the metabolic health, assessed as the blood lipid profile and insulin sensitivity, was not affected by the observed mitochondrial aberrations in the oxidative muscle tissue, mitochondrial function declines in the process of aging due to the buildup of mutations and oxidative damage (Bentov et al., 2011; Chistiakov et al., 2014). Therefore, aging of women can serve as a second hit on the mitochondrial aberrations detected in the muscle of offspring born to OB mothers, that may still lead to metabolic health issues later in life. In addition, mitochondrial dysfunction has been suggested to play a role in the development of cancer due to their important role in many key processes for optimal cellular function (Garg et al., 2014; Rickard et al., 2023). Moreover, mitochondrial ultrastructural abnormalities are seen as a hallmark of cancer development. Mitochondria in cancer cells predominantly show a vacuolated matrix with disarranged cristae and myelin-like structures, including changes in the matrix density and increased presence of electron dense foci (Gabriel, 2009). Even though maternal OB diet consumption as such did not negatively affect the metabolic profile of offspring and even induced some metabolic improvements, future studies may still consider looking at later timepoints and expand the examined outcome parameter list to include more somatic tissues, representing a broader metabolic screening. Either way, we could confirm that these mitochondrial defects occur in OB-born offspring at adulthood, regardless of the offspring's current diet. Therefore, our results may suggest that daughters born to obese mothers should be advised to follow more efficient changes in their lifestyle that may prevent (further) deterioration of somatic cell mitochondrial functions. These interventions should aim at activating mitophagy, since this process helps maintaining mitochondrial quality by removing the dysfunctional mitochondria (Mishra and Thakur, 2023). Stimulating mitophagy can be done by intensive exercise (Wang et al., 2023), fasting (Mehrabani et al., 2020), or by mitophagy activating therapy (Tang et al., 2023). The efficiency of such interventions and the potential impact on metabolic health and fertility in offspring born to obese mothers is yet to be examined.

Regarding our observations in the oocytes, we clearly show that the offspring are not born with mitochondrial aberrations (ultrastructural abnormalities such as loose inner membranes or mitochondrial degeneration) in the oocytes of the primordial follicle pool. This information is crucial, because it illustrates that disturbances in gonadal (ovarian) functions reported in females born to obese mothers (Aiken et al., 2016; Cinzori and Strakovsky, 2022) may not be derived or associated with an inborn reduction in oocyte quality. The offspring hypothalamic-pituitary-gonadal (HPG) axis is first activated during fetal life, crucial for sex determination and genital development, but continues to have significant influences on offspring sexual development and reproductive health throughout the offspring's life (Cinzori and Strakovsky, 2022). Maternal obesity may affect the offspring's HPG axis via altered leptin and

insulin concentrations during pregnancy (Hauguel-de Mouzon et al., 2006; Aung et al., 2020), thereby influencing the offspring's folliculogenesis later in life, a process where oocyte mitochondrial metabolic capacity also plays a role (McLaughlin and McIver, 2009; Kidder and Vanderhyden, 2010; Ernst et al., 2017). Our results suggest a premature activation of the mitochondrial bioenergetic capacity and different metabolic needs as the oocyte continues to develop, without inducing mitochondrial damage or dysfunction in these oocytes. Indeed, in mature oocytes collected after ovulation, the pyruvate consumption was significantly different due to maternal obesity, and complex V was downregulated, showing that the oocyte is trying to limit its metabolic activity to control stress levels. This is confirmed by the normal MMP and ROS levels detected in these oocytes compared to controls, despite clear effects of the offspring OB diet. Practically, this can imply that in human IVF, oocytes from patients with a history of maternal obesity may require an adapted IVF protocol aiming at reduced metabolic activity in the oocytes, for example by limiting the nutrient availability, or by adapting O_2 levels. Nevertheless, further studies are needed to achieve such finely optimized protocols. In addition, the live birth rates after ART remain relatively low as many steps during this process can create potential stressors that may impact the developmental competence of the oocyte (Brinsden, 1993; Frydman et al., 2000; Pudakalakatti et al., 2013; Hansen, 2020). Moreover, the success rate of ART is even more reduced in obese women, since it appears to have a synergistic effect with maternal obesity on pregnancy and offspring risk factors such as for example preeclampsia and the risk of cancer development (Gonzalez et al., 2022). These risks could be, at least in part, oocyte-born and the results provided here constitute the first steps towards addressing patient-specific oocyte needs.

We show that offspring oocyte mitochondrial functions appear not to be affected by maternal OB diet consumption at early adulthood, despite the small increased proportion of mitochondrial ultrastructural abnormalities. However, again, mitochondrial functions are known to be deteriorated by age, which may suggest that the oocytes of offspring born to obese mothers may undergo earlier deterioration by aging. Women with a history of maternal obesity may be advised not to delay their planning to get pregnant. In addition, the small increase in mitochondrial ultrastructural abnormalities induced by maternal OB diet consumption may lead to an increased sensitivity to cryopreservation. A severe reduction of the mitochondrial membrane potential was noticed in cryopreserved oocytes after thawing (Jones et al., 2002), associated with alterations in the calcium signaling and oxidative stress that hampers the developmental competence after fertilization (Jones et al., 2004). These effects may further deteriorate if oocyte mitochondrial ultrastructural alterations are present. Oocyte activation protocols artificially induce calcium release following intracytoplasmic sperm injections (ICSI) to stimulate processes such as mitotic cleavage and early embryo development (Tsai et al., 2022). Since mitochondria are involved in the cellular calcium signaling, even small changes in the mitochondrial structure or function may hamper their response to such techniques as well. Therefore, even though the oocyte structural abnormalities induced by the maternal OB diet in our study are limited and not accompanied by an increased oxidative stress at that stage, it is possible that oocytes of women having a history of maternal obesity may be less countered to the effects of cryopreservation and oocyte activation protocols after ICSI, compared to daughters born to lean mothers. Especially since a certain threshold exists in the balance of ROS that can easily affect the rather limited capacity to maintain cellular homeostasis in oocytes (FitzHarris and Baltz, 2009; Wakai et al., 2019; Warzych and Lipinska, 2020). However, all these raised points involving ART optimization and PCCI are rather speculative and clearly, more trials are need to better shape reproductive management strategies of the women at risk. Nevertheless, as we are the first to show that the few ultrastructural abnormalities induced by maternal OB diet consumption in oocytes of adult offspring are not inborn in outbred Swiss mice, a possible window of protection may be created from birth until early offspring adulthood to anticipate on the changed oocyte mitochondrial ultrastructure in adult daughters born to OB mothers.

Lastly, our results highlight the major negative effect of the offspring's OB diet on the offspring's health and oocyte quality. With this study, we are the first to also show that even the mitochondrial quality of

the dormant primordial follicle pool is clearly hampered by the direct effect of an OB diet, in association with a reduced quality of the corresponding mature ovulated oocytes (C»OB and OB»OB). The fact that the offspring's OB diet induces mitochondrial ultrastructural damage in the dormant primordial follicle pool cannot be overlooked when designing proper PCCI in OB women aiming to become pregnant. Often, women are concerned about the oocyte quality and the pregnancy successes once they plan to have children (Fowler et al., 2024). However, PCCI or attempts to improve oocyte quality may have to start earlier. In a recent retrospective cohort study performed by Kranjac et al. (2024), the prevalence of childhood obesity in the United States was more than doubled from 1999 to 2018. In this study it was also noted that up to 90 % of obese children aged 3-4 years also suffer from obesity at adulthood, and it was highlighted that the obesogenic home environment and exercise patterns will dictate the lifestyle environment of the entire household (Kranjac et al., 2024). This means that the children's caretakers determine the nutritional intake of the children, that will eventually contribute to the health of their offspring later in life (Kranjac et al., 2024). Therefore, it remains extremely important that parents acknowledge the risks of childhood obesity, including the corresponding effects on fertility at adulthood. Since the effect of an offspring OB diet already shows after one week (weight) and will affect the dormant primordial follicle pool, a healthy and balanced post-weaning diet for children is of the utmost importance. Therefore, lifestyle interventions of obese women aiming to become pregnant must endure after a successful pregnancy, thereby preventing the dormant primordial follicle pool from being affect by the offspring's OB diet consumption, and to halt the intergenerational cycles of maternal obesity.

7.3 Limitations of the study

7.3.1 Windows of sensitivity

We aimed to mimic the natural (non-IVF) human situation the best way possible, where exposure to a maternal OB environment takes place during all phases of development starting in the preconception period (during oocyte growth and maturation), through pregnancy and lactation. Our data show that the direct effect of an OB diet on oocyte mitochondria is not transmitted through the female germline, and imply that maternal OB diet consumption does not induce mitochondrial dysfunction at birth, while more changes in mitochondrial biogenesis and dynamics are noticed after nursing by an OB mother. However, no specific insight is obtained to dissect the relative importance of all separate aspects where dietary insults may impact offspring health and fertility. Therefore, whether or not breastfeeding should be avoided when a mother is obese is a question that we cannot answer based on our findings. Nevertheless, our study design allowed us to estimate the inborn effect of maternal OB diet and even though no cross-fostering was used, some additive effects of the maternal OB diet during lactation could be detected. However, for full comprehension of separate timeframes, techniques involving e.g. embryo transfer and cross-fostering have to be implemented despite the fact that such techniques are prone to introducing stress-related bias (Matthews et al., 2011; Sasaki et al., 2013).

7.3.2 Sex-related differences

Sex-related differences in response to OB diets are reported in both human studies and in studies using animal models (Samuelsson et al., 2008a; Keleher et al., 2018; Xu et al., 2023). Women and men have different fat distribution, as men tend to have increased visceral fat depositions, while women have increased subcutaneous fat deposition (Cheng et al., 2010). Therefore, the waist-to-hip ratio is often increased in men compared to women, with an increased prevalence of cardiovascular diseases in men compared to women (Cheng et al., 2010). Pre-menopausal women are suggested to be protected against obesity by estrogen (Acharya et al., 2023) and this connection between estrogen and obesity is bidirectional. On one hand, a deficiency in estrogen can lead to excessive fat accumulation and impair adipocyte function. On the other hand, the adipose tissue of obese individuals is marked by altered expression of estrogen receptors and key enzymes involved in estrogen synthesis (Acharya et al., 2023), and in post-menopausal women, or women with ovariectomy, an increased risk of obesity was noticed (Kurylowicz, 2023). In rats, females tend to have a delayed response in terms of weight gain to an OB diet containing 60 % fat, having less metabolic complications compared to the males. In contradiction, in mice, females had exacerbated visceral fat increase in response to the same OB diet, compared to males (Kurylowicz, 2023). Not much research addresses these sex-related differences at F_0 level, and even less at F_1 level. Samuelsson et al. (2008a) noticed exacerbated obesity in male and female offspring born to OB mothers using inbred mice, but the male offspring showed reduced glucose tolerance compared to female offspring. Keleher et al. (2018) showed sex-related differences in offspring born to OB mothers, with an increased effect of the maternal OB diet on the weight of the female offspring, compared to the male offspring. Contrarily, maternal OB diet consumption induced more differentially expressed genes in the liver of the sons compared to the daughters (Keleher et al., 2018).

Recently, a call for action was made for research involving studies focusing on female obesity in rodent models, as they are limited (Maric et al., 2022). Therefore, our study focusing on female offspring born to either C or OB fed mothers is very important in this DOHaD field of research. The main focus of this PhD was to study the effect of both offspring and maternal OB diets and their interaction on the oocyte quality of the offspring, and hence only samples of female offspring were collected. However, including the comparison between male and female offspring would have been interesting and of additive value, especially when looking at the intergenerational transmission of disease susceptibility as investigated in Chapter 3. However, oocytes are affected by a maternal OB diet even before fertilization, while

spermatozoa are not. In addition, we focused on the role of the mitochondria in this intergenerational transmission, and since these mitochondria are exclusively maternally inherited (Van Blerkom, 2008), the transmission of the mitochondria towards the next generation would stop once a son is produced. Therefore, investigating female offspring is more relevant in studies focusing on intergenerational diseases susceptibility and the role of the mitochondria in this phenomenon.

7.3.3 The age of offspring

In the present study, adult offspring samples were collected at 10 weeks of age. As mentioned above, we did not detect any negative effect of maternal OB diet consumption on the offspring's metabolic profile or the offspring's oocyte quality. Despite the increased mitochondrial abnormalities in the muscles tissue and in the mature ovulated oocytes, it was clear that these alterations were signs of adaptation, rather than dysfunction. Nevertheless, the potential additive effect of the offspring's age on the adaptive changes detected at adulthood need to be taken into account.

Dutta and Sengupta (2016) reported a method to link the age of mice with human age and following this comparison, the murine age in our study is comparable to a human age situated between 19 and 26.5 years. A woman's optimal reproductive age is situated between the late teens and the late twenties. It is suggested that by the age of 30, human female fertility starts to gradually decline. By the age of 45, the ability to conceive naturally becomes unlikely (Forman et al., 2011). As we aimed to study intergenerational effects in our mouse model, this murine age of 10 weeks fits best as here, since optimal fertile age is reached without age-related bias on mitochondrial morphology and function.

We must be aware that eventually, the mitochondrial ultrastructural changes induced or influenced by a maternal OB diet may still lead to metabolic diseases and reduced oocyte quality in the ageing offspring. Especially since offspring aging is associated with mitochondrial dysfunction (Sun et al., 2016; Srivastava, 2017). In muscle tissue, but also in oocytes, the volume, integrity, and functionality of mitochondrial DNA declines in the process of aging due to the buildup of mutations and oxidative damage caused by ROS (Bentov et al., 2011; Chistiakov et al., 2014) and thus, aging as a factor can still contribute to the development of maternal OB diet induced effects on offspring health and oocyte quality later in the offspring's life. For example, Moeckli et al. (2022) noted that control diet fed OB-born C57BL/6 mice showed increased serum ALT but no reduced glucose tolerance at 24 weeks of age, while severe metabolic dysregulation and liver pathology was present at an offspring age of 40 weeks (Moeckli et al., 2022). Masuyama and Hiramatsu (2012) noticed that the systolic blood pressure in OB-born mice was significantly elevated at the age of 24 weeks, but not at the age of 12 weeks. In addition, a large-scale human study examined global trends and correlations between maternal obesity and offspring health outcomes and found that maternal obesity is linked to higher risks of obesity, type 2 diabetes, and cardiovascular diseases in offspring during adulthood, even if these conditions were not evident during childhood (Ezzati et al., 2017).

In research, older mice are often used to study the link between muscle mitochondrial function and the insulin resistance syndrome, even up until the age of 52 weeks (Fraulob et al., 2010; Ravichandran et al., 2019; Lee et al., 2021). Muscle have the capacity to take up and metabolize 80 % of the postprandial circulating glucose and account for 60-70 % of insulin-stimulated glucose uptake (Sergi et al., 2019). Therefore, a resistance to insulin may be visible in skeletal muscle way before hyperglycemia and pancreatic failure are present (Fraulob et al., 2010; Ravichandran et al., 2019; Lee et al., 2021). It could be that the mitochondrial alterations seen in muscle of OB-born offspring will aggravate over time, leading to reduced insulin sensitivity later in life. Therefore, it would be interesting to include older offspring in follow-up intergenerational research.

7.3.4 The comparison with inbred mice

Diet-induced obese mouse models are invaluable for investigating the pathogenesis of long-term health impacts of obesity across generations. Multigenerational human studies are often confronted with difficulties including ethics, randomization, equalization and long term follow-up. Mouse models help circumventing some of these challenges, while retaining pathophysiological relevance (Brehm et al., 2010; Tuttle et al., 2018). The more commonly used C57BL/6 mice are suggested to respond differently to metabolic stress (Freeman et al., 2006; Nicholson et al., 2010) while carrying a high rate of ultrastructural abnormalities in their oocyte mitochondria (Marei et al., 2020). Therefore, we used outbred mice to increase the pathophysiological relevance to human and to avoid strain related bias.

Outbred mice are bred from genetically diverse populations. They are intentionally maintained to have genetic variability, and are proposed as good subjects for biomedical research. On top, outbred mice often show better fertility with larger litters, compared to inbred mice strains (Tuttle et al., 2018). Previous research conducted at our laboratory noted that Swiss females fed an OB diet exhibit obesity, hyperglycemia, hypercholesterolemia and reduced insulin sensitivity with adverse effects on the quality of the oocytes (Marei et al., 2020; Smits et al., 2021; Smits et al., 2022). However, even with the Swiss mouse model being a good model for our research, the higher genetic diversity in Swiss mice compared to inbred C57BL/6 mice (Saul et al., 2019) may have increased the variation in our results. Knowing that such variation exists, a power calculation was done in advance using P&S (https://biostat.app.vumc.org/wiki/Main/PowerSampleSize) to account for this aspect, and no outliers were removed to reduce data variation or to increase the significance, if no technical errors occurred during the processing of the samples. Since our research involved an *in vivo* animal study, all mice were monitored very accurately the entire time. Only in case of illness, the animal would have been excluded from the study. Luckily, no animals got sick during our trial. This approach increased the validity of all significant differences and conclusions we make in this study.

The variation between mice was found to be similar between groups in the development of obesity, the corresponding metabolic profile and most oocyte-related outcome parameters. The highest variation was present in the Western Blot analysis of muscle and oocytes, even though it is assumed that the genetic variation would be reduced in the Western Blot analysis of the oocytes because oocytes from different mice (sisters) were pooled. Therefore, this high variation may be explained by the technique itself, rather than the variation between mice. Western Blot analysis is prone to various technical errors such as experimental conditions, differences in protein load and background signals related to the membrane that may affect the reproducibility of results (Liu et al., 2014). To encounter these technical difficulties, the band intensity was measured directly from the membranes and was corrected for background signals. The relative expression was calculated using β-actin blotting on the same membrane after stripping to block for differences in sample load, and the experimental conditions were always equal for all treatment groups. The variation was also relatively high in the mitochondrial ultrastructural analysis, as this technique involves a subjective classification of the highly variable mitochondrial morphology. However, to account for this limitation, the mitochondrial ultrastructure was performed by 2 researchers blind to the corresponding treatment groups (except for mitochondrial ultrastructure at birth), and merged after testing interrater reliability using interclass correlation coefficient, as described by Koo and Li (2016).

The results of our study contradict findings of other researchers using inbred mice. Therefore it would be of additive value to implement direct comparisons between outbred and inbred mice in future intergenerational studies similar as ours, to confirm our findings and suggestions. In the current PhD study, this would have been practically unfeasible. Nevertheless, some aspects can be tackled in follow-up studies where direct comparison between inbred and outbred mice is implemented.

7.3.1 The obesogenic phenotype

We wanted to investigate the effect of the offspring and maternal OB diet, and the effect of their interaction on its own on offspring health and oocyte quality. Therefore, no mice were excluded based on body weight and both responders and non-responders were included. Non-responders maintain equal body weight and metabolic health compared to lean mice, even when exposed to the same OB diet as obesity-responsive counterparts (Tarasco et al., 2018). It is a well-known fact that consumption of OB diets as such causes a systemic dyslipidemia (Klop et al., 2013), associated with metabolic disorders and reduced oocyte quality (Valckx et al., 2014; Chen et al., 2023). Our lab has shown that in mice, an OB diet consumption leads to increased LDC in the mature oocyte (also stained using BODIPY®) already after 24 h of feeding (Moorkens et al. 2024). This timeframe is sufficient to induce lipid accumulation in the oocyte, but is not sufficient to cause obesity as our own data showed that at least 1 week on an OB diet is needed to induce an increased weight gain compared to C fed offspring (Chapter 3 and 4, C»OB compared to C»C). Furthermore, a murine study focusing on brain development showed that the offspring's brain development and their energy metabolism was affected by increasing (amongst other factors) the offspring's blood pressure already after a maternal OB diet consumption for only 3 days during pregnancy (Ojeda et al., 2023). Therefore, we decided in advance that no cut-off value for the weight of the mothers would be implemented in our study.

7.3.2 The diet

As described earlier, free living rodents normally need a diet containing 10 % kJ of fat, and often a minimum of 45 % dietary fat intake is used to induce obesity in mice (Speakman, 2019). Based on previous research performed at our laboratory using the same outbred mice, we opted for a diet containing 60 % kJ of fat, with beef tallow as the primary source of fat and 20 % kJ fructose adjusted to the drinking water. Beef tallow is rich in saturated fatty acids such as palmitic acid (PA, C:16) and stearic acid (SA, C:18). A diet high in such saturated fatty acids is known to severely affect the metabolic profile in mice (Wang et al., 2020) and corresponding oocyte quality (Marei et al., 2020; Smits et al., 2022). Saturated fatty acids like PA and SA can directly exert negative effects on cell viability and increase apoptosis in oocytes and corresponding embryos (Van Hoeck et al., 2011). On top, they are also detrimental for the offspring's health, as an increased fatty acid intake is positively correlated with the development of gestational diabetes (Dayrit, 2023).

Adding 20 % fructose to the drinking water, as we did in our study, closely resembles drinking 2-3 soda cans a day for humans (Ruff et al., 2013). High fructose intake is associated with increased body weight, adiposity, increased systemic TG and a reduced sensitivity to insulin (Johnson et al., 2007b), and is reported to affect female fertility by hampering the hormonal cycles in rats (Komori et al., 2017) and reducing the pregnancy rates in mice (Saben et al., 2016a). As fructose reduces satiety (Pereira et al., 2017), it is possible that the amount of food intake was increased in the OB-fed mice in our study, that may have acted as a confounder. However, due to the very high food spillage of the OB-fed mice, it was impossible to adjust for the actual food-intake in our study. Nevertheless, the diet used in our study closely resembles the human OB diet.

Even though the used OB diet + fructose adjusted to the drinking water closely resembles the human diet high in fat and sugar, some questions remain about the dietary content of the control diet, containing 10 % kJ of fat and 7 % kJ sucrose. We have chosen this specific diet based on previous studies focusing on the effect of OB diets on oocyte quality and developmental competence performed in our lab (Marei et al., 2020; Smits et al., 2021; Moorkens et al., 2022; Smits et al., 2022). The main benefit of this C diet is that these C and OB diets only differed in fat and sugar content. All other ingredients are equal, allowing us to say that the results detected in our study are solely due to the increased fat and sugar intake in OB diet fed animals, and not due to differences in for example anti-oxidant concentrations and other

microminerals that protect against the negative effects of OB diets (Shahrokhi and Naeini, 2020; Almoraie and Shatwan, 2024). However, often a normal chow diet is used as a control diet. Chow diets contain roughly 7 % sugar and 3 % of fat which is less than the control diet we used. Therefore, the relative high amount of fat and sugar in our C diet may have contributed to the rather small differences between treatment groups (e.g. no changes in TG and ALT in C»OB vs C»C), and may have contributed to the relative high cholesterol levels detected in all C fed offspring (Chapter 3). However, chow diets are reported to contain phytoestrogen content derived from soy that can vary in content due to environmental conditions such as the weather, and economic choices of the company providing these diets (Warden and Fisler, 2008). Not only can these dietary phytoestrogens influence the animal's dietary intake, behavior and activity, but they can also influence an individual's fat deposition, leptin levels, lipogenesis and lipolysis (Warden and Fisler, 2008). Therefore, we believe that our choice of the specific C and OB diets were fit for our hypothesis and experimental design.

7.3.3 Functional interpretation of the data

The maternal OB diet did not negatively impact offspring health and few signs indicative for adaptation were present at the level of the muscle mitochondria. Therefore, it would have been interesting to follow the causes of these mitochondrial adaptations. However, we focused on the OXPHOS active soleus muscles, a small muscle providing limited amount of material and thereby limiting the number of outcome parameters that can be assessed. We studied mitochondrial ultrastructure, a commonly used technique, because it allows evaluation of mitochondria individually and *in situ*, and allows separate assessment of subsarcolemmal and intermyofibrillar mitochondria having different capacities and functions (Mailloux and Harper, 2011; Crescenzo et al., 2014). Mitochondrial ultrastructural abnormalities have been directly associated with other mitochondrial and metabolic functions and phenotypes such as altered mitochondrial bioenergetic functions and ATP production, activation of mitophagy or biogenesis (Cook et al., 2014). In addition, ultrastructural abnormalities were detected in muscle tissue of mice suffering from insulin resistance (Hayden, 2022). In addition, since previous studies on this topic using inbred models found that detrimental effects of diet on offspring health were associated with (inheritance of) mitochondrial ultrastructural abnormalities in muscles tissues (Saben et al., 2016b), our results facilitate comparisons with previous reports. While the combination of morphological analysis and the Western blot analysis of the ETC complex marker expression do not provide a direct measurement of mitochondrial respiratory functions, they are good proxies for estimating alterations of functions (Lee et al., 2021; Hayden, 2022).

Seahorse analysis on muscle tissue directly estimating the mitochondrial function was practically not possible because the mice euthanasia and sample collection was done sequentially from 4 treatment groups divided across several replicates, and also included collection of oocytes. However, new techniques have been described by Acin-Perez et al. (2020) to use frozen muscle tissue for Seahorse analysis, even despite the mitochondrial uncoupling due to freeze-thaw cycles and the unresponsiveness of snap frozen mitochondria to mitochondrial substrates that may reduce the accuracy of measuring respiration in frozen samples (Acin-Perez et al., 2020). Genome sequencing may also provide additive information as in human, this technique is described as a useful diagnostic test in patients with suspected mitochondrial disorders, and enables diagnosis of pathogenic mutations that affect the mtDNA and the nuclear genome (Schon et al., 2021). However, no detrimental effects of the maternal OB diet on offspring health were detected in our study, and further exploration of mitochondrial functions related to these small signs of adaptation should also be done both in outbred and inbred models to study the differences in these mitochondrial adaptations. Therefore, investigating how mitochondrial ultrastructure and complex expression affects mitochondrial and cellular metabolic functions may not be relevant for this specific study. Nevertheless, investigating the causes of these mitochondrial adaptations would be very interesting for follow-up research.

In our study, oocyte quality was only assessed at the level of the mature oocyte and at different developmental stages before. The developmental competence was not investigated as we prioritized analyzing outcome parameters in different developmental stages (primordial/primary/secondary follicles and mature oocytes) and at different time points (birth/weaning/adulthood) that better fit our hypothesis focusing on the intergenerational transmission of OB diet induced effects from the mother to the daughter, through the mitochondria. Parallel associations have been made by our lab and by others between the outcome parameters we have used on mature ovulated oocytes (such as increased LDC, oxidative stress, mitochondrial dysfunction), and the subsequent reduced early embryo developmental capacity. These studies were performed in murine studies, but also in studies using bovine models, and show that if for example the oocyte LDC or the oxidative stress is increased, the corresponding developmental competence after fertilization is reduced (Marei et al., 2019; Smits et al., 2022).

Increased ROS accumulation is associated with spindle defects and chromosomal abnormalities, which is a major cause of fertility decline and miscarriages in women (Sasaki et al., 2019). Excessive ROS generation during oocyte maturation and embryo culture disrupts multiple cellular processes, and thus, maintaining the balance between ROS and antioxidants is crucial for preserving oocyte competence (Torres-Osorio et al., 2019). Even though oxidative stress is widely recognized as a major disruptor of oocyte quality, oocyte quality as such involves more than just accumulation of ROS. In bovine oocytes, a reduced pyruvate dehydrogenase, catalyzing oxidative decarboxylation of pyruvate to acetyl-CoA, NADH and CO_2, coincided with increased oocyte spindle defects. However, these findings were also associated with increased ROS, affected mitochondrial distribution and MMP (Zhang et al., 2023), which was not the case in our study. Also, a reduced mtDNA copy number is associated with reduced developmental competence (Otten et al., 2020), but this was not affected by a maternal OB diet in our present study. In addition, the mitochondrial clustering, in our study increased by a maternal OB diet, was reported by others not to affect the oocyte spindle formation (Lee et al., 2024). Maternal OB diet did increase the mitochondrial ultrastructural abnormalities, but this mainly involved mitochondria showing signs of degeneration (in mature ovulated oocytes at adulthood) and the overall percentage of abnormalities remained limited to maximum 10 %, while only an increase of over 20 % is suggested to affect the oocyte's developmental competence (Simsek-Duran et al., 2013; Muhammad et al., 2021). The expression of the complex III and V markers were reduced by a maternal OB diet, but in pairwise comparisons only confirmed for complex V in offspring fed a mismatching C diet, and the slightly reduced expression of complex III in OB»OB oocytes may even be a beneficial compensatory mechanism for the oocytes to control ROS production. Since MMP was not affected, no consequence for energy production is expected (referring to Chapter 4 for in depth explanation of our findings). Therefore, we believe, based on our findings and the parallel observations performed by others in our lab (Marei et al., 2019; Smits et al., 2022), that a maternal OB diet would not affect the offspring oocyte developmental competence in an outbred mouse model. Nevertheless, extra follow-up studies directly addressing offspring oocyte developmental competence may be of additive value to confirm our thoughts. Consequently, the effect of the maternal OB diet on the next generation of offspring (F_2) would be very interesting, providing better insight in the maternal OB diet induced alterations and the long-term effect of the few significant interactions that we detected in F_1 offspring.

Even though we do not expect a reduced oocyte quality due to a maternal OB diet, some aspects require further investigation. We only investigated mitochondrial ultrastructure in the preantral follicles at adulthood. Therefore, the functional consequences of the altered markers detected either at birth or at weaning remain unelucidated and we do not know if this affects folliculogenesis as such, and lead to a different rate of follicular atresia or follicle depletion. The increased PGC-1α expression at weaning and the increased mitochondrial matrix density in the primordial follicle oocytes may indicate that the dormant primordial follicles are activated earlier. Eventually, this may lead to and increased follicle depletion rate. We did not detect differences in number of ovulated oocytes at adulthood between offspring born to OB or C mothers and therefore the amount of ovulated oocytes is equal between groups. However, follicles may undergo atresia even before they reach the pre-ovulatory stage (Telfer et al.,

2023). Therefore, including follicle counts on all timepoints could have been of important additive value. In addition, we do not know if our findings at birth and at weaning are associated with an impact on energy demanding mechanisms such as epigenetic programming or chromosomal segregation during subsequent nuclear maturation. Especially since Tang et al. (2024a) recently discovered that a maternal HF/HS diet induces meiotic defects and epigenetic programming during fetal oocyte development. These questions remain open and require extensive investigation in future research.

Due to limited time and funding to support rather the time-consuming and expensive outcome parameters, assessment of follicular depletion rate, epigenetic (re)programming and chromosomal segregation were not included in this PhD study. Especially since already a lot of other outcome parameters were addressed that enabled answering our research questions. However, luckily, ovarian samples at birth, adulthood and to a limited extend also at weaning, are still available for further analysis focusing on follicular apoptosis and follicle depletion rate. Since our mice trial was finished over two years ago, we do not have fresh oocytes of adult offspring left, but snap frozen pools of adult offspring oocytes of all treatments groups are still available that can be used for further investigation.

7.4 Conclusions and key messages

With this study using outbred mice, we show that the maternal OB diet as a factor did not affect the weight of the pups at birth, but clearly increased the offspring abdominal fat and body weight at weaning. However, this effect disappeared relatively early post-weaning and remained absent until adulthood. Contradicting to what has been described by others using inbred mice strains, the maternal OB diet did not affect the offspring's health, or influenced or exacerbated the effects of an OB diet on the offspring's health in outbred Swiss mice. Furthermore, the maternal OB diet even improved the offspring's metabolic profile, despite the accompanied mitochondrial aberrations in oxidative muscle tissue.

We find no evidence that offspring born to obese mothers exhibit signs of oocyte mitochondrial dysfunction at birth, suggesting that OB diet induced mitochondrial dysfunction may not persist through the female germline in outbred Swiss mice. However, offspring oocytes do respond to the effects of the maternal OB diet from the very beginning of life, but these alterations seem to be adaptive or reparative. Pups weaned from OB mothers (and thus having pregnancy and lactation under OB conditions) have more apparent changes in mitochondrial biogenesis and mitochondrial dynamics, potentially due to an additive effect of the lactation window. However, the corresponding oocytes of OB-born offspring at adulthood showed no signs of increased oxidative stress or mitochondrial dysfunction, but even had an increased mitochondrial energy efficiency. Nevertheless, deterioration in oocyte mitochondrial ultrastructure appears to occur during folliculogenesis and at adulthood.

Although further research is required, with our findings we hope to bring crucial nuances to this specific DOHaD field of research, showing that in outbred Swiss mice, the effect of a maternal OB diet on offspring health and oocyte quality is not unfavorable, thereby highlighting important differences between mouse models and experimental set-ups that need to be taken into account when interpreting data from intergenerational research.

Key messages

In adult offspring:

1) The direct effect of the offspring's OB diet was confirmed to perturb the adult offspring's metabolic profile in outbred Swiss mice. Considering the relatively young offspring age (10 weeks) that we have investigated, the maternal OB diet did not hamper the offspring's metabolic profile, or increased the offspring's sensitivity to an OB diet. Maternal OB diet consumption even improved the offspring's metabolic profile, despite the changes present in the offspring muscle mitochondria.

2) The direct effect of the offspring's OB diet was confirmed to hamper the adult offspring's mature oocyte quality in outbred Swiss mice. However, up to the relatively young offspring age that we have investigated, maternal OB diet consumption did not lead to any apparent effect on offspring's oocyte quality, and did not interact with the impact of the offspring's diet on the oocytes.

3) We are the first to describe that the direct effect of the offspring's OB diet perturbed the adult offspring's primordial follicle oocyte mitochondrial quality by inducing ultrastructural damage in outbred Swiss mice. However, maternal OB diet consumption did not induce ultrastructural damage in their offspring's primordial follicle oocytes (assessed at 10 weeks of age), and did not interact with the offspring diet effects on the primordial follicle oocytes.

At birth and at weaning:

1) Maternal OB diet did not increase cellular stress levels in the primordial follicle oocytes but reduced TFAM expression both at birth and at weaning, which may affect or regulate mtDNA replication. More prominent effects of the maternal OB diet were detected in the offspring primordial follicle oocytes after lactation, specifically impacting PGC-1α expression and markers of fusion and fission. The functional implications of these changes on oocyte quality during subsequent development are yet to be determined.

7.5 References

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Samenvatting

In deze studie onderzochten we het effect van een obesogeen (OB) dieet van zowel de moeder als de dochter op de metabole gezondheid en de eicelkwaliteit (van zowel de eicellen van primordiale follikels als van de rijpe eicellen na ovulatie) van de dochters, en gingen we na of het effect van het OB dieet van de dochters afhankelijk was van het dieet van de moeder. We onderzochten ook of er reeds schade was aan de mitochondriën van deze eicellen bij de geboorte en bij het spenen. Ons onderzoek is cruciaal, aangezien dochters van moeders met een OB dieet meer kans hebben om het onevenwichtige voedingspatroon van de moeders over te nemen en meer kans hebben om zelf obesitas te ontwikkelen. De effecten van beide OB diëten zouden elkaar mogelijks kunnen versterken, wat kan leiden tot nefaste gevolgen voor de gezondheid en vruchtbaarheid van de dochters met een maternale voorgeschiedenis van obesitas. Bovendien is het belangrijk te weten tijdens welke levensfase van de dochters deze eicel schade kan optreden, en tijdens welke fase in de ontwikkeling van ovariële follikels deze het meest uitgesproken is. Deze fundamentele kennis is noodzakelijk om gepast advies te kunnen geven aan patiënten met een maternale voorgeschiedenis van obesitas, met oog op het verbeteren van de vruchtbaarheid.

In **hoofdstuk 1** worden de effecten van een OB dieet op de gezondheid en eicelkwaliteit van de vrouw besproken. In dit hoofdstuk staan de mitochondriën centraal, gezien hun cruciale rol in de pathogenese van metabole stoornissen en verminderde eicelkwaliteit veroorzaakt door de schadelijke effecten van een OB dieet. De reeds gekende effecten van een maternaal OB dieet op de gezondheid en vruchtbaarheid van de kinderen, en de factoren die bijdragen tot de intergenerationele effecten van maternaal OB dieet consumptie worden vervolgens toegelicht. Tot slot worden de onbekende factoren, de zogenaamde ‘gaps in knowledge’, benoemd waardoor het belang van onze studie wordt benadrukt.

In **hoofdstuk 2** worden de hypotheses en doelstellingen toegelicht. Kort samengevat worden de effecten van een maternaal OB dieet, de effecten van een OB dieet van de nakomelingen, en het effect van de interactie tussen beide diëten, onderzocht aan de hand van verschillende parameters gerelateerd aan de metabole gezondheid en eicelkwaliteit van de nakomelingen. Dit onderzoek werd gedaan in een 2 x 2 factoriaal ontwerp, waarbij gebruik werd gemaakt van een Zwitsers muizenmodel. Stalen van de vrouwelijke nakomelingen werden verzameld bij de geboorte (leeftijd minder dan 1 u), bij het spenen (leeftijd 3 weken) en op volwassen leeftijd (leeftijd 10 weken).

In het eerste resultaten hoofdstuk, **hoofdstuk 3,** onderzochten we het effect van een maternaal OB dieet, het effect van een OB dieet van de vrouwelijke nakomelingen, en het effect van de interactie tussen beide diëten op de metabole gezondheid van de nakomelingen. De effecten op de nestgrootte, de groeicurve van de pups, het abdominaal vet, de metabole gezondheid en de gevoeligheid voor insuline, samen met de ultrastructuur van de mitochondriën en de mitochondriale oxidatieve fosforylatie (OXFOS) in spiercellen werden geëvalueerd en gerapporteerd. We toonden aan dat, in een Zwitsers muizenmodel, de nestgrootte, het geslacht van de pups en het geboortegewicht van de pups niet werd beïnvloed door een maternaal OB dieet. Het lichaamsgewicht van de nakomelingen was wel sterk verhoogd bij spenen, maar dit normaliseerde zich opnieuw en was afwezig in jong volwassen nakomelingen (leeftijd 10 weken). Bovendien leek het abdominaal vet zelfs te zijn verminderd. Ondanks de afwijkingen van de mitochondriale ultrastructuur en de verhoogde expressie van OXFOS merkers, was het metabool profiel van de volwassen nakomelingen niet verslechterd door het maternaal OB dieet. Sterker nog, het metabool profiel van deze nakomelingen was zelfs verbeterd in vergelijking met de nakomelingen van een controle

(C) gevoede moeder. Dit uitte zich in een verhoogde insuline gevoeligheid en een verlaagd cholesterolgehalte in het bloed. Nakomelingen van de OB»C groep, waarbij het maternaal OB dieet verschilt van het C dieet van de nakomelingen, hadden het meest afwijkende, maar wel het beste metabool profiel met de hoogste insulinegevoeligheid. Bovendien konden er geen additieve effecten worden vastgesteld in de OB»OB groep, waarbij nakomelingen van OB gevoede moeders zelf ook een OB dieet kregen. De het gehalte vrije vetzuren in het bloed van OB»OB muizen was zelfs lager dan dit van C»OB muizen. Hierdoor lijken de nakomelingen van OB gevoede moeders beter bestand tegen de nadelige effecten van een OB dieet, of lijken ze te zijn aangepast aan een vet en suikerrijk voedingspatroon. Onze resultaten sluiten aan bij de 'thrifty phenotype hypothesis', waarbij gesteld wordt dat nakomelingen zich tijdens de dracht en kort erna aanpassen aan de nutritioneel energetische beschikbaarheid van de moeder. In onze studie zijn deze aanpassingen dus (nog) niet noodzakelijk nadelig voor het vrouwelijk nageslacht. De schadelijke effecten van het OB dieet van de nakomelingen zelf was wel duidelijk zichtbaar, aangezien de nakomelingen die zelf een OB dieet kregen een lagere insulinegevoeligheid hadden, samen met een verhoogd vetprofiel in het bloed. Dit ging gepaard met een abnormale mitochondriale ultrastructuur in de spiercellen. Een interactie tussen het maternale OB dieet en het OB dieet van de nakomelingen beïnvloedde enkel de ultrastructuur van de spiercel mitochondriën. Onze resultaten verschillen van eerdere studies waarbij inteelt muizenrassen werden gebruikt, en benadrukken het belang van het muizenmodel bij het ontwerpen en interpreteren van intergenerationele studies. In ons Zwitsers muizenmodel draagt het maternale OB dieet op zich niet bij tot de verderzetting van familiale obesitas in de volgende generaties.

Ook al was het metabool profiel niet verslechterd door een maternaal OB dieet, we vroegen ons af wat het effect zou zijn op de eicelkwaliteit van deze volwassen nakomelingen. Daarom onderzochten we in **hoofdstuk 4** het effect van een maternaal OB dieet, het effect van een OB dieet van de vrouwelijke nakomelingen, en het effect van de interactie tussen beide diëten op de eicelkwaliteit van de nakomelingen. Hier bekeken we de vetdruppels in de eicellen, en deden we kwalitatieve en kwantitatieve metingen op de mitochondriën van de eicel, waaronder de bio-energetische activiteit en de ultrastructuur, en bekeken we het metabolisme van de cel. Het maternaal OB dieet zorgde voor een verhoogd aantal ultrastructureel afwijkende mitochondriën, maar dit zorgde niet voor een verhoogde membraanpotentiaal (MMP), oxidatieve stress (ROS), lactaat productie of aantal mtDNA kopieën, en zorgde niet voor een veranderde actieve mitochondriale distributie doorheen de cel. Dit was wel geassocieerd met een verminderde pyruvaat consumptie en een verminderde expressie van OXFOS complex III en V merkers. De meerderheid van de mitochondriën met abnormale ultrastructuur was gedegenereerd, en bevatte geen afwijkingen gelinkt met dysfunctie, zoals membraan defecten of ringstructuren. Dit suggereert dat, althans in ons Zwitsers muizenmodel, beschadigde mitochondriën worden verwijderd zonder de hoeveelheid mtDNA te veranderen. De gedaalde pyruvaat consumptie en lagere OXFOS expressie, zonder bijhorende wijzigingen in de MMP, ROS of lactaatproductie impliceert dat deze veranderingen geen, of slechts weinig, functionele consequenties hebben. In tegenstelling tot de effecten van het maternaal OB dieet zorgde het OB dieet van de nakomelingen wel voor een stijging in de vetdruppels, MMP en ROS, samen met een afwijkende ultrastructuur en een gedaalde pyruvaat consumptie. Het aantal mtDNA kopieën en de lactaat productie bleven opnieuw onveranderd. Een significante interactie tussen beide diëten beïnvloedde enkel de morfologie van de mitochondriën, en benadrukt de sterke mitochondriale flexibiliteit. Opnieuw spreken onze resultaten de reeds eerder beschreven effecten van maternale obesitas op de nakomelingen in inteelt muizenrassen tegen. Extrapolatie van data over de eicel mitochondriale functies bekomen uit inteelt muizenrassen in intergenerationele studies moet daarom met de nodige voorzichtigheid en nuancering gebeuren.

Kleine veranderingen waren zichtbaar in de mitochondriale ultrastructuur van rijpe geövuleerde eicellen van nakomelingen van OB gevoede moeders. We vroegen ons af of deze veranderingen ook al zichtbaar

waren in de eicellen van de primordiale follikels van volwassen nakomelingen. In **hoofdstuk 5** onderzochten we het effect van het maternaal OB dieet, het OB dieet van de vrouwelijke nakomelingen en de interactie tussen beide diëten op de mitochondriale ultrastructuur in preantrale follikels van de nakomelingen. We toonden aan dat de eicellen van de primordiale follikels geen directe schade ondervinden van de effecten een maternaal OB dieet, maar er waren wel een paar kleine veranderingen in vorm en mitochondriale matrix densiteit zichtbaar in deze eicellen van nakomelingen van OB gevoede moeders. Deze veranderingen waren indicatief voor een verhoogde capaciteit van de mitochondriale oxidatieve fosforylatie. Het maternale OB dieet zorgde niet voor een stijging in abnormaliteiten zoals kapotte membraanstructuren en ringformaties, en het beïnvloedde het effect van het OB dieet op de eicel mitochondriale ultrastructuur van de nakomelingen niet. Dit is opnieuw in tegenstelling tot het directe effect van het OB dieet van de nakomelingen. Dit dieet zorgde voor gebarsten en kapotte membraanstructuren die eerder al werden gelinkt aan mitochondriale malfunctie en een verminderde eicelkwaliteit. Verder onderzoek is nodig naar de functionele implicaties van onze bevindingen, maar onze resultaten tonen aan dat de preantrale follikels gevoelig zijn voor de effecten van zowel een maternaal OB dieet als een OB dieet van de nakomelingen, waar rekening mee moet gehouden worden bij het op punt stellen van protocollen met oog op het verbeteren van de vruchtbaarheid bij vrouwen.

Aangezien de primordiale follikels reageerden op het maternale OB dieet, vroegen we ons af of deze veranderingen al aanwezig waren van bij de geboorte, en of deze beïnvloed werden door lactatie bij een OB gevoede moeder. In **hoofdstuk 6** onderzochten we of het maternaal OB dieet de mitochondriale ultrastructuur, biogenese en dynamiek veranderde in eicellen van primordiale follikels van de nakomelingen bij de geboorte en bij het spenen. Zo gingen we na of pups van OB gevoede moeders geboren worden met mitochondriale dysfunctie of adaptatie, en of deze effecten zouden wijzigen na lactatie bij een OB gevoede moeder. We toonden aan dat de ultrastructurele schade veroorzaakt door het rechtstreeks effect van een OB dieet niet aanwezig was van bij de geboorte in pups van een OB gevoede moeder, althans in ons Zwitsers muizenmodel. De mitochondria van deze pups vertoonden wel tekenen van een verminderde mitochondriale transcriptie (TFAM) en een veranderde mitochondriale dynamiek (Drp1 en OPA1 (cytosol)). Pups gespeend van een OB gevoede moeder vertoonden duidelijke veranderingen in PGC-1α gerelateerde mitochondriale biogenese en mitochondriale dynamiek (Mfn2, OPA1 (nucleus en cytoplasma)), vermoedelijk door het additieve effect van de lactatie. Er werden echter geen tekenen waargenomen van een verhoogde stress in de eicel bij de geboorte en bij het spenen. Ook zagen we op volwassen leeftijd geen tekenen van verhoogde stress in de eicellen van nakomelingen van een OB gevoede moeder (hoofdstuk 4). Daarom vermoeden we dat de veranderingen geïnduceerd door een maternaal OB dieet bij de geboorte en bij het spenen tekenen zijn van herstel of aanpassing, en niet van schade. De eicellen in primordiale follikels van nakomelingen van OB gevoede moeders vertoonden (nog) geen tekenen van verstoorde cellulaire homeostase, maar verder onderzoek blijft noodzakelijk om de functionele implicaties van onze bevindingen na te gaan in de verdere stadia van de follikel ontwikkeling.

Tot slot werden in **hoofdstuk 7** al deze resultaten gebundeld en afgetoetst met de beschikbare literatuur. Daaruit konden we in dit onderzoek besluiten dat:

- een maternaal OB dieet geen nadelige gevolgen heeft voor de gezondheid van de nakomelingen, en de effecten van het OB dieet van de nakomelingen niet versterkt. Het metabool profiel van de nakomelingen van OB gevoede moeders lijkt zelfs te zijn verbeterd, ondanks de aanwezigheid van mitochondriale defecten in het spierweefsel.

- pasgeboren pups van OB gevoede moeders geen tekenen van een verslechterde mitochondriale ultrastructuur vertonen in de eicellen van primordiale follikels. De eicellen van deze pups

reageren wel op een maternaal OB dieet vanaf het prille begin, maar vertonen eerder tekenen van aanpassing en niet van schade.

- pas gespeende pups van een OB gevoede moeder meer mitochondriale aanpassingen vertonen in hun eicellen in vergelijking met de pasgeboren pups van een OB gevoede moeder. Dit komt vermoedelijk door het additief effect van de lactatie.

- volwassen nakomelingen van OB gevoede moeders geen tekenen van oxidatie schade of mitochondriale dysfunctie vertonen in hun rijpe eicellen na ovulatie. Echter, de ultrastructuur van de mitochondriën in eicellen wordt wel aangetast tijdens de ontwikkeling van de ovariële follikels en op volwassen leeftijd.

Summary

In this thesis study we aimed to investigate if the metabolic health of adult offspring and the quality of their oocytes was affected by the maternal OB diet, the offspring's OB diet, and the maternal x offspring diet interaction. Oocyte quality was assessed in ovulated oocytes as well as at the primordial and preantral follicle stage. In addition, we aimed to investigate if maternal OB diet induced oocyte mitochondrial alterations are inborn, or further aggravate during lactation from obese mothers. Such fundamental knowledge is crucial in our contemporary society where most obese women are born to obese mothers. Knowing when in the offspring's life these effects may occur is important to design accurate preconception care strategies for women having a family history of obesity. In addition, determining the stage during adult folliculogenesis at which oocytes are affected by the maternal and offspring OB diet can help further develop strategies for improving the offspring's fertility.

In **Chapter 1**, a general introduction about the current state-of-the-art is presented, describing the effects of OB diet consumption on the individual's health and fertility, focusing on oocyte quality in particular. In this chapter, mitochondria take center stage due to their key role in the pathogenesis of metabolic disorders and reduced oocyte quality associated with diet induced obesity. Furthermore, the known consequences of maternal OB diet consumption on the health and fertility of the offspring are described, and the factors that mediate the intergenerational effects of maternal obesity are reported. Most importantly, the current gaps in knowledge are highlighted and defined.

In **Chapter 2**, the hypotheses and aims are stated. Briefly, we aimed to investigate the effect of the maternal OB diet, the offspring's OB and the maternal x offspring diet interaction on several outcome parameters related to offspring health and oocyte quality. For that, a 2 x 2 factorial design was designed using outbred Swiss mice. Offspring samples were collected at birth (age 1h), at weaning (age 3wks) and at adulthood (age 10wks).

In the first results chapter, **Chapter 3**, we investigated the effect of the maternal OB diet, the effect of the offspring's OB diet, and the interaction between the effect of both diets on the offspring's health at adult age. Effects on litter characteristics, offspring growth patterns, abdominal fat deposition, metabolic health and insulin sensitivity, as well as on mitochondrial structure and functions in oxidative skeletal muscle of adult offspring are evaluated and reported. We showed that the maternal OB diet did not affect the litter size, offspring sex ratio and offspring weight at birth. Lactation from an obese mothers, and not the prenatal development exposure, appeared to affect offspring weight since the offspring body weight and the abdominal fat weight was increased by the maternal OB diet at weaning (offspring age 3 weeks). However, this effect was absent at adulthood (offspring age 10 weeks), and was associated with a tendency to a reduction in abdominal fat weight. Even though the maternal OB diet coincided with abnormalities in offspring muscle mitochondrial ultrastructure and OXPHOS complex marker expression, the maternal OB diet did not negatively affect the metabolic profile of the offspring. The maternal OB diet even induced some improvements as offspring blood cholesterol levels were reduced and the insulin sensitivity tended to be improved in OB-born offspring. Moreover, offspring born to obese mothers and fed a mismatching normal diet (OB»C) had the most distinct, yet the most optimal metabolic profile during early adulthood, compared to all the other treatment groups. Furthermore, no additive effects (increased sensitivity) of the maternal OB diet to a matching OB offspring diet were observed in offspring born to obese mothers (OB»OB vs C»OB). The NEFA levels in OB»OB were even lower compared to C»OB. These results are in line with the Thrifty Phenotype Hypothesis, suggesting that OB-born offspring are better

adapted towards an environment with a high energy availability later in life. When looking at the effect of the offspring OB diet, we confirmed that offspring OB diet majorly impacted offspring health by impairing the offspring's serum glucose and lipid profiles, associated with abnormal muscle mitochondrial ultrastructure. A significant maternal x offspring diet interaction only influenced the mitochondrial ultrastructure in muscle. Our results differ from previous reports using inbred mice, highlighting the importance of the model when designing and interpreting intergenerational studies. Using a murine outbred Swiss model, maternal OB diets as such do not embark upon female familial obesity in the following generations.

The maternal OB diet did not negatively affect the offspring's metabolic profile, but it was still interesting to determine the quality of the corresponding oocytes. In **Chapter 4**, we investigated the effect of the maternal OB diet, the effect of the offspring's OB diet, and the interaction between the effect of both diets on the offspring's oocyte (mitochondrial) quality at adult age. Here, we evaluated oocyte lipid content and oocyte mitochondrial qualitative and quantitative measures, including mitochondrial bioenergetic activity, ultrastructure and cell metabolism. The maternal OB diet resulted in an increase in offspring's oocyte mitochondrial ultrastructural abnormalities without altering the lipid droplet content, lactate production, MMP, active mitochondrial distribution, mitochondrial DNA copy numbers, or ROS production. This was associated with reduced mitochondrial complex III and V marker expression and reduced pyruvate consumption. The majority of abnormal oocyte mitochondria in OB-born offspring were degenerated, not containing abnormalities linked with mitochondrial dysfunction such as membrane defects or rose-petal shaped mitochondria, suggesting that, at least in this outbred model, defective mitochondria can be removed and replaced without compromising mitochondrial DNA copy numbers. Reduced oocyte pyruvate consumption and lower OXPHOS marker expression without a significant change in MMP, ROS and lactate production indicates that these adaptations may have no, or limited, functional consequences. Contradicting the results of the maternal OB diet, the offspring OB diet increased oocyte lipid droplet content, mitochondrial activity and ROS levels, altered mitochondrial ultrastructure and reduced oocyte pyruvate consumption. Mitochondrial DNA copy numbers and lactate production remained unaffected. Mitochondrial ultrastructure was the only factor where a significant interaction between maternal and offspring diet effect was detected, highlighting the extreme metabolic flexibility of the mitochondria. In this Swiss outbred model, while offspring OB diet had the largest functional impact on oocyte mitochondrial features, the mitochondrial changes due to the maternal OB diet appear to be adaptive or compensatory rather than dysfunctional. These results are in contrast with previous reports using inbred models and highlight marked differences in responses between inbred and outbred mice strains. Therefore, extrapolation of data from inbred mouse models related to oocyte mitochondrial functions should be done with caution.

Small but significant ultrastructural changes were present in the oocytes of OB-born offspring after ovulation. However, it is not known if these effects are also present in the preantral follicle oocytes. Therefore, in **Chapter 5** we investigated the effect of the maternal OB diet, the effect of the offspring's OB diet, and the interaction between the effect of both diets on the offspring's oocyte mitochondrial ultrastructure in primordial and preantral ovarian follicles at adult age. We showed that the maternal OB diet increased the mitochondrial matrix density in the primordial follicle oocytes, suggesting an earlier increase in bioenergetic (OXPHOS) capacity. Interestingly, the maternal OB did not induce oocyte mitochondrial abnormalities and defects such as membrane damage or rose-petal shaped mitochondria in preantral follicles of their offspring, and did not influence the direct effect of an offspring OB diet. In contrast, only offspring OB diet increased the oocyte mitochondrial abnormalities in the primordial follicles, indicative for mitochondrial damage. While further functional investigations are required, the insights provided here strongly suggest that preantral folliculogenesis is a crucial window of sensitivity of

the oocyte for the effects of maternal and offspring OB diets, which may require attention when developing or optimizing reproductive managing protocols for obese patients.

Since the mitochondria within primordial follicle oocytes responded to the effects of maternal and offspring OB diets, we wondered if such effects were also present in OB-born offspring at birth, and if these effects would change after lactation to OB mothers. In **Chapter 6**, we examined the effect of the maternal OB diet on oocyte mitochondrial morphology, biogenesis and mitochondrial dynamics at birth and at weaning, thereby investigating if offspring from OB mothers are born with mitochondrial dysfunction or adaptational mechanisms, and if an additive effect of lactation is involved. We showed that the ultrastructural abnormalities induced by the direct effect of an OB diet are not inborn in outbred Swiss mice. However, primordial follicle oocytes do respond to the effects of a maternal OB diet from the beginning of life, since the expression of TFAM, Drp1 and cytosolic OPA1 was reduced, indicative for reduced mitochondrial transcription and altered mitochondrial dynamics. Pups weaned from OB mothers have more apparent changes in (PGC-1α related) mitochondrial biogenesis and mitochondrial dynamics (Mfn2, cytosolic and nuclear OPA1), potentially due to an additive effect of the lactation window. However, since no signs of increased cellular stress were detected at birth, weaning and at adulthood, the mitochondrial alterations detected in primordial follicle oocytes of young OB-born offspring from birth until weaning, seem to be adaptive or reparable in nature. The primordial follicle oocytes of offspring born to OB mothers at birth and at weaning are not disrupted in their cellular homeostasis (yet), but further investigation is still necessary to determine the functional implications of these changes during subsequent stages of folliculogenesis.

In general discussion chapter, **Chapter 7**, we compile and discuss the results of all the experimental chapters together, and focus on the main observations, described in the following statements:

- the maternal OB diet did not exacerbate the effects of an OB diet on the offspring's health in outbred Swiss mice, but even improved the offspring's metabolic profile despite the accompanied mitochondrial aberrations in oxidative muscle tissue.

- offspring born to OB mothers do not exhibit signs of affected mitochondrial ultrastructure in the primordial follicle oocytes at birth. However, offspring primordial follicle oocytes do respond to the effects of the maternal OB diet from the very beginning of life, but these alterations seem to be adaptive or reparable in nature.

- pups weaned from OB mothers have more apparent changes in oocyte mitochondrial biogenesis and mitochondrial dynamics compared to OB newborns, potentially due to an additive effect of the lactation window.

- the maternal OB diet did not increase oxidative stress or induced mitochondrial dysfunction in the mature (ovulated) oocytes of offspring. Nevertheless, deterioration in oocyte mitochondrial ultrastructure appear to occur during folliculogenesis and at adulthood.

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- Oral presentation at AETE 38th scientific meeting, Utrecht, The Netherlands

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- Oral presentation Student Competition at AETE 40th scientific meeting, Brescia, Italy
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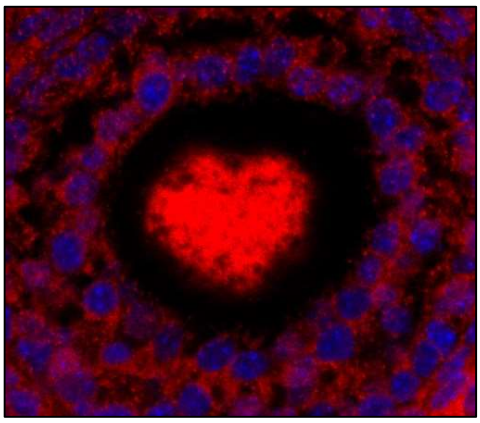

Bedankt!