ABOUT THE COVER

The figure on the cover represents an obese mouse on the left and a non-obese mouse on the right. Obesity is known to have a significant negative impact on fertility, and more specifically, oocyte quality. This is depicted by the red color scheme in the obese mouse. In healthy mice, oocytes are of good quality, as represented by the green color scheme in the right mouse. In this PhD thesis, we investigated the impact of dietary preconception care interventions in obese mice with the aim to result in weight loss, improve metabolic health and oocyte quality, similar to the healthy mouse.

Special thanks goes to my ex-colleague Dr. Jessie De Bie for her big help with the design of this cover. Without your artistic skills, this cover would not exist. With your expertise on the impact of metabolic disorders on oocyte quality, you immediately understood the message I wanted to tell.

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Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Department of Veterinary Sciences

OPPORTUNITIES FOR IMPROVEMENT OF OOCYTE QUALITY IN METABOLICALLY COMPROMISED CONDITIONS:

From fundamental discoveries in the well until the development of preconception care strategies in an obese mouse model

MOGELIJKHEDEN VOOR HET VERBETEREN VAN EICELKWALITEIT IN METABOOL GECOMPROMITTEERDE OMSTANDIGHEDEN:

Van fundamentele ontdekkingen in de schaal tot de ontwikkeling van preconceptie strategieën in een obees muizenmodel.

Dissertation for the degree of Doctor in Veterinary Sciences (PhD) at the University of Antwerp to be defended by

ANOUK SMITS

Supervisors
Prof. Dr. Jo Leroy
Prof. Dr. Diane De Neubourg

Antwerp, 2022

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LIST OF ABBREVIATIONS

ACI	Apoptotic cell index
ALA	Alpha linolenic acid
ALT	Alanine aminotransferase
AO	Antioxidants
ART	Assisted reproductive technology
AST	Aspartate aminotransferase
ATP	Adenosin triphosphate
AUC	Area under the curve
BMI	Body mass index
BSA	Bovine serum albumin
B6	C57BL/6
CC	Cumulus cell
COC	Cumulus oocyte complex
CR	Caloric restriction
CRP	C-reactive protein
CTRL	Control
DGA	Dietary guidelines for Americans
DIO	Diet-induced obesity
DRP	Differentially regulated proteins
eCG	Equine chorionic gonadotropin
ER	Endoplasmic reticulum
ER	Elimination rate
EV	Extracellular vesicles
FA	Fatty acid
FAO	Fatty acid β-oxidation
FBS	Fetal bovine serum
FF	Follicular fluid
FFA	Free fatty acids
FSH	Follicle stimulating hormone
GLUT	Glucose transporter
GnRH	Gonadotropin releasing hormone
GO	Gene ontology
GSH	Glutathione
GTT	Glucose tolerance test
GPx	Glutathione peroxidase
HBP	Hexosamine biosynthetic pathway
hCG	Human chorionic gonadotropin
HDL	High-density lipoprotein
HF	High fat
HFD	High fat diet

HF/HS	High fat/high sugar
HSL	Hormone sensitive lipase
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IL	Interleukin
IR	Insulin receptor
ITS	Insulin-transferrin-selenium
ITT	Insulin tolerance test
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
LD	Lipid droplet
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LPL	Lipoprotein lipase
MMP	Mitochondrial membrane potential
mtDNA	Mitochondrial DNA
NAFLD	Non-alcoholic fatty liver disease
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
OA	Oleic acid
PA	Palmitic acid
PBS	Phosphate buffered saline
PCCI	Preconception care intervention
PCOS	Polycystic ovarian syndrome
PFA	Paraformaldehyde
p.i.	Post insemination
PPP	Pentose phosphate pathway
PUFA	Poly-unsaturated fatty acids
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RCT	Randomized controlled trials
ROS	Reactive oxygen species
SA	Stearic acid
SGLT	Sodium-glucose linked transporter
SHBG	Sex hormone binding globulin
SOF	Synthetic oviductal fluid
TCA	Tricarboxylic acid
TE	Trophectoderm
TEM	Transmission electron microscopy

TG	Triglycerides
TNF	Tumor necrosis factor
UPR	Unfolded protein response
VLDL	Very-low density lipoproteins
VLED	Very low energy diet
WHO	World Health Organisation
WHR	Waist-hip ratio

CHAPTER 1: STATE OF THE ART

CONTENT

STATE OF THE ART: THE IMPACT OF OBESITY ON METABOLIC HEALTH AND FERTILITY

1. THE IMPACT OF OBESITY: FACTS AND FIGURES

The prevalence of obesity has increased worldwide in the past 50 years, reaching pandemic levels. The World Health Organisation (WHO) defines obesity as excessive body fat accumulation that might impair health and is diagnosed at a BMI ≥30 kg/m^2. In 2016, the WHO estimated the worldwide prevalence of adult obesity at 13% (WHO, 2018). In Belgium, 49.3% of the adult population is overweight (BMI ≥25), 15.9% is obese (BMI ≥30) (Sciensano). In addition to BMI, waist circumference, a more relevant method to measure abdominal adiposity, has become an increasingly important tool for classifying obesity (Hu, 2007). Cut-off points for waist circumference were set at 80 and 94 cm for women and men, respectively (Alberti et al., 2007). It is considered a more reliable predictor for metabolic disorders than BMI. Recently, abdominal obesity is defined as a waist-to-hip ratio (WHR) above 0.90 for men and above 0.85 for women (World Health Organisation, 2008).

Obesity represents a major health challenge because it substantially increases the risk of metabolic diseases such as type 2 diabetes mellitus and fatty liver disease. Furthermore, it increases the risk of cardiovascular diseases, musculoskeletal disease (osteoarthritis), Alzheimer disease, depression and some types of cancer, thereby contributing to a decline in both quality of life and life expectancy (Bluher, 2019). 2.8 million people die every year from complications associated with overweight, in particular diabetes and cardiovascular diseases (WHO). More importantly, the World Obesity Federation has declared obesity a chronic progressive disease clearly distinct from being just a risk factor for other diseases (Bray et al., 2017).

Obesity is also associated with unemployment, social inequality and reduced socio-economic productivity, thus creating an economic burden. In Belgium, the costs resulting from overweight and obesity vary between 0.7% and 8% of annual healthcare expenditure (Sciensano). In 2021, it was estimated that every year at least €4.5 billion are spent to cover the direct and indirect costs related to overweight and obesity (Gorasso V. et al, Health care costs and lost productivity costs related to excess weight in Belgium 2021, In preprint). Based on a decision-analytic model, a one unit BMI-reduction in the total overweight and obese Belgian population was estimated to result in a (direct and indirect) societal cost saving of €2.8 billion (Verhaeghe et al., 2016).

The fundamental cause of obesity is a long-term energy imbalance between too many calories consumed and too few calories expended. Obesity is the result of the interplay between heterogenic factors linked to a person's eating behaviour, physical activity and individual energy expenditure determinants. There is wide consensus that changes in the global food system (Roberto et al., 2015; Swinburn et al., 2011) combined with sedentary behaviour (Hu et al., 2003; Rissanen et al., 1991) seem to be the main causes

of the worldwide rise in obesity prevalence over the past 50 years. In addition, hormonal, metabolic and psychological factors also clearly have a role in the pathophysiology of obesity (Hebebrand et al., 2017). Under this main assumption, the UK Foresight Programme 'Tackling Obesities' project identified seven main clusters that play an important role in the development of obesity for an individual or a group (summarized by (Science, 2007); Figure 1). These clusters include the following: physiology, individual psychology, individual physical activity, food consumption, food production, social psychology and physical activity environment.

Energy expenditure due to physical activity has decreased significantly over the past years as a consequence of fewer manual jobs, increased sedentary employment, increased TV viewing and longer work hours, resulting in limited opportunities to exercise. Nowadays, in modern and developed societies, there is a psychological conflict between what people want (e.g. fatty, sweet foods) and their desire to be healthy and/or slim. In addition, social and cultural factors are influenced by family dynamics, school/work policies and the impact of the media. Furthermore, access to (highly processed) food (so-called fast food) and drinks from supermarkets but especially from takeaways and restaurants is significantly increasing. High quality, healthy food is often more expensive, resulting in limited access for low-income families.

The major dietary determinants linked to obesity include high energy dense food, diets high in **fat** and the consumption of sugar-rich drinks. Fast food is high in saturated fat, fructose, glycemic index and very energy dense (Guthrie et al., 2002).The worldwide consumption of **sugar**-sweetened beverages has increased in parallel with the obesity pandemic (Malik et al., 2010; Schulze et al., 2004). In Western diets, added sugars account for about 14% to 17% of the total caloric intake, which is above the recommended level of 10% of the total caloric intake, according to the WHO guidelines. Consumption of fructose and other sugar-sweetened beverages has been associated with insulin resistance, intrahepatic lipid accumulation, and hypertriglyceridemia. In the long term, these risk factors may contribute to the development of type 2 diabetes and cardiovascular diseases (Taskinen et al., 2019). Dietary fat is a strong predictor of weight gain (Sherwood et al., 2000). Increases in fast food intake were associated with increases in BMI and development of insulin resistance (Pereira et al., 2005). It is currently recommended that dietary fat has to be reduced, from the present 35–45% of the total energy content in most Western diets, to below 30% (2001; Health., 2000). In addition to the amount of dietary fat, the **composition of the fatty acids** in the diet also plays an important role in the development of obesity. A typical fast food meal consists of 85% of the recommended daily fat intake and 73% of the recommended saturated fat intake. It has been shown in animal models that a diet high in saturated fat has a more pronounced effect on increase in body fat and impaired insulin sensitivity than a poly-unsaturated (PUFA) rich diet (Botchlett et al., 2016; Botchlett and Wu, 2018). Early human studies already reported a positive correlation between saturated fat intake and BMI (Colditz et al., 1992). Another study observed a significant

increase in waist circumference in men in the upper quartile of saturated fat intake, whereas high intakes of PUFA had no effect on adiposity (Doucet et al., 1998). When people with metabolic syndrome were given a daily dose of 1 gram PUFA's (omega-3 fatty acids) for six months, a significant reduction in body weight and improved serum lipid profile was present (Ebrahimi et al., 2009). Increasing the intake of unsaturated fatty acids at the expense of saturated fatty acids, proteins, and carbohydrates showed beneficial effects on body weight and obesity (Beulen et al., 2018).

Currently, the Dietary Guidelines for Americans (DGA) suggests higher intake of unsaturated compared with saturated fats. Specifically, adults should consume less than 10% of daily calories from saturated fat, and ingest a variety of unsaturated fats, including mono- and polyunsaturated fatty acids. The evidence so far suggests that adopting a Mediterranean diet may prevent type 2 diabetes (Esposito and Giugliano, 2014).

Moreover, observations from twin and adoption studies (Borjeson, 1976; Stunkard et al., 1990) suggested that obesity might be an inherited disorder of energy homeostasis. The heritability of BMI has been estimated as 40–70% (Borjeson, 1976; Stunkard et al., 1990). However, obesity-associated genetic variants explain only a small proportion of BMI variance (Hebebrand et al., 2010; Speliotes et al., 2010) and changes in population genetics cannot explain the rise of obesity prevalence in just 40 years.

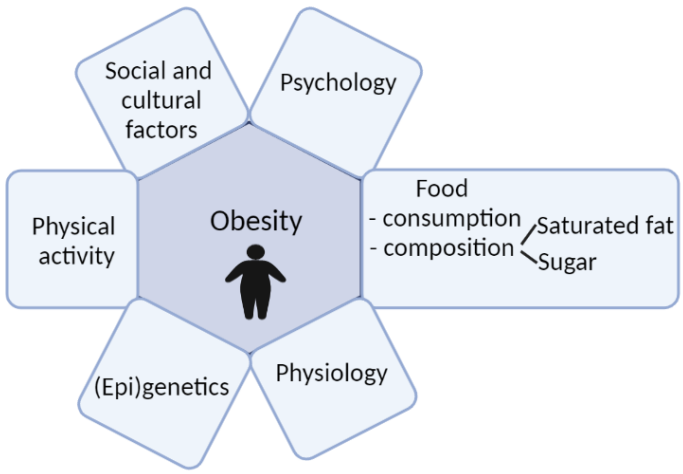

Figure 1. Main clusters that play an important role in the development of obesity (Made with Biorender)

The **Thrifty phenotype hypothesis** proposes that the epidemiological associations between poor prenatal environment and a subsequent rich postnatal environment results in permanent changes in glucose-insulin metabolism, developing type 2 diabetes and the metabolic syndrome as a result (Hales and Barker, 2001). The adaptations to the poor nutritional environment are regulated through the integration

of epigenetic and cellular mechanisms (Fleming et al., 2015). Environmental factors can result in alterations in the critical balance of epigenetic regulation and have an impact on specific target genes and/or at the whole genome level.
One of the most known studies to confirm this hypothesis is the Dutch Hunger Winter (1944-1945). Due to a food embargo, sudden famine emerged in a part of The Netherlands. As a result, embryos or foetuses were exposed to malnutrition during the first or the second and third trimester of pregnancy, respectively. However, this severe food deprivation only lasted for seven months and the babies born grew up in an environment with normal food availability. Findings showed that maternal malnutrition during pregnancy had important effects on offspring health (Roseboom et al., 2006; Roseboom et al., 2000). Interestingly, the effects on metabolic health were dependent of the timing of undernutrition during gestation, as especially the early gestation showed to be a vulnerable period. Individuals exposed to famine during this timeframe were more likely to become obese, whereas those exposed as a foetus during late gestation were less likely to become obese (Ravelli et al., 1976). In addition, exposure to famine during the early stage of gestation was also associated with glucose intolerance, an increased risk of coronary heart disease, a more atherogenic lipid profile, disturbed blood coagulation and increased stress responsiveness (Roseboom et al., 2006).
Even though the Dutch famine was almost 80 years ago, the Thrifty phenotype hypothesis is still relevant in our present society and especially in developing countries. Due to increased economic growth and welfare in these countries, foetuses that adapted in utero to a life with food shortages, are less able to cope with high-calorie diets later in life and display aberrant metabolic health during adulthood (Prentice and Moore, 2005).

The WHO 'Global Action Plan for the Prevention and Control of Noncommunicable Diseases 2013–2020' defines strategies that should be implemented to prevent a further increase in the worldwide prevalence of obesity. So far, progress in tackling obesity has been too slow and chances are slim that the WHO goals will be achieved within the near future. Intervention strategies to treat and prevent obesity, both at the individual and population level, have so far only limited effectiveness. On the positive side, **knowledge regarding the main causes of obesity and their modulating factors has increased significantly. The challenge remains to translate this information into effective actions.** It was suggested that reducing the obesity burden requires approaches that combine individual interventions with changes in the environment and society (Bluher, 2019).

2. IMPACT OF OBESITY ON METABOLIC HEALTH

It is well known that obesity and overweight are linked with an increased prevalence of metabolic complications. The primary metabolic complications associated with obesity are clustered under the term **metabolic syndrome**, also known as insulin resistance syndrome or syndrome X (Grundy, 2000; Saklayen, 2018). Several definitions exist but metabolic syndrome is most often defined as a cluster of several metabolic abnormalities including abdominal adipositas, hyperglycemia, hypertriglyceridemia, low high-density lipoprotein (HDL) cholesterol and hypertension (Alshehri, 2010). In addition, increased free fatty acid (FFA) and cholesterol concentrations and hypertriglyceridemia are common alterations in serum lipid parameters associated with human obesity (Klop et al., 2013). It also involves an imbalance of low-density lipoprotein (LDL) cholesterol and HDL cholesterol.

2.1. DYSLIPIDAEMIA

Triglycerides (TG), resulting from dietary intake, are digested in the intestinal lumen to free fatty acids which are taken up by the enterocytes while cholesterol is taken up via a specific cholesterol transporter. Once in the enterocyte, cholesterol is transformed into cholesterol-esters. Triglycerides are re-synthesized and packaged together with cholesterol-esters in chylomicrons (Hussain, 2014). In addition, very-low density lipoproteins (VLDL) are produced in the liver (Klop et al., 2012). VLDL increases postprandially when TG and FFA, derived from the food, reach the liver. These chylomicrons and VLDL reach the blood stream where they interact with lipoprotein lipase (LPL). LPL is the primary enzyme for TG lipolysis in the circulation and catalyses the release of FFA which diffuse across the endothelial cells and resynthesize in triglycerides in adipose, mammary, muscle and other cells. The activity of LPL is stimulated by insulin which increases postprandially (Karpe et al., 2011; McQuaid et al., 2011). This postprandial increase results in the inhibition of hormone sensitive lipase (HSL), a key enzyme for hydrolysis of intracellular lipids. During the fasting state, FFA mobilization from the adipose tissue takes place, which is also regulated by insulin (Karpe et al., 2011).

During the process of lipolysis, chylomicrons and VLDL shrink to form chylomicron remnants and dense **LDL**, respectively. The liver takes up the chylomicron remnants and breaks them down to FFA, cholesterol, glycerol and amino acids. LDL is primarily taken up by the liver via the LDL receptor (Goldstein and Brown, 2009; Lambert et al., 2012). The liver also plays an important role in the synthesis of **HDL** particles. HDL promotes the uptake of cholesterol from peripheral tissues and returns cholesterol to the liver.

Lipid metabolism is highly dynamic and depends on numerous factors like the postprandial state, energy expenditure, insulin levels and sensitivity and adipose tissue function (Klop et al., 2013).

The typical dyslipidaemia of obesity consists of increased TG and FFA, decreased HDL-C with HDL dysfunction and normal or slightly increased LDL-C with increased small dense LDL.

The role of dyslipidaemia in obesity has been thoroughly reviewed by Klop et al. (2013). In obesity, lipolysis of TG-rich lipoproteins is impaired due to reduced LPL expression levels or activity in adipose tissue and skeletal muscle, respectively, or due to competition for lipolysis between VLDL and chylomicrons (Klop et al., 2012). The presence of excess lipids in the blood leads to elevated levels of FFA, resulting in activation of LPL (Karpe et al., 1992; Peterson et al., 1990). LPL might remain attached to VLDL, resulting in further depletion of TG. The exchange of TG from these remnants for cholesterol-esters from HDL ultimately results in the formation of small, dense LDL particles (Capell et al., 1996; Hokanson et al., 1995). In the presence of high TG, the cholesterol-ester content of LDL decreases while the TG content of LDL increases. However, this increased TG content is hydrolysed by hepatic lipase, resulting in dense LDL particles.
HDL metabolism is also affected by obesity due to increased number of chylomicron remnants and VLDL together with impaired lipolysis. This will ultimately result in lower HDL-cholesterol levels and reduction of circulating HDL particles (Deeb et al., 2003).

Adipose tissue plays an important role in metabolic disorders. In normal situations, hormone sensitive lipase (HSL) moves from the cytosol to the surface of lipid droplets and interacts with perilipin-1 and neutral lipids. Triglycerides are then hydrolysed into glycerol and non-esterified fatty acids (NEFAs). In the blood, NEFAs will bind to albumin to facilitate their transport to different target cells (Bolsoni-Lopes and Alonso-Vale, 2015). Obesity is characterized by an ineffective inhibition of hormone sensitive lipase (HSL)-mediated lipolysis in adipose tissue (Lewis et al., 1993).
In obese individuals, high levels of circulating NEFAs originate from adipocytes undergoing uncontrolled lipolysis but also from the diet. Under conditions of increased caloric intake, adipocytes store more TGs, but as adipocytes grow, their capacity to limit lipolysis in response to insulin drops. When lipolysis increases, the production of adipokines by adipose tissue increases significantly (Arner, 2005). Adipokines have a big impact on the metabolism of tissues and organs, especially liver, muscle and pancreas.
Adipocyte hypertrophy results in infiltration of adipose tissue with macrophages and T-helper cells. These macrophages secrete high amounts of adipokines that affect normal metabolism. They display pro-inflammatory characteristics and synthesize high levels of pro-inflammatory cytokines (TNF-alpha, IL-6, IL1β) (Snider and Wood, 2019). TNF-alpha alters the normal insulin signalling pathway, stimulates lipid breakdown by altering the action of perilipin, decreases the insulin receptor substrate-1 activity and its substrate phosphorylation and decreases the glucose transporter GLUT4 synthesis and membrane translocation in the adipose tissue. Overall, this further increases the

plasma NEFA concentration, leading to increased lipid deposition in skeletal muscle, liver, pancreas and heart (Bergman and Ader, 2000; Wajchenberg, 2000). This promotes insulin resistance and is referred to as **lipotoxicity**. High NEFA concentrations are able to disturb cell physiology by affecting energy uptake and processing pathways, inducing oxidative stress and ... (see further).

Growing insulin resistance and increased body fat mass upregulate the expression of TNF-alpha resulting in reduced adiponectin levels, an anti-inflammatory cytokine (Mihalopoulos et al., 2020). In vitro studies have shown that TNF-alpha downregulates expression of the adiponectin gene via suppression of adiponectin-induced nuclear factor NFκβ (Moradi et al., 2020). Vice versa, adiponectin has an anti-inflammatory effect, supports insulin sensitivity and is able to suppress the production of proinflammatory cytokines TNF-alpha and IL6 (Kumada et al., 2004). Obese individuals secrete less adiponectin than lean individuals and weight loss restores adiponectin to normal levels (Goldfine and Kahn, 2003; Yang et al., 2001).

Another important adipokine is leptin, a hormone involved in hunger and satiety signals (Ge et al., 2020). Leptin, released into circulating blood, is transported to the brain and bound to its receptors in the hypothalamus (Ge et al., 2020; Guan et al., 1998). This results in decreased appetite and reduced food intake with subsequent body fat reduction and increased energy expenditure, which finally leads to body mass decrease (Ge et al., 2020). In obese individuals, high leptin concentrations are associated with accompanying leptin resistance in the hypothalamus, resulting in a deficient appetite control (Pandey et al., 2015).

Lipolysis is not only relevant in obesity and in the case of high dietary (fat) intake but has also been associated with weight loss or long-term fasting. Due to reduced food availability, the lipolysis is upregulated and the rate of re-esterification of NEFAs in the adipose tissue is downregulated (Vernon, 2005). In this way, extreme weight loss can also lead to lipotoxicity resulting in altered insulin sensitivity and disturbed cell physiology.

2.2. REDUCED INSULIN SENSITIVITY

In normal, healthy situations, insulin lowers blood glucose concentrations by suppressing hepatic glucose production and increased glucose uptake in muscle and fat (Kahn and Flier, 2000).

The term **insulin resistance** is defined as the resistance of an insulin dependent cell to the effects of insulin on glucose uptake, metabolism or storage. Insulin resistance in obesity is manifested by decreased insulin-stimulated glucose uptake and metabolism in adipocytes and skeletal muscle, and by impaired suppression of hepatic glucose output (Kahn and Flier, 2000). It is a key feature of type 2 diabetes, obesity, and metabolic syndrome.

The release of NEFAs has been defined as the single most crucial factor in modulating insulin sensitivity (Kahn and Flier, 2000). Other factors that are known to participate in insulin resistance are hormones, proinflammatory cytokines and reactive oxygen species (ROS), all of which are present in obese conditions (Houstis et al., 2006; Shoelson et al., 2006).

Several risk factors play an important role in the development of insulin resistance (Figure 2). One of the major components are **lifestyle factors**: 1) **diet composition, caloric excess**; and 2) reduced **physical activity** due to an increase in sedentary lifestyle (Figure 2). The combination of both a fat load and a glycemic load appears to exaggerate the insulin response and promote weight gain (Harris et al., 2003). In a human study, increases in dietary saturated fatty acids were associated with increases in fasting insulin and insulin secretion after a glucose tolerance test (Isganaitis and Lustig, 2005). In rats, glucose-stimulated insulin secretion is altered by fat intake and the composition of fat in the diet (Dobbins et al., 2002). The intake of high glycemic food results in increased postprandial serum glucose concentrations leading to high β-cell insulin secretion. Although the increased insulin response leads to high clearance of high-glycemic-index meals, the insulin surge continues to exert metabolic effects. Diets with a high glycemic index may impair long-term β-cell function via different mechanisms: glucotoxicity, lipotoxicity and overstimulation (Ludwig, 2002). In rats, food with a high glycemic index has shown to accelerate fat deposition and lead to obesity (Ludwig, 2002).
In relation to that, abdominal adiposity, increased body fat and overall obesity are known factors for insulin resistance (Gokulakrishnan et al., 2011; Racette et al., 2006) due to high amount of FFA and pro-inflammatory cytokines released from the fat tissue, as stated above (Rytka et al., 2011). This also emphasizes the role of **inflammation** due to pro-inflammatory cytokines like TNF-alpha in the development of insulin resistance (Figure 2) (Jin et al., 2014; Zorena et al., 2020).
Interestingly, a change in diet has shown to be effective in improving insulin resistance (Mason et al., 2011), showing the importance of dietary factors in the development of insulin resistance. A study conducted in overweight and obese (middle aged) woman showed that a diet change was able to reduce insulin resistance by 24% (Mason et al., 2011). Diet combined with physical activity reduced insulin resistance by 26%. Furthermore, poly-unsaturated fatty acids (PUFA's) are inversely correlated with insulin resistance (Haugaard et al., 2006).
Besides diet and exercise, the **genetic component** also plays an important role in the development of insulin resistance and/or type 2 diabetes. Insulin resistance patients are often associated with family history of type 2 diabetes. Although single gene mutations (like insulin receptor and PPAR-γ) may cause rare forms of insulin resistance, the more common forms of insulin resistance are expected to be polygenic and heterogenous (Mercado et al., 2002). In addition, the role of certain **hormones** (like leptin and resistin) cannot be ignored as studies show that leptin deficiency/ reduced

leptin activity is linked with obesity and insulin resistance (Elmquist et al., 1998). Furthermore, some **medical conditions**, like polycystic ovarian syndrome (PCOS) and non-alcoholic fatty liver disease (NAFLD), are also associated with insulin resistance (Nafiye et al., 2010).

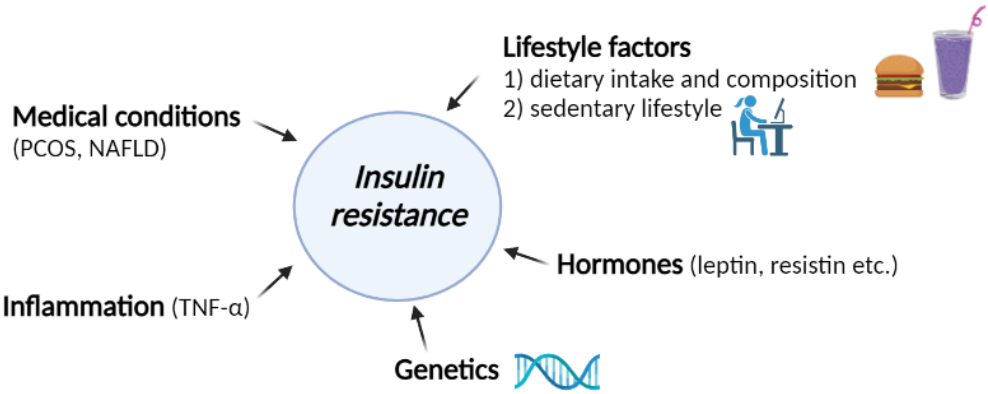

Figure 2. Risk factors involved in the development of insulin resistance (Made with Biorender)

The core pathophysiological defects associated with insulin resistance are represented by three major organs, the so-called triumvirate: muscle, liver and pancreas (Defronzo, 2009).

In metabolically compromised, overweight and obese individuals, the insulin resistant state will have a big impact on the **liver** and the action of insulin in this organ will be impaired. As a consequence, insulin fails to inhibit gluconeogenesis and to reduce the hepatic glucose output (Defronzo, 2009). In addition, there would be a failure to promote glycogen synthesis; to suppress fatty acid oxidation and ketogenesis. As mentioned above, obesity is characterised by increased FFA flux to the liver, resulting in hepatic accumulation of TG. However, the storage of fat in liver cells can become toxic and ultimately lead to non-alcoholic fatty liver disease and steatohepatitis in obese patients. This has been supported by the observation that many patients with obesity display abnormal liver function tests (raised concentrations of transaminases) (Brolin et al., 1998).

Next to the liver, the **β-cells** of the pancreas also play an important role in insulin resistance and diabetes type 2. In non-insulin resistant states, the β-cell is able to release insulin via a controlled mechanism to maintain normal glucose tolerance. Under obese, non-diabetic conditions, the β-cells are able to increase the insulin release sufficiently to overcome the reduced efficiency of insulin action, thereby maintaining normal glucose tolerance but resulting in hyperinsulinemia (Kahn et al., 1993; Perley and Kipnis, 1966; Polonsky et al., 1988). However, when sensitivity to insulin further drops, the β-cells are not able to compensate anymore and their function reduces significantly. When extreme β-cell dysfunction is present, impaired glucose tolerance and at the extreme, type 2 diabetes results. When patients are diagnosed with type 2

diabetes, the β-cells are only operating at 25% or less of their capacity (Roder et al., 1998). Interventions such as weight loss and medication such as thiazolidinedione that mobilize fat out of the β-cell are expected to reverse lipotoxicity and restore or preserve remaining β-cell function.

Increased lipid disposition in the **muscle** reduces the translocation of GLUT4 to the plasma membrane, resulting in the development of insulin resistance and finally diabetes type 2 (DeFronzo and Tripathy, 2009). Furthermore, increased lipid content in the muscle causes mitochondrial dysfunction, impaired glucose metabolism and decreased ATP synthesis (Brehm et al., 2006; Perseghin et al., 1999; Szendroedi and Roden, 2008). In humans, the triglyceride content of the muscle and the fatty acid composition of muscle phospholipids correlates directly with reduced insulin sensitivity (Borkman et al., 1993). It was demonstrated that muscle insulin resistance accounts for over 85–90% of the impairment in total body glucose disposal in type 2 diabetic subjects (DeFronzo et al., 1985; Pendergrass et al., 2007).

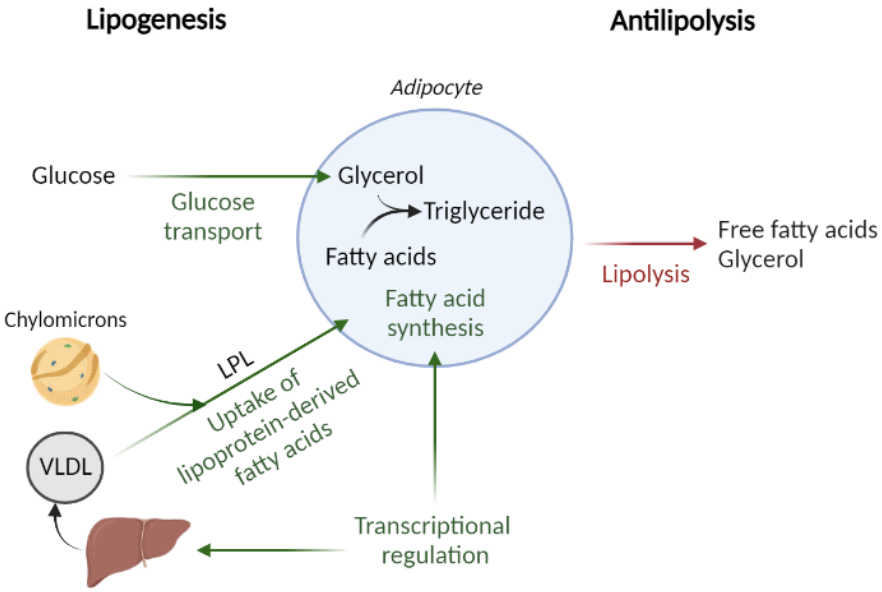

Figure 3. Effects of insulin to promote adipose storage (adapted from Kahn and Flier 2000)

Over the past years, the triumvirate has been expanded to the "Ominous Octet", adding five additional players, involved in the pathogenesis of type 2 diabetes (Defronzo, 2009). These players consist of the fat cell, gastrointestinal tissue, α-cells of the pancreas, kidney and brains (for a detailed review: see (Defronzo, 2009)).
Insulin normally promotes triglyceride storage in the **adipocytes** by different mechanisms. It both stimulates lipogenesis as well as inhibits lipolysis (reviewed by Kahn and Flier (2000); Figure 3). Lipogenesis is promoted by stimulating glucose uptake

and uptake of fatty acids derived from circulating lipoproteins by promoting LPL (as earlier described). In addition, insulin also regulates genes involved in promoting fatty acid synthesis and lipogenesis. The PI3-kinase signalling pathway plays an important role in the regulation of these pathways.
There are two main intracellular insulin pathways leading to metabolic or mitogenic actions through PI3K-Akt/PKB or MAPK, respectively (Figure 4). Akt/PKB protein kinases control actions involved in lipid-, protein- or glycogen synthesis, cell survival and proliferation, glucose transport and receptor endocytosis. MAPK regulates mitogenic functions: gene expression, transcription factor activation, growth and differentiation, intracellular vesicle transport and actin organization (reviewed by Denise Laskowski (Laskowski, 2017)). Insulin action is mediated through its binding to both the insulin receptor and the insulin-like growth factor (IGF)-1 receptor.

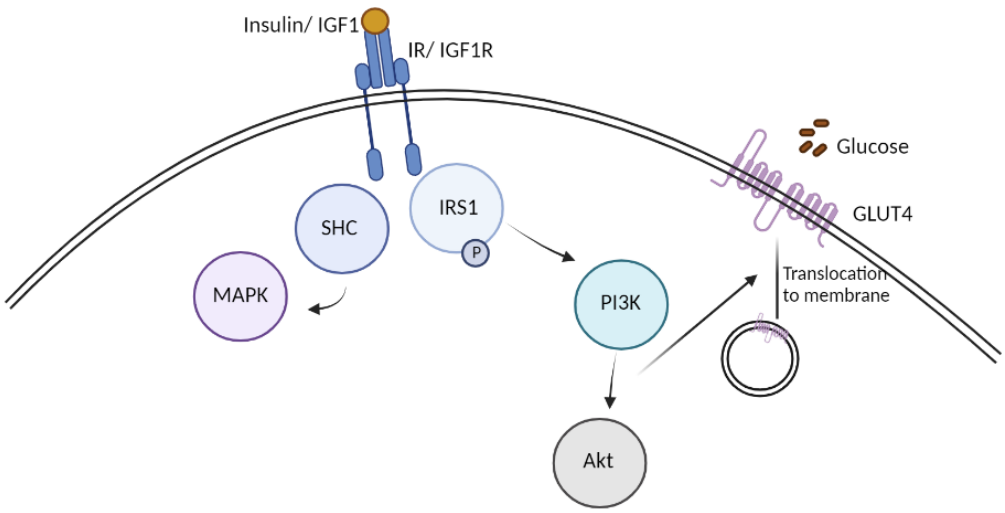

Figure 4. Insulin signalling pathways (made with Biorender)

When sensitivity to insulin is significantly reduced, **fat cells** are characterized by a deranged adipocyte metabolism and altered fat topography. Next to the lipid overflow to the muscle, liver and pancreatic β-cells, as discussed above, 1) Fat cells are resistant to the anti-lipolytic effect of insulin, ultimately resulting in uncontrolled lipolysis and elevated plasma FFA concentrations. 2) These chronically increased FFA levels stimulate gluconeogenesis, induce hepatic/muscle insulin resistance and impair insulin secretion. 3) Excessive production of adipokines that increase insulin resistance and decreased production of insulin-sensitizing adipokines (Defronzo, 2009).

The **gastro-intestinal tissue** is characterized by an incretin deficiency or resistance, a group of metabolic hormones that normally stimulate a decrease in blood glucose levels. They are released after a meal and stimulate the secretion of insulin from the pancreatic β-cells. The **α-cells of the pancreas** play an important role for the

production of glucagon. Research in diabetic individuals showed elevated rates of hepatic glucose production, correlating closely with an increase in fasting glucagon concentrations. Furthermore, the kidney of healthy individuals reabsorbs all of its filtered glucose by its SGLT1 and SGLT2 transporters in the proximal tubule**,** resulting in no glucose in the urine. However, in diabetic individuals, the maximal renal tubular re-absorptive capacity for glucose is increased. Instead of releasing glucose in the urine to correct the present hyperglycemia, the kidney holds on to it. This is alarming as the response of the **kidney** to meet the energy demands of the body, especially the brain, becomes maladaptive in these individuals. Last, but not least, the **brain**, and more importantly the hypothalamic regions, also play an important role in the obesity pathogenesis as it displays an abnormal regulation of appetite and metabolism.

We can conclude that current knowledge clearly indicates a significant impact of obesity(-associated metabolic disorders) on metabolic health.

3. IMPACT OF OBESITY ON FEMALE FERTILITY

3.1. FACTS AND FIGURES ABOUT SUBFERTILITY AND METABOLIC DISORDERS

Next to a significant impact on metabolic health, reproductive physiology is also impaired in obese and overweight (future) mothers. This has been reported in many epidemiological studies (Gesink Law et al., 2007; Loy et al., 2018; Wise et al., 2010).

Hippocrates already wrote in his Essay on the Scynthians that 'People of such constitution cannot be prolific… fatness and flabbiness are to blame. The womb is unable to receive the semen and they menstruate infrequently and little' (J. Chadwick, 1983).

Infertility is defined as the absolute inability to conceive a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009) and it affects one in seven couples (Evers, 2002; Talmor and Dunphy, 2015). Female factors account for 23% of subfertility aetiologies and the risk of subfertility is threefold higher in obese women compared to non-obese women (Evers, 2002; Talmor and Dunphy, 2015). This is true for all subgroups of women, including those with regular cycles, suggesting the impact of obesity is independent of ovulatory status (Gesink Law et al., 2007). Interestingly, this reduction in fertility is particularly associated with high waist circumference and increased WHR (Gesink Law et al., 2007; Wise et al., 2010). A 0.1 unit increase in WHR led to a 30% decrease in probability of conception per assisted reproductive technology (ART) cycle (hazard ratio 0.706; 95% CI 0.562-0.887) (Zaadstra et al., 1993).

Obese women account for a larger percentage seeking medical help to become pregnant compared to normal weight women (Vahratian and Smith, 2009). In Belgium, the majority of the costs associated with an assisted reproductive technology cycle are covered by the Federal Government (up to 6 cycles). Only recently, the average assisted reproduction cost of a life birth (singleton) in Belgium was estimated at 12.000 euro (Peeraer et al., 2017). This figure does not reflect all the extra costs associated with the higher number of in vitro fertilization (IVF) cycles needed to achieve pregnancy, as pregnancy and obstetrical issues are seen much more frequently in obese mothers. Furthermore, these studies never took into account the important domino effects into the next generations. Indeed, there is now also overwhelming scientific evidence showing that this maternal obese condition directly impacts on the health of the offspring. Few recent studies demonstrated that subfertility in the offspring may be programmed by the maternal metabolic condition at its conception (Aiken et al., 2016; Rodriguez-Gonzalez et al., 2015). All this will lead to exponentially increasing health care costs in the future. Next to a financial burden, it is important to also acknowledge the impact of sub-/infertility on the mental well-being of the couple who is desperate to conceive.

Reproductive failure in obese women is a complex health problem and cannot be restricted to a single aspect but is manifested at several levels. Anovulation, menstrual irregularities, reduced conception rate, longer times to conception, increased miscarriage rates, increased risk of pregnancy complications, fetal abnormalities and dystocia are all fertility consequences of a disturbed metabolic state (Robker, 2008; Zain and Norman, 2008).

Overweight and obese women have a higher incidence of **menstrual dysfunction** and anovulation. Menstrual disturbances were found to be fourfold more common in obese women (Rogers and Mitchell, 1952). Several human studies have shown an association between anovulatory infertility and obesity (Bolumar et al., 2000; Green et al., 1988; Jensen et al., 1999). Hartz et al. (1979) reported that women with anovulatory cycles longer than 36 days and hirsutism were at least 13.6 kg heavier than women with no menstrual abnormalities. Best and Bhattacharya (2015) concluded that this effect is dose dependent: greater degrees of obesity over longer periods tend to be associated with worse clinical outcomes.

The duration required to achieve a spontaneous **pregnancy rate** is increased and pregnancy rates are decreased in obese women, including regular ovulatory obese women (Gesink Law et al., 2007; Wise et al., 2010). van der Steeg et al. (2008) followed a large cohort of subfertile couples across different ART centres and found that a linear decline in the probability of spontaneous pregnancy occurred above a BMI of 29. Each unit increase in BMI was associated with a decrease of 4%–5% pregnancy chance (van der Steeg et al., 2008). Shah et al. and Bellver et al. reported that obese women undergoing ART showed lower pregnancy and live birth rates. Interestingly, both pregnancy and live birth rates were reduced progressively with each unit of BMI with a significant association of 0.984 and 0.981, respectively. In addition, the cumulative pregnancy rate after four IVF cycles was reduced as BMI increased (Bellver et al., 2010).

Although an increase in the prevalence of **miscarriage** in case of obesity has been reported several times (Bellver et al., 2003; Bussen et al., 1999; Hamilton-Fairley et al., 1992; Lashen et al., 2004), not all studies confirm this (Lashen et al., 1999; Loveland et al., 2001). Bellver et al. (2003) found a rise in the incidence of spontaneous miscarriage with increasing BMI in patients undergoing ART. Miscarriage rates were 38.1% in obese women, whereas this rate was only 13.3% in patients with a normal BMI. Furthermore, Metwally et al. (2007b) also found a higher risk of early, late, and recurrent miscarriage in obese women.

In addition, success rates of **assisted reproductive technologies,** such as in vitro fertilization and intracytoplasmic sperm injection, are decreased in obese women compared to women with a normal BMI (Dokras et al., 2006; Luke et al., 2011). It has

been reported that the likelihood of having a live birth following the first ART cycle is reduced with 68% in obese women compared to non-obese women (Sim et al., 2014).

The mechanisms of how obesity affects the reproductive function are complex and multifactorial.

3.2. THE ENDOCRINE LINK BETWEEN OBESITY AND SUBFERTILITY

Metabolic disorders have a significant impact on the **hypothalamic-pituitary-ovarian axis**. The hypothalamus is the so-called command centre where the mechanisms controlling metabolic state (somatotropic axis) and reproductive function (gonadotropic axis) collide (Donato et al., 2011). Gonadotropin-releasing hormone (GnRH) is produced by the hypothalamus, triggering the production of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, which in turn regulates oestradiol and progesterone production in the ovary. Modulation in the axis, as a result of an aberrant metabolic state, may affect reproductive processes such as follicular development and ovulation which may ultimately result in a decreased fertility (Jain et al., 2007; Schneider, 2004; van der Steeg et al., 2008). Lower GnRH, LH and FSH concentrations have been observed in obese women, potentially leading to oligo- or anovulation, aberrant follicle recruitment and poor oocyte quality (for review, see (Michalakis et al., 2013)).

Metabolic disorders are also associated with abnormalities in the **sex steroid balance**. Overall, the major effect of obesity on steroid hormones is hyperandrogenism secondary to hyperinsulinemia (Metwally et al., 2007a).

The **adipose tissue** is an important site for steroid production and metabolism, converts androgens to estrogens and estradiol to estrone and stores a lot of steroids. The steroid pool in obese individuals is greater than in normal-weight individuals (Gambineri et al., 2002). In addition, an increase in body weight and fat tissue is associated with sex steroid balance abnormalities (Pasquali et al., 2003) and altered delivery of androgens and estrogens to target organs. Next to androgens and estrogens, these alterations also involve their carrier protein, **sex hormone binding globulin** (SHBG). SHBG is impacted by body fat distribution and is inversely proportional to waist-hip-ratio. In obese individuals, a decrease in SHBG concentrations has been observed. Insulin has an inhibitory capacity on SHBG and elevated insulin levels are able to inhibit hepatic synthesis of SHBG (Norman and Clark, 1998). Furthermore, a reduction in SHBG leads to elevated circulating free sex steroids such as testosterone, resulting in an increased metabolic clearance of these hormones. There is, however, a compensatory increased androgen synthesis by the ovaries resulting in **hyperandrogenism**. Hyperandrogenism has a pathophysiological effect on ovarian function and contributes to menstrual disturbance and oligo-anovulation.

Adipose tissue further affects reproductive function by the secretion of adipokines, such as leptin, adiponectin, ghrelin and resistin, peripheral metabolic cues which may

impact on the hypothalamo-pituitary axis as well as on ovarian follicles and early embryos in women (for review, see Michalakis et al. (Michalakis et al., 2013)).

Next to its impact on important organs like muscle, liver and pancreas, **insulin** also plays an important role on the pituitary and the regulation of reproductive organs like the ovary. In the ovary, insulin acts via the insulin receptor or the insulin-like growth factor (IGF) 1 receptor which has been detected in granulosa, theca and ovarian stromal tissue. Insulin stimulates ovarian steroidogenesis and enhances the stimulatory effect of LH through LH receptor ovulation. Insulin also acts at the level of the pituitary where it increases the sensitivity of pituitary gonadotrophins to GnRH action (Poretsky et al., 1999) and to reinforce the ovarian steroidogenic response to gonadotrophins. Obesity mediates an altered insulin metabolism (insulin resistance and hyperinsulinemia), leading to reduced SHBG, hyperandrogenism and perturbation to the functionality of the IGF system, thereby increasing likelihood of increase menstrual and ovulatory disturbance in obese women. Weight loss of ≥5% in obese subjects has been demonstrated to decrease insulin and IGF, and to increase SHBG concentrations and menstrual cyclicity in women with polycystic ovarian syndrome (PCOS) (Butzow et al., 2000).

PCOS is a metabolic disorder characterized by hyperandrogenemia and has a prevalence of 5-7% in western countries (Ehrmann, 2005). PCOS has been associated with increased adipokines such as TNF-α but also with decreased anti-inflammatory adipokines such as adiponectin. Current data also show that PCOS is related to an increased risk of metabolic disorders such as insulin resistance, hyperinsulinism, impaired glucose intolerance, and obesity, resulting in a significant impact on their fecundity (Cirik and Dilbaz, 2014).

Obesity mediated insulin resistance has been related to FFA, TNF-alpha and leptin, as discussed more detailed previously. In addition to its metabolic actions, there is also strong evidence for a close interplay between **leptin** and the HPO axis in humans. Obesity-associated hyperleptinemia may represent an additional factor involved not only in the development of insulin resistance, but also in the impairment of ovarian function (Brannian and Hansen, 2002).

3.3. THE IMPACT OF OBESITY ALL THE WAY TO THE LEVEL OF THE OOCYTE

The quality of oocytes plays a key role in proper embryo development. An oocyte that has not completed maturation is of poor quality, and thus unable to successfully complete normal developmental processes. Therefore, high quality oocytes are needed to successfully produce well-developing embryos. In humans, oocytes of poor quality may be the cause of women infertility and an important obstacle in successful IVF. The increased rates of miscarriage and very early pregnancy loss seen in obese women have been attributed to a reduction in **oocyte quality** (Fedorcsak et al., 2004; Lashen et al., 2004; Robker, 2008).

Many human studies demonstrated a reduction in oocyte quality, maturity or size with elevated BMI (Carrell et al., 2001; Dokras et al., 2006; Marquard et al., 2011; Wittemer et al., 2000). Research on women undergoing ART using autologous oocytes showed that success declined with increasing BMI, fewer normally fertilised oocytes were observed in women with BMI above 35 (Shah et al., 2011). Oocytes from obese or overweight women undergoing ART were also reported significantly smaller in diameter than oocytes from women with a normal BMI. The smaller oocytes from overweight and obese women were less likely to reach the blastocyst stage (Leary et al., 2015). Oocytes that failed to fertilize in IVF cycles of morbidly obese women showed disarrayed meiotic spindles with misaligned metaphase chromosomes (Machtinger et al., 2012). In obese women undergoing IVF, elevated levels of FFA in the follicular fluid (FF) correlated with abnormal cumulus-oocyte-complex morphology (Jungheim et al., 2011). Embryos derived from the oocytes of obese women have been shown to be of poorer quality than those derived from normal-weight women (Carrell et al., 2001; Metwally et al., 2007a).

The importance of oocyte quality was further investigated using the oocyte donation model, in which donor oocytes were implanted in female overweight recipients who were unable to successfully conceive. Based on the results, the hypothesis was stated that BMI does not affect uterine receptivity and that poor-quality oocytes and resulting embryos are probably the cause of poor reproductive outcomes in overweight women undergoing fertility treatment (Styne-Gross et al., 2005; Wattanakumtornkul et al., 2003; Zenke and Chetkowski, 2004). A very large study reported that increasing obesity (based on BMI) is associated with decreased clinical pregnancy rates from autologous oocytes but not donor oocytes, indicating that the oocyte quality alone can affect pregnancy (Luke et al., 2011). Although other research does report an impact of obesity on the endometrium, it was still suggested that although the endometrium may play a part, oocyte quality is more likely to be the more influential factor (Brewer and Balen, 2010).

To investigate the impact of obesity on oocyte quality more in depth, animal models were used. The murine model has shown to be very valuable and a lot of research has been performed using diet-induced obesity (DIO) models. Female obese mice, put on a high fat diet for 16 weeks were less likely to ovulate compared with lean, control animals. Oocytes that did ovulate were in vitro fertilized and cultured and showed slower developmental competence (Minge et al., 2008). Ovaries collected from high fat diet-exposed obese mice contained significantly more apoptotic ovarian follicles and smaller and fewer mature oocytes compared to controls (Jungheim et al., 2010). In addition, oocytes collected from a DIO mouse model displayed higher rates of meiotic aneuploidy with fragmented disorganized meiotic spindles and chromosomes that were not properly aligned on the metaphase plate (Luzzo et al., 2012).

Next to the impact of the obese state, the possible direct effects of diets and different diet compositions on oocyte quality cannot be ignored (Ashworth et al., 2009). In 2012, Leese underlined that maternal dietary intake is the main source of nourishment for the oocyte and the early embryo (Leese, 2012).
Inadequate maternal vegetable and fruit intake during the periconception period was associated with reduced quality and developmental morpho kinetics of human pre-implantation embryos (Hoek et al., 2020). In contrast, early first trimester maternal 'high fish and olive oil and low meat' dietary patterns were associated with accelerated human embryonic development (Parisi et al., 2018). The addition of linoleic acid, to bovine and murine cumulus oocyte complexes, significantly inhibited cumulus cell expansion and retarded development of the oocytes to the metaphase II stage in a dose-dependent manner and adversely affected its subsequent development (Marei et al., 2010). The addition of 6% fat in the diet has shown to alter the fatty acid profile both in serum and FF in cows. Furthermore, the change in FF fatty acid composition was reflected in the fatty acid content and profile of the cumulus oocyte complex (COC) (Adamiak et al., 2005). Dietary intake of sucrose by healthy monkeys in a dose consistent with human consumption inhibits oocyte maturation and early embryo gene expression (Chaffin et al., 2014).

To obtain obesity in DIO mouse models, mice were exposed to different diets with different compositions, going from a high fat diet, high fat/high sugar diet to a high sugar diet, Western type cafeteria diet and so on, for different time periods (Panchal and Brown, 2011). It is important to keep in mind that the different composition of these diets can have a differential impact on oocyte quality.

Based on this information, it is clear that **obesity and obesogenic diets have a significant impact on fertility, and that quality of the oocyte plays a significant role. Therefore, any intervention to improve fertility should take place during the preconception period, as it is expected to have a big impact on oocyte quality. However, the potential of preconception health care interventions on oocyte quality is difficult to investigate and significantly underexplored.**

4. LINK BETWEEN MATERNAL METABOLIC HEALTH AND OOCYTE COMPETENCE AND QUALITY

4.1. DEVELOPMENT FROM FOLLICLE TO OFFSPRING

Oocyte and embryo development consists of several well-orchestrated processes involved in oogenesis, folliculogenesis, fertilization and early embryogenesis. Therefore, a detailed understanding of these processes is essential to get more insights in the influence of maternal metabolic disorders like obesity on oocyte quality and early embryo development and to discover whether there is a possible window of improvement or recovery.

4.2. FOLLICULOGENESIS AND OOGENESIS

The following description of folliculogenesis and oogenesis applies to most mammalian species but is focusing on human physiology (Figure 5).

Oocyte development is initiated in the female mammalian foetus with the differentiation of primordial germ cells (Collado-Fernandez et al., 2013). During the second week of embryonic development, the primordial germ cells migrate towards the genital ridges where they become oogonia that are mitotically active for several cycles (Aerts and Bols, 2010; Collado-Fernandez et al., 2013). During their migration and while present in the gonad, the majority of primordial germ cells will continue to proliferate leading to an estimated maximum number of 7 000 000 in humans from which only 2 000 000 remain at birth as a result of apoptosis (Aerts and Bols, 2010). All oocytes and follicles will arise from this pool through oogenesis and folliculogenesis until exhausted. This means that the reproductive life span is finite (Kerr et al., 2013). After several rounds of division, the oogonia enter meiosis I where they are arrested at the diplotene of the first meiotic prophase (germinal vesicle stage) and are called primary oocytes (Aerts and Bols, 2010; Collado-Fernandez et al., 2013). The primary oocytes will remain in the prophase and will not complete their first meiotic division until sexual maturity is reached (Sadler, 2009). They are surrounded by one layer of flattened pre-granulosa cells and a basement membrane forming the primordial follicle (Aerts and Bols, 2010; Collado-Fernandez et al., 2013). From sexual maturity on, a pool of **primordial follicles** starts to mature during each cycle and will pass through several follicular developmental stages (Aerts and Bols, 2010). The entire duration of human folliculogenesis, from primordial to pre-ovulatory or Graafian follicle, has been estimated to take approximately 3-4 months. In mice, folliculogenesis takes only 3 weeks (Clarke, 2017).

The primordial follicles are stimulated to grow and differentiate during primordial follicle activation.

This process, where primordial follicles leave their dormant state and enter the growth phase, is tightly controlled by multiple factors. However, despite the progress that has been made, many molecular mechanisms regulating this process are not fully

understood (Sánchez and Smitz, 2012). After activation, the flattened pre-granulosa cells surrounding the primordial follicle transform into a single layer of proliferating cuboidal granulosa cells. Together with the primary oocyte, this entity is called the **primary follicle** (Collado-Fernandez et al., 2013; Fair, 2003). The progression to the **secondary follicle** stage is characterized by the presence of 2 or more **layers** of granulosa cells surrounding the oocyte and by the appearance of the zona pellucida, a layer of glycoproteins (Aerts and Bols, 2010; Fair, 2003; Sadler, 2009). Follicles are called **tertiary or antral** when fluid-filled spaces start to appear between the granulosa cells that merge into a large cavity, the antrum (Smitz and Cortvrindt, 2002). The formation of the antrum leads to the separation of granulosa cells into two anatomically and functionally different cell types: the mural granulosa cells, that line the follicular wall and have an important endocrine role; and the cumulus cells, that surround the oocyte and are crucial for the oocyte metabolism and maturation (Collado-Fernandez et al., 2013; Gilchrist et al., 2004). The follicular development up to and including the early antral stage is relatively independent of gonadotropins, FSH and LH (Zeleznik, 2004). Contrary to the early folliculogenesis, FSH is crucial for the antral development (Drummond and Findlay, 1999). This gonadotropin is also responsible for the induction of LH receptors on the granulosa cells allowing the follicle to be responsive to LH (Zeleznik, 2004). Driven by the rising levels of FSH, there will be a continuous shift from late preantral to antral follicles. From this cohort of antral follicles, only one will be selected in mono-ovular species like human and cows to become the dominant follicle (Wallach and Taymor, 1996). The biggest follicle, able to produce the highest concentration of oestradiol will become dominant, while others become atretic. Acquiring dominance is an interplay between changes in the bio-availability of IGFI and IGFII and the increasing granulosa cell dependence of LH (Roche, 1996). In the absence of elevated progesterone concentrations, the follicle will reach the preovulatory stage and will ovulate after the LH surge. In poly-ovular species, like mice, a cohort of antral follicle will become dominant. This will ultimately result into pre-ovulatory or Graafian follicle(s) ready to ovulate the oocyte into the oviduct (Aerts and Bols, 2010).

In parallel with the folliculogenesis, the oocyte within the follicle will grow and mature likewise. The oocyte becomes transcriptionally active from the secondary follicle stage onwards, and intensively accumulates messenger RNA, ribosomes and proteins which are vital for further embryonic development (Fair, 2003). When the oocyte reaches the late growth phase, the gene transcription becomes quiescent and the nucleus reorganizes to an inactive state (Fair, 2009). The stored RNA molecules and proteins in the mature oocyte cytoplasm will support the early embryonic development until embryonic genome activation occurs after fertilization (Gandolfi and Gandolfi, 2001; Russell and Robker, 2007). As the dominant follicle approaches its maximum size, the oocyte increases its lipid content and its organelles undergo several ultrastructural changes. Those changes are referred to as pre-maturation or capacitation of the oocyte. The final oocyte maturation is triggered by the pre-ovulatory LH-surge (Fair,

2009). LH binds to receptors present on the mural granulosa cells, resulting in the production of epidermal growth factor-like proteins that act on the cumulus cells to trigger oocyte maturation (Arroyo et al., 2020; Mehlmann, 2005). In order to acquire developmental competence, the oocyte must undergo two aspects of maturation: nuclear and cytoplasmic maturation (Eppig, 1996). During cytoplasmic maturation, the oocyte prepares its internal structure to facilitate the fertilization and sustain early embryonic development (Bromfield and Piersanti, 2019). This process is characterized by several changes in the organization and dynamics of the oocyte cytoplasm, such as a further increase in lipid content, a reduction of the Golgi compartment, alignment of the cortical granules and rearrangement of mitochondria (Hyttel et al., 1997). Besides cytoplasmic maturation, the oocyte also undergoes nuclear maturation. The process of nuclear maturation encompasses the resumption of meiosis I and the breakdown of the oocyte nucleus membrane (germinal vesicle breakdown) (Collado-Fernandez et al., 2013; Fair, 2003). The completion of meiosis I results in two daughter cells of unequal size, the secondary oocyte and the first polar body. The oocyte enters meiosis II but remains arrested at the metaphase stage until fertilization (Sadler, 2009). Twelve hours after the LH peak, the **cumulus oocyte complex** (COC) is released from the follicle, a process called ovulation. Besides the stimulation of nuclear and cytoplasmic maturation in the oocyte, the LH-surge also induces alterations in the cumulus cells. In response to this gonadotropin surge, the cumulus cells start to produce hyaluronic acid, a non-sulphated glycosaminoglycan which is attached to the cumulus cells by linker proteins. Hydration of the hyaluronic acid causes the spaces between the cumulus cells to expand and the cells become embedded in a sticky, mucified extra-cellular matrix (Eppig, 2001). This process is referred to as cumulus cell expansion (Nevoral et al., 2014).

During folliculo- and oogenesis, the growing follicle and oocyte are very sensitive to changes in circulating metabolites in the follicular fluid (Best and Bhattacharya, 2015; Valckx et al., 2014b). Valckx et al. showed that exposure to high NEFA concentrations during murine in vitro follicle development reduced follicle diameter and antrum formation (Valckx et al., 2014b). Furthermore, mRNA abundance of genes involved in energy, fatty acid and steroid metabolism; apoptosis and oxidative stress was altered. Development of resulting oocytes was significantly reduced. In addition, supplementation of human FF during in vitro bovine oocyte maturation significantly reduced oocyte development (Valckx et al., 2015).

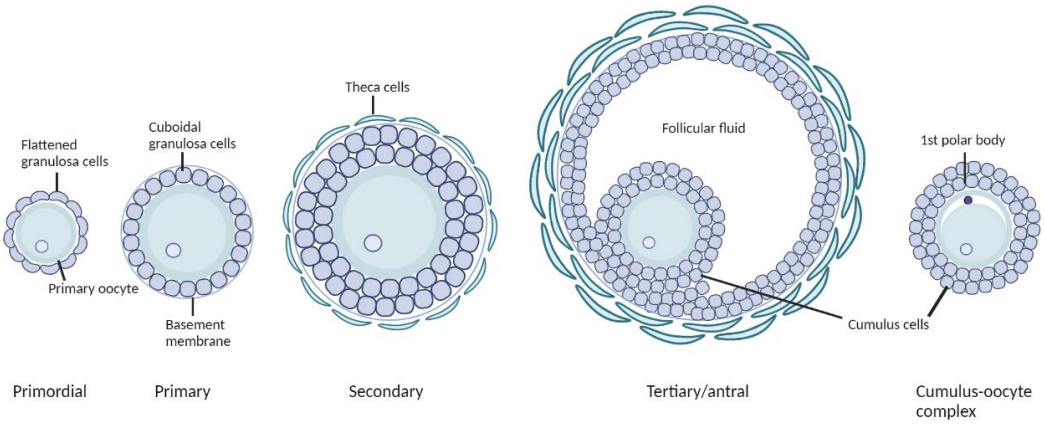

Figure 5. Folliculo- and oogenesis (Made with Biorender)

4.3. FERTILIZATION

Several hours after ovulation, fertilization occurs in the ampulla of the fallopian tube (summarized by Sadler (2009) and Boron and Boulpaep (2005)). Before spermatozoa are able to fertilize the oocyte, they need to mature further by means of capacitation in the female reproductive tract. Capacitation is the process by which spermatozoa acquire the ability to penetrate the zona pellucida of the oocyte (Boron and Boulpaep, 2005). Fertilization itself occurs in different steps. First, the sperm cell head attaches to the zona pellucida which surrounds the oocyte. As a result, the spermatozoa will undergo the acrosome reaction; releasing hydrolysing enzymes that are necessary for the sperm cell to penetrate through the zona pellucida. When the sperm cell has penetrated the zona, the cell membranes of the sperm cell and the oocyte fuse and the oocyte undergoes the cortical granule reaction to prevent polyspermy. The oocyte then completes its second meiotic division with the formation of the second polar body and finally, the male and female haploid pronucleus fuse, to form the diploid zygote (Boron and Boulpaep, 2005).

4.4. EARLY EMBRYO DEVELOPMENT

One or two days after fertilization the zygote will reach the two-cell stage and will then further divide mitotically in the fallopian tube (summarized by (Sadler, 2009) and (Boron and Boulpaep, 2005)). **Embryonic genome activation** (also called maternal to zygotic transition) occurs at the two-cell stage in mice and around the four- to eight-cell stage in humans and 8-16 cell stage in bovine embryos (Duffie and Bourc'his, 2013). This is an important event since embryonic development comes under the exclusive control of the zygotic genome, instead of the maternal genome from the oocyte (Lee et al., 2013).

When the zygote has reached the **morula** stage (16 cells), it is moved via cilia to the lumen of the uterus and transforms into a **blastocyst**. Blastocysts have a fluid-filled

cavity; the blastocoel, which is surrounded by the trophoblast. The trophoblast consists of trophectoderm cells (TE) which will develop into supporting structures like the amnion, the yolk sac and the foetal portion of the placenta. Attached to the trophoblast, on one side of the cavity, there is an inner cell mass (ICM) which will develop into the embryo itself (Boron and Boulpaep, 2005). Based on blastocoel formation and size, blastocysts are classified in vitro in young, normal, expanded, hatching and hatched blastocysts.

The human blastocyst floats freely in the uterus for approximately 72 hours before it will hatch (i.e. break through the zona pellucida) and implant in the endometrium (Niakan et al., 2012). Implantation of the blastocyst thus occurs 6-7 days following ovulation in humans and 4.5-5 days in mice (Boron and Boulpaep, 2005; Matsumoto, 2017). **It is important to know that the pre-implantation embryo itself is also vulnerable to changes in metabolites in the micro-environment and this can influence embryo quality and resulting pregnancy rates (Best and Bhattacharya, 2015; Pinto et al., 2002).** More and more evidence is emerging, indicating a significant epigenetic impact on the development and health of the offspring.

4.5. CUMULUS-OOCYTE COMPLEX INTERACTIONS

As previously described, the pre-ovulatory oocyte is surrounded by several layers of specialized somatic cells, cumulus cells, that significantly contribute to oocyte maturation and fertilization (Collado-Fernandez et al., 2013).

Cumulus cells contain cytoplasmic processes that cross the zona pellucida and penetrate the oocyte membrane, forming the cumulus-oocyte complex (COC). Gap junctions present at the ends of these processes allow for transportation of small molecules including amino acids, glucose metabolites, nucleotides, ions, glutathione (GSH), small regulatory molecules and RNA to the growing oocyte, supporting the oocyte maturation and development (Eppig, 1991; Gilchrist et al., 2004; Macaulay et al., 2016). On the other hand, the oocyte produces paracrine factors, also termed oocyte-secreted factors, that have a significant role in regulating granulosa cell function by directing processes associated with growth, differentiation and cumulus cell expansion (Gilchrist et al., 2004). Studies have shown that *in vitro* maturation of bovine oocytes in the absence of the associated cumulus cells or in the presence of gap junction inhibitors results in oocytes with a disturbed metabolism and poorer capacity to support fertilization (De La Fuente and Eppig, 2001; Fatehi et al., 2002; Geshi et al., 2000). Similarly, vital processes, such as cumulus expansion, are disturbed or even prevented in the absence of an oocyte (Sugiura et al., 2007; Vanderhyden et al., 1990). The co-dependence between the oocyte and cumulus cells is partially due to the metabolic cooperativity of the COC (Richani et al., 2021b). Evidence suggests that the oocyte developmental competence can be predicted by transcript markers expressed in cumulus cells (McKenzie et al., 2004; Vigone et al., 2013). As cumulus cells are discarded at the time of IVF, analysis of their transcriptomic profile may give a direct indication of the fertility potential of the oocyte without jeopardizing the oocyte's

integrity. As such, cumulus cells may serve as an interesting non-invasive biomarker of the oocyte developmental competence and thus subsequent embryo development (McKenzie et al., 2004).

5. OBESITY AND THE OOCYTE'S MICRO ENVIRONMENT

5.1. INTRAFOLLICULAR CONDITIONS

The follicular fluid (FF) is an ultrafiltrate from the blood in the thecal capillaries and its composition is thus affected by systemic changes in metabolites. Fluid from the bloodstream crosses the blood-follicle barrier. As follicular development progresses, this fluid accumulates in the antrum of the follicle, providing the environment in which the oocyte grows and matures up until release in the oviduct.

Various components of the FF influence oocyte growth and quality. Hormones like FSH, LH, progesterone and estetrol, a natural estrogen (E4), are all found within the follicular fluid (reviewed by (Scaramuzzi et al., 2011)). In addition, metabolites like amino acids, fatty acids, nucleotides and others also play important roles in oocyte growth. Amino acids are involved in cellular functions such as energy production (Sutton et al., 2003; Van Winkle, 2001) and fatty acids accumulate within the oocyte as an energy source. The saturated palmitic (PA; C16:0) and stearic acid (SA; C18:0), and the monounsaturated oleic acid (OA; C18:1) are the most predominant NEFAs both in serum and in FF (Leroy et al., 2005; Valckx et al., 2012). Other important metabolic substrates such as pyruvate and glucose are also present in the follicular fluid (Rieger and Loskutoff, 1994).

Metabolic changes in the blood due to e.g. obesity are reflected in the follicular fluid. It was shown by Leroy et al. (Leroy et al., 2004) that negative energy balance (NEB)-associated elevated NEFA, glucose, β-Hydroxybutyrate etc. concentrations in the blood are reflected in the composition of the FF in cows. Palmitic, stearic and oleic acid were found to be the most predominant NEFA's in FF of both women and cows (Leroy et al., 2004; Leroy et al., 2005; Valckx et al., 2012). When investigating the correlation between FF and serum composition in obese women, Valckx et al. (2012) found that metabolic alterations in the serum are reflected in the FF (Valckx et al., 2012). Observed alterations in the FF of obese women are increased glucose and lactate, increased androgen activity and decreased hCG concentrations (Carrell et al., 2001; Robker et al., 2009). Furthermore, insulin showed elevated concentrations in both serum and FF of obese women (Valckx et al., 2012). All these changes in the composition of the ovarian follicular fluid have a direct impact on oocyte maturation and development but also on the metabolism of the cells that support the developing oocyte, such as cumulus cells (Jungheim and Moley, 2010). In addition, the maternal metabolic state can also have a profound effect on the fatty acid composition of the FF as both TG and NEFA concentrations were affected by BMI (Valckx et al., 2014a). 87% of all fatty acids in the NEFA fraction (including palmitic, stearic and oleic acid) are correlated with BMI. Maturation of murine oocytes in medium supplemented with NEFA/TG rich FF, increased oocyte lipid content and upregulation of genes related to endoplasmic reticulum stress were seen (Yang et al., 2012). Higher BMI was also accompanied by elevated CRP concentrations in both serum and FF. High CRP concentrations are characteristic for increased inflammation and involved in oxidative stress, hereby

affecting oocyte developmental competence and resulting embryo quality (Agarwal et al., 2008; Combelles et al., 2009; Das, 2001). In overweight and obese patients, both pro-inflammatory (CRP, sICAM-1, IL6, TNFα) and anti-inflammatory (adiponectin, IL10) markers were associated with intra-follicular lipid concentrations (thoroughly discussed by (Gonzalez et al., 2018)).

When follicular fluid of obese women was added during *in vitro* maturation of bovine oocytes, oocyte developmental competence and embryo quality were affected (Valckx et al., 2015). Jungheim et al. (2011) associated, in a human ovum pick-up setting, high follicular fluid NEFA concentrations with poor cumulus–oocyte complex morphology.

In the last decade, extracellular vesicles (EVs) have emerged to play a significant role in regulating and facilitating the dialogue between mother and reproductive cell types like oocytes (reviewed by Saadeldin et al. (2021)). To illustrate the function of EVs in the ovary, Uzbekova et al. (2020) profiled the EV proteins within bovine follicles and identified 322 unique proteins, originating from granulosa and other cells. These proteins may be involved in the maintenance of follicular homeostasis and may affect oocyte developmental competence. EVs have been detected in all reproductive biofluids with emerging evidence on their roles in gametogenesis, fertilization, early embryo development, and implantation.

5.2. OVIDUCTAL FLUID ENVIRONMENT

Fertilization and early embryo development takes place in the oviduct. The oviductal fluid is formed by secretions of the oviductal epithelial cells and selective passage due to filtration of plasma components into the oviductal lumen (Aviles et al., 2010; Leese et al., 2001). It is composed of carbohydrates, ions, lipids, phospholipids and proteins, all important substrates for the development of the early embryo. In addition, steroid hormones have an impact on the secretory activity of the oviduct (Leese et al., 2001). Besides important substrates such as vitamins, ions, growth factors and energy substrates (reviewed by (Aviles et al., 2010; Hu and Yu, 2017)), specific conditions such as temperature, pH, osmotic pressure and redox potential contribute to optimal early development.

Information regarding the impact of obesity on the oviductal micro environment is limited. Researchers investigated the effect of nutrition on the oviductal micro-environment via embryo transfer in the oviducts of metabolically compromised cattle. They found that the oviductal environment in metabolically stressed lactating and intensively fed dairy cattle was less supportive for embryo survival compared to heifers (Rizos et al., 2010) and to non-lactating cows fed a normal diet (Maillo et al., 2012). Bovine embryos exposed to elevated NEFA concentrations during the *in vitro* culture period showed a lower developmental potential (Desmet et al., 2016). This confirms

that the conditions in the reproductive tract define its ability to sustain early embryo development (Maillo et al., 2012; Matoba et al., 2012; Rizos et al., 2010).

Successful implantation requires synchronous development of embryo and endometrium. Endometrial receptivity results from progesterone- induced differentiation of endometrial cells, generally achieved during the mid-secretory phase of the cycle. Failure to properly develop receptivity results in failed or inadequate implantation and hence no ongoing pregnancy. The blastocyst undergoes final development, apposition, attachment and initiates invasion of the endometrial epithelium within the uterine cavity. Thus, the microenvironment provided by uterine fluid, particularly glandular secretions, is essential for implantation. Analysis of endometrial fluid has identified cytokines, chemokines, proteases, antiproteases and other factors that modulate blastocyst functions relevant to implantation. Exosomes/microvesicular bodies released from the endometrium (and likely also the embryo) are present in uterine fluid. These can transfer miRNA, proteins and lipids between cells, thus providing endometrial–embryo communication in the peri-implantation period. Understanding the uterine microenvironment, and its effects on endometrial–embryo interactions, will provide opportunities to modify current infertility treatments to improve success rates.

5.3. UTERINE FLUID ENVIRONMENT

After the embryo has passed through the oviduct, it enters the uterus. Synchronous development of both the embryo and the receptive endometrium are required for successful implantation. The microenvironment of the uterus, the uterine fluid is essential for implantation. It contains cytokines, chemokines, antiproteases and other factors that modulate blastocyst functions relevant to implantation. In addition, exosomes/microvesicular bodies are released from the endometrium and provide endometrial-embryo communication (reviewed by (Salamonsen et al., 2016)). Research investigating the impact of obesity on the uterine environment, discovered that uteri from obese rats during the estrous phase developed insulin resistance through mechanisms that involve the induction of uterine hypoxia and the down-regulation of the insulin receptor gene (Bazzano et al., 2018). However, the impact of metabolic disorders on the uterus is beyond the scope of the present thesis.

6. THE OOCYTE TAKES CENTRE STAGE: IMPACT OF OBESITY ON OOCYTE AND EMBRYO QUALITY – MECHANISTIC INSIGHTS

Based on the information described in the chapter above, the impact of obesity on oocyte quality is clear. The past years, fundamental knowledge has increased significantly regarding this topic as research investigating the key mechanisms behind it, has expanded substantially.

6.1. IMPACT OF OBESITY ON OOCYTE DEVELOPMENTAL COMPETENCE EN RESULTING EMBRYO QUALITY

The developmental competence of the oocyte is defined as the oocyte's capacity to resume meiosis, to cleave following fertilization, to develop until the blastocyst stage, to induce a pregnancy, and to give rise to a healthy offspring (Sirard et al., 2006).
Different murine studies demonstrated an impairment in embryo developmental rate in oocytes derived from DIO female mice. Until the eight-cell stage, embryos derived from obese females developed at a slower rate (approximately one hour later) compared to embryos derived from female mice on a control diet (Binder et al., 2012; Finger et al., 2015). Furthermore, reduced cleavage and blastocyst rates were also reported (Finger et al., 2015; Igosheva et al., 2010; Sohrabi et al., 2015). Resulting blastocysts showed reduced number of trophectoderm cells and total cell numbers (Binder et al., 2012; Finger et al., 2015). Human blastocysts, resulting from oocytes collected for ART, contain fewer cells notably in the trophectoderm (P = 0.01) when BMI was high.

Earlier research showed that bovine oocytes exposed to pathophysiological concentrations of the most predominant NEFAs (PA, SA and OA) during in vitro maturation for only 24h, already showed significantly reduced developmental competence compared to the basal NEFA exposed counterparts (Aardema et al., 2011; Leroy et al., 2005; Van Hoeck et al., 2013a). Both cleavage and resulting blastocyst rates were lower. In addition, De Bie et al (De Bie et al., 2017) reported significant lower ≥ 4-cell embryos ratios and significant increased fragmented/ ≥ 4-cell embryos percentage. Besides a reduced developmental competence, surviving bovine blastocysts also contained a significantly lower total cell number and increased apoptotic cell index (Marei et al., 2017; Van Hoeck et al., 2011). Furthermore, DNA methylation patterns in the resultant blastocysts were altered (Desmet et al., 2016). In a recent investigation, morphologically good-quality day 7 bovine blastocysts, produced in vitro from metabolically compromised oocytes, were transferred to a healthy uterine environment. Day 14 embryos were recollected and clearly exhibited retarded growth, abnormal cell metabolism and persistent abnormal gene transcription patterns. Production of signaling molecules for maternal recognition of pregnancy (IFN-τ in cows) was significantly reduced. This illustrates the long lasting carry-over effects of the oocyte's maturation environment (Desmet et al., 2020). Thereby, metabolic

perturbations, induced in the oocyte, may not simply result in suboptimal conception rates, but can also result in persisting affects during foetal development or become visible after birth as stated by Vickers (2014).

6.2. CUMULUS-OOCYTE COMPLEX AND EMBRYO METABOLISM

During oocyte growth, maturation and early embryo development, the COC, zygote and blastocyst exploit a multitude of metabolites from its microenvironment to meet its energetic and anabolic needs (Collado-Fernandez et al., 2013) (figure 6). Consequently, this also makes the COC and early embryo vulnerable to any alterations of available nutrients or substrates in the microenvironment as occurs in women suffering from metabolic disorders.

Gene expression and functional data suggest that bovine embryos, resulting from NEFA-exposed oocytes, have altered metabolic strategies, suggesting a mechanism of metabolic dysregulation appearing in the pre-implantation embryo as a consequence of elevated NEFA concentrations peri-conception (Van Hoeck et al., 2013a) (reviewed by Leroy et al. (2017)). These dynamic changes in energy metabolism serve as a valuable independent marker for developmental potential (Gardner et al., 2011).

6.2.1. GLUCOSE METABOLISM AND THE IMPORTANCE OF INSULIN

a) Glucose metabolism in the cumulus-oocyte-complex

Glucose is an essential metabolite to sustain oocyte maturation and subsequent development to the blastocyst stage. Glucose can be metabolized via 4 major metabolic pathways: glycolysis, pentose phosphate pathway (PPP), hexosamine biosynthetic pathway (HBP) and the polyol pathway (Figure 6). A large proportion of the glucose present is metabolized via the **glycolytic pathway**, producing energy in the form of ATP and glycolytic products, pyruvate and lactate (Sutton-McDowall et al., 2010). Pyruvate can be metabolized through the tricarboxylic acid (TCA) cycle in the mitochondria, followed by oxidative phosphorylation to yield higher quantities of ATP than glycolysis (Richani et al., 2021b). Lactate is important for redox regulation in the oocyte (Dumollard et al., 2007). High lactate concentrations were present in the FF of obese women (Robker et al., 2009) and might indicate a compensatory response to control the observed increase in oxidative stress.

Despite the expression of facilitative glucose transporters in the oocyte, the oocyte's capacity to take up and metabolize glucose is poor (Sutton-McDowall et al., 2010). As a result, the oocyte almost completely relies on the surrounding cumulus cells for the uptake of glucose and for the supply of its metabolites pyruvate and lactate (Richani et al., 2021b). In addition, a small proportion of the absorbed glucose is metabolized via the **PPP** resulting in the production of NADPH, which plays a major role in maintaining the redox state through the reduction of glutathione, and precursors essential for the synthesis of nucleotides (Richani et al., 2021b; Sutton-McDowall et al., 2010). As such,

the PPP is crucial for resumption of meiotic maturation. Reduced glucose metabolism via the PPP during murine oocyte maturation has been associated with lower developmental potential (Downs et al., 1998). Glucose can also be metabolized via the **HBP** which plays a major role in producing substrates for matrix synthesis (Sutton-McDowall et al., 2010). The end product of this pathway, UDP-N-acetyl glucosamine, can either be utilized for O-linked glycosylation of proteins or hyaluronic acid synthesis in cumulus cells (Thompson, 2006). During cumulus cell expansion, cumulus cells abundantly synthetize hyaluronic acid which serves as the structural backbone of the mucified extracellular matrix that entraps and disperses the cells. In order to meet the increased demand of hyaluronic acid, the cumulus cell expansion is accompanied by a significant increase in glucose consumption (Thompson, 2006). Bovine COC's exposed to high lipotoxic conditions during in vitro maturation showed an inhibited cumulus cell (CC) expansion with an increased CC apoptosis (De Bie et al., 2017; Marei et al., 2017).

b) Glucose metabolism changes in the pre-implantation embryo

The cleavage stage embryo also relies on pyruvate and lactate to provide energy as it still has a limited capacity to use glucose. However, once the blastocyst stage is reached, the embryo displays a high capacity to use glucose as an energy source. Blastocysts from obese women consumed significantly less glucose compared with blastocysts from normal weight women (Leary et al., 2015). In contrast to a lot of other cell types, the blastocyst converts approximately half the glucose consumed to lactate, even in the presence of sufficient oxygen to support its complete oxidation (Gardner and Leese, 1990). This phenomenon is referred to as aerobic glycolysis or **Warburg effect.** Glucose is turned into pyruvate by glycolysis, after which the pyruvate is transformed into lactate with the formation of NAD+. This mechanism provides the embryo with a growth advantage. This conversion of pyruvate to lactate produces glycolytic intermediates. These glycolytic intermediates allows the cell to meet critical metabolic requirements other than ATP production, such as production of nucleic acid precursors and redox regulation via the PPP (Collado-Fernandez et al., 2013; Krisher and Prather, 2012).
Next to that, it has also been suggested that the considerable amount of lactate produced by the blastocyst could facilitate several key processes involved in the implantation process by creating a receptive microenvironment around the embryo (for an overview: (Gardner and Harvey, 2015)).

Leese et al. (2007) proposed the concept of **'quiet embryo metabolism'**, suggesting an optimal range of embryo metabolic activity consistent with successful developmental progression. Changes in metabolic activities were linked to morphological progression of the embryos and a middle 'optimum' range of depletion for pyruvate has been correlated with high viability (Guerif et al., 2013). Metabolic activity below or above the optimum rate may indicate a compromised metabolic state. So far, little information is

known whether the metabolic phenotype of the early embryo is sensitive to the maternal metabolic state.

c) The important role of insulin in glucose metabolism of oocyte and embryo

Throughout oocyte and embryo development, different GLUT transporters were expressed during different stages of pre-implantation development. Although literature is not consistent on which GLUT transporters were expressed during which developmental stage, it involves both insulin independent (GLUT1 and GLUT3) as insulin dependent (GLUT4 and GLUT8) transporters (Roberts et al., 2004), causing the rate of glucose transportation into cells to be primarily reliant on insulin and insulin-like growth factor 1 (IGF1) concentrations (figure 6) (Sutton-McDowall et al., 2010). Early research reported that the insulin receptor (IR) is absent in murine oocytes. In contrast, recent research confirms the presence of IR and IGF1R in mouse oocytes and embryos (Acevedo et al., 2007; Zheng et al., 2007).
In the ovarian environment, insulin is a key regulator of folliculogenesis, oocyte maturation and subsequent embryo development (Chaves et al., 2011). As mentioned above, binding of insulin to its receptor, activates two main insulin signalling pathways, the PI3K/Akt and MAPK pathway, which are involved in a variety of ovarian functions of which steroidogenesis, insulin-stimulated glucose uptake, ovulation, resumption of oocyte meiosis, cell growth, proliferation and differentiation are the most important ones (Dupont and Scaramuzzi, 2016; Purcell and Moley, 2011). The PI3-kinase-Akt pathway is present throughout mammalian preimplantation development (Riley et al., 2006; Riley et al., 2005). Inhibiting this pathway leads to a reduced insulin-stimulated glucose uptake, a significant delay in blastocyst hatching and an induction of apoptosis in blastocysts (Riley et al., 2006; Riley et al., 2005). Akt is involved in the transcriptional regulation of the pro-apoptotic Bax protein which was increased in degenerated embryos and the anti-apoptotic protein Bcl-2 (Pugazhenthi et al., 2000) with a decreased expression in fragmented embryos (Exley et al., 1999; Moley et al., 1998).

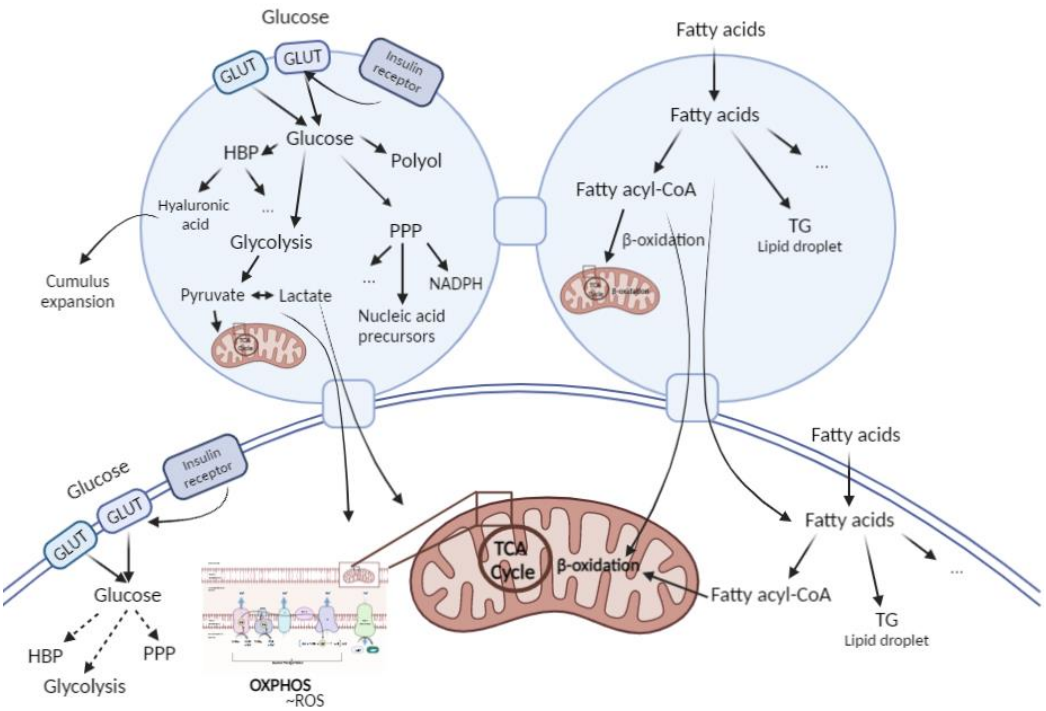

Figure 6. Glucose and fatty acid metabolism in the cumulus-oocyte complex (COC) (Made with Biorender)

Supplementation of media with energy substrates such as glucose or pyruvate has shown the critical role of these substrates for the successful completion of oocyte maturation as well as development of the resulting embryo. However, recent work has shown that fatty acid β-oxidation is another important energy source during oocyte maturation (Paczkowski et al., 2014a).

The **Randle hypothesis**, first described in 1963, suggests a competition between glucose and fatty acids for their oxidation and uptake. As control of the cycle is modified by insulin, it is expected to play a role in insulin resistance. Randle suggested the presence of a biochemical syndrome, the 'fatty acid syndrome', resulting from high concentrations of plasma NEFAs and the relationship to abnormalities in carbohydrate metabolism, including starvation and diabetes (Randle et al., 1963).

6.2.2. THE IMPORTANT ROLE OF FATTY ACIDS DURING OOCYTE AND EMBRYO DEVELOPMENT

Fatty acids are an important source for energy production in the oocyte and serve as an important substrate during oocyte maturation, fertilization and preimplantation development. Previous work has demonstrated that **fatty acid β-oxidation** (FAO) is a crucial metabolic pathway for oocyte maturation and early embryo development

(Downs et al., 2009; Dunning et al., 2010; Paczkowski et al., 2014a). FAO can generate more ATP than glucose and therefore, it is a very efficient source of energy (Richani et al., 2021b). Dunning et al. (2010) illustrated the importance of lipid metabolism in the establishment of murine oocyte developmental competence by evaluating the rate of embryo development following chemical inhibition or activation of the FAO pathway. Whereas inhibition of FAO impaired subsequent embryo development, upregulation of FAO increased oocyte developmental competence as shown by the improved embryo development (Dunning et al., 2010). Accordingly, studies using pharmacological inhibitors have demonstrated that FAO is required for meiotic resumption and maturation in murine oocytes (Downs et al., 2009; Paczkowski et al., 2014a; Valsangkar and Downs, 2013). In contrast, no differences in oocyte developmental competence were reported when FAO was inhibited during bovine and porcine oocyte maturation (Sturmey et al., 2006; Van Hoeck et al., 2013b).

Fatty acids are stored intracellularly within lipid droplets, providing a potent source of energy upon demand. The ability to efficiently store fatty acids in lipid droplets protects the oocyte from lipotoxicity, therefore, it is possible to observe higher numbers of lipid droplets in oocytes from follicular fluid rich in fatty acids (Warzych et al., 2017) (Ferguson and Leese, 2006; Sturmey et al., 2009). Aardema et al. (2011) showed that oocytes actively take up and metabolize NEFAs out of their environment by FAO. This may influence lipid storage in lipid droplets within the oocyte, depending on the type and amount of FA offered. Bovine oocytes exposed to the saturated PA and SA showed less intracellular lipid storage and a hampered oocyte developmental competence. However, this effect was counteracted by the addition of the mono-unsaturated oleic acid.
A remarkable high amount of lipid droplets, however, has a big impact on oocyte and embryo quality. Wu et al. (2010) demonstrated that both oocytes and cumulus cells, collected from DIO mice, contained dramatically increased intracellular lipids. Whether the observed increase in oocyte intracellular lipids is due to increased active uptake from the follicular fluid or due to a shift in energy pathways in the oocyte as a result of the different nutrient availability, is not known yet. However, lipid accumulation in cells other than adipocytes is generally considered the initiating event in lipotoxicity pathways (Szendroedi and Roden, 2009).
This accumulation of intracellular lipids leads to high levels of FFA which causes upregulation of mitochondrial activity, resulting in increased ROS and oxidative stress (Burton et al., 2003; Iossa et al., 2002).

As mentioned before, fatty acids are metabolized by FAO and the tricarboxylic acid (TCA) cycle within the mitochondrial matrix. For this to occur, mitochondria and lipid droplets, the primary source of fatty acids, should ideally reside in close proximity. This has been observed in oocytes of different species, where mitochondria and lipid droplets are seen to associate, forming ‘metabolic units’ which tend to accumulate at

the edge of the oocyte, thus ensuring a steady and readily accessible supply of O_2 for oxidative processes (Sturmey et al., 2006).

6.3. THE IMPORTANT ROLE OF MITOCHONDRIA AND IMPACT OF OXIDATIVE STRESS

As the most prominent organelles in the oocyte and early embryo, mitochondria are of capital importance to guarantee oocyte developmental competence (Van Blerkom, 2004). The primary role of mitochondria is the generation of ATP through oxidative phosphorylation (OXPHOS) in the mitochondrial matrix. The OXPHOS is made up of the TCA and electron transport chain pathways (Grindler and Moley, 2013). This process provides an electron gradient (Chistiakov et al., 2014), ultimately resulting in ATP production (Bentov et al., 2011). However, this process also coincides with the generation of ROS (Menezo et al., 2016). A small portion of electrons in the mitochondrial electron transport chain 'leak' to oxygen and forms superoxide anion (O_2-) (Figure 7) (Kovacic et al., 2005). Therefore, mitochondria need a well-organized protection carried out by antioxidants and enzymatic defence mechanisms.

The balance between ROS production and detoxification is essential for correct embryo development (Halliwell and Aruoma, 1991; Kehrer and Lund, 1994). Adequate amounts of ROS play important roles in multiple physiological activities involved with oocyte development, going from follicle development, the process of ovulation to sperm function during the fertilization process and embryo development (Cardoso et al., 2019). Among the physiologic functions, mitochondrial activity demands a low level of oxidative stress (Cardoso et al., 2019; Singh et al., 2015). The beneficial role of a physiological level of ROS has been suggested by Attaran et al. (2000) who proposed that low concentrations of follicular ROS may be a candidate marker for predicting a successful pregnancy outcome in IVF patients. Oxidative stress occurs when the generation of ROS and other radical species exceeds the scavenging capacity of important antioxidants like SOD, CAT and GPx (figure 7) (Maier and Chan, 2002). This excessive production of ROS induces mitochondrial dysfunction and oxidative damage in the oocyte (Agarwal et al., 2008), negatively influencing the quality of the gametes, oocyte maturation and fertilization (Abbasihormozi et al., 2019; Agarwal et al., 2012; Budani et al., 2017; Da Broi and Navarro, 2016; Santini et al., 2018; Wojsiat et al., 2017) with deleterious impact on embryo development. In vitro maturation of bovine oocytes under lipotoxic conditions induced higher ROS levels in the oocytes and subsequently a lower developmental competence compared to controls (Marei et al., 2017). Resulting blastocysts were triggered by oxidative stress regulating mechanisms and showed signs of mitochondrial dysfunction. Also obese mouse models showed that metabolic stress conditions are associated with oxidative stress and cellular dysfunctions (Grindler and Moley, 2013).

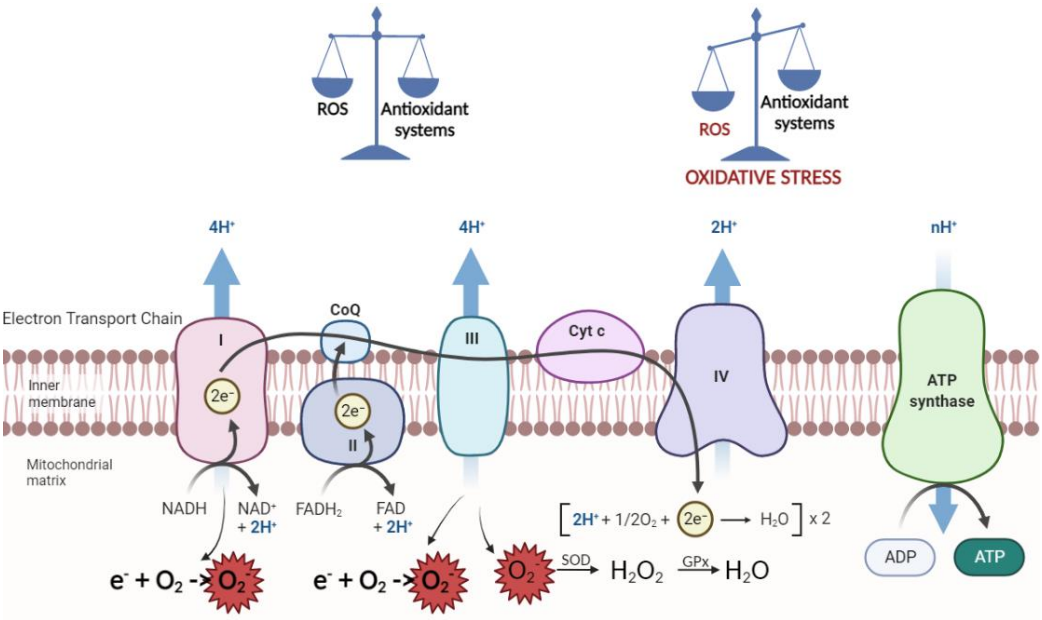

Figure 7. Oxidative stress mechanism and the production of reactive oxygen species (ROS) (adapted from De Bie et al 2017, made with Biorender)

Besides the production of energy, mitochondria also execute a multitude of functions in the oocyte such as calcium signalling, regulation of apoptosis, spindle formation and promoting cell survival and mitochondrial recovery through the unfolded protein response (UPR_{mt}) (Richani et al., 2021b). The UPR acts as a coordinated response via initiation of a global reduction in protein synthesis, induction of ER-associated protein degradation and activation of chaperones (Rutkowski and Kaufman, 2004). In normal conditions the UPR would be activated for a restricted time, to restore the homeostasis of the organelles (Haynes and Ron, 2010). If the activation of UPR persists due to excessive stress levels in the oocyte, the UPR activates apoptosis (Tabas and Ron, 2011). Furthermore, misfolded-proteins accumulate and activate ER stress. COCs derived from DIO mice showed increased expression of ER stress marker genes, increased apoptosis in cumulus cells and decreased fertilization rates compared to COCs of mice fed a control diet. These results suggest that both ER stress and mitochondrial dysfunction resulting from lipotoxic damage in ovarian cells might contribute as a possible cause to subfertility (Wu et al., 2010).

Mitochondria are inherited via the maternal lineage, as sperm mitochondrial proteins are degraded upon entry into the oocyte (Song et al., 2014). Because mitochondrial replication does not occur in the oocyte until blastocyst stage, the preimplantation embryo is completely reliant on the oocyte's mitochondria. Consequently, mitochondrial dysfunction in the oocyte may seriously compromise the early embryo

development (Richani et al., 2021b). Several stressors, such as the maternal metabolic status and food intake are known to affect the mitochondrial activity. In obese women, the poor quality of mitochondria seen in oocytes are the main reason for limited success rates in IVF clinics (Shamsi et al., 2013). Many researchers have shown that oocyte maturation under lipotoxic conditions alters mitochondrial functions and DNA methylation in the resulting pre-implantation embryos (Desmet et al., 2016) and results in significant epigenetic modifications in imprinted and developmentally important genes (Arnaud, 2010; Duranthon et al., 2008; Sinclair and Singh, 2007). This can distort implantation, placentation, and fetal growth rate and compromise postnatal health (Young et al., 1998).

Aberrant mitochondrial function manifests at different levels. For example, mitochondrial activity appears to be influenced by the magnitude of the polarity across the inner mitochondrial membrane. Wu et al. (2010) demonstrated that the **mitochondrial membrane potential** (MMP) in murine oocytes was significantly reduced by a high fat diet. This alteration might affect the oocyte developmental competence since mitochondria are crucial in the generation of ATP and controlling of cellular homeostasis in both the oocyte and early embryo, as stated above. In addition, high NEFA exposure during bovine in vitro maturation reduced mitochondrial inner membrane potential (JC-1 staining) in both CCs and oocytes (Marei et al., 2017). However, other studies report an increased MMP in oocytes collected from obese mice (Igosheva et al., 2010). Both high and low MMP have been linked to reduced oocyte developmental competence (Acton et al., 2004; Wilding et al., 2001). This could be explained by a process called uncoupling. Mitochondrial uncoupling is the loss of inner MMP and occurs in response to elevated oxidative stress. The reduction in MMP happens when oxidative stress levels have reached a threshold at which mitochondrial uncoupling occurs. Uncoupling of inner mitochondrial membrane in somatic cells is an actively regulated process in response to oxidative stress to reduce further ROS production as an attempt for cell survival (Brand, 2000). This may result in a heterogeneous population of hyperactive and inactive mitochondria within the same oocyte. Therefore overall changes in the MMP in oocytes should always be carefully interpreted in the context of other related parameters and considering the associated ROS levels.

The cellular energy demands of the oocyte are achieved by a crosstalk between the nuclear and **mitochondrial DNA**. The oocyte depends on its mitochondria for ATP production and maintenance of cellular homeostasis (Duchen, 2000). For example, if the cellular energy need increases, the nuclear genome transcribes the mitochondrial transcription factor a (*TFAM*) which will start replication and transcription of mtDNA (Anderson et al., 1981). The number and activity of mitochondria are regulated to ensure proper oocyte maturation, fertilization and subsequent embryo development (Cummins, 2002; Reynier et al., 2001). In mice, oocytes with low mtDNA copy number

are more likely to be compromised than oocytes with higher mtDNA copy numbers (Ge et al., 2012). In women, is has been indicated that mtDNA copy numbers are essential to guarantee acceptable fertilization rates. After analyzing the mtDNA content of 142 fertilized and non-fertilized oocytes, they found a significant reduction of 35% in mtDNA copy numbers in the non-fertilized oocytes (Santos et al., 2006). Although, other studies have shown that the relation between mtDNA copy numbers in oocytes and fertility is not consistent (Chiaratti et al., 2010).

Furthermore, the **distribution pattern of mitochondria** throughout the oocyte is considered an important candidate marker of oocyte developmental competence. Van Blerkom et al. (2002) reported that in the mature oocyte, high polarized mitochondria normally occupy a circumferential domain immediately subjacent to the plasma membrane, also described as 'vanguard mitochondrial polarity' (Van Blerkom and Davis, 2006). Pericortical localisation of active, highly polarized mitochondria guarantees the equal distribution of mitochondria among the different blastomeres upon cleavage and supports ATP levels in the suboolemmal cytoplasm during embryo cleavage (Van Blerkom et al., 2003). Furthermore, it is required for successful sperm penetration and cortical granule exocytosis (Van Blerkom and Davis, 2007).
After maturation, oocytes associated with high developmental competence showed larger mitochondrial clusters at the periphery while oocytes with a reduced developmental competence did not (Stojkovic et al., 2001).

In addition, a clear relationship exists between mitochondrial functioning and their **morphology**. In normal conditions, the mitochondria in the oocyte are round shaped with a smooth inner membrane and truncated cristae (Fabozzi et al., 2021). An unbalanced dietary intake, such as high-fat diet, has adverse effects on the morphology of the mitochondria in the oocyte and cumulus cells (Boudoures et al., 2016; Luzzo et al., 2012). Mitochondria from oocytes of obese mice have fewer cristae, increased cristae disarray, decreased electron density of the mitochondrial matrix and a greater number of vacuoles than oocyte mitochondria from mice on the control diet (Luzzo et al., 2012). The altered shape of the oocyte dumbbell-like mitochondria was correlated to disturbances of their dynamics, suggesting the occurrence of fission and fusion (Boudoures et al., 2016). It was suggested that the adverse effects of an unbalanced diet on mitochondrial morphology can be partially compensated by a healthy lifestyle (Boudoures et al., 2016)

6.4. IMPACT OF OBESITY AT THE OOCYTE PROTEOME LEVEL

The past years, the impact of obesity on the proteome has gained more and more attention. It is only since recently that the impact of obesity on the reproductive tissue has been explored at this more functional level.

The study of Marei et al. (2019) analyzed cellular stress responses at the proteome level of bovine oocytes and cumulus cells after short-term *in vitro* exposure to lipotoxic conditions. The results showed a reduced metabolic activity in cumulus cells indicated by the differentially expressed proteins involved in carbohydrate metabolism and fatty acid β-oxidation. In addition, proteins involved in the formation of mitochondrial cristae were downregulated in cumulus cells, possibly leading to abnormalities in the mitochondrial ultrastructure. Also a dysregulation of several ER and ribosomal proteins was observed in cumulus cells as a consequence of oxidative stress.
However, most of these changes were pro-apoptotic. In contrast to the observations in cumulus cells, the proteomic changes in oocytes were predominantly pro-survival and were depicting an adaptive mechanism. This included pro-survival ER and mitochondrial UPRs, redox regulatory and compensatory metabolic mechanisms.

When human FF proteins of overweight women were compared with those from normal weight women, 22 up-regulated FF proteins and 21 down-regulated FF proteins were found in the overweight status women. These altered human FF proteins participated in development, metabolism, immunity, and coagulation (Liu et al., 2020a).

Ovulated oocytes collected from high fat diet (HFD)-fed mice showed a reduced expression of the TIGAR (TP53-induced glycolysis and apoptosis regulator) protein (Wang et al., 2018). Specific depletion of TIGAR in mouse oocytes results in the marked elevation of reactive oxygen species (ROS) levels and the failure of meiotic apparatus assembly.

These data confirm a significant impact of obesity on oocyte quality, substantiated by important mechanistic and fundamental insights. It is not known whether these induced cellular dysfunctions are permanent or whether they can be reversed. Furthermore, the most optimal time frame for any intervention is not clearly described yet. This knowledge is important as it will help us in finding out the most optimal window for interventions.

7. REFERENCES

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CHAPTER 2: GENERAL INTRODUCTION

CONTENT

INTRODUCTION - INTERVENTIONS TO IMPROVE FERTILITY IN OBESE WOMEN WITH A FOCUS ON OOCYTE AND EMBRYO QUALITY

1. HOW TO IMPROVE FERTILITY

As clearly stated earlier, obesity has a significant effect on metabolic health and overall fertility, including oocyte and embryo quality and development. Therefore, more and more researchers emphasize the importance of optimizing fertility and safeguarding maternal health. Nowadays, a short internet search results in thousands of hits with many (non-scientific) advices on how to improve female fertility. However, there is a lack of uniform guidelines and there are still a lot of (fundamental) questions that need to be answered.

1.1. SUPPORTIVE EMBRYO CULTURE CONDITIONS ('POST' CONCEPTION CARE INTERVENTION)

Nowadays, obese women who suffer from fertility problems often decide to undergo ART as the next step in their fertility process (Talmor and Dunphy, 2015). However, research demonstrates that oocyte quality, developmental competence and resulting pregnancy chances in obese women undergoing IVF treatments are significantly lower compared to non-obese women (Pandey et al., 2010; Wittemer et al., 2000). In the most conventional way of ART, follicles grow *in vivo* by a pre-treatment with GnRH, after which oocyte retrieval takes place through ovum pick-up to start *in vitro* fertilization and culture. **This means that there is a possible intervention window during embryo culture to improve/repair/rescue embryo quality from a potential harmful maternal metabolically-compromised background**, as stated by Rizos et al. (2002) (Figure 1).

For example, in patients with recurrent production of highly fragmented IVF embryos, early transfer of zygotes to the uterus could rescue these zygotes and improve pregnancy and birth rates (Sermondade et al., 2012). Addition of antioxidants to the in vitro culture medium improved mitochondrial activity, gene expression and development in embryos of older female mice (Silva et al., 2015). Thus, it appears that modifying the culture conditions can be beneficial to certain embryos originating from compromised oocytes.

Given the limited success of IVF procedures, novel approaches are continuously tested with the aim of improving IVF outcomes. Therefore, using supportive embryo culture conditions, like **antioxidants**, have been introduced as a possible strategy to improve fertility in women subjected to ART fertility treatments (Xu et al., 2018) although the extents of its effect have not been fully elucidated. Antioxidants (AO) are scavenging molecules that neutralize ROS to prevent oxidative damage and cellular dysfunction (Agarwal et al., 2005). Several antioxidants are known to protect oocytes from ROS-induced damage. Recently, Budani et al. (Budani et al., 2017; Rodriguez-Varela and Labarta, 2020), summarized the effect of AO supplementation on embryo

developmental competence and quality. Based on information gained from experimental studies, AO supplementation may have beneficial effects on IVF outcomes in terms of quality and cryotolerance of in vitro produced embryos, together with positive effects on in vitro oocyte maturation and early embryonic development. However, further research is necessary to characterize the potential clinical value of supportive embryo culture conditions in human IVF treatments from patients with metabolic disorders. At the moment, coenzyme Q10 supplementation has shown to be a promising approach to rescue oxidative stress-induced damages. Some of the obesity-induced effects on murine oocytes were prevented by CoQ10 supplementation, with improvements seen in mitochondrial distribution, spindle formation, and chromosome alignment (Boots et al., 2016).
Very recently, a study was published investigating the reparative or rescuing effects of AO treatment during embryo culture of metabolically compromised bovine oocytes (Marei et al., 2019). In that study, Marei et al. (2019) revealed that the addition of MitoQ (an AO specifically targeting mitochondria) to the in vitro embryo culture medium reduced oxidative stress in bovine embryos derived from lipotoxicity induced metabolically compromised oocytes in vitro, leading to higher blastocyst rates and lower blastomere apoptosis.

During routine in vitro culture, media (such as synthetic oviductal fluid (Gardner et al., 1994; Tervit et al., 1972) contains different factors that provide essential support for the developing embryo. Previously, culture media were often supplemented with **serum** as it has been shown to improve embryo development by its embryotrophic factors and inactivation of embryotoxic agents (George et al., 2008; Mesalam et al., 2019). However, the presence of serum during culture has been linked to excessive lipid accumulation in the embryo through an increased lipid uptake from the medium and/or through disturbance of the mitochondrial metabolism (Abe and Hoshi, 2003; Abe et al., 1999; Ferguson and Leese, 1999; Kim et al., 2001). Besides lipid accumulation, serum also increases sanitary risks (including potential for Hepatitis B infections) and is suspected to contribute to the large offspring syndrome in cows through epigenetic modifications (Farin et al., 2001). It is responsible for accelerated kinetics of development, increased percentage of blastocysts obtained and higher total number of cells in blastocysts (Van Langendonckt et al., 1997).
Nowadays, serum is often replaced by **serum albumin** (bovine or human) during in vitro culture. Albumin is a large, high soluble protein that is found in the blood and it is the most prevalent protein in the mammalian reproductive tract (Kinsella and Whitehead, 1989). It acts as a carrier and may provide beneficial factors to the culture environment, including energy substrates, amino acids, and growth factors. However a major biological role of serum albumin is to scavenge ROS as it represents the major antioxidant in plasma (Roche et al., 2008). A big part of its antioxidant activity results from its ligand-binding capacities. Serum albumin binds free iron thus preventing its reaction to form a hydroxyl radical (Roche et al., 2008). A disadvantage of BSA is the

fact that its antioxidant capacity varies from one batch to another (Otsuki et al., 2013) and sanitary risks also exist (Guerin et al., 1997). Other synthetic macromolecules such as polyvinylpyrrolidone (PVP) are used in cell culture protocols (Eckert et al., 1998; Krisher et al., 1999), however, blastocyst development is significantly reduced in the absence of bovine serum albumin (BSA) (Carolan et al., 1995). Addition of BSA to the in vitro culture media also resulted in better cryosurvival and differential relative abundance of developmentally important gene transcripts compared to embryos cultured in serum-containing media (Rizos et al., 2003).

BSA is often added in combination with **insulin-transferrin-selenium** (ITS) during *in vitro* bovine culture. ITS is routinely used during embryo culture when serum-free media are used. When examining the effect of adding ITS during culture, multiple researchers reported an increase in blastocyst rate after oocytes maturation under standard conditions (Bowles and Lishman, 1998; George et al., 2008; Mesalam et al., 2019). These effects can be due to antioxidant properties of transferrin and selenium and/or due to the mitogenic and anti-apoptotic effects of insulin.

Transferrin and selenium are antioxidants and may neutralize ROS. Selenium prevents oxidative damage by operating at the GPx activity (Barnes and Sato, 1980). It promotes GPx activity which removes free radicals by converting H_2O_2 to H_2O (Sun et al., 1997). When iron is present, transferrin is able to remove iron from the media before its breakdown to a ferrous state (Gutteridge, 1989). Transferrin acts as an iron binding protein for transportation into the cells and as a detoxifying protein by removing toxic metals from the medium (Barnes and Sato, 1980). **It is not known whether the presence of these antioxidants during culture may compensate for or even cure the ROS effects induced at the level of the oocyte by an aberrant metabolic state (e.g. obesity) which may result in an enhanced development of embryos resulting from these oocytes.**

Insulin on the other hand can exert mitogenic and anti-apoptotic activities in bovine preimplantation embryos so supplementation of insulin to the embryo culture may increase rates of cleavage, blastocyst development and the total number of blastocyst cells by alleviating metabolic stress in developing embryos (Augustin et al., 2003).
A very recent study recovered two-cell and eight-cell embryos from obese and control mice and cultured them in medium supplemented with insulin until blastocyst formation. Expanded control blastocysts showed increased mean cell numbers an increased inner-cell-mass/trophectoderm ratio and a reduced level of apoptosis. In contrast, embryos originating from obese mice were significantly less sensitive to insulin; no difference was recorded in any tested variable between the embryos exposed to insulin and those cultured in insulin-free medium. Furthermore, there was a significant increase in the amount of insulin receptor transcripts in blastocysts recovered from obese dams (M et al., 2021). **Based on these results, it seems that**

there is a significant impact of the maternal metabolic state on the mitogenic and anti-apoptotic responses of preimplantation embryos to insulin.

Can the addition of anti-apoptotic, antioxidative and mitogenic factors (namely, Insulin-Transferrin-Selenium (ITS) or serum) to embryo culture media rescue developmental competence and quality of embryos derived from metabolically compromised oocytes?

1.2. IS IT BETTER TO IMPROVE OOCYTE QUALITY BEFORE CONCEPTION BY APPLYING PREVENTIVE STRATEGIES? IS THIS POSSIBLE?

Based on the current available literature, supplementing the in vitro culture medium, so-called post-conception care intervention, might be an interesting option to improve oocyte developmental competence and resulting blastocyst quality in metabolically-compromised women (Figure 1). However, after in vitro culture, the possible rescued blastocyst will be transported back into the aberrant in vivo maternal micro-environment. Furthermore, obese woman are often not allowed access to ART by their fertility specialist before they reach a certain amount of weight loss or BMI, as weight loss has been shown to improve their metabolic health and fertility. This means that assisted reproduction and possible rescue during in vitro culture is not an option for them**. In addition to that, prevention instead of rescuing might be a better option to improve oocyte developmental competence and quality before conception** (Figure 1). A preconception care intervention strategy might impact early in the timeframe or before folliculogenesis and prevent the development and recovery of bad quality oocytes.

In fact, many studies have shown that the period when the oocyte is growing and maturing in the ovarian follicle and the first few weeks of embryo development are particularly sensitive to changes in the maternal environment.

One possibility to protect the oocyte from detrimental effects, is to provide the obese women with **dietary** (antioxidant (AO)) **supplements** before conception. Research using animal models showed that AO supplementation is reflected in the composition of the FF (De Bie et al., 2016; Dobbelaar et al., 2010). At oocyte level, the lipotoxic effects of saturated fatty acids during in vitro bovine maturation could be reversed with the addition of alpha linolenic acid (ALA), mainly by its protective effects at the level of the bovine cumulus cells (Marei et al., 2017). However, Trolox treatment simultaneous with the metabolic stress insult during bovine IVM or in vitro culture (IVC) could not improve embryo developmental competence (De Bie et al., 2021). In contrast, melatonin supplementation to high-fat diet-induced obese mice markedly reduced oxidative stress in oocytes (Han et al., 2017). In the fertility clinic, an omega-3 fatty acid

rich preconception diet in women has been associated with improved embryo morphology before transfer (Hammiche et al., 2011).
To date, there are a few studies regarding this approach, but no uniformity in the results is seen (Collins and Rossi, 2015; Youssef et al., 2015).

Nowadays, obese women who are having issues with getting pregnant are advised by their fertility specialist to lose weight. Advising obese women to lose weight before conception might not only improve their metabolic health and the response to gonadotrophin treatment but may also positively impact their oocyte quality and overall fertility. However, to date there is no evidence-based strategy to guide preconception weight loss (Matusiak et al., 2014). Up until now, Belgian fertility centers determine their own criteria while other countries will not allow facilitated treatment unless the patient's BMI is below a certain level (Legro, 2016).
Weight loss can be achieved through different methods. **Bariatric surgery** is considered as one of the most effective therapies to treat severe obesity (Legro, 2017), leading to a more pronounced weight loss compared to nonsurgical measures such as behavioural and pharmacological interventions (Merhi, 2009). Although evidence suggests that bariatric surgery leads to improvements in menstrual irregularities, ovulation rate and pregnancy outcomes (Maggard et al., 2008), it is considered an invasive and expensive technique linked with a high morbidity related to surgical complications (Legro, 2017). In addition, pregnancy must be postponed with ±12 months after surgery in order to adapt to nutritional deprivation and to avoid intrauterine fetal growth retardation (Merhi, 2009). This can be conflicting with an advanced maternal age. Next to surgery, a number of **drugs** are available for the treatment of obesity. However, most of these drugs lack data about direct reproductive toxicity in women (Legro, 2017).
Due to these limitations, **lifestyle interventions**, such as dietary modifications (optimization of composition and quantities) and regular physical activity, are still considered the first step in preconception care interventions aiming at improved metabolic health and thus fertility (Best et al., 2017; Legro, 2017).
Recent human studies in obese women revealed that increased **physical activity** alone without any weight loss intervention is sufficient to achieve significant improvement in metabolic health. Boudoures et al. (2016) studied ad libitum exercise wheels in obese mice for six weeks and reported improved, but not restored, metabolic health and oocyte quality. Last but not least, the impact of **dietary interventions** has been studied extensively in human studies. It is an easy accessible, non-expensive method to lose weight and the preconception care intervention of choice for this thesis. Therefore, more detailed information, emphasizing the relevance and importance of these lifestyle interventions, will be discussed below.

1.3. DIETARY INTERVENTIONS AS A PRECONCEPTION CARE INTERVENTION

1.3.1. IMPACT ON METABOLIC HEALTH

Although a limited amount of weight loss (3-5%) can already partly improve metabolic health in humans, more significant weight losses are needed for complete recovery (Clamp et al., 2017; Jensen et al., 2014). A significant correlation was shown between the amount of weight loss during three months of dieting and the decrease in fasting plasma glucose over the same time period in type 2 diabetic people (Group, 1990). A meta-analysis human study reported that a weight loss of 1 kg decreases serum cholesterol by 2.28 mg/dL, LDL cholesterol by 0.91 mg/dL, and triglycerides by 1.54 mg/dL (Dattilo and Kris-Etherton, 1992).

Such weight loss can be induced by diet normalization, where a change in diet composition is made by switching from a Western type diet to an ad libitum healthy, control diet. Reynolds et al. (2015) showed in obese mice undergoing diet normalisation for a period of eight weeks that body weight and metabolic health parameters returned to normal. However, another, more severe method of diet change is a caloric restriction diet. Generally, a caloric restriction (CR) diet refers to a 30-40% decrease in daily caloric intake and to a more healthy diet composition (Aksungar et al., 2017). Switching to this diet was effective in improving body composition, blood pressure, plasma lipids and insulin sensitivity in metabolically compromised individuals (Aksungar et al., 2017; Andersen and Fernandez, 2013; Vangoitsenhoven et al., 2018). Obese mice that switched to a CR diet showed restored leptin, adiponectin and cholesterol concentrations. In addition, parameters involved in liver function (ALT and AST) were also normalized (Cui et al., 2013; Vangoitsenhoven et al., 2018). However, maintaining this severe calorie restricted diet for a long time has proven to be very challenging. In addition, extreme weight loss results in lipid mobilization leading to more free fatty acids in the bloodstream (Chusyd et al., 2016). As explained before, these high concentrations of free fatty acids are reflected in the follicular fluid and might hamper oocyte quality and subsequent developmental competence through lipotoxicity associated pathways. Severe weight loss is often seen in high producing dairy cows during the first weeks after calving. The high energy demand for milk cannot be compensated for through dietary intake. The resulting negative energy balance coincides with massive lipid mobilisation and it has clearly been shown that this can directly hamper oocyte quality (for overview, see Leroy et al. (2015)). To our knowledge, very limited information is known regarding the impact of (severe) weight loss on oocyte quality in obese women. Does significant weight loss, as a preconception care intervention, have a positive impact on oocyte quality and/or might diet normalisation be sufficient to optimize fertility outcome?

1.3.2. IMPACT ON FERTILITY

Recently, two very large human randomized controlled trials (RCT) were performed, not reporting any significant improvements in pregnancy rates following weight loss. Mutsaerts et al. (2016a) randomized more than 600 obese infertile women to a six month lifestyle intervention before 18 months of infertility treatment or to immediate infertility treatment. Although the average weight loss in the intervention group was 4,4 kg, no improvements in live birth rates were reported. This is in accordance with the results of another large RCT in which 317 obese women scheduled for IVF were randomized into a control group and an intervention group (Einarsson et al., 2017a). Patients in the intervention group were subjected to a low-calorie liquid formula diet (880 kcal/day) for 12 weeks resulting in an average weight loss of 6,6 kg. The results of this study failed to demonstrate improved live birth rates via IVF after preconception weight loss via dietary interventions. However, a significant increase in spontaneous pregnancies was recorded. Several other human studies indicate that weight loss via dietary and lifestyle interventions prior to ART lead to an improved fertility, displayed by a significant increase in pregnancy and live birth rates in obese women (for a review: see (Best et al., 2017; Price et al., 2019; Sim et al., 2014)). Furthermore, ovulation rates and menstrual irregularities were improved and the number of ART cycles and cycle cancellation rates decreased (Sim et al., 2014).

As indicated above, **results of human lifestyle intervention studies are often inconsistent**. These studies are often confronted with a lot of confounders, ranging from lack of sufficient power to a possible effect of the patients' clinical history and societal and lifestyle background. As a complete switch from an obesogenic diet to a healthy lifestyle is often not feasible, high drop-out rates are present. They also often lack a control group (Cancer, 2005).

Current clinical guidelines recognize the importance of preconception weight loss for obese patients but there is **no evidence-based strategy to guide this preconception weight loss**. Dietary interventions are often recommended as the first line therapy. Yet, general instructions do not mention the amount, the rate or method of weight loss that is most suitable to improve reproductive outcomes (Matusiak et al., 2014; Price et al., 2019). Price et al. (2019) concluded that a modest weight loss of 3-5% improves fertility and causes no harm to the offspring (Glazer et al., 2004; Mutsaerts et al., 2016a; Pasanisi et al., 2001). These data are substantiated by other studies, who reported that 5% weight loss is already sufficient to re-establish normal ovulation in obese women (Best and Bhattacharya, 2015; Kiddy et al., 1992; Kort et al., 2014; Ryan and Yockey, 2017). However, the impact of substantial weight loss is less clear. Currently there are only a few small human trials investigating the impact of a very low energy diet (VLED) on pre-pregnancy weight loss but no information regarding the impact of VLED-induced weight loss on pregnancy outcomes (Price et al., 2019). Currently, excessive weight loss in a short period of time has been discouraged in clinical settings as it has been reported to negatively affect the outcome of ART (Legro, 2017; Tsagareli et al., 2006) and may

negatively impact the long-term health of the offspring (Matusiak et al., 2014). Modest weight loss, due to diet normalisation, might be a more suited method (Price et al., 2019). **However there is a lack of clear evidence-based research confirming this. Before we apply preconception guidelines, it is important to obtain more fundamental scientific insights**.

To our knowledge, **very limited information is available regarding the specific impact of a dietary intervention (diet normalization or a caloric restriction diet) on oocyte quality**.
Reynolds et al. (2015) investigated in an inbred mouse model whether the adverse effects on oocyte quality caused by a western type diet, could be reversed by diet normalisation for a period of eight weeks and concluded that oocyte quality remained unaltered. This included meiotic spindle and chromosomal abnormalities, abnormal lipid distribution, mitochondrial damage and abnormalities in mitochondrial distribution. These data indicate that, although metabolic health returned to normal, the aberrant oocyte quality was not reversed. A small pilot study reported a negative outcome on *in vitro* fertilization rates after exposing obese women to a short term very low calorie diet of 4-6 week, resulting in an average weight loss of 5.6 kg (Tsagareli et al., 2006). It is not known if a longer duration of dietary restrictions before conception might be more efficient to improve oocyte quality.

These data clearly indicate that there is a lack of crucial scientific information on the impact of preconception dietary interventions on oocyte quality (Figure 1)**.**
As human studies are difficult to set up and are confronted with several limitations, there is a clear need for more fundamental, well-controlled research. Therefore, using a strategically designed animal model to investigate the impact of dietary interventions on metabolic health and oocyte quality, might provide us with fundamental insights.

Besides the amount of weight loss, **the most optimal interval of dietary intervention to induce an improvement in oocyte quality remains unknown**. The duration of published non-surgical weight loss studies varies from 3 to 6 months but the longer duration studies did not necessarily achieve more weight loss (Price et al., 2019). This might suggest that weight loss interventions should occur over a well-defined short period.
Another important aspect to keep in mind when focusing on the best time period for preconception care intervention, is the length of the folliculogenesis (figure 1). As clearly stated above, the negative impact of obesity might already have happened early in the process of folliculogenesis or even at the level of the dormant pool of primordial follicles. This further builds on the hypothesis stated by Britt (1992), 30 years ago. It was suggested that the developmental competence of the oocyte is determined by its biochemical microenvironment during a long period of follicular growth before

ovulation. Primary follicles exposed to adverse metabolic health conditions during this period, are likely to contain an inferior oocyte, which will then be ovulated. This might indicate that a preconception intervention should last at least one cycle of folliculogenesis (which is 3-4 months in human).
In addition, it is important to emphasize that this intervention period should be as short as possible to avoid the potential negative effects of advancing maternal age on ovarian reserve, oocyte quality and reproductive capacity (Lan et al., 2017; Sim et al., 2014).

Are diet normalization or caloric restriction, as preconception care interventions, able to improve metabolic health and oocyte quality in obese individuals?

What is the most optimal timeframe of intervention to induce improvements in metabolic health and fertility?

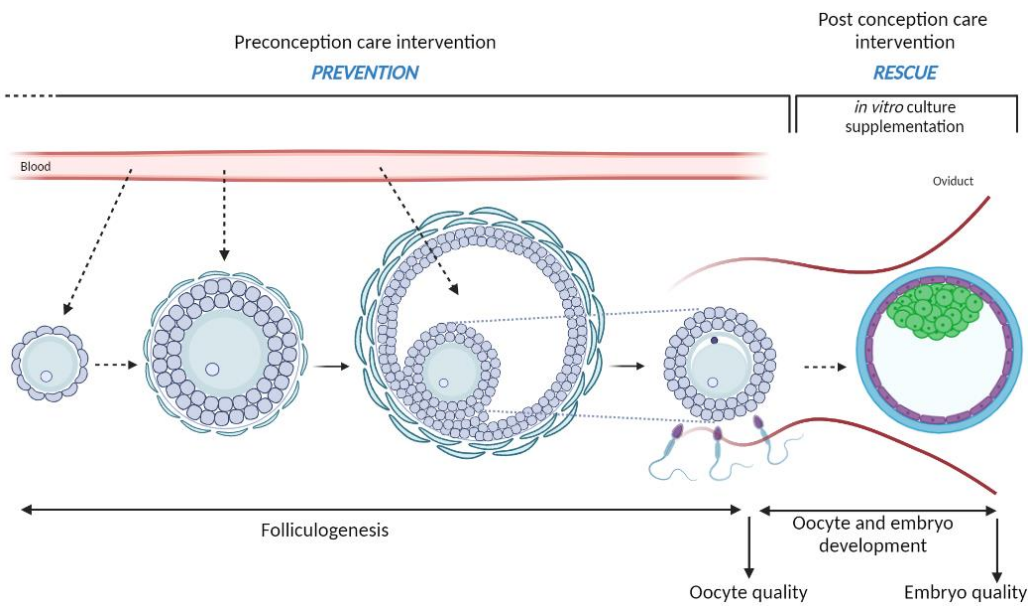

Figure 1. Possible impact windows of pre- or postconception care interventions on fertility, and more specifically during folliculogenesis, oocyte and embryo development. (Made with Biorender)

2. MODELS TO STUDY IMPACT OF OBESITY AND THE POWER OF PRECONCEPTION CARE

2.1. IN VIVO *VS.* IN VITRO

In vitro studies are a vital tool in research to gain mechanistic insights. It allows a tight control of the chemical and physical environment and does not require ethical approval. However, as researchers, we have to be critical when translating the expertise generated using in vitro research to the more complex in vivo situation.

The first part of this thesis focuses on in vitro embryo production (IVP) using oocytes matured, fertilized and cultured under in vitro conditions. This has been a common and valid practice for research purposes for many years. However, is it known that there are differences between in vitro and in vivo produced embryos. Embryos obtained through IVP exert differences in their phenotypic features compared to in vivo derived embryos (Khurana and Niemann, 2000; Rizos et al., 2002). Nevertheless, the IVP model allows the researcher to focus on the impact of one specific factor on important pathways and significantly increases mechanistic insights. This is not possible when performing in vivo research. Furthermore, to improve fundamental knowledge on the possible impact of post conception care treatments, the best option is to investigate this in vitro. This way, any possible confounders due to a different (maternal) history of the oocyte or environment will be eliminated. Therefore, in vitro research has extensively been used in research to understand what happens during embryo development, both in normal and aberrant conditions. Of course, extrapolating these results must be done with caution.
In addition, as stated before, obese women often undergo ART due to their reduced fertility. As a result, the in vitro culture period is a possible window to increase their pregnancy chances, making the use of an in vitro model is very relevant to study the concepts of the potential of in vitro embryo culture medium supplementation.
In vivo research is performed in the context of a living organism and entails the full complexity of the human or animal body. As diet exposure and an aberrant metabolic condition has both direct and indirect effects of the associated metabolic changes on the oocyte, zygote and embryo, in vivo research gives researchers the complete picture. The second part of this thesis investigates the impact of different preconception care interventions in an obese in vivo mouse model, focusing both on metabolic health and fertility.

2.2. ADVANTAGES AND APPLICATIONS OF BOVINE AND MURINE RESEARCH MODELS AND THE IMPORTANCE OF THE MOST-SUITED MOUSE STRAIN

Studying the relation between female (in)fertility and metabolic disorders like obesity is often hindered by the limitations associated with research on human material. With regards to oocyte and embryo quality, human data can only provide us with information regarding morphological evaluation and developmental competence of oocytes and embryos in relation to the metabolic status of the mother. More in depth

research requires ethical approval and human samples are scarce and difficult to obtain. Therefore, researchers mostly rely on animal models. In the experimental studies described in this thesis, two animal models were used, namely a bovine and a murine model. The **bovine model** is often used for fertility research as there are many similarities between bovine and human follicular physiology, oocyte maturation and early embryo development (Adams et al., 2012; Menezo and Herubel, 2002). Bovine oocytes are retrieved from slaughterhouse ovaries and give researchers the opportunity to investigate in vitro embryo production in more detail. As said before, dairy cows are post-partum often confronted with negative energy balance, a disturbed metabolic state (Leroy et al., 2008), which also makes these cows a relevant model to study the link between maternal metabolic disorders and fertility. However, the bovine model is often not the best choice for long-term in vivo research focusing on diet-induced obesity and fertility. Cows are large animals, housing is complex and well-controlled long-term in vivo research is practically very challenging and expensive. Furthermore, they are mono-ovulatory animals and have a long gestation period of 9 months.

Therefore, a **mouse model** might be a better alternative for in vivo research regarding this topic. Rodents are the most commonly used animals to investigate metabolic syndrome (Wong et al., 2016). Mice are small, housing is easy and they have a short gestational period. However, it is important to keep in mind that differences exist between human and rodent reproductive physiology as the period of oocyte recruitment is much shorter in mice than in women (Clarke, 2017) and the mouse is a poly-ovulatory animal. In addition, the correct choice of a mouse **strain** for your research is of vital importance (Figure 2). To investigate the impact of obesity on metabolic health and fertility, mouse strain options are almost unlimited, going from specific monogenic knock-out strains to different polygenic inbred or outbred strains. The ob/ob mouse strain is a commonly used strain, which becomes obese due to mutations in the gene responsible for leptin production, resulting in leptin deficiency. Next to obesity, these mice display hyperglycemia, glucose intolerance, and elevated plasma insulin concentrations. However, they are often subfertile, making them less suited for research where fertility takes a central role in the scientific hypothesis (Charles River). Inbred mice are genetically homogenous and there is little variation or heterogeneity. As these mice are all genetically similar, this may reduce experimental variability and increase reproducibility. Currently, the B6 strain is the most commonly used polygenic strain for studies focusing on the impact of obesity/high fat(-high sugar) diet exposure on metabolic health and fertility. It was the first strain to have its genome completely sequenced (Gregory et al., 2002) and is relatively susceptible to diet-induced obesity (Champy et al., 2008; Collins and Rossi, 2015). However, different C57BL/6 substrains exist. So far, many obesity-related research used the C57BL/6J strain. The Jackson laboratory reported that there are ten thousands of entries in Pubmed that use the J-substrain while only thousands of entries use the C57BL/N substrain (The Jackson Laboratory, 2019). However, with regards to obesity research,

the correct choice of the C57BL/6 substrain is very important. C57BL/6J mice carry the Nnt mutation, a gene that encodes a mitochondrial protein involved in β-cell mitochondrial metabolism, ultimately resulting in impaired glucose-mediated insulin secretion (Freeman et al., 2006). In contrast, the C57BL/6N substrain does not have the Nnt deletion and might therefore be more suited to investigate the impact of obesity on metabolic health.

However, the C57BL/6 strain is characterized by low fertility, small litter size and cannibalism of pups, making this strain less suitable for research focusing on fertility. In addition, relying on an inbred strain might limit the translation to human (outbred) physiology (Ellacott et al., 2010). Outbred mice are bred to maximize genetic diversity and heterozygosity within a population (Ellacott et al., 2010). Just like the human population, no two individuals are genetically identical within an outbred strain. They also tend to be better breeders than inbred mice. More and more scientists agree that there is a need for experiments comparing variations between commonly used inbred and outbred rodents (Ahima et al., 1996; Ellacott et al., 2010). A recent paper even concluded that outbred mice might be a better subject for biomedical research (Tuttle et al., 2018). To our knowledge, literature comparing the effect of high fat diet on fertility in commonly used inbred and outbred strains is not available. However, research focusing on the impact of a different genetic background on reproductive parameters all concluded that effects on fertility are strain dependent and that strain differences should be considered when interpreting reproductive experiments using mouse models (Ibanez et al., 2005; Kaleta, 1977). In addition, a study investigated variation in experimental endpoints relevant to diabetes and obesity research, hereby focusing on insulin dosing and metabolic effects. They showed that phenotypic variability in most of the typical metabolic readouts was comparable between inbred and outbred strains of mice (Jensen et al., 2013). Swiss mice have been shown to be susceptible to diet-induced obesity (Alexenko et al., 2007; Rosenfeld et al., 2003) as they develop hypercholesterolemia, obesity, hyperinsulinemia and insulin resistance. In addition, the Swiss outbred strain is perfectly fertile and displays excellent nurturing abilities.

Therefore, using an outbred strain, like Swiss mice, might be more relevant when investigating the impact of obesity on metabolic health and fertility as they mimic the genetic diversity in the human population.

Does high fat diet-induced obesity have a similar effect on metabolic health and oocyte quality of outbred Swiss mice compared to, the often used, inbred C57BL/6N mouse?


C57BL/6 mouse (inbred)	Swiss mouse (outbred)
Genetically homogenous - Reduced experimental variability - Increased reproducibility	Genetic diversity - Increased experimental variability - Decreased reproducibility
Limit translation to human (outbred) physiology	More relevant to human physiology
Susceptible to diet-induced obesity → Currently most commonly used	Susceptible to diet-induced obesity
Low fertility	Highly fertile - Good breeders
Small litter size	Big litter size
Pup cannibalism	Excellent nurturing abilities

Figure 2. Characteristics of inbred versus outbred mice

2.3. DIET-INDUCED OBESITY: HIGH FAT VERSUS HIGH FAT-HIGH SUGAR DIET

There are many ways to induce obesity in rodent models. From a scientific and ethical point of view, it is important that both the phenotype and pathogenesis of the animal's condition resemble the human disease examined as close as possible. Looking at the polygenic nature of the humane metabolic syndrome, it seems that studies examining the monogenic or pharmacologically induced obesity models must be interpreted with care. To answer the research questions proposed in this thesis, a mouse model was used in which obesity was induced by a high fat or a high fat-high sugar diet, the so called **diet-induced obesity (DIO) models**. These models better demonstrate the interaction between disease, environment and genetics (reviewed by Fuchs 2018 (Fuchs et al., 2018)).

Reviews published by Panchal and Brown (2011) and Wong (2016) (Panchal and Brown, 2011; Wong et al., 2016) compared different diets used to induce metabolic syndrome in rodent models. Rodents exposed to a **high fat (HF) diet** developed cardiac and renal complications. In addition, body weight increased significantly and hyperglycaemia and impaired glucose tolerance was reported. Diets consisting of lard as major fat source also increased liver triglyceride, plasma triglyceride, free fatty acid and insulin concentrations. Diets consisting of beef tallow as fat source also increased plasma insulin, lipid and leptin concentrations in addition to hepatic steatosis. Beef tallow is characterized by a more saturated fatty acid composition compared to lard. It contains more palmitic and stearic acid, the two most predominant fatty acids in serum and FF. Interestingly, the in vitro bovine oocyte exposure model is based on the addition of these NEFAs to mimic the state of metabolic stress. This in vitro NEFA exposure model has clearly indicated significant impacts on lipotoxicity pathways in the oocyte (Leroy

et al., 2017). Therefore, **if we would like to dissect the specific effects of lipotoxicity on oocyte quality in an in vivo model, then most probably, HF diets should be selected.** This also validates the use of beef tallow as fat source in the high fat diet. **Panchal and Brown (2011) concluded that although a high fat diet induces most of the symptoms of human metabolic syndrome in rodents, it does not resemble the diet causing metabolic syndrome and associated complications as the human diet is more complex than a high fat diet alone.**

A diet high in fat and carbohydrates might mimic the Western Type human diet more closely due to the addition of sugar. The most common carbohydrates are **fructose** and sucrose (Panchal and Brown, 2011; Wong et al., 2016). Over the past years, is has been suggested that high fructose consumption is related to the development of the metabolic syndrome (Aydin et al., 2014; Pereira et al., 2017). It reduces satiety hereby increasing caloric intake (Pereira et al., 2017). Based on the knowledge regarding fructose metabolism, it is believed to be superior for inducing metabolic syndrome compared to glucose or starch. High intake of dietary fructose leads to increased body weight, adiposity, hypertriglyceridemia, hyperlipidaemia, hypertension, glucose intolerance and decreased insulin sensitivity in animal models (Johnson et al., 2007; Schulze et al., 2004). Fructose has been traditionally used to develop DIO rodent models (Crescenzo et al., 2015; Panchal and Brown, 2011; Panchal et al., 2011). Furthermore, studies investigating the impact of fructose consumption on fertility reported a 30% decrease in pregnancy rates in female mice after six weeks on a 60% fructose diet (Saben et al., 2016a). Female rats on a high fructose diet has longer oestrus cycles than control-fed animals (Ko et al., 2017).

Diets rich in both fructose and fat increased body weight and plasma concentrations of triglycerides, cholesterol, FFA and leptin. It also caused hyperinsulinemia, insulin resistance, impaired glucose tolerance, increased abdominal fat deposition, hepatic steatosis and inflammation. **Panchal and Brown (2011) concluded that since high-carbohydrate high-fat diet-fed rodents develop all the complications present in human metabolic syndrome and the diet is similar to Western type human diets, this model is probably the best to study the human metabolic syndrome.** Moreno-Fernandez et al. (2018) compared metabolic syndrome alterations in a high fat diet to a high fat-high glucose (HF/HG) and a high fat-high fructose diet (HF/HF). Animals from all three diets showed increased fasting plasma glucose and insulin levels although the greatest increase was present in rats on the HF/HF diet. In addition, both triglyceride and cholesterol levels were increased. In addition, Della Vedova et al. (2016) also compared the symptoms associated with a high fat diet (chicken fat) to those linked to a high fat-high fructose diet. They reported that mice on a HF/HF diet had more caloric intake and gained more weight. In addition, they showed higher cholesterol, fasting glycemia, insulin resistance and hypertension. They also showed steatohepatitis and systemic oxidative stress and inflammation.

To our knowledge, research comparing the effects of different diets, used to induce metabolic syndrome, on fertility and more specifically oocyte quality, is very limited. Although, Fabozzi et al. (2021) summarized the impact of a high fat or a high fat/high sugar diet on oocyte mitochondrial function, he did not compare or discussed possible differential effects elicited by these two different diet exposure models. **Therefore, more knowledge regarding this topic is needed.**

Does a high fat diet elicit different effects on metabolic health and, more importantly, fertility than a high fat/high sugar diet? Furthermore, if preconception care interventions are applied, do these interventions yield the same beneficial effects?

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CHAPTER 3: AIMS OF THE STUDY

AIMS OF THE STUDY

More and more research confirms the significant impact of metabolic health disorders (like obesity) on female fertility. More specifically, the importance of reduced oocyte and embryo quality in this subfertility problem together with the underlying mechanisms have been studied in detail. The oocyte is directly affected by alterations in its environment leading to (most often) lipotoxicity associated oxidative stress and cellular dysfunctions. As a result, there is a growing focus towards research investigating possible interventions to improve fertility in metabolically compromised women. **In general, we hypothesized that pre- and/or post-conception care interventions may improve metabolic health and fertility in obese, metabolically compromised women**. Such strategically planned intervention is hypothesized to prevent damage or to improve and/or recover oocyte quality leading to an optimized oocyte developmental competence and restored fertility.

To test the hypothesis of oocyte quality recovery during the **post-conception** window, it was important to first set-up an in vitro experiment to investigate this biological concept. For this, we used an established in vitro bovine exposure model where metabolically compromised oocytes were used, resulting from high NEFA exposure during maturation (as seen in metabolically compromised women). Therefore, we aimed to investigate:

1) whether supportive in vitro embryo culture conditions supplemented with anti-apoptotic, antioxidant and mitogenic factors such as serum or insulin-transferrin-selenium can rescue the developmental capacity of metabolically compromised bovine oocytes and can restore the quality of the resulting embryos (CHAPTER 4).

In vivo research investigating the impact of obesity on metabolic health and fertility is often performed in **mice.** Nowadays, the inbred C57BL/6 mouse is often used in diet-induced obese mouse models. However, inbred strains are characterized by a reduced fertility and might limit translation to human (outbred) physiology, especially when fertility is the research focus. Therefore, using an outbred strain might be more relevant to investigate the impact of obesity on metabolic health and fertility. Therefore, we aimed to investigate:

2) if high fat diet-induced obesity has a differential effect on fertility and more specifically on oocyte quality in the outbred Swiss mouse, compared to the routinely used inbred C57BL/6N mouse strain (CHAPTER 5).

Next to a post-conception care intervention, where the newly formed embryo originating from a compromised oocyte, is “treated”, more prevention oriented research investigating the impact of **preconception care interventions** (PCCI) is gaining more and more attention. Of these methods, lifestyle interventions, like diet

improvements, are considered as the first step. These interventions aim to result in weight loss and significant improvements in metabolic health. However, results of human lifestyle intervention studies focusing on the impact on fertility or on oocyte quality more specifically, are often inconsistent, resulting in a lack of scientifically-substantiated guidelines to be implemented in the clinic. Furthermore, such interventions span over a period of many months or even years and may thus conflict with the impact of an advancing maternal age. Therefore, using an (outbred) mouse model to study the impact of strategically designed dietary interventions is necessary to provide us with fundamental knowledge. Not only the choice of the dietary intervention strategy but also the **most optimal length of the intervention period** remains unknown when it comes to improvement of oocyte quality and fertility. Therefore, we aimed to investigate:

3) whether the efficiency of a preconception care intervention (PCCI) in high fat diet-induced obese outbred mice to improve metabolic health, oocyte quality and fertility depends on the method of diet change (diet normalization or caloric restriction) and on the duration of that intervention (CHAPTER 6).

Evidence is emerging that in our Western society, next to the detrimental effect of high, especially saturated fat diets, the additional impact of a high **sugar content** (and especially high fructose) on metabolic health and fertility cannot be ignored. Therefore, as a next step, we aimed to investigate:

4) whether additional or differential negative effects of a high fat/high sugar diet on metabolic health and oocyte quality could be detected compared to the findings in CHAPTER 6. (CHAPTER 7A)
5) whether the efficiency of a diet normalization or dietary restriction to improve metabolic health and oocyte quality in high fat/high sugar diet-induced obese outbred mice yielded different results compared to the findings in CHAPTER 6. (CHAPTER 7A)
6) the in depth cell biology functions at the proteome level affected by such a high fat high sugar diet and how these cellular pathway deviations may be altered again by the preconception care interventions. (CHAPTER 7B)

CHAPTER 4:

SUPPORTIVE EMBRYO CULTURE CONDITIONS

RESCUE POTENTIAL OF SUPPORTIVE EMBRYO CULTURE CONDITIONS ON BOVINE EMBRYOS DERIVED FROM METABOLICALLY COMPROMISED OOCYTES

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1. ABSTRACT

Elevated non-esterified fatty acid (NEFA), predominantly palmitic acid (PA), concentrations in blood and follicular fluid are a common feature in maternal metabolic disorders such as obesity. This has a direct negative impact on oocyte developmental competence and the resulting blastocyst quality. We use NEFA-exposure during bovine oocyte in vitro maturation (IVM) as a model to mimic oocyte maturation under maternal metabolic stress conditions. However, the impact of supportive embryo culture conditions on these metabolically compromised zygotes are not known yet. We investigated if the addition of anti-apoptotic, antioxidative and mitogenic factors (namely, Insulin-Transferrin-Selenium (ITS) or serum) to embryo culture media would rescue development and important embryo quality parameters (cell proliferation, apoptosis, cellular metabolism and gene expression patterns) of bovine embryos derived from high PA- or high NEFA-exposed oocytes when compared to controls (exposed to basal NEFA concentrations). ITS supplementation during in vitro culture of PA-exposed oocytes supported the development of lower quality embryos during earlier development. However, surviving blastocysts were of inferior quality. In contrast, addition of serum to the culture medium did not improve developmental competence of PA-exposed oocytes. Furthermore, surviving embryos displayed higher apoptotic cell indices and an aberrant cellular metabolism. We conclude that some supportive embryo culture supplements like ITS and serum may increase IVF success rates of metabolically compromised oocytes but this may increase the risk of reduced embryo quality and may thus have other long-term consequences.

2. INTRODUCTION

Maternal metabolic disorders like obesity are known to affect reproductive physiology, ultimately leading to a disappointing fertility. Incidences of these metabolic health disorders are dramatically increasing worldwide and have been strongly linked to a significant loss of reproductive capacity (2015; WHO, 2006).

Maternal metabolic disorders are associated with upregulated lipolysis and elevated non-esterified fatty acid (NEFA) concentrations in the blood and in the ovarian follicular fluid (FF) (Jungheim et al., 2011a; Leroy et al., 2005; Robker et al., 2009; Valckx et al., 2012). It has been shown that elevated NEFA concentrations in the FF directly affects oocyte developmental capacity (Leroy et al., 2005), and has been associated with poor cumulus-oocyte-complex morphology in humans (Jungheim et al., 2011b). Pasquali et al. (2003) reported a significant reduction in oocyte quality of obese patients. When FF of obese patients was added during bovine in vitro oocyte maturation (IVM), oocyte developmental competence and embryo quality were negatively affected (Valckx et al., 2015). The predominant NEFAs that increase during metabolic disorders are the lipotoxic saturated palmitic (PA; 16:0) and stearic (SA; 18:0) acids and the mono-unsaturated oleic (OA; 18:1) acid (Valckx et al., 2014). A retrospective study demonstrated that pregnancy results after intracytoplasmic sperm injection (ICSI) were negatively associated with the PA concentration in the FF upon oocyte pick-up (Mirabi et al., 2017). Furthermore, the increased concentration of PA in human FF was linked to lower oocyte nuclear maturation and embryo cleavage rates (O'Gorman et al., 2013). In animal models, exposure to a mixture of predominant NEFAs (PA, SA and OA) or to PA only at pathophysiological concentrations during IVM increased apoptosis in cumulus cells (Leroy et al., 2005), altered expression of genes related to oxidative stress in oocytes, reduced embryo development to the blastocyst stage, reduced blastocyst cell numbers and increased blastocyst apoptotic cell indices compared to NEFA-free controls (Aardema et al., 2011; Marei et al., 2017b; Van Hoeck et al., 2013; Van Hoeck et al., 2011).

There is a growing evidence that oxidative stress plays a key role in the pathogenesis of reduced oocyte developmental competence under lipotoxic conditions. The affected oocytes have higher reactive oxygen species (ROS) content and exhibit upregulation of reduction-oxidation (REDOX)-related genes (Burton et al., 2003; Marei et al., 2019a; Van Hoeck et al., 2013). Importantly, it has been demonstrated that embryos derived from PA-exposed oocytes exhibited persistent oxidative stress and loss of mitochondrial activity despite in vitro culture in standard, fatty acid-free conditions (Marei et al., 2019b). In addition, the produced blastocysts, resulting from high NEFA-exposed oocytes, displayed a less active oxidative metabolism, altered DNA methylation and transcriptomic fingerprints compared to blastocysts exposed to basal NEFA concentrations during IVM (Desmet et al., 2016; Van Hoeck et al., 2011). Together, these data illustrate the negative impact of exposure to lipotoxic conditions

during maturation on early embryo development and on embryo quality (Marei et al., 2017a; Van Hoeck et al., 7-8 Sept. 2012).

On the other hand, the microenvironment in which early embryos grow also has a significant impact on developmental rate and quality. Rizos et al. (2002) stated that the in vitro culture (IVC) system is a major determinant of blastocyst quality, whereas the developmental capacity itself is mainly determined during maturation. Nevertheless, it was illustrated that transferring in vitro produced embryos to sheep oviducts yielded significantly higher rates of embryonic development compared to those cultured in simple in vitro conditions (Lazzari et al., 2010). In humans, early transfer of zygotes to the uterus could rescue embryo development and improve pregnancy and birth rates in patients with recurrent production of highly fragmented in vitro fertilization (IVF) embryos (Sermondade et al., 2012).

Routine in vitro embryo production (IVP) protocols use culture media (such as synthetic oviductal fluid (SOF)) that provide a supportive microenvironment during early embryo development. This can be beneficial as it may provide a recovery potential to the growing embryo originating from a low-quality oocyte. This suggests that supportive embryo culture conditions, for example, enriched with antioxidants, may be used to reduce oxidative stress and prevent aggravation of cell damage during in vitro embryo development when oocytes are collected from metabolically compromised obese patients.

Culture media are often supplemented with serum which is known to improve oocyte developmental competence due its embryo-trophic effects and due to its protective effects against embryo-toxic agents (Bavister, 1995; George et al., 2008; Mesalam et al., 2019). To date, serum is still used to increase IVP efficiency in some bovine IVP laboratories both for research and commercial purposes. However, serum supplementation has been shown to have several disadvantages such as excessive lipid accumulation in the embryo, various alterations of embryo morphology, ultrastructure and kinetics of development (Van Langendonckt et al., 1997), increased sanitary risks (including potential for Hepatitis B infections) and long-lasting epigenetic alterations, such as those leading to large offspring syndrome in cows (Abe and Hoshi, 2003; Abe et al., 1999; Ferguson and Leese, 1999; Kim et al., 2001). Therefore, in human and some bovine IVP protocols, serum is replaced by other macro-molecules such as human or bovine serum albumin (BSA). Albumin acts as a carrier, provides substrates and growth factors and scavenges ROS (Roche et al., 2008).

In addition to albumin, insulin-transferrin-selenium (ITS) is commonly supplemented during routine serum-free bovine IVC due to the antioxidant properties of transferrin and selenium and for the mitogenic and anti-apoptotic effects of insulin (Augustin et al., 2003; Barnes and Sato, 1980; Goovaerts et al., 2012; Gutteridge, 1989). Some commercially available human IVC media contain insulin (Morbeck et al., 2014).

In the present study, we hypothesized that supportive embryo culture conditions supplemented with anti-apoptotic, antioxidant and mitogenic factors such as serum or ITS can reduce oxidative stress levels and rescue the development and quality of embryos derived from metabolically compromised oocytes.

To test this hypothesis, we used NEFA-exposure during bovine oocyte IVM as a model to mimic oocyte maturation under maternal metabolic stress conditions. Oocytes were exposed to either a high PA concentration (PA) or to a combination of high concentrations of NEFAs (PA, SA and OA; HCOMBI) compared to basal concentrations of NEFA (BASAL). After IVF, zygotes were cultured in SOF culture medium containing only bovine serum albumin (BSA) or supplemented with ITS or serum. We investigated the effects on developmental competence as well as on important embryo quality parameters: cell proliferation, apoptosis, embryonic cell metabolism and expression of a selected number of genes related to oxidative stress, mitochondrial unfolded protein response, endoplasmic reticulum stress and mitochondrial biogenesis.

3. MATERIALS AND METHODS

All laboratory materials were purchased from Sigma-Aldrich (Overijse, Belgium) unless otherwise stated.

3.1. EXPERIMENTAL DESIGN

Metabolically compromised oocytes were generated by exposure to different pathophysiological concentrations of NEFAs during IVM, a model that has been previously established and validated in our laboratory (Leroy et al., 2017; Van Hoeck et al., 2013; Van Hoeck et al., 2011). Subsequently, presumptive zygotes were cultured in vitro in different conditions: SOF media containing only BSA as a macromolecule, or BSA supplemented with ITS (as a mitogenic, anti-apoptotic and antioxidative supplement). This was compared to a group cultured in SOF media supplemented with serum (which naturally contains albumin and embryo-trophic factors but is less chemically defined).

Bovine COCs were matured for 24 h in media supplemented with different concentrations of NEFAs, as measured in the FF of normal weight and obese women (Valckx et al., 2014) and in cows during negative energy balance (Leroy et al., 2005): (1) A combination of basal concentrations of PA, SA, and OA (BASAL; 23, 28 and 21 µM, respectively; 72 µM total NEFA) as a physiological control, (2) high pathophysiological PA (150 µM) concentration together with basal SA and OA concentrations (PA) and (3) a combination of high, pathophysiological concentrations of PA, SA and OA (HCOMBI; 150, 75 and 200 µM, respectively; 425 µM total NEFA).

After fertilization, presumptive zygotes from each maturation condition were cultured in modified synthetic oviductal fluid (mSOF) supplemented with either (1) 2% BSA, (2) 2% BSA and ITS containing 10 µg/mL insulin, 5.5 µg/mL transferrin and 6.7 ng/mL selenium (Fisher Scientific, Merelbeke, Belgium), or (3) 5% Fetal Bovine Serum (FBS), resulting in nine treatment groups in a 3 × 3 factorial design (see Figure 1).

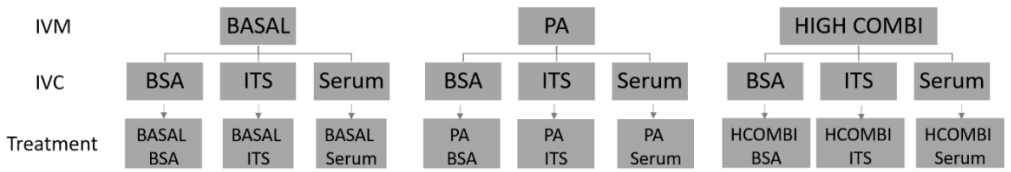


Figure 1. Overview of the nine treatment groups: BASAL-BSA, BASAL-ITS, BASAL-serum, PA-BSA, PA-ITS, PA-serum, HCOMBI-BSA, HCOMBI-ITS, HCOMBI-serum.

Embryo developmental competence was assessed by recording cleavage rate (48 h p.i.) and blastocyst rate at day 7 and 8 p.i. (n = 3737). Blastocyst quality was assessed by examining total cell count and apoptotic cell index (n = 498). Furthermore, the metabolic profile (glucose and pyruvate consumption, lactate production; n = 389) and

expression of a selected number of genes of interest (7–16 blastocysts/pool/treatment group) were also analyzed (see Figure 2). The number of replicates and COCs or blastocysts used for each outcome parameter are indicated in the results section.

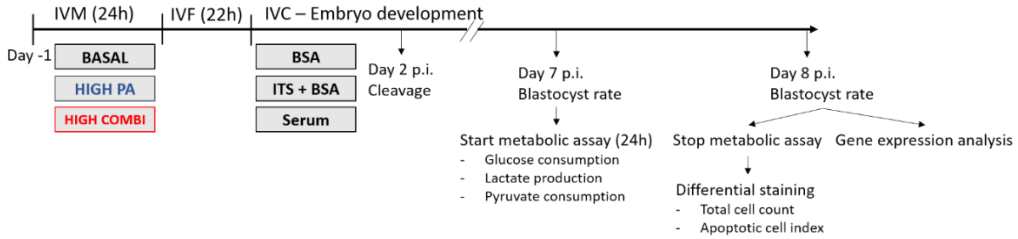


Figure 2. Overview of the experimental design.

3.2 PREPARATION OF NEFA STOCKS AND NEFA-SUPPLEMENTED IN VITRO MATURATION MEDIA

Stock solutions of SA, PA and OA were prepared in absolute ethanol. These stock solutions were added to maturation medium to obtain the final desired NEFA concentrations. Ethanol was adjusted to the same concentration (0.5%) in all treatment groups. Previous studies in our laboratory have shown that addition of ethanol (0.5%) or BASAL NEFA concentrations during IVM did not have any significant impact on embryo cleavage or development to the blastocyst stage compared to standard solvent-free and FFA-free (standard lab control) conditions when cultured in the presence of ITS (Marei et al., 2017b) or serum (Van Hoeck et al., 2011). We have also confirmed this in the presence of BSA alone (Supplementary Table S3). Therefore, based on these results, exposure to BASAL NEFA concentrations during IVM was used as a physiological control in the present study.

3.3. IN VITRO EMBRYO PRODUCTION PROCEDURE

3.3.1. OOCYTE COLLECTION AND IN VITRO MATURATION (IVM)

IVM of bovine oocytes was performed for 24 h as previously described by Desmet et al. (2016). Briefly, immature COCs surrounded by five or more compact cumulus cell layers (quality grade I) were retrieved from slaughterhouse ovaries and selected for serum-free IVM. The COCs were washed and cultured in 500 mL maturation medium supplemented with 20 ng/mL epidermal growth factor (EGF) stock II in groups of 50–60 COCs for 24 h in humidified air with 5% CO2 at 38.5 °C. Maturation medium consisted of TCM199 (Life Technologies, Merelbeke, Belgium) supplemented with 0.4 mM L-glutamine, 0.2 mM sodium pyruvate, 0.1 µM cysteamine and 50 µg/mL gentamicin. Maturation medium was supplemented with different concentrations of NEFAs, as mentioned in the experimental design.

3.3.2. IN VITRO EMBRYO PRODUCTION

After maturation, COCs were in vitro fertilized using frozen-thawed semen of the same ejaculate from a proven-fertile bull. Straws were thawed in warm sterile water (37 °C for 30 s) and their content was centrifuged on a discontinuous Percoll® gradient (90% and 45%, Amersham Biosciences, Roosendaal, The Netherlands) to select the viable, motile spermatozoa. COCs were co-incubated in groups of 100–120 in fertilization medium (Fert-TALP (Tyrode albumin lactate pyruvate) containing heparin as a capacitating agent) in a final concentration of 10^6 sperm cells/mL for 22 h at 38.5 °C and 5% CO2 in a humidified incubator (Leroy et al. (2005)).

At 22 h of IVF, presumptive zygotes were denuded by vortexing (3 min). Zygotes were then washed and cultured in groups of 25 ± 4 in 75 µL mSOF medium supplemented with or without 2% BSA, ITS or 5% serum, as described in the experimental design. SOF-medium consisted of 2.77 mM myoinositol, 0.72 mM sodium pyruvate, 1.5% (v/v) minimum essential medium (MEM) 50X, 1% (v/v) non-essential amino acids (MEM-NEAA) 100X, 0.4 mM L-glutamine, 50 µg/mL gentamycin, 108 mM NaCl, 70 mM KCl, 120 mM KH2PO4, 10 mM MgSO4, 7 H2O, 60% lactate, 20 mM NaHCO3, 0.01% phenol red, 180 mM CaCl2, 2H2O and 10 mg/mL trisodium citrate. Culture plates were incubated at 38.5 °C, 5% CO2, 5% O2 and 90% N2 (maximum humidity) until day 7 or 8 p.i., according to the outcome parameter that was assessed (Harvey, 2007).

3.4. OUTCOME PARAMETERS

3.4.1. ASSESSMENT OF CUMULUS CELL EXPANSION

As described by Marei et al. (2012), cumulus cell expansion was evaluated following 24 h of IVM. Cumulus expansion was scored (0–3) using an Olympus SZX7 stereomicroscope: not expanded (score 0), poorly expanded (score 1), partially expanded (score 2) or fully (maximum) expanded (score 3) (Figure 3). An average score of all COCs was calculated for each treatment group.

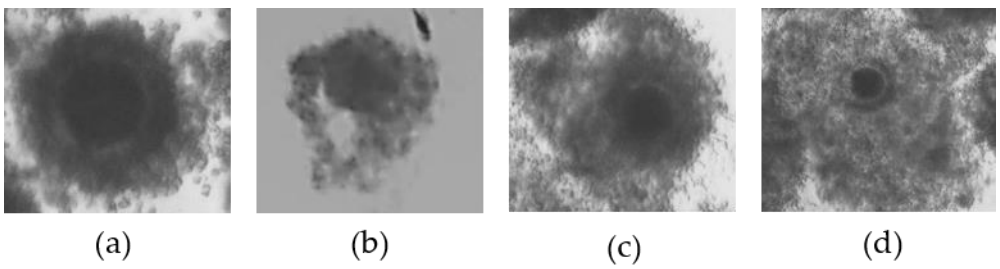


Figure 3. Grades of cumulus cell expansion in cumulus-oocyte complexes (COCs) after 24 h incubation in maturation media. (a) unexpanded COC (score 0), (b) poorly expanded COC (score 1), (c) partially expanded COC (score 2) and (d) and full y expanded COC (score 3). Scale bar = 100 µm.

3.4.2. EMBRYO DEVELOPMENTAL COMPETENCE

Total cleavage rate, two-cell block, number of embryos with ≥4-cells and fragmentation were recorded at 48 h p.i. Zygotes were categorized as fragmented if ≥20% of their cellular mass was fragmented (Stringfellow et al., 2010). Cleavage rate and blastocyst yield was assessed at days 7 and 8 p.i. using an inverted Olympus CKX41 microscope (Olympus, Aartselaar, Belgium). Blastocyst rates were presented as the number of blastocysts per total number of oocytes used.

3.4.3. BLASTOCYST ENERGY METABOLISM: DETERMINATION OF PYRUVATE AND GLUCOSE UPTAKE AND LACTATE PRODUCTION

Assessment of embryo metabolism was performed on individual bovine blastocysts using an ultra-micro-fluorometric technique (the Tecan Infinite M200 spectrophotometer) as described in detail by De Bie et al. (2017), a protocol adapted from Guerif et al. (2013). Briefly, individual day 7 embryos were incubated for 24 h in analysis medium (AM) with defined physiological concentrations of glucose and pyruvate. The AM resembled the standard SOF medium mentioned above but with 0.5 mM glucose, 0.4 mM pyruvate and no lactate. Day 7 blastocysts were cultured individually in 8 µl droplets of AM for 24 h under equilibrated mineral oil in 60 mm dishes at 38.5 °C, 5% CO2 and 5% O2 until day 8. A few droplets were left without embryos and were used as blanks. Blastocyst morphological stage was scored at the start and end of the metabolic assay. Blastocysts were then removed and the dishes containing AM droplets were sealed and stored at −80 °C until analysis.

After removal, the blastocysts were washed in phosphate-buffered saline with polyvinylpyrrolidone (PBS-PVP) and transferred individually to a labelled 96-well plate containing paraformaldehyde (PFA) 4% for fixation (20 min), then washed and stored at 4 °C for differential staining to determine their quality (see below).

Absolute concentrations of glucose, pyruvate and lactate in spent media droplets were determined using enzymatic reactions and standard curves (0–0.5 mM), as described by Guerif et al. (2013). To determine the rate of consumption or production for each metabolite by each embryo, we calculated the difference in metabolite concentration in its spent medium droplet compared to that in the blank droplets. All samples were measured in duplicate. Lactate production and glucose and pyruvate consumption were expressed as pmol/embryo/h. In addition, lactate:(2 glucose) ratios (1 mol glucose produces 2 mol lactate) were calculated to estimate the metabolic pathway by which glucose was preferentially metabolized. For each measure, the coefficient of variance (CV) was monitored: glucose displayed an intra- and inter-assay CV of 7.0% and 7.0% respectively, while CV's of lactate were 8.4% and 8.2% and CV's of pyruvate were 7.8% and 7.9%, respectively.

3.4.4. ASSESSMENT OF BLASTOCYST CELL NUMBER AND APOPTOTIC CELL INDEX

Fixed blastocysts were immuno-stained with anti-cleaved caspase-3 antibody (Asp 175; Cell Signaling Technology) to determine the number of apoptotic cells, and all nuclei were counter-stained with Hoechst for total cell counting. Briefly, fixed day 8 blastocysts were permeabilized overnight at 4 °C using 1% Triton X-100 and 0.05% Tween 20 in PBS. Blocking was performed for 2 h at 4 °C in 10% normal goat serum in 0.05% Tween 20-PBS. Caspase-3 labelling was done using a rabbit anti-cleaved caspase-3 (1:250) in blocking solution overnight at 4 °C. Embryos were then washed in PBS-PVP and incubated with the secondary goat anti-rabbit fluorescein isothiocyanate (FITC) antibody (1:200) in blocking solution. Counter-staining was performed using PBS-PVP containing Hoechst 33342 (50 µg/mL) for 10 min at room temperature. After staining, the blastocysts were washed in PBS-PVP and mounted on slides in a drop of 1% 1,4-diazabicyclo[2.2. 2]octane (DABCO) (in 90% glycerol and 10% PBS). The blastocysts were examined under a fluorescence microscope (Olympus IX71, X-cite series 120 Q) with 4',6-diamidino-2-phenylindole (DAPI) and FITC filters at 200× magnification and images were acquired using Cell Sens-Standard software at the same exposure and gain settings. Total cells and caspase-3-positive cells were counted.

3.4.5. BLASTOCYST RNA EXTRACTION, REVERSE TRANSCRIPTION AND QUANTIFICATION OF GENE EXPRESSION BY QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Pools of at least ten day 8 blastocysts from each treatment were washed and transferred to a 1.5 mL vial in minimal volume of 0.1% PBS-PVP and snap-frozen and stored at −80 °C until further processing.

Total RNA from each replicate was extracted and purified using the PicoPureTM RNA Isolation Kit (Thermo Fisher Scientific, Asse, Belgium). RNA was isolated following the Manufacturer's instructions, with minor modifications. Extracted RNA was treated with DNase (Qiagen, Venlo, The Netherlands). The RNA concentration and purity were checked using a BioAnalyzer (Agilent). After extraction, cDNA synthesis was performed using a Sensiscript reverse transcriptase (RT) kit as described by Marei et al. (2019b).

Gene transcripts were quantified by quantitative Polymerase Chain Reaction (qPCR) using SYBR green (SsoAdvanced SYBR Green supermix, Bio-Rad, Temse, Belgium). All samples were analyzed in duplicate. Quantification was normalized using the geometric mean of three housekeeping genes: 18 S, H2AFZ and YWHAZ, calculated by geNorm software (geNorm, Camberley, UK). The comparative quantification cycle (Cq) method, '2–ΔΔCq', was used to quantify the relative expression level of each gene, as described by Livak and Schmittgen (2001b). Fold-changes of all studied genes were calculated compared to the control as a reference (= 1 fold-change) and expressed as fold-change ± standard error of the mean (S.E.M.).

Transcript abundance of genes involved in mitochondrial unfolded protein responses (HSP10 (official name: HSPE1) and HSP60 (official name: HSPD1)), oxidative stress (GPx, catalase (CAT) and superoxide dismutase 2 (SOD2)), endoplasmic reticulum stress (ATF4 and ATF6) and mitochondrial biogenesis (TFAM) were analyzed. Primer details are shown in Supplementary Table S4.

3.5. STATISTICAL ANALYSIS

Statistical analysis was performed using IBM Statistics SPSS 25. Categorical data of cleavage and blastocyst rates were compared between the same maturation or culture groups using a binary logistic regression model. Interaction between treatment (fixed factor) and repeat (random factor) effects was evaluated, and since they were not significant, this interaction term was omitted from the final model. Numerical data of total cell numbers, apoptotic cell index, gene expression, glucose and pyruvate consumption and lactate production of blastocysts were compared between relevant groups for equality of variance and normality of distribution and analyzed using a linear mixed model. When equal variance could not be assumed, a non-parametric Kruskal–Wallis and Mann–Whitney test were performed for each comparison. A post hoc test was performed for multiple comparison with a Bonferroni correction. Data are presented as mean ± S.E.M. p-values < 0.05 are considered significant.

4. RESULTS

4.1. THE IMPACT OF HIGH NEFA SUPPLEMENTATION DURING IVM

4.1.1. CUMULUS CELL EXPANSION

Cumulus cell expansion was significantly ($p < 0.05$) reduced in cumulus oocyte complexes (COCs) exposed to PA (1.90 ± 0.02) and HCOMBI (1.61 ± 0.02) compared with the BASAL control (physiological NEFA concentrations) (2.83 ± 0.001).

4.1.2. EMBRYO DEVELOPMENT

A total of 3737 bovine COCs were matured in either BASAL, PA or HCOMBI NEFA concentrations during maturation and then cultured in BSA, ITS (+BSA) or serum. The effect of NEFAs on developmental competence was highly dependent on the culture condition, as shown in Table 1, Supplementary Figure S1 and described below. Supplementation of culture media with ITS or serum did not significantly influence cleavage and blastocyst rates of BASAL control oocytes when compared to IVC in BSA only ($p > 0.1$, see Supplementary Table S1 for statistical comparison).

Table 1**.** The effect of insulin-transferrin-selenium (ITS) or serum supplementation during in vitro culture (IVC) on the developmental competence of oocytes matured under metabolic stress conditions (PA (high palmitic acid) or HCOMBI NEFAs (a combination of pathophysiological, high concentrations of non-esterified fatty acids).

Maturation	Culture	Oocytes Used	Cleaved Embryos	Two-Cell Stage	Four-Cell Plus	Fragmented Embryos	Day 7 Blastocysts	Day 8 Blastocysts
BASAL	BSA	515	395 ^{a} (76.69%)	48 $^{a\$}$ (9.32%)	255 ^{a} (49.51%)	63 (12.23%)	105 ^{a} (20.38%)	125 $^{a\$}$ (24.27%)
PA	BSA	270	176 ^{b} (65.18%)	42 ^{b} (15.55%)	81 ^{b} (30.00%)	35 (12.96%)	30 $^{b\$}$ (11.11%)	37 ^{b} (13.70%)
HCOMBI	BSA	527	377 ^{ab} (71.53%)	63 $^{ab\$}$ (11.95%)	221 ^{c} (41.93%)	65 (12.33%)	87 $^{ab\$}$ (16.50%)	96 $^{ab\$}$ (18.21%)
BASAL	ITS	510	406 $^{\$\$}$ (79.6%)	63 (12.35%)	250 ^{x} (49.01%)	56 $^{\$}$ (10.98%)	124 (24.31%)	139 (27.25%)
PA	ITS	278	200 $^{\$}$ (71.94%)	43 (15.46%)	110 ^{y} (39.56%)	30 (10.79%)	52 (18.7%)	68 (24.46%)
HCOMBI	ITS	525	387 $^{\$}$ (73.71%)	46 (8.76%)	235 ^{xy} (44.76%)	84 $^{\$}$ (16.00%)	101 (19.23%)	120 (22.85%)
BASAL	Serum	488	348 $^{jk\$}$ (71.31%)	73 ^{j} (14.95%)	182 ^{j} (37.29%)	55 (11.27%)	119 ^{j} (24.39%)	136 ^{j} (27.86%)
PA	Serum	247	156 $^{j\$}$ (63.15%)	54 ^{k} (21.86%)	53 ^{k} (21.45%)	30 (12.14%)	30 ^{k} (12.14%)	45 ^{k} (18.21%)
HCOMBI	Serum	377	275 ^{k} (72.94%)	55 ^{jk} (14.58%)	149 ^{j} (39.52%)	41 (10.87%)	88 ^{j} (23.34%)	91 ^{jk} (24.13%)

% values refer to proportions from total number of the used oocytes. Values with different superscripts within the same in vitro culture group and column are significantly different at $p < 0.05$. Values labeled with a, b and c are used within BSA IVC; x, y is used for culture in the presence of ITS; j, k is used within Serum IVC. Values labeled with "§" or "$" tend to be different from each other at $p < 0.1$ and > 0.05.

In basic BSA culture media, PA-exposure during IVM significantly reduced embryo cleavage rate, increased arrest at the 2-cell stage and reduced rates of development to the ≥4-cell stage at 48 h post-insemination (p.i.), and to the blastocyst stage at days 7 and 8 ($p < 0.05$) compared with BASAL control (Table 1). Culture of PA-exposed oocytes in serum-supplemented medium did not alleviate any of its negative impact on developmental competence when compared to the BASAL control group cultured in serum, as similar differences where observed. In contrast, development of PA-exposed oocytes was relatively improved with ITS supplementation where, despite lower 4 cell+ proportions, blastocyst rates on days 7 and 8 were similar to those observed in the BASAL-ITS control ($p > 0.1$) and significantly higher than those in the PA-BSA group ($p < 0.05$, see Supplementary Table S1).

Exposure to HCOMBI NEFAs during IVM also reduced developmental competence in basic culture (BSA) compared to BASAL NEFAs, with increased arrest at the 2-cell stage ($p < 0.1$), lower proportions of 4 cell+ embryos ($p < 0.05$) and day 8 blastocysts ($p < 0.1$). This negative impact however was smaller compared with the effects seen after maturation in PA (Table 1). In contrast, supplementation of either ITS or serum during IVC alleviated the negative impact of HCOMBI exposure on embryo development as there was no difference anymore with the corresponding BASAL control groups ($p > 0.1$).

4.2. EMBRYO QUALITY

4.2.1. TOTAL CELL COUNT AND APOPTOTIC CELL INDEX

The effect of BSA, ITS + BSA or serum supplementation during IVC on total cell numbers and apoptotic cell index (ACI) of blastocyst resulting from metabolically compromised oocytes was determined in 7 replicates using 498 embryos (Figure 4).

Overall, total cell counts of day 8 blastocysts were not influenced by PA or HCOMBI exposure in any of the IVC conditions compared to the corresponding BASAL controls ($p > 0.1$), and were not influenced by the ITS or serum supplementation either. As an exception, HCOMBI-ITS exhibited significantly higher total cell numbers compared to BASAL-ITS (137 ± 6 vs. 116 ± 5, $p < 0.05$) (Figure 4).

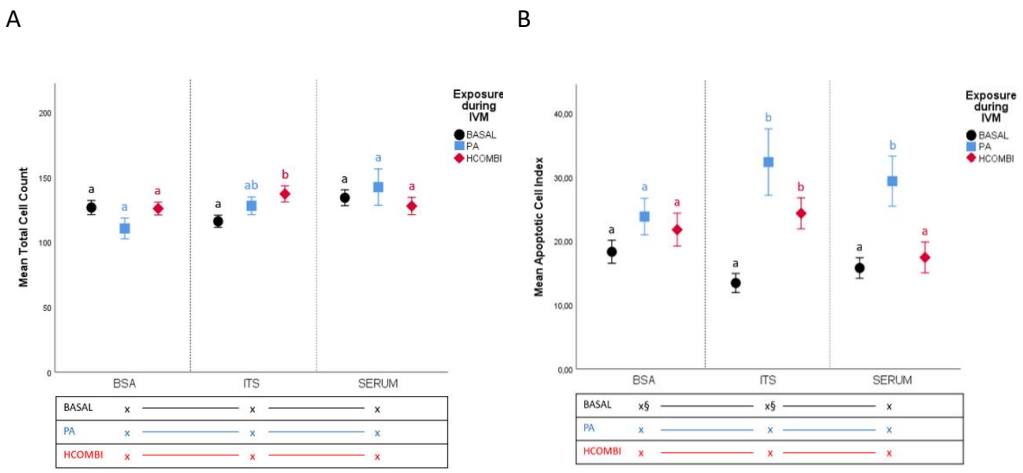


Figure 4. Mean total cell count (A) or apoptotic cell index (B) of Day 8 blastocysts resulting from oocytes exposed to BASAL (physiological NEFA concentrations), PA and HCOMBI NEFAs during maturation and cultured in the presence or absence of bovine serum albumin (BSA), ITS or serum. Data are presented as mean percentage ± SEM. Vertical superscripts (a, b) indicate significant differences between maturation conditions within the same IVC condition ($p < 0.05$). Horizontal superscripts (x indicate significant difference between culture conditions within the same in vitro maturation (IVM) condition ($p < 0.05$). Values labeled with "§" tend to be different from each other at $p < 0.1$ and > 0.05.

Apoptotic cell index (Figure 4) in day 8 BASAL control embryos was not significantly influenced by ITS and serum supplementation compared to BSA culture ($p > 0.05$). ACI in PA-BSA was similar to that in BASAL-BSA blastocysts ($p > 0.1$), however, ACI was significantly higher in PA-ITS and PA-Serum compared to BASAL-ITS (32.32 ± 5.2 vs. 13.43 ± 1.5, $p < 0.05$) and BASAL-Serum groups, respectively (29.34 ± 3.9 vs. 15.78 ± 1.6, $p < 0.05$). ACI of HCOMBI-derived blastocyst was similar to BASAL controls in BSA and serum culture, but it was significantly increased in ITS culture (24.31 ± 2.4 vs. 13.43 ± 1.5, $p < 0.05$).

4.2.2. METABOLIC ACTIVITY OF SURVIVING BLASTOCYSTS

Metabolic assays were performed on 389 blastocysts (in individual culture from day 7 p.i. for 24 h) in four replicates. The morphological stage of each blastocyst was scored on days 7 and 8.

At the start of the metabolic assay, no significant differences were present when comparing day 7 blastocyst stages between the different treatment groups. The percentage of blastocysts that further developed from one morphological stage to another during the 24 h of single culture was similar among treatment groups ($p > 0.05$).

Within the BASAL control group, it was noticed that embryo culture in ITS-supplemented media did not influence blastocyst metabolic activity, while serum supplementation in embryo culture significantly increased lactate production (33.4 ± 2.1 vs. 22.2 ± 1.2 pmol/embryo/hour, Figure 5) and lactate:(2 glucose) ratio (1.31 ± 0.32 vs. 0.43 ± 0.07 pmol/embryo/hour) compared to the BASAL-BSA group ($p < 0.05$).

In ITS-free and serum-free culture, PA-BSA blastocysts exhibited similar glucose and pyruvate consumption and lactate production compared to BASAL-BSA controls ($p > 0.1$). In contrast, HCOMBI-derived blastocysts consumed significantly less pyruvate under BSA culture compared to BASAL controls (11.1 ± 3.6 vs. 25.3 ± 2.4 pmol/embryo/hour, $p < 0.05$), while glucose and lactate metabolism were not influenced ($p > 0.1$).

In ITS culture, PA-derived blastocysts tended to consume lower pyruvate (17.2 ± 2.8 vs. 26.1 ± 2.6 pmol/embryo/hour, $p < 0.1$) and exhibited a significantly lower lactate:(2 glucose) ratio (0.20 ± 0.1 vs. 0.46 ± 0.03 pmol/embryo/hour, $p < 0.05$) compared to the BASAL-ITS group. Serum supplementation also reduced pyruvate consumption (14.9 ± 3.3 vs. 26.7 ± 2.9 pmol/embryo/hour) and lactate:(2 glucose) ratio (0.49 ± 0.1 vs. 1.31 ± 0.32 pmol/embryo/hour) of PA-derived embryos and significantly increased their glucose consumption (45.5 ± 6.0 vs. 27.8 ± 3.3 pmol/embryo/hour) compared to the BASAL-serum control group ($p < 0.05$).

In contrast, HCOMBI-derived blastocysts consumed significantly less pyruvate under BSA culture compared to BASAL controls (11.1 ± 3.6 vs. 25.3 ± 2.4 pmol/embryo/hour, $p < 0.05$), while glucose and lactate metabolism were not influenced ($p > 0.1$). Furthermore, with ITS and serum supplementation during culture, the metabolic activity of HCOMBI blastocysts including pyruvate consumption were all similar to the corresponding BASAL controls ($p > 0.1$). HCOMBI-serum blastocysts consumed significantly less glucose (19.8 ± 1.7 pmol/embryo/hour) and displayed higher lactate:(2 glucose) ratio (1.11 ± 0.27 pmol/embryo/hour) compared to HCOMBI-BSA (35.6 ± 2.6, 0.39 ± 0.04 pmol/embryo/hour, respectively) and HCOMBI-ITS (32.5 ± 3.4, 0.57 ± 0.05 pmol/embryo/hour, respectively) blastocysts ($p < 0.05$). Pyruvate consumption was only higher when compared to HCOMBI-BSA blastocysts.

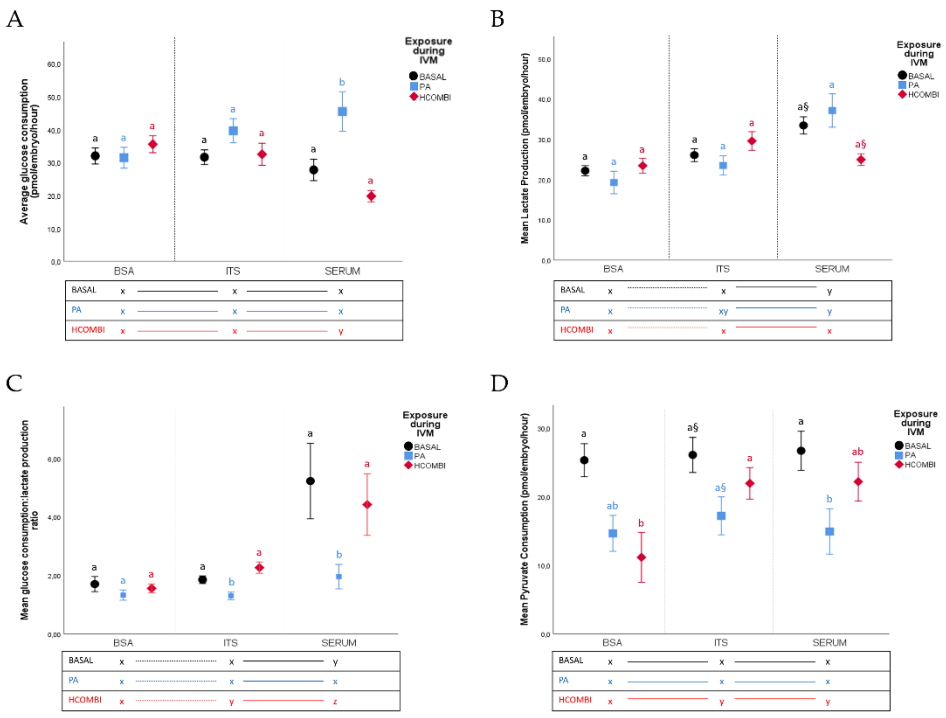


Figure 5. The effect of BSA, ITS or serum supplementation during IVC on (A) average glucose consumption, (B) lactate production, (C) lactate:(2 glucose) ratio and (D) pyruvate consumption (pmol/embryo/hour) of blastocysts resulting from BASAL-, PA- or HCOMBI-exposed oocytes. Data are presented as mean percentage ± SEM. Vertical superscripts (a, b) indicate significant differences between maturation conditions within the same IVC condition ($p < 0.05$). Horizontal superscripts (x, y, z) indicate significant difference between culture conditions within the same IVM condition ($p < 0.05$). Values labeled with "§" tend to be different from each other at $p < 0.1$ and > 0.05.

4.2.3. GENE EXPRESSION ANALYSIS OF DAY 8 BLASTOCYSTS

Gene expression analysis was performed on a total of 7–16 blastocysts/pool/treatment, with 4 pools/treatment group. A general overview of the results is displayed in Supplementary Tables S2a, b.

PA-BSA blastocysts had a significantly higher expression of heat shock protein 60 (HSP60) (a marker of mitochondrial unfolded protein responses (UPR)) compared to BASAL-BSA ($p < 0.05$, Figure 6). This effect was completely alleviated in ITS- and serum-supplemented groups. PA-serum tended to have a lower expression of Catalase (antioxidant) compared to BASAL-serum ($p < 0.1$). The expression patterns of other genes of interest were not affected by PA-exposure in any of the culture conditions

compared to BASAL controls. Within the PA-exposed group, embryos cultured in the presence of ITS displayed lower HSP60 expression compared to those cultured in BSA ($p < 0.1$) or serum ($p < 0.05$). ITS supplementation also resulted in relatively higher mitochondrial transcription factor A(TFAM) expression (involved in mitochondrial biogenesis) in PA-ITS blastocysts compared to PA-BSA and PA-serum ($p < 0.1$).

On the other hand, exposure to HCOMBI did not influence the gene expression pattern in any of the culture conditions compared to the corresponding BASAL controls ($p > 0.1$).

Regardless of the IVM condition, addition of serum during in vitro culture significantly reduced glutathione peroxidase (GPx) expression of embryos when compared to those cultured in the presence of either BSA, ITS (with BSA) or both.

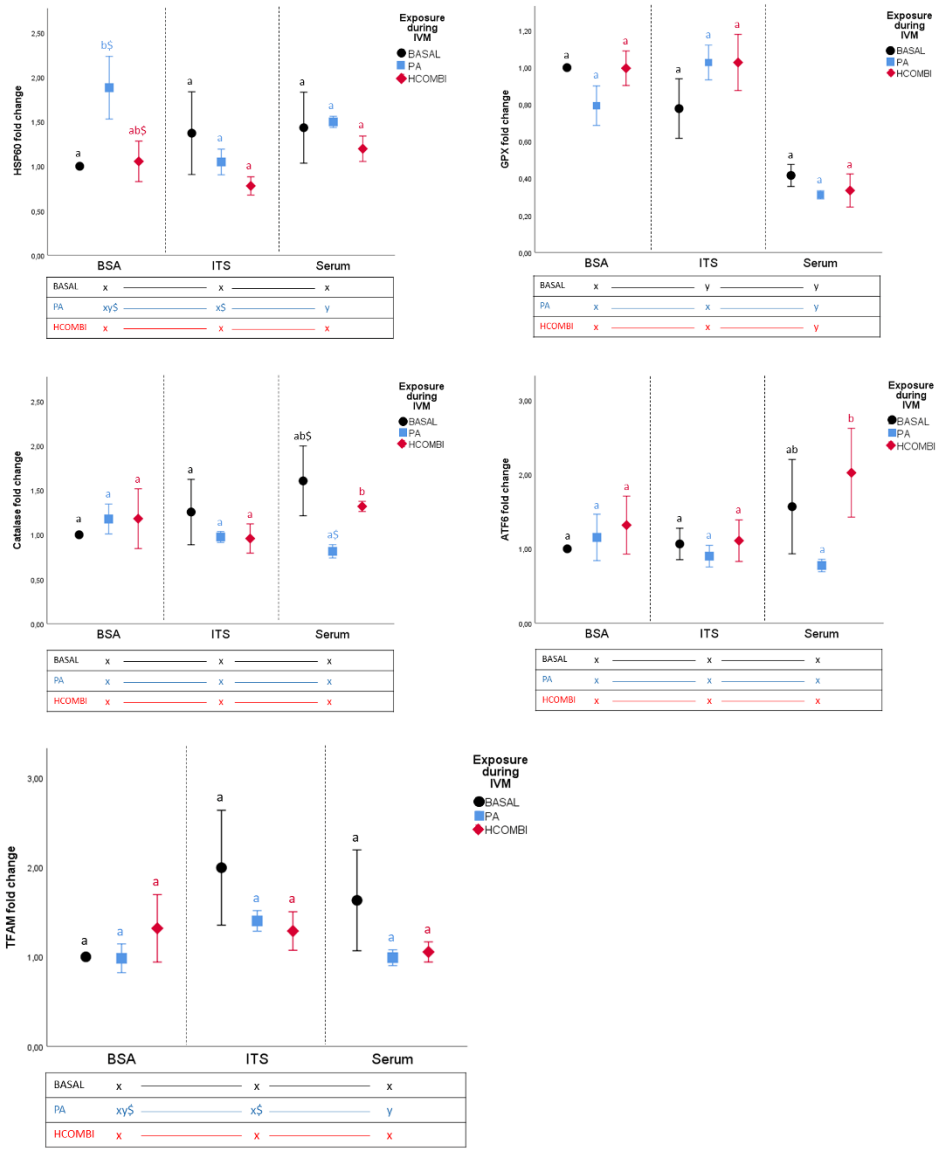


Figure 6. mRNA expression (fold change) of genes related to cellular stress of blastocysts resulting from BASAL-, PA- or HCOMBI-exposed oocytes cultured in serum, BSA and/or ITS. Data are presented as fold change ± SEM. Vertical superscripts (a, b) indicate significant differences between maturation conditions within the same IVC condition ($p < 0.05$). Horizontal superscripts (x, y) indicate significant difference between culture conditions within the same IVM condition ($p < 0.05$). Values labeled with "$" tend to be different from each other at $p < 0.1$ and > 0.05.

5. DISCUSSION

The aim of this study was to examine the effect of supportive culture conditions (ITS or serum supplementation) on development and quality of embryos derived from oocytes matured under lipotoxic conditions. We confirmed that a lipotoxic environment during IVM significantly reduced embryo developmental competence when cultured in basic SOF medium supplemented only with BSA. ITS-supplementation during in vitro culture, however, was able to alleviate the negative effects of a lipotoxic maturation environment on oocyte developmental competence. Nonetheless, surviving PA-derived blastocysts were overall lower in quality, as they displayed higher apoptotic cell indices, an aberrant cellular metabolism and few alterations in expression of genes involved in mitochondrial biogenesis and unfolded protein responses (UPRs). Addition of serum to the culture medium did not improve developmental competence of PA-exposed oocytes. However, similar to ITS, serum supplementation to PA-exposed embryos resulted in higher apoptotic cell indices and an aberrant cellular metabolism.

5.1. HIGH NEFA CONCENTRATIONS DURING IVM AFFECT SUBSEQUENT EMBYRO DEVELOPMENT BUT NOT QUALITY (APOPTOSIS, CELLULAR METABOLISM AND GENE EXPRESSION ANALYSIS) WHEN CULTURED IN NON-SUPPORTIVE CONDITIONS (BASIC SOF-BSA MEDIUM)

In the present study, maturing oocytes in both PA or HCOMBI reduced cumulus cell expansion compared to the BASAL-exposed COCs in all experiments, confirming what De Bie et al. (2017) and Marei et al. (2017b) observed. Leroy et al. (2005) linked the observation of poorly expanded COCs after maturation in the presence of PA to a high degree of late apoptotic and even necrotic cumulus cells. In line with these observations, COCs collected from obese patients have been shown to be inferior in quality with bad morphology and reduced cumulus cell expansion (Jungheim et al., 2011b), which was linked to low maturation rates (Wittemer et al., 2000).

Oocytes exposed to elevated concentrations of PA during maturation and cultured in basic BSA showed significantly lower developmental competence compared to BASAL-exposed oocytes. This is in line with Paczkowski et al. (2014b), Aardema et al. (2011) and Marei et al. (2019b). HCOMBI-BSA embryos also displayed reduced developmental competence when compared to the BASAL control group, although to a lesser extent, with a relatively higher proportion of good quality (≥four-cell) embryos and higher blastocysts rates compared to PA-exposed oocytes. The fact that PA exposure had a more detrimental impact on embryo development compared to HCOMBI has been observed in previous studies in our laboratory also under ITS- and serum-free culture conditions (De Bie et al., 2017; Marei et al., 2019b; Marei et al., 2019a). As demonstrated in a human clinical study, PA concentration in the follicular fluid may increase without a concomitant increase in OA, which was associated with negative pregnancy results following ICSI (Mirabi et al., 2017). The presence of OA, as a

monounsaturated FFA, has been shown to partially protect the oocyte against the lipotoxic effects of saturated FFAs, such as PA and SA, leading to relatively higher embryo development rates (Aardema et al., 2011). OA also increases lipid storage, hereby compensating for the adverse effects of PA and SA by deviating the fatty acid overload of the mitochondria away towards lipogenesis and accumulation of lipid droplets (Aardema et al., 2011).

After maturation in HCOMBI or PA and culture in BSA, we could not detect any reduction in quality of the surviving blastocysts compared to BASAL controls. Total cell numbers, ACI and embryo metabolism were similar to the controls. In addition, with the exception of higher HSP60 expression (a marker of mitochondrial UPR), mRNA transcription of the tested endoplasmic reticulum (ER) stress, UPR, mitochondrial biogenesis and oxidative stress markers were also not affected. This might indicate that the cellular stress level present in these surviving blastocysts was relatively low as no other marks of oxidative stress or apoptosis were present. It may furthermore indicate that the embryos with high cell stress levels were arrested before reaching the blastocyst stage and only the best quality embryos survived.

This is an important finding as it indicates that non-supported IVC conditions reduce the chance of a metabolically compromised oocyte to develop to the blastocyst stage. However, those that did develop further were of overall good quality based on the evaluated parameters. This notion is further confirmed when looking at the ITS and serum effects, as discussed later.

It was noticed that the apoptotic cell index of BASAL-BSA control embryos was relatively higher (18.31% ACI) than what is expected under standard conditions. Therefore, as an extra validation, we compared BASAL NEFA exposure to FA-free and solvent (0.5% ethanol) IVM control groups (Supplementary Table S3). Embryo cleavage and blastocyst rates in both solvent and BASAL treatments during maturation showed no significant differences in developmental competence and total cell count when compared to control blastocysts. However, we noticed a significantly higher ACI (13.67% and 14.70% vs. 5.80%, respectively). These data suggest that the addition of ethanol to the in vitro maturation medium caused an increase in blastocyst apoptosis, explaining the high ACI in BASAL-exposed control oocytes. It has been shown previously that addition of ethanol to the IVM medium led to increased expression of caspase-3 (Lee et al., 2014).

5.2. SUPPLEMENTATION OF IVC MEDIUM WITH ITS SUPPORTS THE DEVELOPMENT OF LOWER QUALITY OOCYTES THAT OTHERWISE WOULD NOT HAVE DEVELOPED FURTHER

It has been reported previously that development of embryos derived from oocytes matured under standard conditions could be significantly improved by ITS supplementation during IVC (Bowles and Lishman, 1998; George et al., 2008; Mesalam et al., 2019; Shamsuddin et al., 1994). We could not confirm this in our BASAL matured

oocytes as ITS supplementation did not result in increased developmental competence rates. However, our data showed that supplementation of ITS to the IVC medium of metabolically compromised oocytes (PA exposure) significantly improved developmental competence and yielded similar blastocyst rates compared to the BASAL controls. This shows that ITS, in contrast to IVC in basic media (only supplemented with BSA), supports the development of metabolically compromised oocytes that would not have cleaved or developed to a blastocyst in the absence of ITS. This effect was already visible when recording cleavage rates at 48 h p.i. (24 h after starting the ITS treatment). In addition, the ratio of blastocyst/cleaved embryos was significantly higher in PA-ITS- compared to PA-BSA-treated embryos, further confirming the supportive effect of ITS on oocyte developmental competence.

Developmental competence is an important non-invasive assessment parameter indicating the ability of an oocyte to undergo successful cytoplasmic and nuclear maturation, fertilization and embryo development. In addition to developmental competence, it is also important to look at embryo quality as it determines further development (Rizos et al., 2002). When focusing on embryo quality, apoptotic cell indices in PA-ITS and HCOMBI-ITS embryos were significantly higher compared to BASAL-ITS-treated embryos. This does not support our hypothesis of the possible anti-apoptotic effect of insulin at this stage. Together with the results of oocyte developmental competence, this leads to a very important conclusion: ITS might be able to support the development of low-quality oocytes to the blastocyst stage that would have otherwise been arrested during earlier development. Although the produced embryo is morphologically normal, the high apoptotic rates put the subsequent developmental capacity after transfer of such embryo at risk.

This notion was further supported when looking at embryo metabolism. PA-ITS embryos displayed lower pyruvate consumption and lower lactate:(2 glucose) ratio compared to BASAL-ITS embryos, which may indicate a decrease in Warburg metabolism. Warburg metabolism is a metabolic phenotype observed in healthy embryos during pre-implantation development allowing rapid cell proliferation and other important metabolic requirements like redox regulation and the production of biosynthetic molecules (Krisher and Prather, 2012). Lactate (a product from Warburg metabolism or aerobic glycolysis) production has been shown to be vital for embryo development as embryo development was reduced when the conversion from pyruvate to lactate was inhibited (Xie et al., 2016). Therefore, reduced lactate production may contribute to the defective development of PA-derived embryos under ITS culture. Lactate production by the pre-implantation embryo also plays and important role in the first embryo-maternal cross-talk. It has been reported to facilitate several key functions during implantation, including biosynthesis and endometrial tissue breakdown (Gardner and Harvey, 2015).

Within ITS culture conditions, no significant differences were found when comparing the expression of genes related to mitochondrial UPRs and biogenesis, ER stress and oxidative stress of PA- and HCOMBI-derived blastocysts to the BASAL control group. This means that the significant increase in HSP60 (a marker of UPR) detected in PA-blastocysts cultured in non-supplemented media (BSA) could be alleviated by ITS. This conclusion is further substantiated by a tendency to decrease HSP60 expression in PA-ITS embryos when compared to PA-BSA.

Furthermore, PA-ITS embryos displayed an increased TFAM expression when compared to those cultured in BSA or serum, suggesting an increase in mitochondrial biogenesis. Khera et al. (2015) reported that supplementation of selenium protects cells from mitochondrial stress through the upregulation of antioxidant systems and mitochondrial biogenesis. When matured in the presence of high NEFAs (PA or HCOMBI), embryos cultured in ITS-supplemented culture medium displayed significantly higher GPx expression when compared to those cultured in serum. ITS consists of selenium, which is known to promote GPx activity. Furthermore, it has been shown that ITS supplementation can significantly increase glutathione content in oocytes and display an insulin-like function to raise the developmental potential of oocytes (Jeong et al., 2008). These data may explain our observation that supplementing in vitro culture medium with ITS can support the development of lower quality oocytes that otherwise would not have developed further, by promoting its antioxidative activity in mitochondrial biogenesis.

5.3. SUPPLEMENTATION OF IVC MEDIUM WITH SERUM DID NOT IMPROVE DEVELOPMENTAL COMPETENCE AND QUALITY OF PA-EXPOSED OOCYTES

Supplementation of serum to the culture medium did not further improve nor deteriorate developmental competence of PA-exposed oocytes when compared to PA-oocytes cultured in non-supplemented BSA media. In contrast to the effects of ITS supplementation, the addition of serum resulted in reduced developmental competence of PA-exposed oocytes when compared to BASAL-serum oocytes, hereby confirming earlier findings (Desmet et al., 2020; Van Hoeck et al., 2011). Furthermore, similar to the observations after culture in ITS medium, PA-exposed oocytes cultured in the presence of serum displayed significantly higher ACI compared to BASAL- and HCOMBI-exposed oocytes, indicating a decreased quality of the surviving blastocysts. Furthermore, glucose consumption was significantly higher in PA-serum blastocysts compared to BASAL- and HCOMBI-serum blastocysts, resulting in the observed decrease in the lactate:(2 glucose) ratio. This increase in glucose consumption is most likely due to an inhibition of the fatty acid oxidation (FAO) cycle, caused by the increased concentration of saturated PA (Bhattacharjee et al., 2015). This inhibition of the FAO has been shown to upregulate glucose metabolism, suggesting an adjustment towards oxidative phosphorylation to compensate for the loss of optimal ATP production via the FAO cycle (Ferguson and Leese, 2006; Sturmey and Leese, 2008).

Interestingly, glucose consumption of HCOMBI-serum blastocysts was significantly lower compared to PA-serum blastocysts and not significantly different from BASAL-serum controls. OA has been shown to increase the expression of genes linked to the FAO pathway. As a result, OA potentially accelerated the rate of complete FAO, hereby replacing the increased consumption of glucose (Lim et al., 2013).

It has been demonstrated before that gene expression in the developing embryo was changed when embryos were cultured in medium supplemented with serum (Wrenzycki et al., 1999). In the present experiment, GPx (= antioxidant function) expression was significantly lower when embryos were cultured in serum compared to IVC in BSA or ITS, regardless of their exposure during in vitro maturation. Together with the other oocyte quality parameters, this decrease in GPx antioxidant gene expression confirms the reduced quality of the PA-serum surviving blastocysts.

Interestingly, when morphologically good-quality blastocysts, resulting from PA-exposed oocytes cultured in serum, were transferred to healthy cows, surviving post-hatching embryos still showed decreased quality, indicated by growth retardation, altered metabolism and altered transcriptomic profile after 7 days of in vivo culture (14 days p.i.) when compared to the BASAL control group, indicating long-lasting effects (Desmet et al., 2020). Post-hatching development of HCOMBI embryos was not tested in this study.

5.4. SUPPLEMENTATION OF SERUM TO IVC MEDIA OF HCOMBI- AND BASAL-EXPOSED OOCYTES ALTERS CELLULAR METABOLISM IN SURVIVING BLASTOCYSTS

In our experimental set-up, supplementation of serum during IVC alleviated the negative impact of HCOMBI exposure on embryo development as there was no difference anymore with the corresponding BASAL control groups. With regards to embryo metabolism, HCOMBI-serum embryos displayed significantly lower glucose consumption compared to IVC in BSA or ITS. Furthermore, both pyruvate consumption and lactate:(2 glucose) ratio were increased, suggesting a preference towards Warburg metabolism in this group. BASAL-serum embryos displayed a significantly higher lactate production and lactate:(2 glucose) ratio when compared to IVC in the presence of BSA alone or ITS, also indicating a preference towards Warburg metabolism. These results may suggest that the addition of serum to the culture medium may support this metabolic feature. Warburg metabolism spares glucose from being used for energy production and shunts it towards other metabolic pathways such as the pentose phosphate pathway (PPP) (Krisher and Prather, 2012). During the PPP, many reducing equivalents are being produced. As such, we propose that the addition of serum leads to an increased production of reducing equivalents available for biosynthesis and production of reduced glutathione, a key intracellular antioxidant (Gardner and Harvey, 2015). In line with this hypothesis, it was shown before that human hepatocellular carcinoma cells cultured in the presence of fetal bovine serum (FBS) typically display a

Warburg-like metabolic profile. When replacing the FBS, metabolic analysis shows that the Warburg-like metabolic profile was restored to oxidative metabolic features of normal liver cells (Steenbergen et al., 2018). To summarize, BASAL- and HCOMBI-serum blastocysts displayed a preference towards Warburg metabolism, resulting in a possible increased production of reduced glutathione. Together with the displayed reduced GPx gene expression in these treatment groups, this might indicate that additional antioxidant defense might not be necessary, as those blastocysts displayed overall good quality.

CONCLUSIONS

We confirmed that high NEFA exposure during IVM significantly reduced embryo developmental competence when cultured in basic SOF medium (supplemented only with BSA). ITS supplementation during in vitro culture of PA-exposed oocytes supported the development of lower quality embryos during earlier development. However, this increase in blastocyst yields (which may be due to supporting defective embryos to develop) had negative consequences on the quality (expressed as apoptotic cell index, cellular metabolism and gene expression analysis) of the surviving blastocysts. In contrast, addition of serum to the culture medium did not significantly improve developmental competence of PA-exposed oocytes. Furthermore, serum supplementation to PA-exposed embryos resulted in higher apoptotic cell indices and an aberrant cellular metabolism.

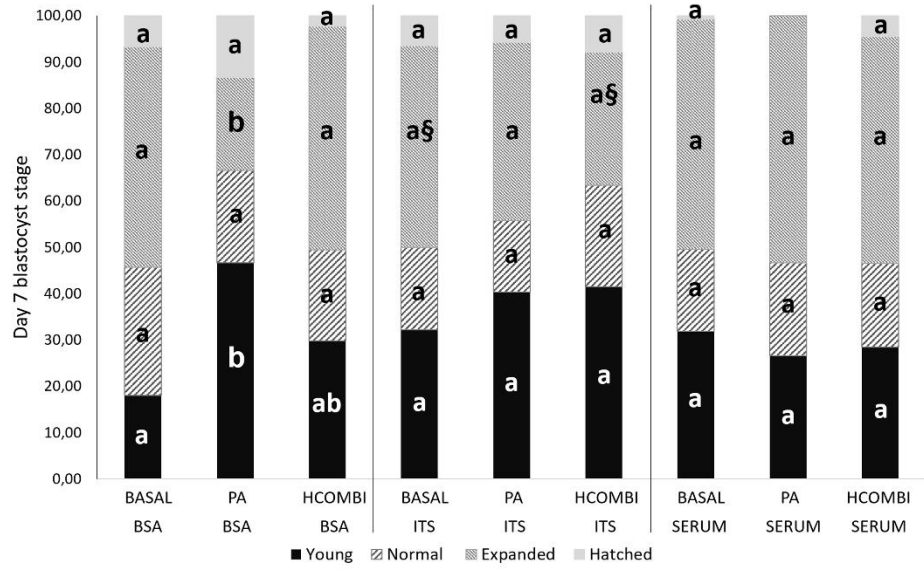
Based on these data, we conclude that some of the routinely used supportive embryo culture supplements like ITS and serum may increase IVF success rates of metabolically compromised oocytes but this may increase the risk of reduced embryo quality and may thus have other long-term consequences on establishment of pregnancy or on offspring health. These results highlight the importance of embryo quality assessment and long-term follow-up of developmental programming and postnatal health before approving new applications of any in vitro supportive treatments in IVF clinics. Further research is required to unravel this.

6. SUPPLEMENTARY MATERIALS

Suppl Table 1**.** The effect of ITS or serum supplementation during IVC on developmental competence within the same *in vitro* maturation group (BASAL, PA and HCOMBI):

Maturation	Culture	Oocytes used	Cleaved embryos	Two-cell stage	Four-cell plus	Fragmented embryos	Day 7 blastocysts	Day 8 blastocysts
Basal	BSA	515	395^{ab} (76.69%)	48^{a} (9.32%)	255^{a} (49.51%)	63 (12.23%)	105 (20.38%)	125 (24.27%)
Basal	ITS	510	406^{a} (79.6%)	63^{ab} (12.35%)	250^{a} (49.01%)	56 (10.98%)	124 (24.31%)	139 (27.25%)
Basal	Serum	488	348^{b} (71.31%)	73^{b} (14.95%)	182^{b} (37.29%)	55 (11.27%)	119 (24.39%)	136 (27.86%)
PA	BSA	270	176 (65.18%)	42 (15.55%)	81$^{xy\$\S}$ (30.00%)	35 (12.96%)	30^{x} (11.11%)	37^{x} (13.70%)
PA	ITS	278	200§ (71.94%)	43 (15.46%)	110$^{x\$}$ (39.56%)	30 (10.79%)	52^{y} (18.7%)	68^{y} (24.46%)
PA	Serum	247	156§ (63.15%)	54 (21.86%)	53^{y§} (21.45%)	30 (12.14%)	30^{xy} (12.14%)	45^{xy} (18.21%)
HCOMBI	BSA	527	377 (71.53%)	63 (11.95%)	221 (41.93%)	65 (12.33%)	87^{j} (16.50%)	96§ (18.21%)
HCOMBI	ITS	525	387 (73.71%)	46 (8.76%)	235 (44.76%)	84§ (16.00%)	101^{jk} (19.23%)	120 (22.85%)
HCOMBI	Serum	377	275 (72.94%)	55 (14.58%)	149 (39.52%)	41§ (10.87%)	88^{k} (23.34%)	91§ (24.13%)

% values refer to proportions from total number of used oocytes. Values with different superscripts within the same *in vitro* **maturation** group are significantly different at P<0.05. Values labeled with a, b are used within BASAL IVM; x, y is used for maturation in the presence of PA; j, k is used within HCOMBI IVM. Values labeled with "§" or "$" tend to be different from each other at P<0.1 and >0.05.



Suppl. Figure 1. Proportions (%) of different stages of blastocysts resulting from oocytes exposed to BASAL, PA and HCOMBI NEFAs during maturation and cultured in the presence of absence of BSA, ITS or serum. Values with different superscripts per embryo stage within the same *in vitro* **culture** group are significantly different at P<0.05. Values labeled with "§" tend to be different from each other at P<0.1 and >0.05.

Suppl. Table 2a. The effect of ITS or serum supplementation during IVC on mRNA expression (fold change) of genes related to cellular stress of blastocysts within the same *in vitro* maturation group (BASAL, PA and HCOMBI). Data are presented as mean fold change ± SEM.

Matur-ation	Culture	HSP60 fold change	GPx fold change	Catalase fold change	ATF6 fold change	TFAM fold change	HSP10 fold change	SOD2 fold change	ATF4 fold change
BASAL	BSA	1	1[a]	1	1	1	1	1	1
BASAL	ITS	1.37±0.47	0.78±0.16 [b]	1.26±0.37	1.07±0.21	2.00±0.64	1.95±0.85	1.16±0.69	1.02±0.20
BASAL	Serum	1.43±0.40	0.42±0.06 [b]	1.61±0.39	1.57±0.64	1.63±0.56	1.88±0.66	1.56±0.78	0.80±0.12
PA	BSA	1.88±0.35 [xy§]	0.79±0.11 [x]	1.18±0.17	1.15±0.31	0.98±0.16 [xy§]	1.44±0.4	1.18±0.33	0.82±0.24
PA	ITS	1.05±0.14 [x§]	1.03±0.09 [x]	0.98±0.06	0.90±0.15	1.40±0.12 [x§]	1.27±0.10	1.09±0.09	0.91±0.19
PA	Serum	1.50±0.06 [y]	0.31±0.02 [y]	0.81±0.08	0.77±0.08	0.99±0.09 [y]	1.03±0.07	0.80±0.04	0.60±0.08
HCOMBI	BSA	1.06±0.23	1.00±0.09 [j]	1.18±0.34	1.32±0.39	1.32±0.38	1.45±0.35	1.46±0.53	1.28±0.29
HCOMBI	ITS	0.78±0.10	1.03±0.15 [j]	0.96±0.16	1.11±0.28	1.29±0.22	1.15±0.12	0.97±0.14	1.02±0.05
HCOMBI	Serum	1.20±0.14	0.34±0.09 [k]	1.32±0.06	2.03±0.60	1.05±0.11	1.61±0.37	0.95±0.10	0.89±0.14

Values with different superscripts within the same *in vitro* **maturation** group are significantly different at P<0.05. Values labeled with a, b are used within BASAL IVM; x, y is used for maturation in the presence of PA; j, k is used within HCOMBI IVM. Values labeled with "§" or "$" tend to be different from each other at P<0.1 and >0.05.

Suppl Table 2b**.** mRNA expression (fold change) of genes related to cellular stress of blastocysts resulting from BASAL, PA or HCOMBI exposed oocytes cultured in serum, BSA and/or ITS. Data are presented as mean fold change ± SEM.

Matur-ation	Culture	HSP60 fold change	GPx fold change	Catalase fold change	ATF6 fold change	TFAM fold change	HSP10 fold change	SOD2 fold change	ATF4 fold change
BASAL	BSA	1[a]	1	1	1	1	1	1	1
PA	BSA	1.88±0.35 b§	0.79±0.11	1.18±0.17	1.15±0.31	0.98±0.16	1.44±0.41	1.18±0.33	0.82±0.24
HCOMBI	BSA	1.06±0.23 ab§	1.00±0.09	1.18±0.34	1.32±0.39	1.32±0.38	1.45±0.35	1.46±0.53	1.28±0.29
BASAL	**ITS**	1.37±0.47	0.78±0.16	1.26±0.37	1.07±0.21	2.00±0.64	1.95±0.85	1.16±0.69	1.02±0.20
PA	ITS	1.05±0.14	1.03±0.09	0.98±0.06	0.90±0.15	1.40±0.12	1.27±0.10	1.09±0.09	0.91±0.19
HCOMBI	ITS	0.78±0.10	1.03±0.15	0.96±0.16	1.11±0.28	1.29±0.22	1.15±0.12	0.97±0.14	1.02±0.05
BASAL	**Serum**	1.43±0.40	0.42±0.06	1.61±0.39 jk§	1.57±0.64 jk	1.63±0.56	1.88±0.66	1.56±0.78	0.80±0.12
PA	Serum	1.50±0.06	0.31±0.02	0.81±0.08 j§	0.77±0.08 j	0.99±0.09	1.03±0.07	0.80±0.04	0.60±0.08
HCOMBI	Serum	1.20±0.14	0.34±0.09	1.32±0.06 k	2.03±0.60 k	1.05±0.11	1.61±0.37	0.95±0.10	0.89±0.14

Values with different superscripts within the same *in vitro* **culture** group and column are significantly different at $P < 0.05$. Values labeled with a and b are used within BSA IVC; x is used for culture in the presence of ITS; j and k are used within Serum IVC. Values labeled with "§" or "$" tend to be different from each other at $P < 0.1$ and > 0.05.

Suppl. Table 3. The effect of 0.5% ethanol (Solvent) or BASAL NEFA concentrations during IVM on developmental competence:

Maturation	Culture	Oocytes used	Cleaved embryos	Day 8 blastocysts
Control	BSA	84	63 (72.9%)	25 (30.4%)
Solvent	BSA	79	51 (63.9%)	21 (26.6%)
BASAL	BSA	84	59 (71.2%)	21 (25.1%)

% values refer to proportions from total number of the used oocytes. None of the displayed differences were significant (P>0.1).

Suppl Table 4. Details of primers used for qPCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)	Gene Bank accession number
SOD2	TGCAAGGAACAACAGGTCTTATC	CTCAGTGTAAGGCTGACGGTT	181	NM_201527.2
GPX1	AACGTAGCATCGCTCTGAGG	TCTCCTCGTTCTTGGCGTTT	145	NM_174076.3
CAT	CTATCCTGACACTCACCGCC	GAAAGTCCGCACCTGAGTGA	268	NM_001035386.2
HSP10	CAACGGTGGTAGCTGTTGGA	TGGTGCCTCCATATTCTGGG	108	NM_174346.2
HSP60	CTACTGTACTGGCACGCTCT	CAATCTCTTCGGGGGTTGTC	159	NM_001166608.1
ATF4	TTTCTGAGCAGCGAGGTGTT	AAGCATCCTCCTTGCTGTTG	214	NM_00134341.2
ATF6	CGAGGATGGGTTCATAGGCA	GACGGCCATGAGCTGAGAAT	126	BC120388.1
TFAM	GCCAAGCTATGGAGGGAACT	AGCTTTACCTGTGATGTGCCA	293	NM_001034016.2
18S	AGAAACGGCTACCACATCCA	CACCAGACTTGCCCTCCA	169	AF176811.1
YWHAZ	GCATCCCACAGACTATTTCC	GCAAAGACAATGACAGACCA	120	BM446307.1
H2A	CGGAATTCGAAATGGCTGGC	TCTTTCGATGCATTTCCTGCC	238	NM_174809.2
BAX	AGCAGATCATGAAGACAGGG	TCAGACACTCGCTCAGCTTC	141	NM_173894.1

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CHAPTER 5:

OBESITY AND OOCYTE QUALITY – INBRED *vs.* OUTBRED MICE

DIFFERENTIAL EFFECTS OF HIGH FAT DIET-INDUCED OBESITY ON OOCYTE MITOCHONDRIAL FUNCTIONS IN INBRED AND OUTBRED MICE

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1. ABSTRACT

Maternal obesity can cause reduced oocyte quality and subfertility. Mitochondrial dysfunction plays a central role here, and most often inbred mouse models are used to study these pathways. We hypothesized that the mouse genetic background can influence the impact of high fat diet (HFD)-induced obesity on oocyte quality. We compared the inbred C57BL/6 (B6) and the outbred Swiss strains after feeding a HFD for 13w. HFD-mice had increased body weight gain, hypercholesterolemia, and increased oocyte lipid droplet (LD) accumulation in both strains. LD distribution was strain-dependent. In Swiss mouse oocytes, HFD significantly increased mitochondrial inner membrane potential (MMP), reactive oxygen species concentrations, mitochondrial ultrastructural abnormalities (by 46.4%), and endoplasmic reticulum (ER) swelling, and decreased mtDNA copy numbers compared with Swiss controls ($P<0.05$). Surprisingly, B6-control oocytes exhibited signs of cellular stress compared to the Swiss controls ($P<0.05$); upregulated gene expression of ER- and oxidative stress markers, high mitochondrial ultrastructural abnormalities (48.6%) and ER swelling. Consequently, the HFD impact on B6 oocyte quality was less obvious, with 9% higher mitochondrial abnormalities, and no additive effect on MMP and stress marks compared to B6 control ($P>0.1$). Interestingly, mtDNA in B6-HFD oocytes was increased suggesting defective mitophagy. In conclusion, we show evidence that the genetic background or inbreeding can affect mitochondrial functions in oocytes and may influence the impact of HFD on oocyte quality. These results should create awareness when choosing and interpreting data obtained from different mouse models before extrapolating to human applications.

2. INTRODUCTION

The prevalence of obesity and metabolic syndrome is increasing and is currently affecting millions of women in reproductive age worldwide. This has been inextricably linked with infertility. Obese women have a lower chance of spontaneous pregnancy, higher risks of early pregnancy losses and miscarriage (Sebire et al., 2001; Wang et al., 2002). In addition, success rates following assisted reproductive treatments are usually lower as compared to normal body mass index (BMI) cohorts (Dokras et al., 2006; Luke et al., 2011).

Over the last decade, several studies have correlated obesity with reduced oocyte quality using animal models, and elucidated some of the underlying causative mechanisms (Jungheim et al., 2010; Jungheim et al., 2012; Marei et al., 2016; Valckx et al., 2014b; Wu et al., 2011). High fat diet (HFD) is commonly used to induce obesity in mouse models. This results in hyperlipidemia, systemic lipotoxicity and oxidative stress, which is reflected in the ovarian follicle microenvironment (Reynolds et al., 2015b). Mimicking such lipotoxic conditions *in vitro* was shown to inhibit the growth of murine secondary ovarian follicles (during 13 days exposure) and substantially reduced oocyte developmental capacity and the quality of the resulting blastocysts (Valckx et al., 2014b). Importantly, mitochondrial dysfunction clearly plays an important role in the pathogenesis of reduced oocyte quality. HFD increased mitochondrial ultrastructural abnormalities in oocytes, altered mitochondrial inner membrane potential (MMP) and ATP production, and altered mitochondrial biogenesis and mtDNA copy numbers, compared to control diet in mice (Igosheva et al., 2010; Wu et al., 2010; Wu et al., 2011; Wu et al., 2015). The direction and extent of most of these changes are inconsistent among different studies as discussed later in this manuscript.

Most HFD-induced obese mouse models use the C57BL/6 strain. This is an inbred strain which progressively gain weight (Cheong et al., 2014; Igosheva et al., 2010; Jungheim et al., 2010) and develop hyperlipidemia and inflammatory responses when supplemented with HFD. The genetic homogeneity in inbred strains minimizes variability in experimental settings. However, the C57BL/6 strain is characterized by low fertility, small litter size and cannibalism of pups, which makes it unreliable for studies focusing on fertility outcomes. Furthermore, inbreeding increases the risk of genetic drift and persistent undiscovered mutations, which may confound responses to experimental factors (Nicholson et al., 2010). After all, extrapolation of the data and conclusions acquired from inbred models to human physiology is almost impossible. In contrast, outbred strains such as Swiss mice are more fertile and display better nurturing behavior. They are also metabolically sensitive to HFD, and develop hypercholesterolemia, obesity, and insulin resistance (Frediani Brant et al., 2014; Sikder et al., 2014). However, due to the wide genetic variability, more variation is seen in responses to changes in environmental conditions.

The effect of the genetic background on reproductive parameters has been examined only in a few studies comparing inbred and outbred mice and focusing on sensitivity to hormonal stimulation and oocyte developmental competence (Ibanez et al., 2005; Kaleta, 1977; Spearow and Barkley, 1999; Suzuki et al., 1996). However, the potential interplay of genetic background and the response to HFD-induced obesity at the oocyte level has not been described yet.

Interestingly, it has recently been shown that the mitochondrial genetic background modulates bioenergetics and susceptibility to metabolic diseases. By cross insertion of mtDNA from C57BL/6 mice to C3H/HeN mice, Fetterman et al. (2013) showed that mitochondria containing the C57BL/6 mtDNA generate more reactive oxygen species (ROS) and have a higher MMP relative to those having the C3H/HeN mtDNA. Oxidative stress and mitochondrial dysfunction are known to cause serious detrimental consequences on oocyte developmental competence, but it is not known yet whether this detrimental effect may vary depending on the genetic background of the mouse strain studied.

In addition, mitochondria also act as biological sensors of cellular stress and regulate unfolded protein responses (UPR) and signaling cascades to promote cell survival or initiate apoptosis, similar to those regulated by the endoplasmic reticulum (ER) (Runkel et al., 2014). Recent data suggest that mitochondrial dysfunction may affect UPR signaling in oocytes and embryos following short term *in vitro* exposure to lipotoxic conditions (Marei et al., 2019b). However, there is no data available about the activation of these mechanisms in oocytes in obese individuals following long-term exposure to oxidative stress or lipotoxicity. These responses may also be strain dependent.

Therefore, we hypothesize that HFD-induced obesity has a differential effect on oocyte quality and mitochondrial functions in the inbred C57BL/6 strain as compared to the outbred Swiss mice. We also hypothesize that in addition to the alteration in the mitochondrial ultrastructure and MMP, HFD may also alter UPR signaling in oocytes. To test these hypotheses, we exposed C57BL/6 (hereafter referred to as “B6”) and “Swiss” mice to a long-term high fat diet (13w) to induce obesity. Weight gain and blood composition were evaluated and related to oocyte lipid content, mitochondrial ultrastructure and function, ROS production, mtDNA copy numbers and UPR-related gene expression.

3. MATERIALS AND METHODS

3.1. ANIMALS, DIET AND EXPERIMENTAL DESIGN

All procedures in this study were approved by the ethical committee of the University of Antwerp and performed accordingly (ECD nr. 2014/57), and all methods were performed in accordance with the relevant guidelines and regulations. Seven-week-old female outbred Rj:Orl Swiss (n=32, hereafter referred to as "Swiss") and inbred C57BL/6N (n=32, hereafter referred to as "B6") mice (Janvier labs) were used. Mice of each strain were randomly divided into two main groups with ad libitum access to either a control (D12450J, Research Diets) or a high fat diet (HFD; D12492, Research Diets) for a period of thirteen weeks. The HFD was composed of 60 kcal% fat from lard, 20 kcal% carbohydrate and 20 kcal% protein. The matched, purified control diet contained 10 kcal% fat from lard, 70 kcal% carbohydrate and 20 kcal% protein. The energy density of these diets were 5.21 kcal/g and 3.82 kcal/g, respectively. Access to water was provided ad libitum. Mice were put on the diet in subgroups of 3-4 animals per treatment (4 replicates) with an interval of few days between replicates to facilitate handling and sample collection. Mice were weighed weekly. Two B6-HFD and one B6-Control mice developed disease symptoms (eye inflammation and skin lesions) and were excluded from the experiment because their food intake was affected.

At 13 weeks, mice received intraperitoneal injections of 10 IU equine chorionic gonadotropin (eCG, Synchrostim**;** Ceva Santé Animale) followed, 48 h later, by 10 IU human chorionic gonadotropin (hCG, Pregnyl; Organon) to induce and synchronize ovulations. Mice were sacrificed 13-14 h after hCG injection by decapitation to allow collection of the maximal volume of blood. After euthanasia, oocytes were collected and further examined as described below.

3.2. OOCYTE AND CUMULUS CELL COLLECTION AND PREPARATION FOR SUBSEQUENT ANALYSES

In vivo matured oocytes were obtained from the oviducts immediately after euthanasia. Each oviduct was dissected together with the ovary and a part of the uterine horn and transferred to a collection tube containing L15 medium (Thermofisher Scientific) supplemented with 50 IU /mL penicillin G sodium salt, and 10% Fetal Bovine Serum.

The cumulus oocyte complexes (COCs) collected from both oviducts of the same animal were pooled. According to the total number of COCs available, COCs from each mouse were distributed for downstream analysis as follows: one whole COC per mouse was fixed in glutaraldehyde solution for transmission electron microscopy (TEM). The remaining COCs were completely denuded by repeated pipetting through 100µm Stripper tips fitted on EZ-grip (Origio) in a droplet of L15 medium supplemented with

0.3mg/mL hyaluronidase. Denuded oocytes were transferred to a fresh drop. Two denuded oocytes (per mouse) were immediately transferred to JC-1 and CellROX Deep Red staining to determine mitochondrial activity and intracellular ROS content. One to two oocytes per mouse were fixed in paraformaldehyde 4% for determination of lipid droplet content. The remaining oocytes from each subgroup (n > 15 oocytes from 3-4 animals) were pooled and washed in PBS containing 1mg/mL PVP and snap frozen in a 1.5mL tube in a minimum volume for simultaneous RNA and DNA extraction for qPCR and determination of mtDNA content. Meanwhile, the droplets containing the detached cumulus cells from all animals in each subgroup were also immediately pooled, centrifuged, washed in PBS-PVP and snap frozen. All frozen samples were stored at -80°C until further analyses. Some oocytes were lost during mounting. DNA extraction of 1 replicate failed due to technical reasons. The final numbers of animals used to collect data for each outcome parameter are shown in figure legends.

3.3. SERUM COLLECTION AND ANALYSES

Blood was centrifuged 30 min after collection at 2000 rpm for 10 min, and serum was stored at -80°C. Serum analyses were performed in a commercial laboratory (Algemeen Medisch Labo, Antwerp, Belgium). Non-esterified fatty acid (NEFA) concentrations were determined using a colorimetric assay (Randox Laboratories Ltd, Crumlin, Co. Antrim, United Kingdom) on an IDS iSYS multi-discipline automated instrument (Immunodiagnostic Systems Hld, Tyne & Wear, UK). Triglycerides (TG) and Cholesterol were measured on an Abbott Architect c16000 (Abbott, Illinois, U.S.A).

3.4. ASSESSMENT OF OOCYTE LIPID DROPLET VOLUME

Oocytes were fixed in 4 % paraformaldehyde and stored in 1 mg/ml PBS-PVP at 4°C until staining. Lipid droplets were assessed using BODIPY 493/503 staining (ThermoFisher) followed by confocal microscopy (Van Hoeck et al., 2015)(see appendix A.1 for the detailed method).

3.5. ASSESSMENT OF MITOCHONDRIAL ACTIVITY AND INTRACELLULAR ROS

Mitochondrial inner membrane potential (MMP) and ROS level in oocytes were estimated using a combined JC-1 (Invitrogen) and CellROX Deep Red (ThermoFisher) staining, respectively, followed by confocal microscopy (Marei et al., 2019b) (see appendix A.2 for the detailed staining method).

3.6. TRANSMISSION ELECTRON MICROSCOPY (TEM)

COCs were fixed in glutaraldehyde solution and processed individually for TEM analysis as described in appendix A.3. For each COC, images of at least 5 cumulus cells and at

least 10 random fields in the oocyte, representative for mitochondria- and ER- rich regions, were acquired at 16500-25000X. Mitochondria in the acquired images were morphologically evaluated by an expert blind to the corresponding treatment group. The dimensions of the mitochondria and ER were also measured using Image-J to determine mitochondrial roundness/elongation and ER swelling among the different treatment groups.

3.7. QUANTIFICATION OF GENE EXPRESSION BY QPCR

Total RNA and DNA was extracted from oocytes and cumulus cells using AllPrep DNA/RNA Micro Kit (Qiagen, Venlo, NL) following manufacturers guidelines. Extracted total RNA was treated with RNase–free DNase (Qiagen). The concentration and purity of the isolated RNA samples were determined using a Nanodrop (Thermofisher). Total RNA (50ng) from each sample was reverse transcribed using SensiScript-RT kit (QIAGEN). Negative-RT control samples (missing reverse transcriptase) were included. Transcripts of the target genes of interest were quantified by quantitative Polymerase Chain Reaction (Real-Time PCR; qPCR) using SYBR Green (SsoAdvanced Universal SYBR Green supermix, Bio-Rad, Temse, Belgium). Genes of interest were involved in mitochondrial unfolded protein responses (*HSPD1* and *HDSPE1*), mitochondrial biogenesis (*TFAM*), ER stress (*BiP, Atf4* and *Atf6*) and Redox regulation (*SOD2, CAT, PRDX1, PRDX3, PRDX6* and *NRF2*) Quantification was normalized using the geometric mean of 3 housekeeping genes (*18S, YWHAZ, H2A*) calculated by geNorm software (Camberley, UK). The comparative quantification cycle (Cq) method, '$2^{-\Delta\Delta Cq}$', was used to quantify the relative expression level of each gene, as described by Livak and Schmittgen (2001b).

3.8. RELATIVE CHANGE OF MTDNA COPY NUMBERS

The ratio of mtDNA to nuclear DNA was determined in each oocyte DNA sample by qPCR of the mitochondrial gene (ND4) and the nuclear gene (bACT). The comparative quantification cycle (Cq) method, '$2^{-\Delta\Delta Cq}$', of ND4 vs. bACT was used to quantify the relative mtDNA to nuclear DNA ratio, as described by Livak and Schmittgen (2001b).

3.9. STATISTICAL ANALYSIS

Statistical analysis was performed with IBM SPSS Statistics 26 (for Windows, Chicago, IL, USA). Numerical data, e.g. blood parameter, MMP, and ROS, were checked for normal distribution and homogeneity of variance. The effect of strain, time and diet and their interaction on weight gain was first checked using Multivariate ANOVA, followed by pairwise comparisons of the diet effect at each time point within each strain. For all the described parameters in serum, oocytes and cumulus cells, a two-tailed independent sample T-test was used to compare HFD and control groups of the same strain, and the control groups of the two strains. Gene expression data were not

homogenous and were analyzed using non-parametric independent sample Mann-Whitney test. Categorical data, e.g. proportions of different ultrastructural classifications, were analyzed using Chi square test. The numbers of replicates and oocytes used to generate the data are described in the results section for each parameter. Differences with *P*-values ≤ 0.05 are statistically significant. Differences with *P* values > 0.05 and ≤ 0.1 are not statistically significant and reported as *tendencies*. Data are expressed as means ± S.E.M unless otherwise stated.

4. RESULTS

4.1. HIGH FAT DIET AND BODY WEIGHT GAIN

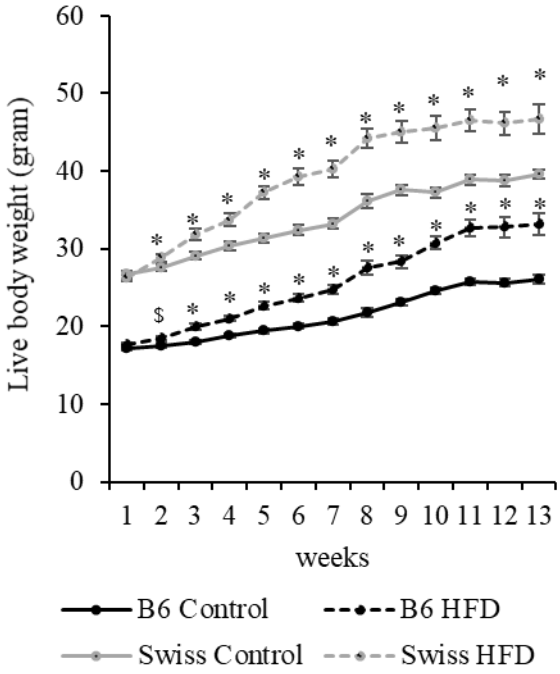


Figure 1. The effect of HFD feeding on body weight gain in B6 and Swiss mice. Data are shown as means ± SEM from 14-16 mice per group. Significant difference ($P < 0.05$) between HFD and Control groups within each strain are indicated by an asterisk (*). Tendencies ($0.05 < P < 0.1$) are indicated by a dollar sign ($).

Swiss and B6 mice fed HFD gained more weight during the 13w-feeding period compared with their corresponding controls of the same strain. Multivariate ANOVA showed that body weight was significantly affected by *Strain* (F = 3277.3, $P < 0.001$), and *Time* (F = 154.1, $P < 00.1$), and their interaction (F = 5.39, $P < 0.001$). The effect of *Diet* (F= 466.8, $P <0.001$) and the interaction *Strain*Diet* (F = 9.3, $P =0.002$) were also significant. Within strain, the increase in weight due to HFD was statistically significant starting from week 2 onwards in the Swiss mice, and from week 3 in the B6 mice ($P<0.05$) compared to controls. At 13 w, the weight of the Swiss and B6 HFD group was on average 18.1% and 27.2% higher than their corresponding controls ($P <0.05$), respectively (Figure 1).

4.2. EFFECT ON BLOOD LIPID PROFILE

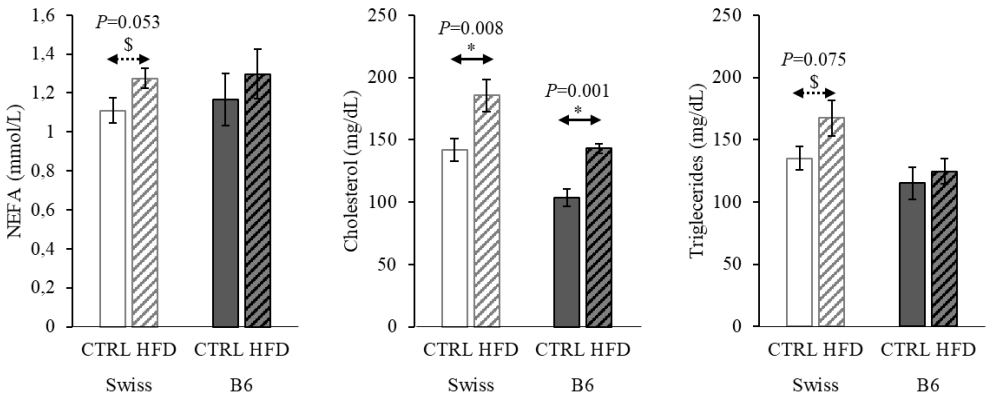


Figure 2. The effect of HFD feeding for 13 weeks on serum lipid profile in B6 and Swiss mice. Data are shown as means ± SEM from 14-16 mice per group. Significant differences ($P < 0.05$) between the groups demarcated by arrows are indicated by an asterisk (*). Tendencies ($0.05 < P < 0.1$) are indicated by a dollar sign ($). Exact *P* values are also shown.

HFD feeding for 13 weeks resulted in a significant increase in blood total cholesterol levels in both Swiss and B6 strains ($P <0.05$). HFD also resulted in a tendency to increase serum triglycerides (TGs) and NEFAs ($P <0.1$) in Swiss mice but not in B6 (Figure 2).

4.3. EFFECT ON OOCYTE RECOVERY FOLLOWING HORMONAL STIMULATION

The average number of oocytes (±SD) recovered from the oviducts following hormonal stimulation tended to be higher in the Swiss control mice compared with the B6 control (14±6.2 vs. 11±3.4 per mouse) however this difference was not statistically significant ($P = 0.065$). HFD did not have any impact on oocyte recovery since the number of oocytes collected from HFD-fed Swiss and B6 were similar to their corresponding controls (13±5.4 and 13±5.9 respectively, $P >0.1$).

4.4. EFFECT ON INTRACELLULAR LIPID DROPLETS (LD) IN OOCYTES

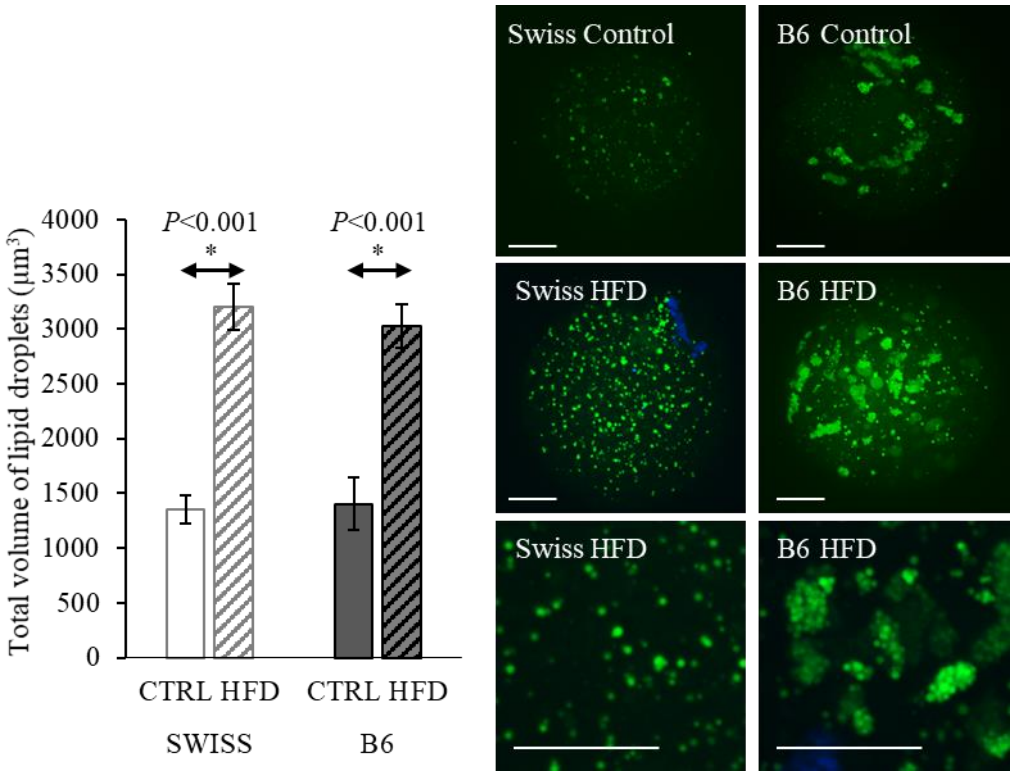


Figure 3. Representative confocal microscope images after BODIPY 493/503 and DAPI staining showing lipid droplets (Green) in oocytes collected from Swiss and B6 mice fed either control or HFD. Each image is a z-stack projection of 40 x 1µm steps. Scale bar = 17µm. Data are presented as means ± SEM from one oocyte per animal and 10-12 oocytes per group. Significant differences ($P < 0.05$) between the groups demarcated by arrows are indicated by an asterisk (*).

Lipid droplets in oocytes were examined using BODIPY 493/503 staining and confocal microscopy. Regardless of the type of feeding, the distribution pattern of LDs in oocytes was strain dependent. In Swiss mouse oocytes, LDs were either not clustered or formed very small aggregates and were evenly distributed throughout the ooplasm. Whereas in B6 oocytes, LDs were mostly found in large aggregated clusters (Figure 3). Quantification of the z-stacks (from a total of 40 oocytes) showed that HFD markedly increased the average total volume of LDs in oocytes compared to control (P <0.001) in both mouse strains.

4.5. EFFECT ON MITOCHONDRIAL ACTIVITY AND ROS IN OOCYTES

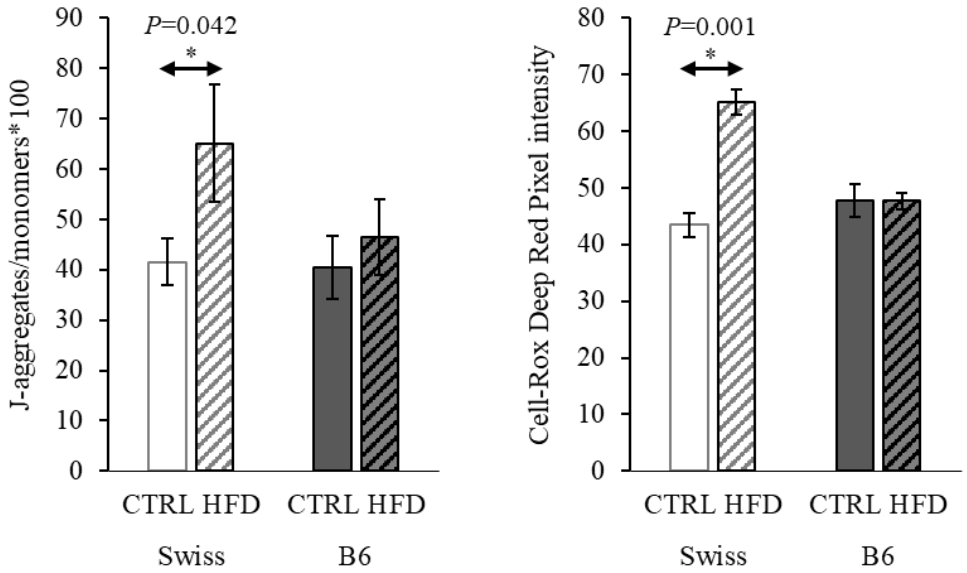


Figure 4. Quantification of mitochondrial activity (JC-1 staining, 590/525nm intensity x 100) and ROS levels (CellROX Deep Red staining, 660 nm intensity) in B6 (n=50) and Swiss (n=67) oocytes from the control and HFD groups. Data are presented as means ± SEM from 1-2 oocytes per animal and 14-16 animals per group. Significant differences ($P < 0.05$) between the groups demarcated by arrows are indicated by an asterisk (*).

Swiss HFD oocytes exhibited higher MMP compared to Swiss controls ($P < 0.05$). This was estimated as the ratio of the J-aggregates (590nm) to the monomers (525nm) after JC-1 staining and confocal microscopy (Figure 4). An increased 660nm intensity of CellROX Deep Red staining was also evident in the HFD Swiss oocytes indicating higher intracellular ROS concentrations compared with Swiss controls ($P < 0.05$). In contrast, the overall MMP and ROS levels were similar in B6-HFD and B6-control oocytes ($P > 0.1$).

4.6. EFFECT ON MITOCHONDRIAL ULTRASTRUCTURE IN CUMULUS CELLS AND OOCYTES

Cumulus oocyte complexes (COCs) were examined with transmission electron microscopy (TEM). In the oocytes, mitochondria were considered to be normal if they were spherical and electron dense, with or without regular vacuoles (Saben et al., 2016b; Wakai et al., 2014). Mitochondria with abnormal morphology were either vacuolated with loose membranous structures, elongated, dumbbell and rose petal in shape, contained highly electron dense foci or were degenerated (supplementary figure 1).

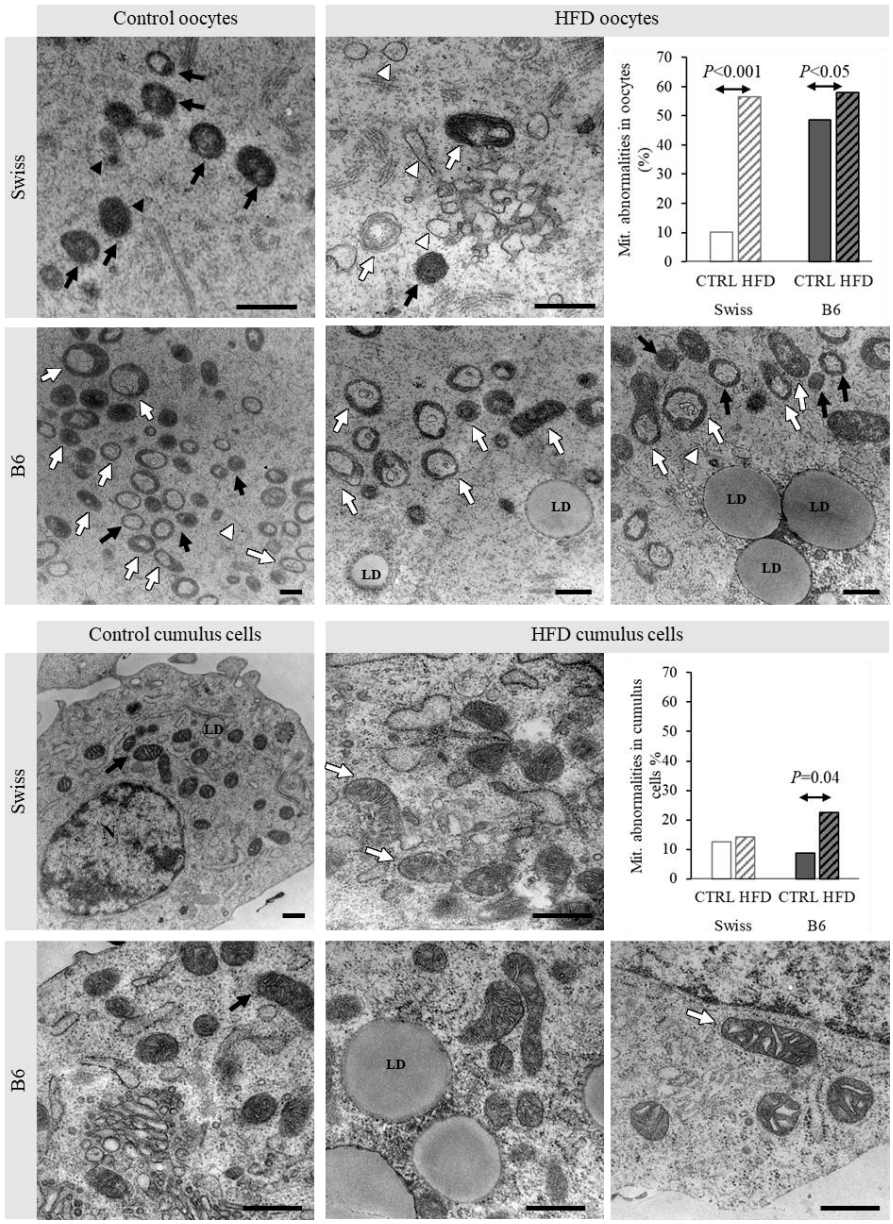


Figure 5. Representative TEM micrographs showing the ultrastructure of mitochondria (arrows) and ER (arrows heads) in oocytes and cumulus cells collected form Swiss and B6 mice fed a control or high fat diet (HFD) for 13 w. Different forms of normal (black arrows) and abnormal (white arrows) mitochondrial structures can be observed as classified in Supplementary figure 1. A quantification of the percentage of mitochondrial abnormalities is also included. Data are presented as proportion percentages from 229, 188, 344, and 746 mitochondria counted in Swiss Control, Swiss HFD, B6 Control and B6 HFD respectively, from 3 COCs (from 3 animals) per group. The *P* values between the groups indicated by the arrows are shown.

In Swiss control oocytes, 89.95% of the mitochondria were normal, 18.3% of which contained regular vacuolated areas (Supplementary table 1). HFD increased the percentage of mitochondrial abnormalities to 56.4%. In contrast, B6-control oocytes exhibited significantly higher proportion of abnormal mitochondria (48.6 %) when compared to Swiss controls ($P < 0.05$). The proportion of mitochondrial abnormalities in B6-HFD (57.9%) was about 9% higher than in B6 control ($P<0.05$) (Figure 5). A detailed quantification of these mitochondrial abnormalities is shown in "Supplementary figure 1" and "Supplementary table 1".

In cumulus cells, normal elongated mitochondria with many well developed cristae could be observed in Swiss and B6 controls. In the B6-HFD group, a significantly higher proportion of mitochondria had distorted shapes and small vacuoles compared to B6-control ($P<0.05$). In both B6 and Swiss HFD groups, it was noticed that many mitochondria had a relatively lower electron density, which was difficult to quantify.

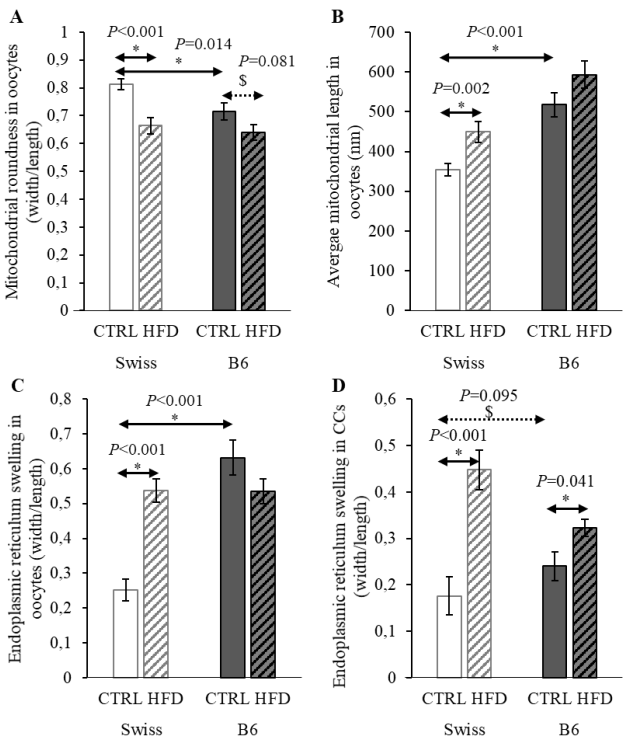


Figure 6. Measurements of the dimensions of mitochondria in oocytes, and ER in oocytes and cumulus cells to estimate the effect of HFD feeding on their ultrastructural morphology in Swiss and B6 mice. Mitochondrial roundness and ER swelling indices were calculated and compared. Data are presented as means ± SEM from the measured organelles in 3 COCs (from 3 animals) per group. Significant differences ($P < 0.05$) between the groups demarcated by arrows are indicated by an asterisk (*). Tendencies ($0.05 < P < 0.1$) are indicated by a dollar sign ($). Exact P values are also shown.

To acquire more insight about the impact of HFD, the dimensions of the mitochondria and ER in the TEM images were measured using Image-J software. The mitochondria were found to be almost spherical in the Swiss control oocytes, and less round in the B6 control oocytes ($P<0.05$) (Figure 6A). Mitochondrial roundness was further reduced by HFD in both strains ($P<0.05$). The average length of the mitochondria was shortest in Swiss control oocytes and was significantly longer in Swiss-HFD oocytes and in both B6 groups ($P<0.05$) (Figure 6B).

ER swelling, calculated as a ratio between ER width and length was lowest in Swiss control oocytes and cumulus cells and was significantly increased in Swiss HFD ($P<0.05$). ER swelling was significantly higher in B6 control and HFD oocytes compared to Swiss controls ($P<0.05$) (Figure 6C and D).

4.7. mRNA EXPRESSION OF MARKERS OF CELLULAR STRESS IN CUMULUS CELLS AND OOCYTES

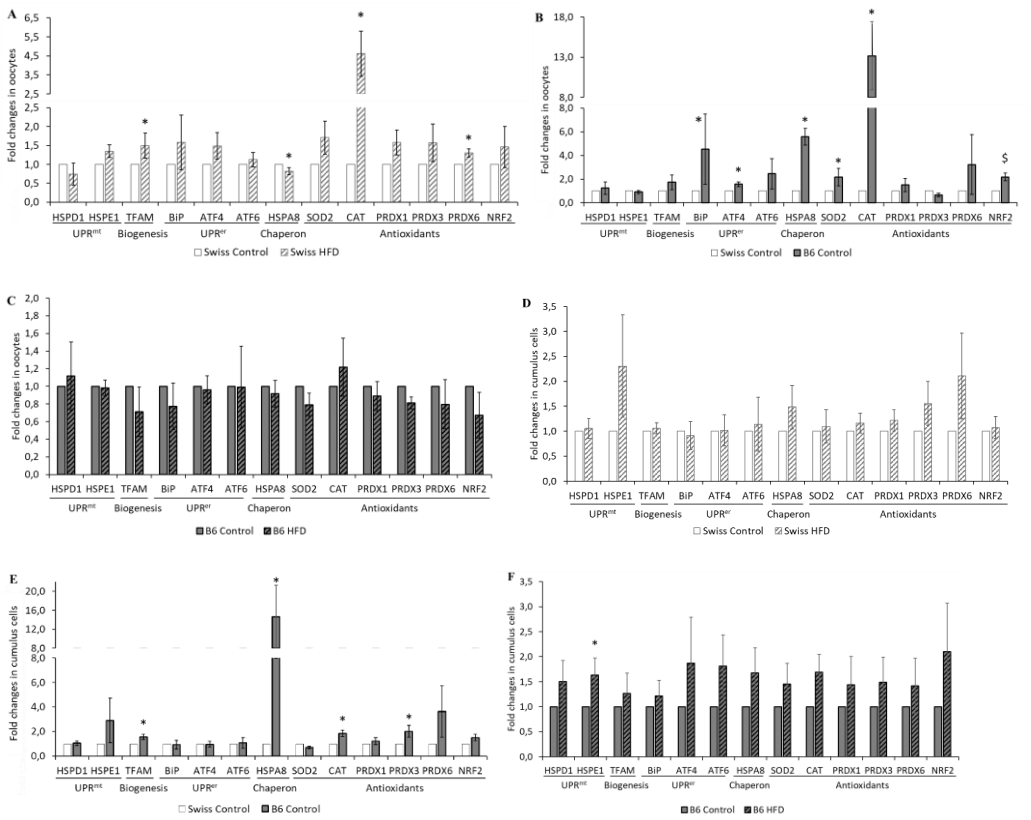


Figure 7. Strain and HFD effects on transcription markers of cellular stress in oocytes (A-C) and cumulus cells (D-F). Marker genes related to mitochondrial and ER UPRs and redox regulatory mechanisms were analyzed in B6 and Swiss mice fed either control or HFD. Data were compared within strain or between the controls of the two strains. Columns display means ± SEM of fold changes relative to housekeeping genes from 4 replicates (pools from 3-4 animals per group each). Significant differences are indicated with an astrisk ($P < 0.05$). Tendencies are indicated by a dollar sign "$" ($P < 0.1$ and >0.05).

In Swiss oocytes, HFD increased *CAT* and *PRDX6* mRNA expression. The expression of other tested genes related to redox regulatory mechanisms (*SOD2*, *PRDX1*, *PRDX3*, *NRF2*) were not significantly different from controls. Importantly, the expression levels of markers of mitochondrial UPRs (*HSPE1* and *HSPD1*) and ER UPRs (*BiP*, *ATF4*, and *ATF6*) in the Swiss HFD oocytes were also similar to the Swiss controls (P >0.1). Nevertheless, *TFAM* expression was higher in the HFD group (P<0.05). *HSPA8* was significantly lower with P<0.05 but the relative difference in fold change was very subtle compared to the controls (Figure 7A).

Surprisingly, several oxidative stress related transcripts were upregulated in control B6 oocytes when compared to the Swiss control group (Figure 7B). This includes an increased expression of *CAT*, *SOD2*, and *HSPA8* (P <0.05), and a tendency to higher *NRF2* transcript levels (P <0.1). ER stress-related transcripts were also upregulated (*ATF4* and *BiP*), whereas mitochondrial UPR-related genes were not differently expressed. HFD did not induce any further difference in the expression level of any of the tested genes in the B6 oocytes (P >0.01) (Figure 7C).

In cumulus cells, the variability across replicates was high in the Swiss HFD group, and despite numerical increase in some stress related genes, none of them were statistically significant compared to the controls (P >0.1) (Figure 7D). However, as observed in the oocytes, cumulus cells from B6 controls showed a significantly higher expression of *CAT*, *HSPA8* and *PRDX3*, as well as *TFAM*, compared with the Swiss control (P<0.05) (Figure 7E). Markers of mitochondrial and ER UPRs were not altered in B6 controls, but the mitochondrial *HSPE1* was upregulated in response to HFD in the B6 cumulus cells compared to B6 controls (P <0.05) (Figure 7F).

4.8. EFFECT ON MTDNA COPY NUMBERS IN OOCYTES

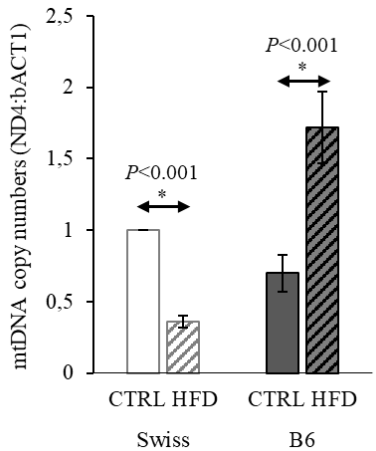


Figure 8. Alteration in mtDNA copy numbers in oocytes collected from Swiss and B6 mice fed a control or high fat diet (HFD) for 13 w. Data are shown as mean ± SEM from 3 replicates (pools from 3-4 animals per group each). Significant differences (P < 0.05) between the groups demarcated by arrows are indicated by an asterisk (*).

mtDNA copy numbers in the control oocytes of B6 and Swiss were similar (P > 0.1). HFD reduced mtDNA copy numbers by 2.81X in Swiss oocytes but resulted in a 2.46X increase in mtDNA copy numbers in B6 oocytes (P<0.05 compared to the corresponding control) (Figure 8).

5. DISCUSSION

This study aimed to investigate mitochondrial dysfunction in oocytes at the structural, functional and molecular level in response to HFD-induced obesity. We studied if inbreeding might influence these alterations. Interestingly, we observed major differences in mitochondrial structure in the inbred B6 oocytes as compared to the outbred Swiss oocytes. This was associated with contrasting responses to HFD-induced metabolic stress. We found that feeding HFD for 13 w resulted in various alterations in mitochondrial abnormalities, MMP, mtDNA copy number and cellular stress signaling in oocytes. These effects were highly dependent on the strain.

As expected, HFD increased weight gain and serum total cholesterol concentrations in both strains, which validates the induction of the required metabolic alteration. Swiss mice also exhibited a tendency to increase blood NEFAs and TGs (not statistically significant at $P<0.05$) while this was not evident in B6 mice. Similar increase in serum cholesterol was described in other studies in C57BL/6 after feeding a HFD, with (Boudoures et al., 2016) or without a concomitant increase in TGs and NEFAs (Williams et al., 2014). The impact of this metabolic stress on oocyte quality focusing on mitochondrial functions was the main focus of this study.

There is a strong evidence that hyperlipidemia is linked with reduced oocyte quality, which is mainly due to a direct impact of altered lipid composition of the follicular fluid (Valckx et al., 2014a). Here, we found that HFD increased intracellular lipid accumulation in the Swiss and B6 oocytes. Similar results were reported in CBA and C57Bl/6J mice (Reynolds et al., 2015b; Wu et al., 2015). This can be due to active lipid accumulation, since murine oocytes can directly incorporate free fatty acids (FFAs) from their microenvironment as demonstrated *in vitro* following direct exposure to FFA-rich media (Yang et al., 2012).

Interestingly, we noticed that the LDs distribution was markedly different in B6 and Swiss strains. Lipid droplets were clustered in large aggregates in B6 but were finely scattered in the Swiss ooplasm. Reynolds et al. (2015a) showed that after 6 weeks of HFD in C57BL/6 the LDs in the oocytes increased in number and became aggregated. This aggregation was persistent even after 8w of subsequent diet normalization. We suggest that LD clustering could be an important phenomenon that may have extended detrimental effects on oocyte quality, however, this notion has not been tested. Here, the aggregated LDs in B6 mice were also noticed in the control oocytes, at a lower volume, showing that this could be a strain effect, and not only induced by diet. LD clustering may be caused by deficient proteins required for LD stabilization (Ta et al., 2012). In contrast, smaller LD aggregates (as in the Swiss strain) might provide more surface area for LD-associated proteins, such as lipases (Ta et al., 2012), and may facilitate LD interaction with other organelles, mainly the mitochondria and ER. The impact of LD clustering on oocyte quality requires further investigation.

Accumulation of LD in non-adipose tissue is a cellular mechanism that prevents lipotoxicity by storing intracellular lipids in a neutral status (Jarc and Petan, 2019). However, under lipotoxic conditions, upregulated lipolysis and lipophagy may result in a nutritive overload, which affects mitochondrial functions, disrupt cellular metabolism, and may induce oxidative stress and apoptosis (Le Lay and Dugail, 2009). In the present study, we found that MMP (JC-1 aggregates) was significantly increased by HFD in Swiss oocytes, but not in the B6. Higher MMP indicates higher mitochondrial activity, possibly due to increased FA β-oxidation. This could be facilitated by the small LD size in Swiss oocytes, while LD clustering in B6 oocytes may render them less consumable.

Changes in mitochondrial activity in oocytes in response to HFD is inconsistent among different studies. Igosheva et al. (2010) reported that HFD significantly increased MMP in oocytes and zygotes collected from obese C57BL/6 mice. In contrast, Wu et al. (2010) showed that HFD rather reduced mitochondrial activity in CBA mouse oocytes. In addition, the interpretation of relative changes in MMP can also be perplexing. Lower MMP in oocytes, e.g. due to maternal aging, was associated with lower ATP production and reduced developmental competence, whereas higher MMP has been linked with higher embryo development (Wilding et al., 2001). In contrast, high MMP can also be detrimental, and was linked with increased embryonic arrest at the 2-cell stage in mice and with high fragmentation rate in 8-cell embryos in humans (Acton et al., 2004). Interestingly, the increase in MMP at the final stage of IVM in bovine oocytes due to lipotoxicity and oxidative stress has been shown to be temporary, and was followed by a subsequent reduction in MMP (uncoupling) during early cleavage stages (Marei et al., 2019a; Marei et al., 2019b). Uncoupling occurs particularly in mitochondria that suffer most from high ROS accumulation, which may result in a heterogeneous population of hyperactive and inactive mitochondria within the same oocyte. Therefore overall changes in the MMP in oocytes should always be carefully interpreted in the context of other related parameters and considering the associated ROS levels.

HFD-Swiss oocytes exhibited higher MMP and also higher intracellular ROS as shown using CellROX Deep Red staining. In contrast, HFD did not influence MMP nor ROS in B6 oocytes compared to B6 control. This may either imply that HFD did not elicit an oxidative stress in B6 oocytes, or that the B6 control oocytes already exhibited high levels of oxidative stress for other reasons. The notion that B6 control oocytes also suffer from oxidative stress was further supported by the gene expression results and other outcome parameters as discussed below.

B6 control oocytes had higher *CAT*, *SOD2*, and *NRF2* mRNA expression compared to Swiss control oocytes. The expression of these genes was equally high in B6-HFD oocytes compared to B6 controls. Similar strain dependent differences in some cellular stress related genes were also observed in the B6-control cumulus cells compared to

Swiss controls. On the other hand, Swiss HFD oocytes clearly exhibited a significantly higher expression of genes related to Redox regulation compared to Swiss controls.

Looking further into the ultrastructural morphology of the oocytes, we found that the majority (about 90%) of the mitochondria in the Swiss control oocytes had normal ultrastructure (Saben et al., 2016b; Wakai et al., 2014). As expected, HFD significantly increased mitochondrial ultrastructural abnormalities (about 60%) in Swiss oocytes. Surprisingly, B6 control oocytes already exhibited very high rates of mitochondrial abnormalities. This feature has not been previously brought into attention. However, in Boudoures et al. (2016) we noticed that only 60% of the mitochondria were reported to be round in control C57BL/6 oocytes, while 40% were categorized as elliptical or dumbbell in shape. Moreover, 20% of the spherical mitochondria were described as rose petal in shape (Boudoures et al., 2016). This relatively high percentage of abnormalities was suggested to be probably attributed to the sedentary type of housing of these mice (Boudoures et al., 2016). Here, the housing was the same for both strains showing that the mitochondrial abnormalities in B6 could rather be genetically driven. Feeding HFD for 4 weeks (Luzzo et al., 2012) or for 12 w (Boudoures et al., 2016) resulted in an additional 10-20% increase in the proportions of abnormal mitochondria in B6 oocytes compared to controls, an effect that was also observed here (9.36% higher than B6 control). This effect was much bigger in the Swiss strain.

Additionally, our data clearly show that the roundness of the mitochondria was highest in Swiss control oocytes and significantly lower in B6 control oocytes, and was further reduced by HFD in both strains. Mitochondrial elongation can be an indication of the activation of mitochondrial fission and fusion (Friedman and Nunnari, 2014) and could therefore be a part of a compensatory reparative mechanism. An increased expression of PGC1α and DRP1, which play an important function in mitochondrial biogenesis, has been described in B6 oocytes in response to HFD (Luzzo et al., 2012). Mitochondrial elongation and reduction in electron density normally occur after the 8-cell stage and become more evident at blastocyst formation (Sathananthan and Trounson, 2000), coinciding with the change in the metabolic activity of the mitochondria towards an increased glucose utilization and oxygen consumption (Grindler and Moley, 2013). Therefore, detection of these changes in HFD oocytes here may indicate a premature alteration in cellular metabolism. Similar ultrastructural abnormalities have been linked with abnormal or delayed meiotic progression of the affected oocytes and abnormal spindle morphology (Boudoures et al., 2016) thus signifying a reduced oocyte developmental competence. Finally, increased ROS production inside the mitochondria could induce mitochondrial fission (Skulachev et al., 2004) which may link the increased mitochondrial elongation and the disruption in Redox regulatory mechanisms observed here in the Swiss HFD oocytes.

ER swelling was increased in the HFD Swiss oocytes, and interestingly in both HFD and control B6 oocytes, compared to Swiss controls. Again, this suggests that the B6 oocytes

exhibit high levels of cellular stress regardless of the HFD. ER swelling is an indication of increased ER stress, which is closely related to the mitochondrial dysfunction and increased risk of apoptosis (Zhao et al., 2017). ER stress has a well described role in mediating the effects of altered lipid metabolism and lipotoxicity in somatic cells. This was also documented in bovine COCs exposed to lipotoxic concentrations of FFAs *in vitro* (Marei et al., 2017; Sutton-McDowall et al., 2016).

Another main aim of the present study was to determine if UPRs occur in the oocytes in response to HFD. Mitochondrial and ER stress in somatic cells are known to stimulate transcription of chaperons and other related factors to control protein misfolding and enhance cell survival (Pagliassotti et al., 2016; Runkel et al., 2014). Therefore, we examined the transcription levels of marker genes for UPR^{er} (*BiP*, *ATF4* and *ATF6*), and UPR^{mt} (*HSPD1* and *HSPE1*). Keeping in mind the limited transcriptional capacity in oocytes specially at the final stage of maturation, we found that the oxidative stress-related mRNA expression of *CAT* and *PRDX6* were significantly increased in the Swiss HFD group. This is in line with the increased oxidative stress levels illustrated by CellROX Deep Red staining, and suggests that the long-term exposure to HFD during oocyte growth could influence gene expression levels while the oocyte is still transcriptionally active. Nevertheless, we could not detect any corresponding change in UPR related genes despite the evident mitochondrial dysfunction and ER swelling. Perhaps, these responses are not fully functional in mature oocytes at the transcriptional level. We have previously shown that exposure of bovine COCs to lipotoxic concentrations during IVM resulted in several UPR-related proteomic changes (Marei et al., 2019b), which were persistent until the blastocyst stage (Marei et al., 2019a). Interestingly, we observed that *BiP* and *ATF4*, as well as *SOD2* and *CAT* were significantly higher in B6 control oocytes compared to the Swiss controls, and were equally high in the B6 HFD group. This is in line with the observed ER swelling and further suggests that these UPRs could also be strain dependent.

Finally it was also important to assess the alteration in mtDNA copy numbers in oocytes in response to HFD. This parameter is linked to mitochondrial dysfunction and oxidative stress. Swiss HFD oocytes exhibited a significantly lower mtDNA, which was associated with a (compensatory) increase in TFAM expression compared to Swiss controls. This could be simply due to mitophagy and removal of damaged mitochondria. In contrast, B6-HFD oocytes had a significantly higher mtDNA content with no difference in TFAM expression compared to B6 controls. A previous study showed a similar increase in mtDNA copy numbers (with a higher TFAM expression) in B6 mice exposed to obesogenic diet compared to lean mice (Igosheva et al., 2010).

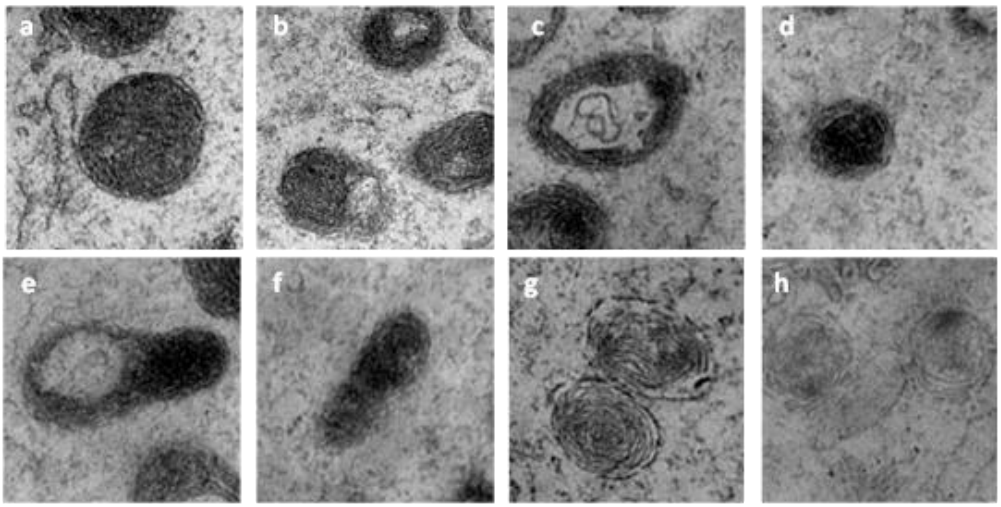
The number of mtDNA copies in oocytes increases during folliculogenesis and reaches its maximum at the end of oocyte maturation (St John, 2014) thus it may be influenced during long-term exposure to altered follicular environment due to the HFD. Short exposure to lipotoxic conditions during IVM (24h) did not influence mtDNA copy

numbers in bovine oocytes despite the evident oxidative stress and mitochondrial dysfunction (Marei et al., 2019a). The mtDNA copy number in an oocyte is a product of a turnover process during which mitochondrial biosynthesis and degradation take place. Therefore, the increased mtDNA copies in HFD-B6, considering the evident mitochondrial ultrastructural damage, may not necessarily only be due to increased mitochondrial biogenesis, but could also be due to defective mitophagy (Lemasters, 2014). This hypothesis requires further investigation. Inhibition of mitophagy using a proteasome inhibitor has been shown to increase mtDNA content in oocytes, which shows that degradation is an important determinant under certain conditions (Sato et al., 2014). Human embryos with relatively high mtDNA copy numbers (MitoScore) have significantly higher risk of failure of implantation (Fragouli et al., 2017). Therefore, the increased mtDNA in B6 HFD oocytes could be detrimental at later stages of development.

Mechanisms regulating mitophagy and mitochondrial biogenesis in oocytes and early embryo development have not been clearly elucidated. Recent studies illustrated that accumulation of damaged mitochondria in obesity-exposed oocytes and their transmission to next generations is due to defective mitophagy (Boudoures et al., 2017; Saben et al., 2016b). These studies were carried out using C57BL/6 mice. However, our results strongly suggest that mitochondrial damage and mitophagy may vary in different strains of mice, possibly due to inbreeding. This is crucial for the correct extrapolation of information described in transgenerational studies based on the C57BL/6 strain.

In conclusion, different important aspects of mitochondrial dysfunction in response to HFD-induced obesity appear to be influenced by the genetic background of the mouse model used, including alteration in MMP, mitochondrial ultrastructural abnormalities and alteration in mtDNA copy numbers (and possibly mechanisms regulating mitochondrial biogenesis and mitophagy). This may be caused by inbreeding. Mitochondrial chaperons in oocytes were not affected by long-term HFD at the transcription level in both B6 and Swiss mice. However, B6 oocytes exhibited some upregulated ER UPR markers again suggesting a strain effect. These results should further build on awareness when designing mouse models to study human fertility and transgenerational effects. Depending on the strain used, conclusions can be significantly different. We showed that the outbred Swiss mouse seems to be more sensitive to HFD-induced reduction in oocyte quality when compared to the inbred B6 mouse. Whereas the B6 oocytes appear to be of low quality regardless of the type of diet, which may explain the reduced fertility observed in this strain.

6. SUPPLEMENTARY INFORMATION



Supplementary figure 1. Different forms of mitochondrial ultrastructure that were observed in mouse oocytes. Mitochondrial structure was considered normal when spherical (a) or spherical with regular vacuoles (b). Mitochondrial abnormalities include vacuolation with loose inner membrane structures (c), electron dense foci (d), dumbbell shapes (e), elongation (f), rose petal appearance (g) or degeneration (h).

Supplementary table 1. Proportions of different categories of normal and abnormal mitochondrial ultrastructure in oocytes collected from Swiss and B6 mice fed a control or a high fat diet (HFD) for 13 w.

	Swiss Control	**Swiss HF**	**B6 Control**	**B6 HF**
Total	229	188	344	746
Normal mitochondria	***206 (89.95%)***	***82 (43.61%)***	***177 (51.45%)***	***314 (42.09%)***
a. Spherical	164 (71.6%)	63 (33.5%)	91 (26.5%)	185 (24.8%)
b. Regular Vacuoles	42 (18.3%)	19 (10.1%)	86 (25.0%)	129 (17.3%)
Abnormal mitochondria	***23 (10.04%)***	***106 (56.38%)***	***167 (48.54%)***	***432 (57.90%)***
c. Membranous Vacuoles*	12 (5.24%)	22 (11.7%)	63 (18.3%)	168 (22.5%)
d. Electron dense foci	0 (0%)	15 (7.98%)	41 (11.9%)	106 (14.2%)
e. Dumbbell-shaped	1 (0.44%)	8 (4.26%)	16 (4.65%)	27 (3.62%)
f. Elongated	6 (2.62%)	26 (13.8%)	24 (6.97%)	33 (4.42%)
g. Rose petal-shaped	2 (0.87%)	14 (7.45%)	19 (5.52%)	35 (4.69%)
h. Degenerated	2 (0.87%)	21 (11.2%)	4 (1.16%)	63 (8.45%)

*Vacuoles containing loose membranous structures. Letters a-h correspond to the representative images shown in supplementary figure 1.

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CHAPTER 6:

HIGH FAT DIET-INDUCED OBESITY AND PRECONCEPTION CARE INTERVENTIONS

DIET NORMALIZATION OR CALORIC RESTRICTION AS A PRECONCEPTION CARE STRATEGY TO IMPROVE METABOLIC HEALTH AND OOCYTE QUALITY IN OBESE OUTBRED MICE

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1. ABSTRACT

Background: Maternal metabolic disorders are linked to reduced metabolic health and oocyte quality. Obese women are advised to lose weight before conception to increase pregnancy chances. However, as human studies show no univocal guidelines, more research is necessary to provide fundamental insights in the consequences of dietary weight loss on oocyte quality. Therefore, we investigated the impact of diet normalization or calorie restricted diet for two, four or six weeks, as preconception care intervention (PCCI), in obese mice on metabolic health and oocyte quality.
Methods: Outbred female mice were fed a control (CTRL) or high-fat (HF) diet for seven weeks (7w). Afterwards, HF-mice were put on different PCCIs, resulting in four treatment groups: 1) control diet up to 13w, 2) HF diet up to 13w (HF_HF), switch from a HF (7w) to 3) an *ad libitum* control diet (HF_CTRL) or 4) 30% calorie restricted control diet (HF_CR) for two, four or six weeks. Body weight, metabolic health, oocyte quality and overall fertility results were assessed.
Results: Negative effects of HF diet on metabolic health, oocyte quality and pregnancy rates were confirmed. HF_CTRL mice progressively improved insulin sensitivity, glucose tolerance, serum insulin and cholesterol from PCCI w2 to w4. No further improvements in metabolic health were present at PCCI w6. However, PCCI w6 showed best oocyte quality improvements. Mature oocytes still showed elevated lipid droplet volume and mitochondrial activity but a significant reduction in ROS levels and ROS: active mitochondria ratio compared with HF_HF mice. HF_CR mice restored overall insulin sensitivity and glucose tolerance by PCCI w4. However, serum insulin, cholesterol and ALT remained abnormal. At PCCI w6, glucose tolerance was again reduced. However, only at PCCI w6, oocytes displayed reduced ROS levels and restored mitochondrial activity compared with HF_HF mice. In addition, at PCCI w6, both PCCI groups showed decreased mitochondrial ultrastructural abnormalities compared with the HF_HF group and restored pregnancy rates.
Conclusions: Diet normalization for four weeks showed to be the shortest, most promising intervention to improve metabolic health. Most promising improvements in oocyte quality were seen after six weeks of intervention in both PCCI groups. This research provides fundamental insights to be considered in developing substantiated preconception guidelines for obese women planning for pregnancy.

2. INTRODUCTION

The prevalence of obesity and metabolic syndrome is significantly increasing worldwide and has been regarded as a major threat to public health (Eckel et al., 2010; Engin, 2017; WHO, 2018). Not only the genetic background but especially a sedentary lifestyle together with a too high caloric intake of diets rich in sugars and (especially) saturated fat are seen as the major causative players (Bluher, 2019).

Very often, obesity coincides with a significant reduction in metabolic health, characterized by an increase in body weight, abdominal fat accumulation, aberrant serum lipid profiles, liver dysfunction, an impaired glucose tolerance and reduced insulin sensitivity (Dahlhoff et al., 2014; Jungheim and Moley, 2010; Samuelsson et al., 2008). Reduced fertility is often seen in these obese patients. Clinical centers for assisted reproduction report a higher incidence of menstrual irregularity or even anovulation, reduced oocyte developmental capacity after *in vitro* fertilization and thus a longer time to successful conception in this cohort of obese patients (Klenov and Jungheim, 2014; Metwally et al., 2007a; Zain and Norman, 2008). Even obese women with a normal ovarian cycle display reduced fertility rates, indicating a negative impact of the disturbed metabolic health on critical peri-conception events that determine oocyte quality and ultimate pregnancy success (Robker, 2008).

In depth research, using not only human but also animal research models, provide growing evidence for the major role that reduced oocyte quality plays in the pathogenesis of subfertility in metabolically compromised women. We and others showed that the oocyte's micro-environment, the follicular fluid (FF), in obese women reflects the disturbed metabolic state (Jungheim and Moley, 2010; Sutton-McDowall et al., 2010). This means that the oocyte directly senses the hyperglycemic and hyperinsulinemic conditions (Jungheim and Moley, 2010) next to the elevated non-esterified fatty acid (NEFA) and triglyceride concentrations (Valckx et al., 2014a). Factors involved in oxidative stress and inflammation also impact on the FF composition and have the potential to reduce oocyte quality (Robker et al., 2011; Snider and Wood, 2019).

Oocytes, collected from obese patients but also from Western Type diet induced obese mice, displayed an impaired quality, indicated by high rates of meiotic spindle abnormalities, increased mitochondrial ultrastructural abnormalities, altered mitochondrial membrane potential and increased cellular oxidative stress levels (Grindler and Moley, 2013; Igosheva et al., 2010; Marei et al., 2020; Wu et al., 2010). Furthermore, mitochondrial DNA (mtDNA) copy number and mitochondrial biogenesis in oocytes from obese mice were altered (Marei et al., 2020) and higher levels of lipid accumulation and abnormal lipid distribution in oocytes were present (Marei et al., 2020; Reynolds et al., 2015; Wu et al., 2010).

Up until now, overweight and obese patients are often advised to lose weight before conception through dietary lifestyle interventions to improve metabolic health, fertility and to increase the chance of a healthy pregnancy (Jungheim and Moley, 2010; Lassi et al., 2014; Pasquali, 2006).

Although a limited amount of weight loss (3-5%) can already partly improve metabolic health in humans, more significant weight losses are needed for complete recovery (Jensen et al., 2014). Such a weight loss and metabolic health improvement can be achieved by a simple diet normalization (Reynolds et al., 2015). However, in a lot of studies, a more strict calorie restricted diet showed to be more effective for weight loss and restoring metabolic health (Aksungar et al., 2017; Andersen and Fernandez, 2013). Extreme weight loss induces extensive lipid mobilization, and thus high NEFA concentrations, which may trigger lipotoxic effects in the oocyte (Chusyd et al., 2016). In depth in vitro research could clearly confirm the importance of these lipotoxicity pathways in explaining the reduced oocyte quality seen in metabolically compromised individuals (Marei et al., 2019). Such direct impact of drastic weight loss regimes on oocyte quality has, to the best of our knowledge, never been studied before.

So far, there are no evidence-based guidelines regarding fertility treatment in overweight and obese infertile women. Several (sometimes underpowered) lifestyle intervention studies that investigated the effect of weight loss before conception on fertility in obese and overweight individuals, observed a significant increase in pregnancy and/or live birth rates (for an overview, see (Sim et al., 2014)). Recent large randomized controlled trials could not confirm this (Einarsson et al., 2017b; Mutsaerts et al., 2016b) and concluded that preconception weight loss via dietary interventions did not improve live birth rates in obese women scheduled for IVF (Einarsson et al., 2017b). However, the authors did detect more spontaneous pregnancies in the lifestyle program group. Advising for a more severe weight loss before conception by applying a very low calorie diet, has recently been discouraged in clinical settings as it is estimated that this results in potential harm to the oocyte which may further lead to an adverse pregnancy outcome (Jensen et al., 2014; Tsagareli et al., 2006).

These conflicting results, together with the limitations of human studies (high drop-out rates, lack of sufficient power, patient clinical history, societal and lifestyle background) lead to a lack of scientifically supported advice (Norman and Mol, 2018). Does significant weight loss, as a preconception care intervention, have a positive impact on oocyte quality and/or might diet normalization be sufficient to optimize fertility outcome? Only very few studies focused on the impact of preconception dietary interventions on oocyte quality in obese women, so in depth research is needed.
Reynolds et al. used an inbred obese mouse model (C57BL/6) for her research and reported that diet normalization for eight weeks could not recover oocyte quality although the metabolic health returned to normal. However, we recently showed that control C57BL/6 mice, in contrast to outbred Swiss mice, already show a high degree of

oocyte mitochondrial abnormalities and a disturbed mitophagy (Marei et al., 2020). Therefore, using an outbred mouse strain might be more relevant for this research and for further translation to human settings. A small pilot study reported a negative outcome on *in vitro* fertilization rates after feeding obese women a short term very low calorie diet for 4-6 weeks (Tsagareli et al., 2006). It is not known if a longer exposure time might be more efficient to improve oocyte quality or whether a long-lasting carry-over effect at the early phase of folliculogenesis should be expected on the quality of the mature oocyte.

This confirms the need for well-controlled and strategically designed outbred animal experiments investigating the specific effect of diet normalization or caloric restriction and the duration of these interventions on oocyte quality. Are a complete normalized weight and metabolic health necessary for oocyte quality to recover? It is important to emphasize that this intervention period should be as short as possible to avoid the potential negative effects of advancing maternal age on ovarian reserve and reproductive capacity (Lan et al., 2017; Sim et al., 2014).

Therefore, in this study we hypothesized that the efficiency of a preconception care intervention (PCCI) in high fat-fed obese outbred mice to improve metabolic health, oocyte quality and fertility depends on the method of diet change (diet normalization or caloric restriction) and on the duration of that intervention.

To test this hypothesis, we aimed to switch high fat-fed obese outbred mice to two different preconception care interventions (PCCIs): 1) an ad libitum control diet or 2) a 30% calorie restricted control diet for two, four or six weeks and to assess the impact on metabolic health, oocyte quality and general fertility results.
To assess the impact on metabolic health we aimed to analyze serum insulin, glucose, cholesterol, triglyceride, NEFA and alanine aminotransferase (ALT) concentrations together with the assessment of glucose tolerance and insulin sensitivity (at PCCI week 0, 2, 4 and 6). Oocyte quality was evaluated by assessing intracellular lipid droplet content, reactive oxygen species (ROS), mitochondrial activity and localization of active mitochondria, as well as mitochondrial ultrastructural abnormalities, and mtDNA copy numbers. In addition, oocyte recovery and pregnancy rates were investigated.

3. MATERIALS AND METHODS

3.1. ANIMALS, DIET AND EXPERIMENTAL DESIGN

Five-week-old female outbred Rj:Orl Swiss (n = 156, hereafter referred to as “Swiss”) mice (Janvier labs) were used. At the start of the experiment, mice were randomly divided into two groups with ad libitum access to either a control (CTRL, E157453-04; Sniff Diets) or a high fat diet (HF, E15741-34, Sniff diets) for a period of seven weeks.

Afterwards, some of the HF-mice were switched to two different preconception care interventions for two, four or six weeks, while the remaining HF and the control mice remained on their corresponding diet for comparison. This resulted in four different treatment groups of equal size: 1) control diet for up to 13 weeks (CTRL_CTRL), 2) high fat diet for up to 13 weeks (HF_HF), 3) high fat diet for seven weeks then a switch to an *ad libitum* control diet for two, four or six weeks (HF_CTRL) and 4) high fat diet for seven weeks then a switch to a 30% caloric restriction diet for two, four or six weeks (HF_CR). An experimental timeline is shown in figure 1.

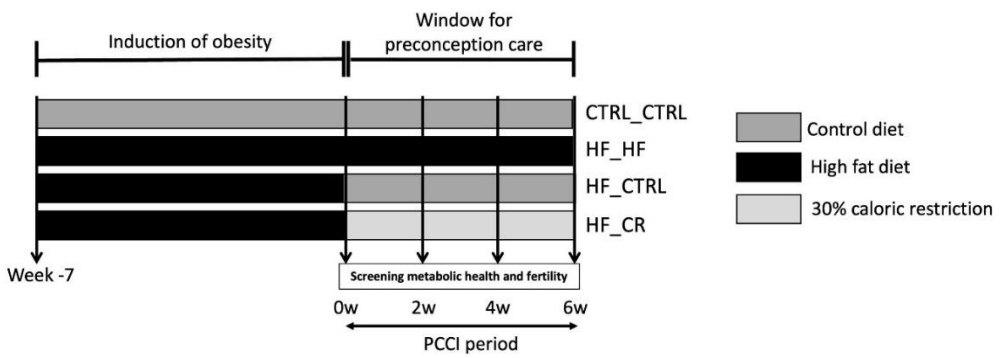


Fig. 1. Overview of the experimental design

The HF diet was composed of 60 kJ% fat from beef tallow, 20 kJ% carbohydrate and 20 kJ% protein. The matched, purified control diet contained 10 kJ% fat, 70 kJ% carbohydrate and 20 kJ% protein. Access to water was provided ad libitum. The 30% caloric restriction diet had the same composition as the control diet, however, the HF_CR mice received only 70% of the amount of food consumed by the CTRL_CTRL mice (paired feeding regimen, calculated on the basis of the daily consumption).

Mice were put on the diet in subgroups of 8 animals per treatment per time point (in 2 replicates) with an interval of few weeks between replicates to facilitate handling and sample collection procedures. Mice were weighted weekly before the start of the PCCI and every four days during the PCCI period to closely follow-up weight changes.

3.2. SERUM COLLECTION AND ANALYSIS

At each time point during the PCCI period, mice were fasted overnight and were sacrificed by decapitation to allow blood collection. Blood was centrifuged 30 min after collection at 2000 rpm for 10 min, and serum was stored at −80 °C until analysis. Insulin concentrations were measured using an ultrasensitive mouse insulin ELISA kit (90080, CrystalChem, The Netherlands). In addition, the following serum analyses were performed in a commercial laboratory (Algemeen Medisch Labo, Antwerp, Belgium): NEFA concentrations were determined using a colorimetric assay (Randox Laboratories Ltd, Crumlin, Co. Antrim, United Kingdom) on an IDS iSYS multi-discipline automated instrument (Immunodiagnostic Systems Hld, Tyne & Wear, UK); Triglycerides, Cholesterol and Alanine aminotransferase (ALT, an overall indicator of liver damage), were measured on an Abbott Architect c16000 (Abbott, Illinois, U.S.A).

3.3. INSULIN AND GLUCOSE TOLERANCE TEST

Mice (n=8 per treatment per time point) were fasted for six hours prior to the glucose (GTT) and insulin tolerance test (ITT). The tip of the tail was cut off and a blood drop was used to measure the basal glycaemia with a glucose stick (One Touch Verio glucose test strips; ADC, Belgium) in a glucose meter (OneTouch Verio; ADC, Belgium) and immediately afterwards the mouse was intraperitoneally injected with 20% glucose solution (2g glucose/kg body weight; Thermo Fisher Scientific, Belgium) for the GTT or with 0,075 IU insulin/kg body weight (Novorapid (Novo Nordisk), ADC, Belgium) for the ITT. The glycaemia was measured again 15, 30, 60, 90 and 120 minutes after the injection. Afterwards, the area under the curve (AUC) and elimination rate (ER) of glucose were calculated for both tests as follows:

$$AUC = ((C_1 + C_2)/2)\ x\ (t_2 - t_1)$$

C_1 and C_2 are the concentrations of glucose at time points t_1 and t_2, respectively (Pruessner et al., 2003). This calculation was made per time frame (0-15min, 15-30min etc.) and total AUC was calculated as the sum of all AUC calculations.

$$ER\ gluc = ((\ln[Pgluc] - \ln[Ngluc])/(tn - tp))x\ 100$$

$Pgluc$ and $Ngluc$ are peak and nadir (lowest) glucose concentrations, while tp and tn are the times of the peak and nadir glucose concentrations, respectively (Rezende et al., 2012).

The same mice were used to perform GTT and ITT with an interval of 2 days. The analysis was repeated using the same mice at zero, two, four and six weeks of PCCI (n=8 per treatment group).

3.4. OOCYTE AND CUMULUS CELL COLLECTION AND PREPARATION FOR SUBSEQUENT ANALYSES

In order to avoid any bias due to the glucose and insulin injections, different mice than those used for the GTT and ITT were selected to determine oocyte quality. At all time points, these mice received intraperitoneal injections of 10 IU equine chorionic gonadotropin (eCG, Synchrostim; Ceva Santé Animale) followed, 48 h later, by 10 IU human chorionic gonadotropin (hCG, Pregnyl; Organon) to induce and synchronize ovulations. Mice were sacrificed 13–14 h after hCG injection. *In vivo* matured oocytes were obtained from the oviducts immediately after euthanasia. Each oviduct was dissected together with the ovary and a part of the uterine horn and transferred to a collection tube containing L15 medium (Thermo Fisher Scientific, Belgium) supplemented with 50 IU/mL penicillin G sodium salt (Merck, Belgium), and 10% Fetal Bovine Serum (Greiner Bio-One, Belgium).

The cumulus oocyte complexes (COCs) collected from both oviducts of the same animal were pooled. Only COCs that met the following selection criteria were used for analysis: oocytes surrounded by an expanded cumulus cell mass with a perfect spherical shape, a regular zona and a translucent, homogeneously colored cytoplasm without inclusions. Morphologically good quality COCs with expanded cumulus cells from each mouse were distributed for downstream analysis, according to the total number of COCs available, as follows: one whole COC per mouse was fixed in glutaraldehyde solution for transmission electron microscopy (TEM) (at 0 and 6w of PCCI). The remaining COCs were completely denuded by repeated pipetting through 150 µm Stripper tips fitted on EZ-grip (Origio, The Netherlands) in a droplet of L15 medium supplemented with 0.3 mg/mL hyaluronidase (Merck, Belgium). Denuded oocytes were transferred to a fresh drop of L15 medium. One or two denuded oocytes (per mouse) were immediately transferred to JC-1 and CellROX Deep Red staining to determine mitochondrial activity and intracellular ROS content. One to two oocytes per mouse were fixed in paraformaldehyde 4% for determination of lipid droplet content. The remaining oocytes were washed in PBS containing 1 mg/ mL PVP and snap frozen per individual mouse in a 1.5 mL tube in a minimum volume for DNA extraction for determination of mtDNA content. All frozen samples were stored at −80 °C until further analyses.

An overview of all outcome parameters at specific time points (week 0, 2, 4 and/or 6 after start of the PCCI) are presented in table 1. The final numbers of animals and oocytes used to collect data for each outcome parameter are described in the figure legends.

Table 1. Overview of the outcome parameters assessed at specific time points during the preconception care intervention period (PCCI).

		PCCI week 0	PCCI week 2	PCCI week 4	PCCI week 6
Metabolic health	Insulin sensitivity test* (n=8 mice/treatment/time point)	✓	✓	✓	✓
	Glucose tolerance test* (n=8 mice/treatment/time point)	✓	✓	✓	✓
	Serum° - Glucose - Insulin - NEFA - Cholesterol - Triglycerides - ALT (n=7-8 mice/treatment/time point)	✓	✓	✓	✓
Fertility	Oocyte recovery rate° (n=8 mice/treatment/time point)	✓	✓	✓	✓
	Lipid droplet volume in oocytes° (n=8 mice/treatment/time point)	✓	✓	✓	✓
	ROS in oocytes° (n=8 mice/treatment/time point)	✓	✓	✓	✓
	Mitochondrial activity in oocytes° (n=8 mice/treatment/time point)	✓	✓	✓	✓
	Localization of active mitochondria in mature oocytes° (n=8 mice/treatment/time point)	✓	✓	✓	✓
	mtDNA copy numbers in oocytes° (n=3-5 mice/treatment/time point)		✓	✓	✓
	Mitochondrial ultrastructural abnormalities (TEM)° in cumulus cells and oocytes (n=3-5 mice/treatment/time point)	✓			✓
	Pregnancy rates (n=8 mice/treatment)				✓

NEFA = non-esterified fatty acids; ALT = alanine aminotransferase; ROS = reactive oxygen species; TEM = transmission electron microscopy

*Same mice were used to determine the selected outcome parameters over all PCCI time points

° Same mice were used per PCCI to analyze the selected outcome parameters

3.5. ASSESSMENT OF OOCYTE LIPID DROPLET VOLUME

Intracellular lipid droplets in the fixed denuded oocytes were examined using BODIPY staining according to Marei et al. (2020). To summarize, oocytes were permeabilized for 30 min in PBS containing 0.1 % (w/v) saponin (Fiers, Kuurne, Belgium) and 0.1 M

glycine. Next, oocytes were incubated in 20 µg/ml BODIPY 493/503 (Thermo Fisher Scientific, Belgium) in PBS for 1h. Oocytes were washed twice in PBS containing 3 mg/mL PVP after each step in the staining procedure. Finally, the oocytes were transferred to droplets of PBS-PVP on glass-bottom dishes and immediately examined under a confocal microscope. High resolution images were obtained using a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Zaventem, Belgium) equipped with 488 nm diode lasers for excitation of green fluorophores, respectively. For each oocyte, a z-stack of 40 µm (with steps of 1 µm) was acquired in the lower half of the oocyte (closest to the objective lens where the image is sharpest). Images were analyzed using Volocity 6.0.1 software (PerkinElmer) to evaluate the differences in lipid droplet content among oocytes in different groups. To exclude background, only particles ≥0.5 μm^3 in size were considered as lipid droplets and included in the analysis.

3.6. ASSESSMENT OF MITOCHONDRIAL ACTIVITY, LOCALIZATION OF ACTIVE MITOCHONDRIA AND INTRACELLULAR ROS

Oocyte mitochondrial activity and intracellular ROS concentrations were assessed using a combined fluorescence staining technique using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Invitrogen) and CellROX™ Deep Red Reagent (Thermo Fisher Scientific, Belgium) as described by Komatsu et al. (2014). This combined staining and simultaneous detection using multilaser was validated and described by De Biasi et al. (2015). Freshly collected oocytes were incubated for 30 min in L15 medium containing JC1 (5 µg/mL) and CellRox deep red (2.5 mM) (from 1000X stock solutions in DMSO) at 6 % CO_2 and 37°C. They were then washed and transferred to L15 medium droplets under mineral oil on a 35 mm dish with a glass bottom. Stained oocytes were immediately examined under a Leica SP8 confocal microscope enclosed in a humid warm chamber (37°C) and equipped with white laser source (Leica WLL) lasers. Mitochondrial activity was measured as the mean grey scale intensity of the J-aggregates (excitation/emission 561/590 nm, which is dependent on mitochondrial inner membrane potential), while intracellular ROS was measured as the mean grey scale intensity of the Cell Rox deep Red (644/665 nm). The ratio of ROS: active mitochondria was calculated as the ratio of grey scale intensity at 665:590. The grey scale intensity in each channel was measured using Leica Application Suite X (LAS X) software.

The regional distribution of active mitochondria was examined. Active mitochondria migrate within the developing oocyte. They reach a pericortical localization in the mature oocyte, which is linked to successful preimplantation development (Wakefield et al., 2008). To determine pericortical localization of active mitochondria, one optical section was taken (excitation/emission 561/590 nm) at the maximum oocyte diameter. Based on its radius, the oocyte was divided into 5 equal circular zones Wakefield et al. (2008), in which the most central zone was labelled as zone 1 and the most peripheral

zone was labelled as zone 5. If mitochondria were localized in zone 5 of the oocyte, they were categorized as “Pericortical”. If not, they were labelled as “Diffuse”. Since this staining is performed on live oocytes, the number of oocytes/mouse used in this outcome parameter was limited to avoid any bias due to increased time of imaging.

3.7. MITOCHONDRIAL ULTRASTRUCTURE - TRANSMISSION ELECTRON MICROSCOPY (TEM)

The ultrastructure of mitochondria in cumulus-oocyte-complexes was only assessed at zero and six weeks of PCCI, according to Marei et al. (2020). Briefly, freshly collected whole COCs were immediately fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5 % glutaraldehyde solution at 4°C for a maximum of 1 month. Individual COCs were then embedded in 2% agarose blocks to enable handling. Afterwards, blocks were washed three times in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5 % saccharose solution. Post-fixation was performed by incubating the blocks for 2 h with 1 % OsO4 solution. After dehydration in an ethanol gradient, samples were embedded in EM-bed812. Ultrathin sections were stained with lead citrate, and examined in a Tecnai G2 Spirit Bio TWIN microscope (Fei, Europe BV, Zaventem, Belgium) at 120 kV. For each COC, images of at least 5 cumulus cells and at least 10 random fields in the oocyte (covering most of the oocyte area), were acquired at 16500–25000×. Mitochondria in the acquired images were morphologically evaluated by an expert blind to the corresponding treatment group and were classified based on their morphology (Supplementary Fig. 1), according to Marei et al. (2020).

3.8. RELATIVE CHANGE OF MTDNA COPY NUMBERS.

DNA extracts from oocyte pools were used to determine the ratio of mtDNA to nuclear DNA by qPCR of the mitochondrial gene (ND4) and the nuclear gene (bACT). The relative mtDNA: nuclear DNA ratio was calculated using the 2–ΔΔCq method described by Livak and Schmittgen (Livak and Schmittgen, 2001b).

3.9. PREGNANCY RATES

As an endpoint assessment (PCCI week 6), mice (n=8 from each treatment group) were mated with Swiss males of proven fertility (maintained on a control diet) to test for pregnancy rates. Two females were housed with one male for four nights. During mating and pregnancy, CTRL_CTRL, HF_HF and HF_CTRL mice stayed on their respective diet. HF_CR mice were offered an ad libitum control diet to provide sufficient nutrients to the fetuses during pregnancy. Pregnancy rates were assessed between 18-21 days after mating.

3.10. STATISTICAL ANALYSIS.

Statistical analysis was performed with IBM SPSS Statistics 26 (for Windows, Chicago, IL, USA).

Numerical data, e.g. weight, blood parameters, mitochondrial activity and ROS, were checked for normal distribution and homogeneity of variance. Within each time point, numerical data were analyzed using One-way ANOVA. Post-hoc LSD was performed in a sequential manner for predefined comparisons based on the null hypothesis for each conditional research question. Following research questions were covered: 1) did exposure to a HF_HF diet induce a change compared with the CTRL_CTRL, if yes: 2) where the PCCIs effective in achieving any IMPROVEMENT compared with the HF_HF group, if yes: 3) were the measurements in the PCCI groups RECOVERED to the level of the CTRL_CTRL group. To test questions 1 and 2, a post-hoc test was performed using the HF_HF group as a reference group. If a significant difference (and thus an improvement) was detected in the PCCI group(s) compared with the HF_HF group, a second post-hoc test was performed using the CTRL_CTRL group as a reference group to check for potential recovery (i.e. considered as no significant difference anymore between a PCCI group and the CTRL_CTRL group). Non-homogenous data were analyzed using non-parametric independent sample Kruskal Wallis and a series of Mann-Whitney t-tests using the same sequential approach. On the other hand, categorical data, e.g. proportions of different ultrastructural classifications in TEM images, were analyzed using a Chi Square test also using the same strategy for comparisons.
The number of mice and oocytes used to generate the data are described in the results section for each parameter. Differences with *P*-values ≤ 0.05 were considered statistically significant. Differences with *P* values> 0.05 and ≤ 0.1 were reported as tendencies. Data are expressed as means ± S.E.M unless otherwise stated.

3.11. SAMPLE SIZE CALCULATION

Sample size calculation was performed using 'PS: Power and Sample Size Calculation version 3.1.2, 2014 (from Vanderbilt University)'. Based on available data from relevant outcome parameters (serum concentrations, glucose tolerance test, staining for oocyte lipid droplet volume), numbers of mice needed to detect statistical differences, averaged between 7-8. Sample size calculation was performed with a type 1 error of 0.05 and a power of 0.9.

4. RESULTS

4.1. WEIGHT GAIN AND LOSS

Feeding a HF diet already resulted in a significantly higher weight in HF_HF mice after only one week when compared with mice fed the control diet (CTRL_CTRL). HF-fed mice kept increasing in weight resulting in 25% more weight than the control group after seven weeks on the HF-diet (Fig. 2a).

As soon as the PCCI period started, mice that switched from a HF to an ad libitum control (HF_CTRL) or a 30% caloric restricted control (CR) diet (HF_CR) started to lose weight (Fig. 2b). After 16 days of PCCI, the HF_CR group showed a mean weight loss of 20.04%, and reached similar weights as the CTRL_CTRL group. As the PCCI continued, HF_CR mice kept losing weight however never significantly below the weight of the CTRL_CTRL mice. In the HF_CTRL group, complete weight recovery was achieved after 24 days of PCCI with a weight loss of 13.34%.

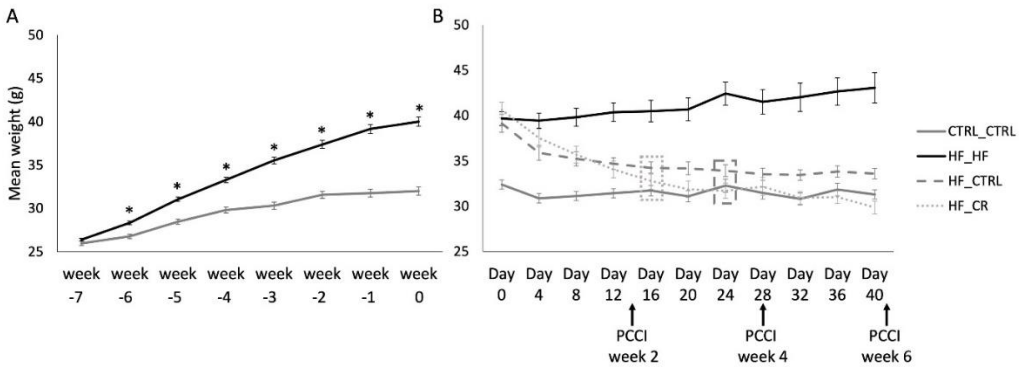


Fig. 2. The effect of high fat diet on **body weight gain during the first seven weeks (A); and body weight changes after the start of the preconception care intervention** (**PCCI) period (B)**. Data of fig 2A are shown as means ± SEM from 156 mice in total. Significant difference ($P < 0.05$) between HF_HF and CTRL_CTRL group are indicated by an asterisk (*). Time point at which HF_CR or HF_CTRL group showed no significant difference anymore with the CTRL_CTRL group are indicated by a rectangle with the same lay-out (Fig. 2B).

4.2. METABOLIC HEALTH

4.2.1. BLOOD SERUM PROFILE

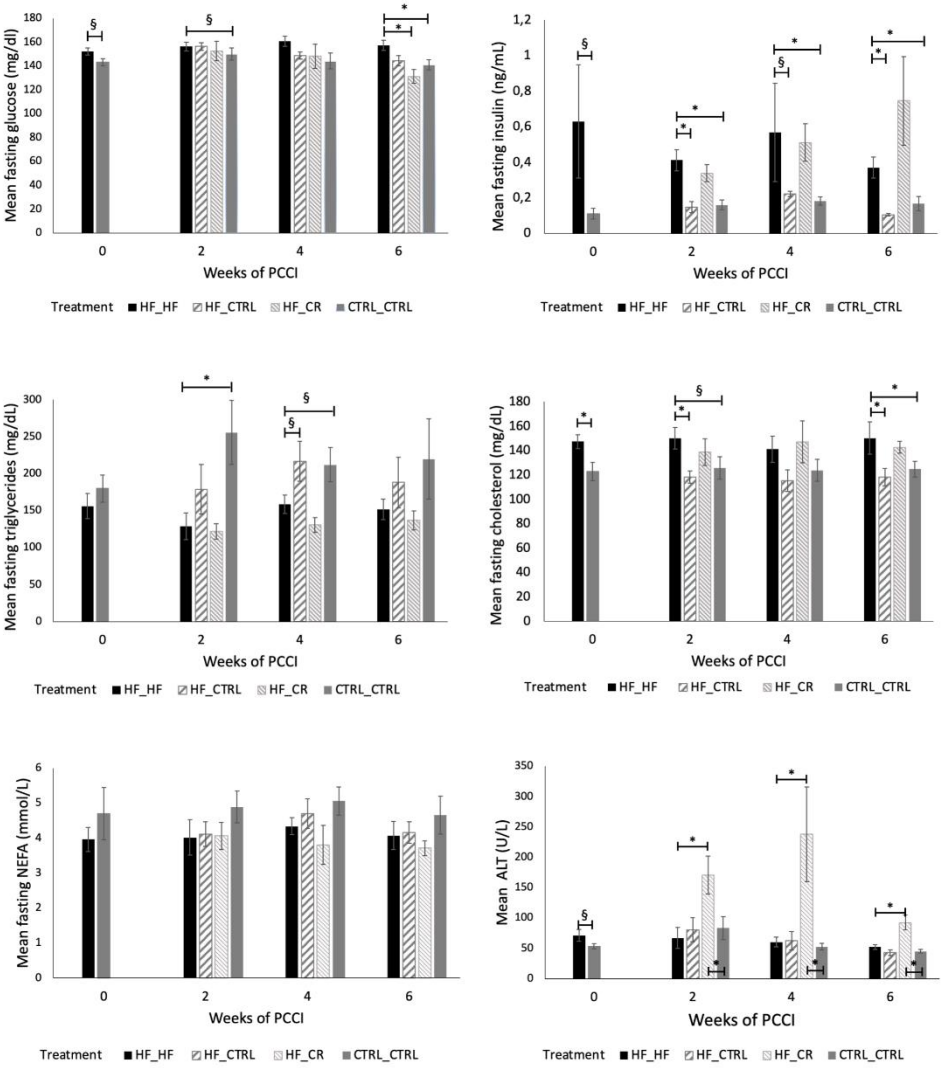


Fig. 3. **Fasting serum** concentrations of glucose, insulin, cholesterol, triglycerides, non-esterified fatty acids (NEFA), and alanine aminotransferase (ALT) among all treatment groups at different time points after starting the preconception care intervention (PCCI). Data are shown as means ± SEM from 7-8 mice per group per time point. Insulin concentrations after zero weeks of PCCI are from 4 mice/treatment group. Asterisks (*) indicate significant differences between the indicated treatment groups within the same PCCI period ($P<0.05$). Values labelled with "§" tend to be different from each other at $0.05 < P < 0.1$.

Feeding a HF diet increased fasting blood glucose concentration compared with the CTRL_CTRL group after zero (P<0.1), two (P<0.1) and six (P<0.05) weeks of PCCI (Fig. 3). This was associated with a marked increase in serum fasting insulin concentrations at all time points. Switching from a high fat to an ad libitum control diet (HF_CTRL) did not improve serum glucose concentrations when compared with the HF_HF group. However, serum insulin concentrations were normalized starting from week 2 (P<0.001 at week 2, P<0.1 at week 4 and P<0.001 at week 6).
In contrast, insulin levels in the HF_CR group remained as high as in the HF_HF mice at all time points (P>0.1) while these mice only displayed significantly improved glucose concentrations after six weeks of PCCI (P<0.05) when compared with the HF_HF group.

When focusing on the blood lipid profile, HF_HF mice displayed higher fasting cholesterol concentrations when compared with the CTRL_CTRL group at almost all time points (PCCI week 0 (P<0.05), week 2 (P<0.1) and week 6 (P<0.05); Fig 3). Mice that underwent diet normalization (HF_CTRL) already showed restored cholesterol concentrations after only two weeks of PCCI (P<0.05). In contrast, HF mice submitted to the CR diet (HF_CR) never showed any improvement of the elevated cholesterol concentrations. No significant differences in fasting NEFA concentrations could be detected in our experimental set-up.
Interestingly, triglyceride concentrations in the HF_HF group were lower after two (P<0.05) and four (P<0.1) weeks of PCCI when compared with the CTRL_CTRL group. Feeding a control diet to obese mice as a PCCI (HF_CTRL) only tended to increase triglyceride concentrations again after four weeks of intervention (P<0.1). A 30% calorie restricted diet (HF_CR) had no impact at all on the lowered triglyceride concentrations seen in the HF_HF group.

Feeding a HF diet had no effect on ALT concentrations, which is an indicator of liver damage. HF_CR mice, however, showed significantly higher ALT concentrations than the control (CTRL_CTRL) and the high fat (HF_HF) group at all time points.

4.2.2. INSULIN AND GLUCOSE TOLERANCE TESTS

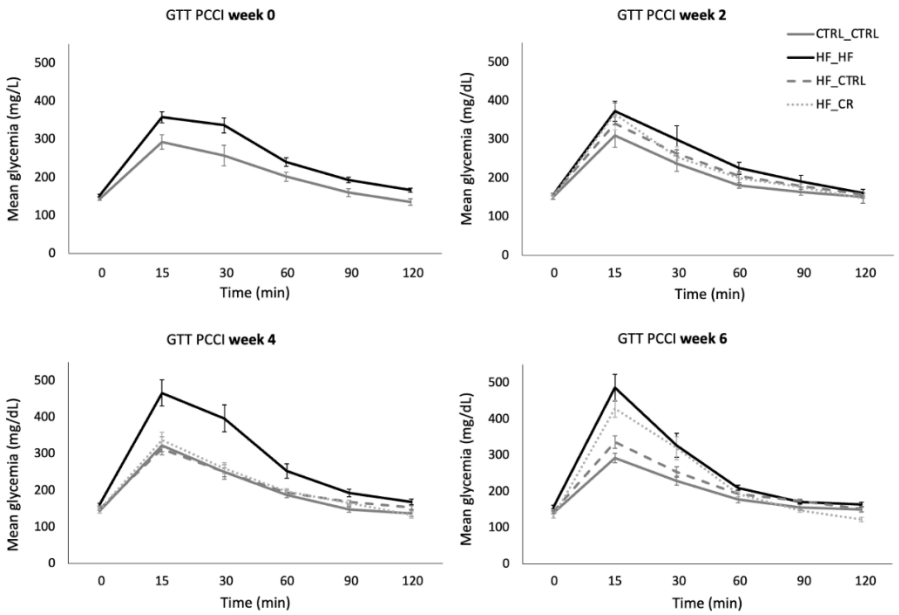


Fig. 4. Serum glucose concentrations during a **glucose tolerance test (GTT)** per treatment group at different time points after starting the preconception care intervention (PCCI) (week 0, 2, 4 and 6). Data are shown as means ± SEM. Per treatment, the same 8 mice were used at each time point.

Glucose tolerance tests (GTT) were performed at all time points on the same mice per treatment group.

Mice fed the HF diet displayed impaired glucose tolerance compared with the CTRL_CTRL mice throughout the experiment (Fig. 4). More specifically, HF_HF mice showed significantly higher AUC (P<0.05) and glucose peak (P<0.05) concentrations compared with the CTRL_CTRL group at all time points. Despite the increased AUC for glucose in the HF_HF mice, the elimination rate (ER) was elevated after four (P<0.1) and six (P<0.05) weeks of PCCI compared with HF_CTRL and CTRL_CTRL mice (Supplementary Fig. 2).

The curves in figure 4 suggests an overall improvement in glucose tolerance in the HF_CTRL mice already after two weeks of PCCI. However only from four weeks of PCCI onwards, HF_CTRL values for AUC, ER and peak glucose concentrations were significantly decreased when compared with the HF_HF mice (P<0.05) and even restored to the level of the control group.

Similarly, HF_CR mice showed a partial improved glucose tolerance after only two weeks of PCCI and a complete recovery after four weeks. However, feeding a calorie

restricted diet for six weeks aggravated glucose tolerance characteristics again together with an upregulated ER, similar to the HF_HF mice.

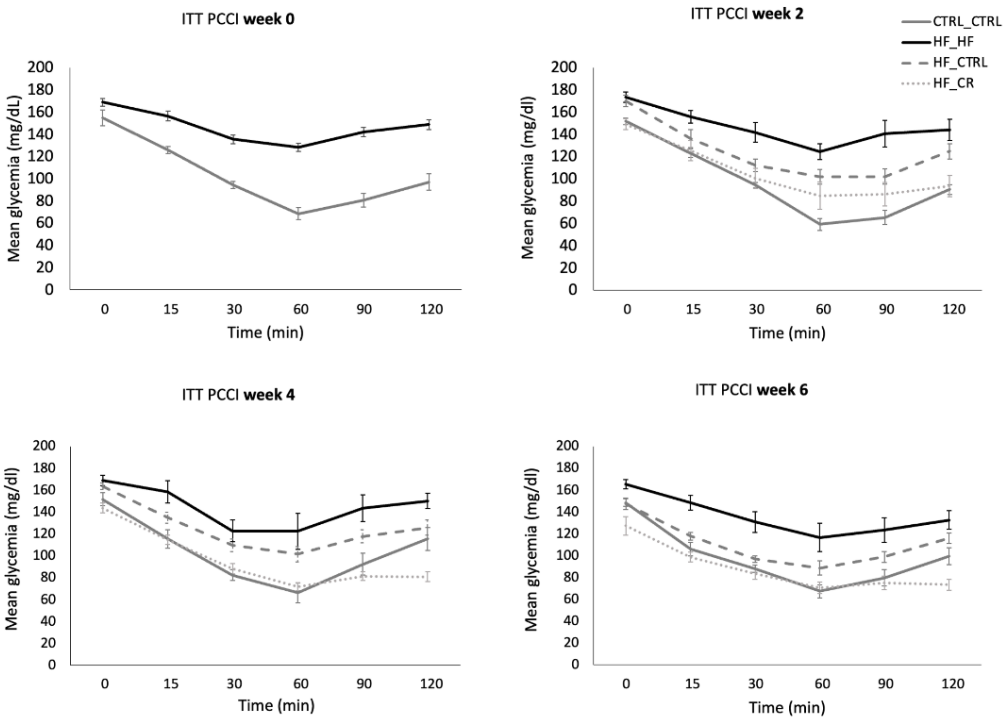


Fig. 5. Serum glucose concentrations during an **insulin sensitivity test (ITT)** per treatment group at different time points after starting the preconception care intervention (PCCI) (week 0, 2, 4 and 6). Data are shown as means ± SEM from the same 8 mice per group per time point. Per treatment, the same 8 mice were used at each time point.

Insulin tolerance tests (ITT) were performed on the same mice at all time points.

HF_HF mice displayed an overall impaired insulin sensitivity compared with CTRL_CTRL mice throughout the experiment (Fig. 5). This was confirmed by a significantly higher AUC and a lower ER for glucose than the CTRL_CTRL group at all time points (Supplementary Fig. 3).

The curve in figure 5 showed a partially improved insulin sensitivity in HF_CTRL mice, illustrated by a steadily improving AUC for glucose while the ER was never statistically different from the HF group (Supplementary Fig. 3). However, the HF_CTRL mice never showed a completely recovered insulin sensitivity, even after six weeks of PCCI.

In contrast, a caloric restriction diet (HF_CR) was able to significantly improve insulin sensitivity after only four weeks of PCCI, substantiated by a significantly lower AUC. At six weeks of PCCI, overall insulin sensitivity of HF_CR mice was still restored, in contrast

to the observed decreased glucose tolerance at this time point. However, ER for glucose did not improve when compared with the HF_HF mice at both time points as the HF_CR mice were not able to restore their glucose concentrations back to the basal levels within the tested timeframe of 120 minutes.

4.3. OOCYTE QUALITY - FERTILITY

4.3.1. EFFECT ON OOCYTE RECOVERY RATE AFTER HORMONAL STIMULATION

The average number of oocytes collected from the oviduct after hormonal stimulation was significantly lower in the HF_HF compared with the CTRL_CTRL group regardless of the PCCI time point (14±5.5 vs. 19±8.9; $P<0.05$).

After two weeks of PCCI, no significant differences between the HF_HF and the PCCI groups were present. However, HF_CR mice yielded significantly more oocytes than both CTRL_CTRL and HF_HF mice after four weeks of PCCI (24±10 *vs*. 15±7; 24±10 *vs.* 12±5 respectively). This was not the case anymore after six weeks of PCCI. No other significant differences were present.

4.3.2. MITOCHONDRIAL ULTRASTRUCTURE IN CUMULUS CELLS AND OOCYTES

Table 2. Proportions of **mitochondria** with normal or abnormal **ultrastructure** (using TEM) in cumulus cells and oocytes from all treatment groups at PCCI week 0 and 6.

		Total mitochondria	Normal mitochondria	Abnormal mitochondria
Oocytes				
PCCI week 0	**HF_HF**	525	347 (66.10%)	178 (33.90%)[a]
	CTRL_CTRL	400	379 (94.75%)	21 (5.25%)[b]
PCCI week 6	**HF_HF**	562	301 (53,56%)	261 (46.44%)[a]
	HF_CTRL	442	398 (90.05%)	44 (9.95%)[b]
	HF_CR	586	530 (90.44%)	56 (9.56%)[b]
	CTRL_CTRL	497	482 (96.98%)	15 (3.02%)[c]
Cumulus cells				
PCCI week 0	**HF_HF**	305	301 (98.69%)	4 (1.31%)
	CTRL_CTRL	193	191 (98.96%)	2 (1.04%)
PCCI week 6	**HF_HF**	446	437 (97.98%)	9 (2.02%)
	HF_CTRL	318	312 (98.11%)	6 (1.89%)
	HF_CR	429	416 (96.97%)	13 (3.03%)
	CTRL_CTRL	504	497 (98.61%)	7 (1.39%)

Data are presented as proportions from total number of mitochondria evaluated, from 3-5 COCs per treatment group (from 3-5 mice per treatment group). Significant differences between the indicated treatment groups at the same PCCI time point are indicated with letters a, b and c ($P<0.05$).

In the CTRL_CTRL oocytes, 5.25% (PCCI week 0) and 3.02% (PCCI week 6) of the evaluated mitochondria were categorized as structurally abnormal. Exposure to a HF diet (HF_HF group) significantly increased that percentage to 33.90% (P<0.001) at PCCI week 0 which further increased to 46.44% at week 6 (Table 2). Both preconception care intervention groups displayed a significant improvement of mitochondrial ultrastructural abnormalities to only 9.95% in the HF_CTRL group and 9.56% in the HF_CR group compared with the HF_HF group. However, they were not completely restored as they still showed significantly higher percentage of mitochondrial abnormalities than CTRL_CTRL oocytes (9.95% and 9.56% versus 3.02%, respectively).

No significant differences in the ultrastructure of cumulus cell mitochondria could be observed at both time points (week 0 and 6 of PCCI) (Table 2).

4.3.3. MITOCHONDRIAL ACTIVITY, LOCALIZATION OF ACTIVE MITOCHONDRIA AND ROS LEVELS IN OOCYTES

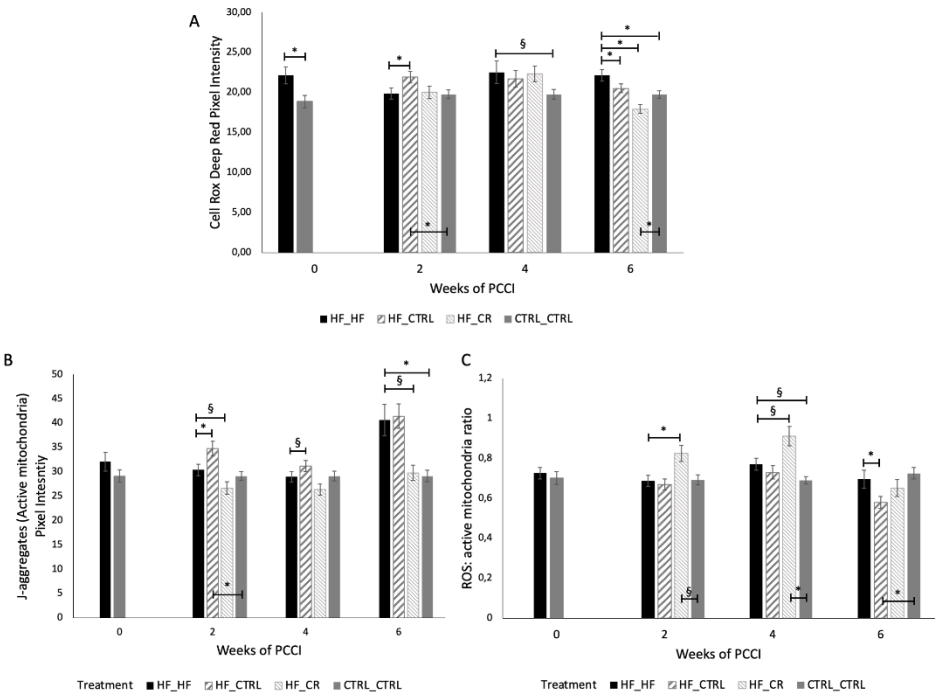


Fig. 6. Quantification of **ROS levels** (Fig. 6A; CellRox Deep Red staining), **mitochondrial activity** (Fig. 6B; JC1- staining) and **ROS: active mitochondria ratio** (Fig. 6C) at different time points after starting the preconception care intervention (PCCI). Data are shown as means ± SEM from 1-3 oocytes per mouse and 6-8 mice per group per time point. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period (P<0.05). Values labelled with "§" tend to be different from each other at 0.05 < P <0.1.

Oocytes collected from HF-fed mice displayed higher ROS levels compared with CTRL_CTRL oocytes at almost all time points (PCCI week 0 (P<0.05), week 4 (P<0.1) and week 6 (P<0.05); Fig. 6A). However, when ROS levels were normalized for total number of active mitochondria, this effect disappeared (Fig. 6C). HF_HF oocytes only showed significantly higher mitochondrial activity than CTRL_CTRL oocytes at PCCI week 6 (P<0.05; Fig. 6B).

HF_CTRL oocytes showed significantly higher ROS levels and mitochondrial activity than both reference groups after two weeks of PCCI (P<0.05). These levels declined as the PCCI period continued. ROS levels were even significantly improved after six weeks of PCCI when compared with the HF_HF group (P<0.05). This was associated with a significantly lower ratio of ROS: active mitochondria (P<0.05).

In the same line as HF_CTRL mice, switching to a caloric restriction diet (HF_CR) significantly improved ROS levels only after six weeks of PCCI (P<0.001), even lower than CTRL_CTRL oocytes (P<0.05). In contrast to HF_CTRL, oocytes collected from HF_CR mice showed reduced mitochondrial activity after two and six weeks of PCCI when compared with the HF_HF group (P<0.1). Interestingly, at two and four weeks, a significant increase was seen in the ROS levels when normalized for total number of active mitochondria. At PCCI week 6, this was not the case anymore.

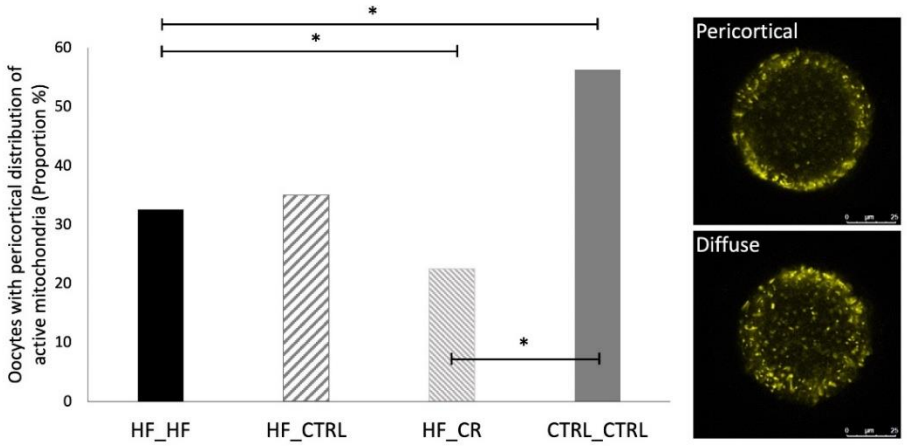


Fig. 7. Percentage of oocytes with **pericortical distribution of active mitochondria** (diffuse/pericortical), pooled per treatment group regardless of time point. Data are shown from 32-43 oocytes (from 20-26 mice) per treatment group. Asterisks (*) indicate significant differences between indicated treatment groups (P<0.05).

Since the number of oocytes used in this live cell confocal imaging was relatively low, data were pooled per treatment group and analyzed regardless of time points. The

proportion of oocytes with pericortical distribution of active mitochondria was markedly lower in HF_HF-fed mice compared with CTRL_CTRL (P<0.05 when using the merged data; Fig. 7). This was not improved in both PCCI groups and this condition was even worse in the HF_CR oocytes compared with the HF_HF oocytes. When dissecting these data for each time point, a similar trend was seen.

4.3.4. OOCYTE mtDNA COPY NUMBER

No significant differences were present between treatment groups after two weeks of PCCI. At PCCI week 4, oocytes from HF_HF mice displayed significantly higher mtDNA copy numbers than oocytes from the CTRL_CTRL group (*P*<0.05; Supplementary Fig. 4). Oocytes collected from HF_CR mice showed a tendency to lower mtDNA copy numbers when compared with the HF_HF group (*P*<0.1). At week 6 of the intervention, no differences between the treatment groups could be seen anymore.

4.3.5. LIPID DROPLET VOLUME – BODIPY STAINING

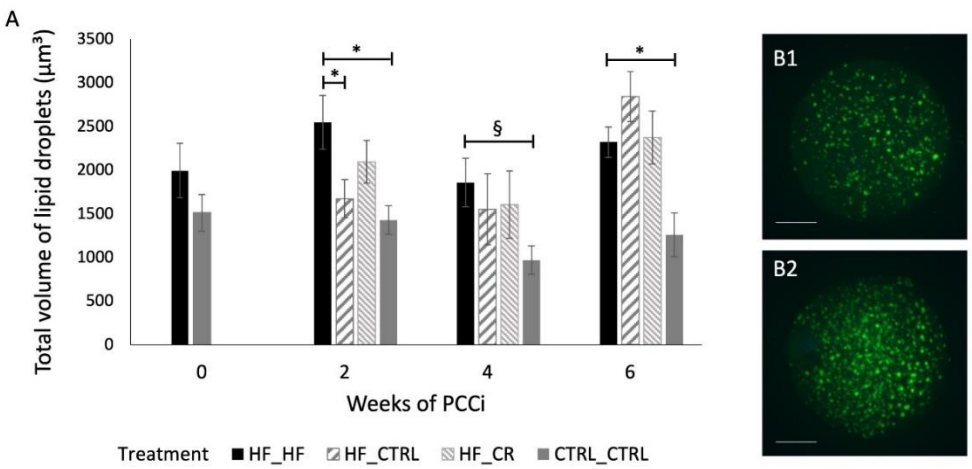


Fig. 8. Representative confocal microscope images after **BODIPY** 493/503 staining showing lipid droplets (green, panel B1 and B2) in oocytes collected from mice of all treatment groups, after 0, 2, 4 or 6 weeks of preconception care intervention (PCCI). Each image is a z-stack projection of 40 ×1 µm steps. Data are presented as means ± SEM from 1-3 oocytes per mouse, collected from 6-8 mice per group per time point (panel A). Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period (*P*<0.05). Values labelled with "§" tend to be different from each other at 0.05 < *P* <0.1.

Oocytes were examined for total lipid droplet volume using BODIPY 493/503 staining. Quantification of the z-stacks showed that oocytes collected from HF_HF mice displayed a higher lipid droplet volume than the CTRL_CTRL mice at PCCI week 2 (*P*<0.05), 4 (*P*<0.1) and 6 (*P*<0.05) (Fig. 8a). Oocytes collected from HF_CTRL mice

showed a significantly lower lipid droplet volume than mice on the high fat diet (HF_HF) after two weeks of PCCI ($P<0.05$). However, at PCCI week 4, this was not the case anymore. Obese mice that switched to a caloric restriction diet (HF_CR) were not able to improve the elevated lipid content in the oocytes.

4.3.6. PREGNANCY RATES (AFTER SIX WEEKS OF PCCI)

Pregnancy rates (defined as proportion %) were significantly lower in the HF_HF mice compared with the control group (12.5% *vs.* 100%; 8 mice per treatment group). Interestingly, this was not the case anymore in mice that switched to a preconception diet intervention as they both showed significantly higher pregnancy rates than mice on the HF diet and similar rates when compared with the CTRL_CTRL group (87.5% in HF_CTRL mice and 100% in HF_CR mice).

It is important to keep in mind that pregnancy rates are based on a timed mating where male and female mice were housed together for four nights.

5. DISCUSSION

The aim of this study was to investigate whether dietary interventions of two, four or six weeks before conception were associated with an improved metabolic health, oocyte quality and fertility in an obese outbred mouse model. We confirmed that a HF diet exposure resulted in an obese phenotype characterized by a hampered metabolic health together with low oocyte quality and reduced fertility. When obese mice were switched from their obesogenic diet to a normal, control diet (HF_CTRL) for only two weeks, overall metabolic health (serum insulin and cholesterol, insulin sensitivity) was clearly improved. Extending the exposure period to four weeks further ameliorated their metabolic health as glucose tolerance was also significantly restored. Submitting obese mice to a 30% calorie restricted diet also improved glucose tolerance and insulin sensitivity after four weeks of PCCI but other parameters measuring metabolic health (serum insulin, cholesterol and ALT) remained abnormal when compared with control mice. Submitting mice to this severely restricted feeding regime for six weeks negatively affected glucose tolerance.
The beneficial effects of both PCCI's on oocyte quality were most prominent after six weeks as indicated by a reduction in ROS and mitochondrial ultrastructural abnormalities. At the end, pregnancy rates were significantly improved in both dietary interventions while oocyte lipid content was still abnormal after six weeks of PCCI.

METABOLIC HEALTH

We could confirm that HF diet feeding significantly increased body weight of Swiss outbred mice, resulting in an obese phenotype that was maintained throughout the whole experiment. In addition to the high body weight, obesity is also linked to reduced glucose tolerance, which we confirmed in this study (Buettner et al., 2007). The reported increased ER and glucose peak concentrations that were detected at PCCI week 4 and 6 can be linked to a renal loss of glucose due to the hyperglycemic state (Liman and Jialal, 2021). Next to that, the pancreas might have responded with a compensatory increased insulin release. To further confirm this hypothesis, insulin sensitivity of the mice was also tested. In addition to the significantly increased fasted serum insulin concentrations, HF_HF mice also displayed a significantly decreased insulin sensitivity at all time points when compared with the CTRL_CTRL group.
These results clearly indicate that HF diet feeding to Swiss outbred mice resulted in a reduced insulin sensitivity, corresponding to a pre-diabetic state.

In addition to the reduced glucose tolerance and insulin sensitivity, HF_HF mice also showed hypercholesterolemia, as reported in many other studies on metabolic syndrome (Marei et al., 2020; Podrini et al., 2013). Serum NEFA concentrations in this study were unchanged, as also reported by Podrini et al. (2013) and Williams et al. (2014). These observations should be interpreted with caution as animal handling and

fasting may rapidly change NEFA concentrations, potentially masking important treatment effects. Interestingly, HF diet decreased serum triglyceride concentrations which has already been described in several murine studies (Biddinger et al., 2005; Guo et al., 2009; Podrini et al., 2013). Also in a human study, decreased circulating triglyceride concentrations were reported when 550 kcal of fat was added to the daily diet (Meugnier et al., 2007). This decrease in circulating triglycerides can be caused by several possible mechanisms, like an inadequate export of triglycerides from the liver, a suppressed triglyceride production and/or an increased triglyceride clearance from the blood stream. In addition, the reduced serum triglyceride concentrations can also be linked to the elevated insulin concentrations observed in the HF_HF group since insulin can acutely inhibit hepatic VLDL secretion (Lewis et al., 1995) and stimulates adipose triglyceride uptake via lipoprotein lipase (Kessler, 1963). In contrast, other studies report the opposite effect (Ginsberg et al., 2005). Clearly, additional research is needed to fully comprehend the mechanisms causing reduced serum triglyceride concentrations during HF diet feeding.

HF_CTRL mice already showed a partial normalization of the aberrant serum triglyceride and cholesterol profile after only two weeks of PCCI. This was paralleled with a clear loss in weight of 11%. Earlier research described a positive linear relationship between cholesterol and weight change (Poobalan et al., 2004). Insulin sensitivity has also been reported to improve in proportion to the degree of weight loss (Ferrannini and Camastra, 1998). While insulin serum concentrations were significantly improved, overall insulin sensitivity was only partially restored at PCCI week 2 as indicated by a decrease in glucose AUC which remained however significantly higher than CTRL_CTRL mice. Both fasting blood glucose concentrations and glucose tolerance were not significantly improved after two weeks of PCCI. These data indicate that switching from a HF to an ad libitum control diet for only a short time frame of two weeks already shows some positive effects on metabolic health with regards to weight, serum lipid profile and insulin sensitivity. However, a complete recovery of the glucose tolerance was only present after four weeks of diet normalization, coinciding with 14% of weight loss. This is in line with the results of Reynolds et al. (2015), who also reported a complete recovery of glucose tolerance in mice that underwent diet normalization, however, these analyses were only performed at eight weeks of PCCI. Although fasting insulin serum concentrations were decreased in the HF_CTRL group at all timepoints, there was no complete recovery of overall insulin sensitivity, even after six weeks of PCCI.

Metabolic health was also assessed in HF_CR mice. Switching from a high fat to a 30% caloric restriction diet (HF_CR) also resulted in a reduction in weight which however continued during the whole PCCI period, ranging from 17% weight loss at PCCI week 2 to 27% weight loss at week 6. In contrast to HF_CTRL mice, HF_CR mice showed a lack of improved cholesterol concentrations at all time points. Earlier research in obese women associated major weight loss with a late rise in serum cholesterol, possibly from

mobilization of adipose cholesterol stores. However, the increased cholesterol resolved when weight loss stabilized (Phinney et al., 1991).
Furthermore, serum collected from HF_CR mice contained significantly higher ALT concentrations than both reference groups at all time points. This has been seen in other studies (Friis et al., 1987; Gasteyger et al., 2008; Hoy et al., 1994; Kreitzman et al., 1984). The rapid mobilization of intra- and extrahepatic fat stores may represent a hepatotoxic factor, explaining the leakage of this liver enzyme in the bloodstream. Such damage of the hepatocytes can, furthermore, cause an inadequate export of triglycerides from the liver which explains the low serum triglyceride concentrations seen in the HF_CR mice at all time points.

With regards to glucose tolerance and insulin sensitivity, HF_CR mice showed a very similar response as HF_CTRL mice. After two weeks of PCCI, no significant improvements in glucose tolerance and only a partial improvement in insulin sensitivity were present.
After four weeks on a calorie restricted diet, the glucose tolerance was completely restored. Furthermore, and in contrast to HF_CTRL mice, HF_CR mice completely restored their insulin sensitivity to the level of the CTRL_CTRL mice. However, calorie restricted mice were not able to recover from the hypoglycemia induced by the insulin injection by the end of the insulin tolerance test (Time 120' on the graph (Fig. 5). As suggested above, liver health is reduced in this HF_CR group (high ALT concentrations), the capacity to restore from this hypoglycemia may be jeopardized due to reduced rates of liver gluconeogenesis and glycogenolysis. Furthermore, the prolonged calorie restricted diet can lead to glycogen depletion in muscle and liver (Xu S et al., 2015).

In addition, maintaining HF_CR mice for six weeks on this restricted diet showed to be detrimental for overall glucose tolerance, accompanied by a higher peak glucose concentration and a faster clearance. Most probably, a higher insulin independent glucose uptake will play an important role in this. Furthermore, this phenomenon can also be explained by a state of pseudo-diabetes, as firstly reported by Claude Bernard in 1848 (Lundbaek, 1948). Although a very low calorie diet has been accepted as a powerful treatment to improve health and to reverse type 2 diabetes in humans, some reports describe the development of a diabetes-like state (Blagosklonny, 2019; Fontana et al., 2010; Koffler and Kisch, 1996). However, the disease profile of pseudo-diabetes is characterized by 5 major symptoms. Of these symptoms, in our experimental set-up, only glucose intolerance and hyperinsulinemia support the hypothesis of a pseudo-diabetes state in the HF_CR mice as they did show normoglycemia and recovered insulin sensitivity after six weeks of PCCI.

Taken together, although serum insulin and cholesterol concentrations of HF_CTRL mice already showed a promising improvement after two weeks of PCCI, this time period is too short to completely restore glucose tolerance and insulin sensitivity in

both HF_CTRL and HF_CR mice. The most promising metabolic improvements were present in HF_CTRL mice (including a restored glucose tolerance) at PCCI week 4, as HF_CR mice still showed an aberrant serum profile. A longer period of intervention did not further improve metabolic health in the HF_CTRL group and was even detrimental in HF_CR mice.

OOCYTE QUALITY

Our results confirm that oocytes collected from HF_HF mice contained a significantly higher lipid droplet volume than oocytes from CTRL_CTRL mice (Marei et al., 2020). Lipids are an important source for energy production in the oocyte (Sutton-McDowall et al., 2012). However, obesity leads to hyperlipidemia, resulting in lipid accumulation and storage in cells other than adipocytes. This ultimately results in lipotoxicity as the intracellular accumulation of lipids causes damage to cellular organelles like mitochondria, resulting in increased ROS and oxidative stress (Suzuki, 2017; Wu et al., 2011). Whether the observed increase in oocyte intracellular lipids is due to increased active uptake from the follicular microenvironment or due to a shift in energy pathways in the oocyte as a result of the different nutrient availability, needs further study. Oocytes collected from HF_HF mice also exhibited higher ROS levels than the CTRL_CTRL group at almost all time points, as shown by Cell Rox Deep Red staining pixel intensity (Marei et al., 2020). Elevated intracellular ROS concentrations and thus increased oxidative stress in oocytes from obese individuals results in mitochondrial dysfunction and reduced oocyte quality (Grindler and Moley, 2013; Marei et al., 2020). Igosheva et al. (2010) linked increased ROS production in oocytes from obese female mice to a higher mtDNA copy number. A high mtDNA copy number in oocytes has been linked with a higher risk of implantation failure in human embryos (Fragouli et al., 2017). Oocytes collected from HF_HF mice contained higher ROS and significantly higher mtDNA copy number than oocytes from CTRL_CTRL oocytes after 11 weeks (at week 4 of the PCCI period) on their respective diet. We suggest that this increase in mtDNA copy number can be linked to a defective mitophagy and/or increased mitochondrial biogenesis during oogenesis to compensate for the increasing number of defective mitochondria (Barrientos et al., 1997; Igosheva et al., 2010; Luzzo et al., 2012). In contrast, at PCCI week 6, a reduction in mtDNA copy numbers was present in oocytes from HF_HF mice. This might indicate that the earlier compensatory feedback that resulted in increased mitochondrial biogenesis is no longer present. At the same timepoint we confirmed a very high proportion of mitochondrial ultrastructural abnormalities (Marei et al., 2020). Although not reported in oocytes yet, persistent nutrient surplus, like e.g. HF diet exposure, has been shown to override the adaptation and can lead to mitochondrial overloading and dysfunction in muscles (Cheng and Almeida, 2014).

The mitochondrial dysfunction in oocytes caused by obesity also manifests at several other levels, ranging from an increased mitochondrial activity (JC-1 pixel intensity) to an improper mitochondrial distribution during final maturation, as confirmed in the

present study (Igosheva et al., 2010; Marei et al., 2020). Increased mitochondrial membrane potential has been linked with reduced oocyte developmental competence in both mice and humans (Acton et al., 2004). In addition, HF_HF mature oocytes displayed a significantly lower percentage of active mitochondria that were pericortically localized when compared with CTRL_CTRL oocytes. Van Blerkom et al. (2002) reported that in the mature oocyte, high polarized mitochondria normally occupy a circumferential domain immediately subjacent to the plasma membrane, also described as 'vanguard mitochondrial polarity' (Van Blerkom and Davis, 2006). This pericortical localization supports ATP levels during embryo cleavage (Van Blerkom et al., 2003), is required for successful sperm penetration and cortical granule exocytosis (Van Blerkom and Davis, 2007). As HF_HF oocytes clearly show a lower percentage of oocytes containing pericortically localized active mitochondria, this might result in decreased fertilization and cleavage rates.
Furthermore, in this study, pregnancy rates of HF_HF mice were also significantly lower, as reported by Skaznik-Wikiel et al. (2016).

Focusing on the HF_CTRL mice, ovulated oocytes exhibited a lower lipid droplet content after only two weeks of PCCI. This was correlated with the reported decrease in blood total cholesterol levels, again suggesting similar biochemical changes in the ovarian follicular fluid. As mitochondrial activity was increased, this suggests an increased use of lipids for fatty-acid β-oxidation to produce energy for the oocyte. Dunning et al. (2010) illustrated that upregulation of β-oxidation increased oocyte developmental competence. The increased mitochondrial activity can also be linked to a possible improved responsiveness of the cumulus cells to glucose after two weeks of diet normalization. This results in increased pyruvate uptake in the oocyte, leading to an increased oxidative phosphorylation (Richani et al., 2021a). In this study, the increased mitochondrial activity also coincided with an increase in ROS, possibly indicating the presence of oxidative stress.
As the PCCI continued beyond the timeframe of folliculogenesis (i.e. three weeks) (PCCI week 4 and 6), there was a clear transition in HF_CTRL oocytes towards (again) a high lipid droplet volume, accompanied by high mitochondrial activity but a significant reduction in ROS levels and ratio of ROS: active mitochondria. As glucose and pyruvate, besides lipids, are critical substrates for successful oocyte and embryo development (reviewed by (Richani et al., 2021a)), we suggest that the HF_CTRL oocyte relies on the carbohydrate metabolism of the cumulus cells and uses its end products as the major energy source, resulting in an increase of intracellular lipids. The ability to efficiently store fatty acids in lipid droplets might decrease lipotoxic effects and can be a method to store energy for the preimplantation development (Ferguson and Leese, 2006; Hillman and Flynn, 1980; Sturmey et al., 2009). These observations may suggest that oocytes from HF_CTRL mice are more metabolically efficient and have a higher antioxidant capacity.

Above mentioned outcome parameters indicate a possibly improved oocyte quality. However, the overall percentage of oocytes with pericortically localized active mitochondria did not improve in HF_CTRL oocytes and was even similar to the percentage reported in HF_HF oocytes.

To summarize, we see a clear shift in oocytes from HF_CTRL mice towards the use of different energy pathways around the timeframe that folliculogenesis (i.e. three weeks) did no longer took place in a high fat environment. This might indicate that the preconception care intervention should be followed for a period longer than what is needed for folliculogenesis. The timeframe of six weeks PCCI showed to be the most optimal.

On the other hand, oocytes collected from the HF_CR group still exhibited a high volume of lipid droplets at each PCCI time point. Weight loss has been linked to increased lipid mobilization from the adipose tissue, resulting in increased NEFA concentrations in the blood stream that were reflected in the FF (Chusyd et al., 2016; Valckx et al., 2012). These increased FF NEFA concentrations might lead to increased fatty acid uptake in the oocyte, resulting in a high lipid droplet volume in the oocyte. In addition to the high lipid content, mean ratio of ROS: active mitochondria was even higher than the HF_HF oocytes after two and four weeks of PCCI. This indicates an upregulation of mitochondrial β-oxidation due to the high lipid content, resulting in excessive ROS production leading to oxidative stress. However, at PCCI week 6, a reduction in total ROS levels and ratio of ROS: active mitochondria was present in HF_CR oocytes, indicating a decrease in oxidative stress.

To our knowledge, nothing is known regarding the effect of a CR diet on mitochondrial activity in oocytes. However, earlier research in other tissues indicated that a restricted calorie intake or an increased physical activity can significantly improve mitochondrial integrity and function and protect against metabolic syndrome (Cheng and Almeida, 2014; Martin-Montalvo and de Cabo, 2013; Toledo and Goodpaster, 2013). Similar to the HF_CTRL mice, overall percentage of oocytes with pericortically localized active mitochondria did not improve in HF_CR mice and was even lower than HF_HF mice. However, after six weeks of PCCI, HF_CR oocytes showed lower mitochondrial activity than HF_HF oocytes. This indicates that switching to a CR diet for six weeks might result in oocytes with an improved mitochondrial function.

As an additional endpoint parameter, oocyte mitochondrial ultrastructural abnormalities were categorized. Oocytes from both HF_CTRL and HF_CR mice showed a significantly reduced proportion of mitochondrial ultrastructural abnormalities at PCCI week 6 compared with HF_HF mice. Along with the other assessed oocyte quality parameters, these data suggest that exposure to a preconception diet intervention for six weeks seems to result in the ovulation of a better quality oocyte that was able to develop in healthier conditions as the complete process of folliculogenesis took place in a non-high fat environment. However, the mitochondrial ultrastructural abnormalities were not fully restored to the level of the control group.

Folliculogenesis lasts 3-4 months in humans and only three weeks in mice (Clarke, 2017). During this process, the growing follicle and the enclosed oocyte are very sensitive to changes in its micro-environment which impacts the quality of the oocyte at the moment of ovulation (Best and Bhattacharya, 2015; Valckx et al., 2014b). This emphasizes the importance of the different PCCI period lengths that were selected in the present study. Seeing oocyte quality significantly improving after four and six weeks of PCCI suggests that the dormant primordial follicle pool is not really affected by the HF dietary insult or that oocyte recovery mechanisms during folliculogenesis are able to repair. However, any remnant negative impact on oocyte quality at PCCI week 4 and 6 may, to some extent, contradict this. Furthermore, the not fully recovered metabolic health of course may still exert negative effects during the growth and maturation of the follicle. From this, it should be clear that more in depth research is needed to better understand these concepts.

As a final validation of our findings regarding oocyte quality, pregnancy rates were assessed at PCCI week 6. Both HF_CTRL and HF_CR mice showed restored pregnancy rates, hereby indicating that switching from a HF to an ad libitum control or 30 % CR diet had a positive effect on overall fertility.

Taken together, a PCCI period of six weeks shows to be the most promising in both PCCI groups. Oocytes from HF_CTRL mice that stayed on the control diet for six weeks seemed to switch towards the use of different energy pathways, which may suggest a more metabolically efficient phenotype with a higher antioxidant capacity. At the end of the PCCI period, oocytes from HF_CR mice displayed reduced ROS levels and restored mitochondrial activity. Additional endpoint parameters assessed at week 6 indicated that mitochondrial ultrastructural abnormalities were drastically lower in both PCCI groups compared with the HF_HF group and also pregnancy rates were restored.

LIMITATIONS AND EXTRAPOLATION POTENTIAL TO THE HUMAN SITUATION

As mentioned before, human studies are often confronted with a lot of possible confounders (high drop-out rates, ethnical and social background,…), leading to a lack of scientifically substantiated guidelines. Therefore, using a mouse model for this research has several advantages and provides us with fundamental information, including the specific impact on oocyte quality. Mice are small, fertile and have a short gestational period. However, in contrast to humans, mice are poly-ovulatory animals and translating the results from this mouse study to the human setting should be done with caution. However, the use of an outbred mouse strain, the specific implementation of PCCI lengths related to the duration of mouse folliculogenesis (i.e. three weeks) together with an in depth study of the weight changes and the metabolic health effects, should help in understanding the potential consequences of our study's fundamental insights for a human fertility setting. Furthermore, ongoing

research in our laboratory is providing deeper insights in potential postnatal health effects of described preconception care interventions.

CONCLUSIONS

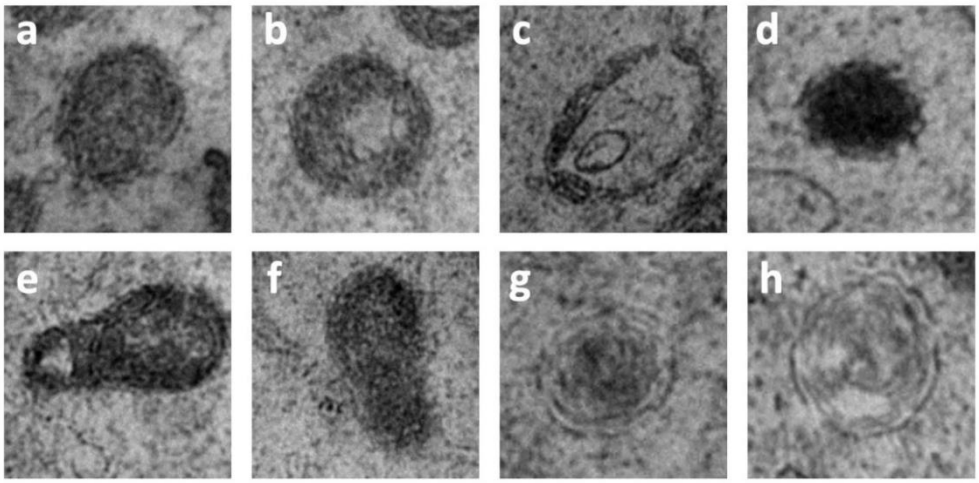
This research is an important step in providing fundamental insights regarding the impact of diet normalization or a calorie restricted diet for two, four or six weeks as preconception care intervention strategies on metabolic health and oocyte quality in HF-diet fed obese outbred mice.

We confirmed that obesity has a detrimental effect on both metabolic health and oocyte quality in a HF diet-fed outbred mouse model.

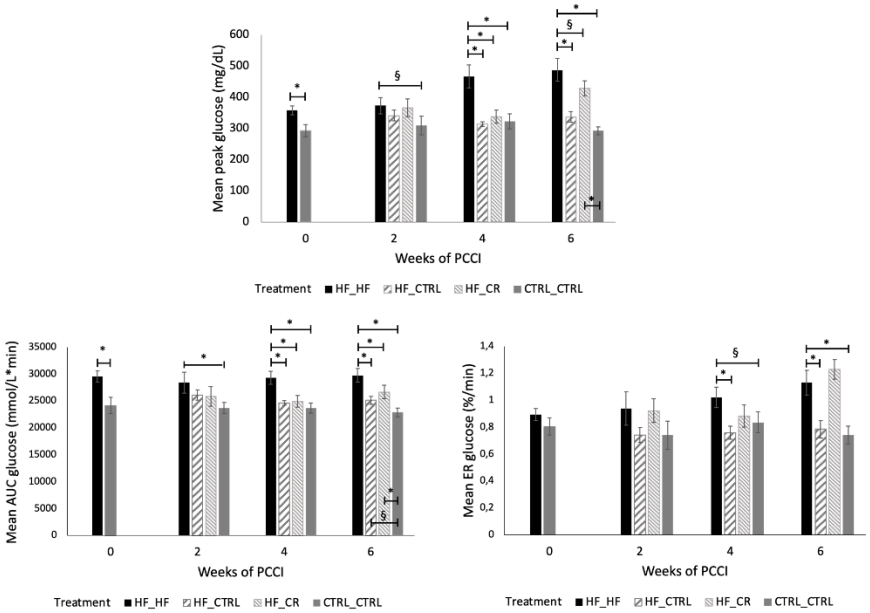
Based on the collected data, switching to an ad libitum control diet (HF_CTRL) for a time frame of four weeks with a weight loss of 14% is a good approach to improve metabolic health. Oocyte quality parameters assessed already showed some promising improvements at PCCI week 4. However, these were further ameliorated at PCCI week 6.

Switching to a 30% caloric restriction diet (HF_CR) for six weeks showed to be a too extreme intervention, due to its negative impact on glucose tolerance, insulin concentrations and liver health and due to the lack of improved serum lipid concentrations. In contrast, oocyte quality parameters in the HF_CR mice only showed a significantly improved quality after six weeks of PCCI as indicated by a restored mitochondrial activity, reduction in ROS and mitochondrial ultrastructural abnormalities.

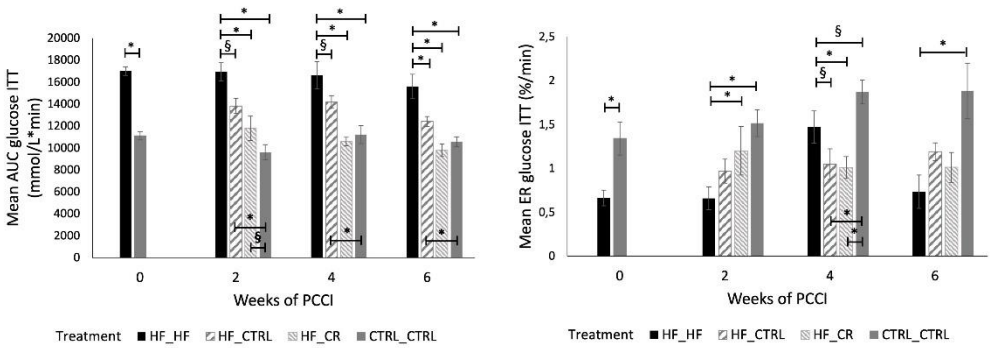
6. SUPPLEMENTARY FIGURES



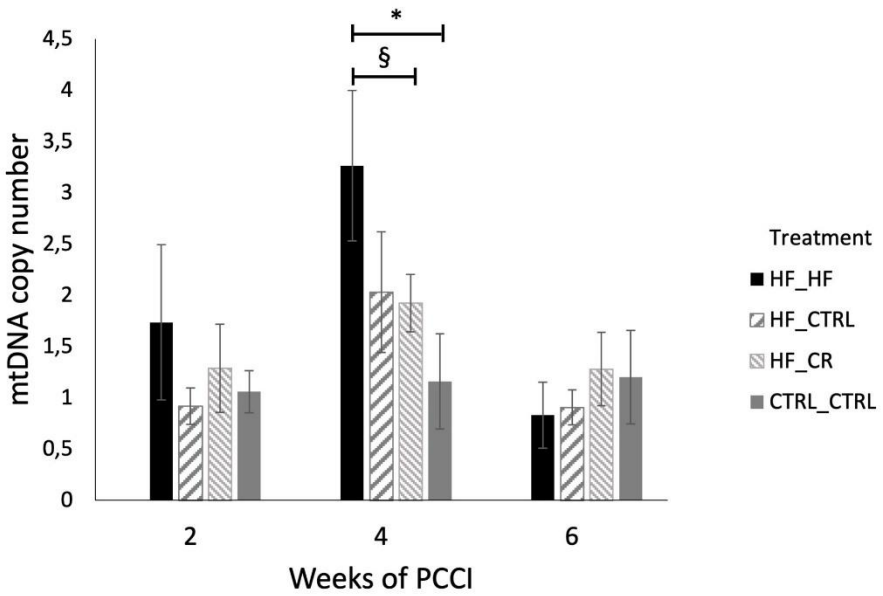
Supplementary Fig. 1. **Classification of different forms of mitochondrial ultrastructure**. Mitochondria were considered normal when spherical (a) or spherical with regular vacuoles (b). Mitochondrial abnormalities include vacuolation with loose inner membrane structures (c), electron dense foci (d), dumbbell shapes with vacuolation (e), dumbbell shapes (f), rose petal appearance (g) or degeneration (h).



Supplementary Fig. 2. **Peak glucose concentration, area under the curve (AUC) and elimination rate (ER)** of the **glucose tolerance test** of all treatment groups at different time points after starting the preconception care intervention (PCCI) (week 0, 2, 4 and 6). Data are shown as means ± SEM from 8 mice per group per time point. Asterisks (*) indicate significant differences between the indicated treatment groups within the same PCCI period (P<0.05). Values labelled with "§" tend to be different from each other at 0.05 < P <0.1.



Supplementary Fig. 3. Glucose **area under the curve (AUC) and elimination rate (ER)** of the **insulin tolerance test** (ITT) of all treatment groups at different time points after starting the preconception care intervention (PCCI). Data are shown as means ± SEM from 8 mice per group per time point. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period (P<0.05). Values labelled with "§" tend to be different from each other at 0.05 < P <0.1.



Supplementary Fig. 4. **mtDNA copy numbers** of all treatment groups at different time points after starting the preconception care intervention (PCCI). Data are shown as means ± SEM from pools of oocytes ($11 \leq n \geq 42$) per treatment group collected from 3-5 mice per group per time point. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period ($P<0.05$). Values labelled with "§" tend to be different from each other at $0.05 < P < 0.1$.

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CHAPTER 7:

HIGH FAT/HIGH SUGAR DIET-INDUCED OBESITY AND PRECONCEPTION CARE INTERVENTIONS

OBESE OUTBRED MICE ONLY PARTIALLY BENEFIT FROM DIET NORMALIZATION OR CALORIE RESTRICTION AS PRECONCEPTION CARE INTERVENTIONS TO IMPROVE METABOLIC HEALTH AND OOCYTE QUALITY

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1. ABSTRACT

Study question: Can diet normalization or a calorie restricted diet for two or four weeks be used as a preconception care intervention (PCCI) in Western-type diet induced obese Swiss mice to restore metabolic health and oocyte quality?
Summary answer: Metabolic health and oocyte developmental competence was already significantly improved in the calorie restricted group after two weeks, while obese mice that underwent diet normalization showed improved metabolic health after two weeks of PCCI and improved oocyte quality after four weeks.
What is known already: Maternal metabolic disorders are linked to reduced metabolic health and oocyte quality, therefore obese women are advised to lose weight before conception to increase pregnancy chances. However, as there are no univocal guidelines and the specific impact on oocyte quality is not known, strategically designed studies are necessary to provide fundamental insights in the importance of the type and duration of the dietary weight loss strategy for preconception metabolic health and oocyte quality.
Study design, size, duration: Outbred female Swiss mice were fed a control (CTRL) or high-fat/high-sugar (HF) diet for seven weeks. Afterwards, HF-mice were put on two different PCCIs for two different time periods (two or four weeks), resulting in four treatment groups: 1) control diet for up to 11 weeks (CTRL_CTRL), 2) HF diet for up to 11 weeks (HF_HF), switch from a HF (7w) to 3) an ad libitum control diet (HF_CTRL) or 4) 30% calorie restricted control diet (HF_CR) for two or four weeks. Metabolic health and oocyte quality (n=8 mice/ treatment/time point) were assessed.
Participants/materials, setting, methods: Body weight changes were recorded. To assess the impact on metabolic health; serum insulin, glucose, cholesterol, triglyceride and alanine aminotransferase (ALT) concentrations were measured and glucose tolerance and insulin sensitivity were analyzed at PCCI week 2 and 4. Quality of in vivo matured oocytes was evaluated by assessing intracellular lipid droplet content, mitochondrial activity and localization of active mitochondria, mitochondrial ultrastructure, cumulus cell targeted gene expression and oocyte in vitro developmental competence.
Main results and the role of chance: Significant negative effects of a high fat/high sugar diet on metabolic health and oocyte quality were confirmed (P<0.05). HF_CTRL mice already restored body weight, serum lipid profile and glucose tolerance to the CTRL_CTRL group after only two weeks of PCCI (P<0.05 compared with HF_HF) while insulin sensitivity was not improved. Oocyte lipid droplet volume was reduced at PCCI week 2 (P<0.05 compared with HF_HF) while mitochondrial localization and activity were still aberrant. At PCCI week 4, oocytes from HF_CTRL mice displayed significantly less mitochondrial ultrastructural abnormalities and improved mitochondrial activity (P<0.05) while lipid content was again elevated. Their in vitro developmental capacity was improved but did not reach CTRL_CTRL levels. HF_CR mice completely restored cholesterol concentrations and insulin sensitivity already after two weeks. Other

metabolic health parameters were only restored after four weeks of intervention with clear signs of fasting hypoglycemia. Although all mitochondrial parameters in HF_CR oocytes stayed aberrant, oocyte developmental competence in vitro was completely restored already after two weeks of intervention.

Limitations, reasons for caution: In this study we applied a relevant high fat/high sugar Western type diet to induce obesity in an outbred mouse model. Nevertheless, translating these results to the human setting should be done with caution. However, the in depth study and follow up of the metabolic health changes together with the strategic implementation of specific PCCI intervals (2 and 4 weeks) related to the duration of the mouse folliculogenesis (three weeks), should aid in the extrapolation of our findings to the human setting.

Wider implications of the findings : Our study results with a specific focus on oocyte quality provide important fundamental insights to be considered when developing preconception guidelines for obese metabolically compromised women planning for pregnancy.

2. INTRODUCTION

The prevalence of obesity and metabolic syndrome is significantly increasing worldwide and has been regarded as a major threat to public health (Eckel et al., 2010; Engin, 2017; WHO, 2018). Obesity can coincide with a significant reduction in metabolic health and is characterized by an increase in body weight, abdominal fat accumulation, aberrant serum lipid profiles, liver dysfunction, an impaired glucose tolerance and reduced insulin sensitivity (Dahlhoff et al., 2014; Jungheim and Moley, 2010; Samuelsson et al., 2008). Obese women often have reduced fertility and there is ample evidence showing a significant negative impact of their disturbed metabolic health on important processes in reproduction physiology before and after conception (Klenov and Jungheim, 2014; Metwally et al., 2007a; Robker, 2008). Reduced oocyte quality plays a key role in the pathogenesis of subfertility in metabolically compromised women. The follicular fluid (FF), the oocyte's micro-environment, reflects the disturbed metabolic state of obese women (Jungheim and Moley, 2010; Sutton-McDowall et al., 2010; Valckx et al., 2012) and has the potential to directly affect oocyte quality (Leroy et al., 2015).

Major causative players for obesity include a too high caloric intake of diets rich in fats and sugars (Bluher, 2019). Dietary fat is a strong predictor of weight gain (Sherwood et al., 2000) and especially a high intake of saturated fats has been shown to significantly increase body fat and impair insulin sensitivity (Botchlett et al., 2016; Botchlett and Wu, 2018; Isganaitis and Lustig, 2005). In obese individuals, high NEFA concentrations originate from the upregulated lipolysis (Klop et al., 2013). A high dietary fat intake further increases the delivery of fatty acids to tissue, leading to the accumulation of lipid intermediates in tissues like liver and skeletal muscle (Bergman and Ader, 2000; Wajchenberg, 2000). This results in cellular dysfunction, plays a major role in the pathogenesis of disorders associated with metabolic syndrome and is referred to as lipotoxicity. Rodents exposed to a diet high in saturated fat also showed aberrant serum triglyceride and NEFA concentrations (Marei et al., 2020a; Smits et al., 2021) which are known to be reflected in the follicular fluid (Valckx et al., 2014a; Valckx et al., 2012), directly impacting on oocyte maturation and development (Jungheim et al., 2011). Bovine oocytes matured in high saturated fatty acid concentrations in vitro showed elevated oxidative stress levels and altered mitochondrial function, leading to a significantly reduced oocyte developmental competence (Marei et al., 2017; Van Hoeck et al., 2013). Oocytes collected from high fat diet induced obese mice also displayed an impaired quality with increased mitochondrial ultrastructural abnormalities, altered mitochondrial membrane potential (MMP), increased cellular oxidative stress levels and higher lipid droplet volume (Marei et al., 2020b; Smits et al., 2021; Wu et al., 2010).

Next to a high (saturated) fat content, Western type diets contain substantial amounts of sugar (Panchal and Brown, 2011). Consumption of fructose and sugar-sweetened

beverages has been associated with insulin resistance, intrahepatic lipid accumulation and hypertriglyceridemia, ultimately contributing to the development of metabolic syndrome (Taskinen et al., 2019). Increased fructose consumption results in its unregulated hepatic uptake and metabolism (Teff et al., 2009), leading to increased de novo lipogenesis (Stanhope et al., 2009) which increases the intra-hepatic lipid supply (Cox et al., 2012; Maersk et al., 2012; Sevastianova et al., 2012). This may result in dyslipidemia, hepatic insulin resistance (Adiels et al., 2008; Morino et al., 2006) and ultimately whole body insulin resistance (described in detail by Stanhope (2016)). Insulin resistance was suggested as an important mechanism between increased sugar-sweetened beverage consumption and the impact on fertility. Both increased serum glucose and insulin concentrations were reflected in the FF of obese women (Robker et al., 2009; Valckx et al., 2012). Oocytes collected from insulin resistant mice showed oxidative stress and mitochondrial dysfunction, affecting oocyte quality and early embryo development (Ou et al., 2012). Studies investigating the impact of fructose consumption itself on fertility reported a decrease in pregnancy rates in mice after six weeks on a 60% fructose diet (Saben et al., 2016a) and in women who consumed ≥7 sugar sweetened beverages per week compared with those who had no sugar sweetened beverage intake (Hatch et al., 2018).

Although high fat-diet induced obesity markedly affects metabolic health, it is clear that fructose itself also has a significant impact. The combination of both a high fat and high sugar load has shown to exaggerate the insulin response (Isganaitis and Lustig, 2005), significantly increasing the risk for metabolic syndrome (Stanhope et al., 2013). Oocytes collected from mice on a high fat/high sugar (HF/HS) diet also displayed an impaired quality when compared to control oocytes. Mitochondrial ultrastructure, distribution and function, the meiotic spindle and oxidative stress levels were clearly aberrant (Boots et al., 2016; Saben et al., 2016b).

Up until now, overweight and obese patients who are having problems with getting pregnant are often advised to lose weight before conception through dietary lifestyle interventions in order to increase the chance of a healthy pregnancy (Jungheim and Moley, 2010; Lassi et al., 2014; Pasquali, 2006). However, so far, there are no evidence-based guidelines regarding preconception care in overweight and obese infertile women. Although most research indicates a positive impact of (modest) weight loss on metabolic health, results on fertility are not consistent. While several (sometimes underpowered) lifestyle intervention studies observed a significant increase in pregnancy and/or live birth rates (for an overview, see (Sim et al., 2014)), two recent large randomized controlled trials could not detect a significant increase in live birth rate prior to IVF while the chance of a spontaneous pregnancy increased (Einarsson et al., 2017b; Mutsaerts et al., 2016b). These conflicting results, together with the limitations of human studies (high drop-out rates, lack of sufficient power, unknown patient clinical history and social background) lead to a lack of scientifically supported advice (Norman and Mol, 2018). How much weight loss is necessary to improve

metabolic health and fertility success? Is diet normalization enough or is a more drastically calorie restricted diet necessary? In addition, what is the shortest intervention window possible to induce substantial positive effects. Factors such as the duration of normal folliculogenesis but also advancing maternal age should be taken into account (Clarke, 2017). Furthermore, very few studies focused on the specific impact of preconception dietary interventions on oocyte quality (Reynolds et al., 2015; Smits et al., 2021; Tsagareli et al., 2006). Oocyte quality is determined throughout folliculogenesis, a process taking several months in human. Can we expect oocyte quality to improve or even restore or should the preconception care period span the entire period of folliculogenesis to recruit oocytes from the dormant primordial follicle pool?

More controlled, fundamental research is needed to investigate the potential positive impact and the necessary minimal duration of diet normalization or caloric restriction as preconception care interventions on oocyte quality in Western type diet induced obese outbred mice.

Therefore, we hypothesized that the efficiency of a preconception dietary intervention in HF/HS-fed obese outbred mice to improve metabolic health and oocyte quality depends on the duration and on the type of this dietary intervention. To test this hypothesis, we aimed to feed female outbred mice a high fat/high sugar diet for seven weeks to obtain an obese phenotype. Afterwards, they were switched to two different preconception care interventions (PCCI): 1) an ad libitum control diet or 2) a 30% caloric restricted control diet. Mice underwent these diet changes for two or four weeks, whether or not covering the duration of one cycle of folliculogenesis (i.e. three weeks in mice). To assess the impact on metabolic health; serum insulin, glucose, cholesterol, triglyceride and alanine aminotransferase concentrations were measured and glucose tolerance and insulin sensitivity were monitored. Oocyte quality was evaluated by assessing intracellular lipid droplet content, proportion and distribution of active mitochondria, proportions of mitochondrial ultrastructural abnormalities as well as oocyte developmental competence in vitro. In addition, cumulus cells were analyzed for mitochondrial ultrastructural abnormalities and for transcript abundance of a selected number of genes of interest related to insulin signaling, glucose and lipid metabolism, cumulus cells expansion and oxidative stress.

3. MATERIALS AND METHODS

3.1. ETHICAL APPROVAL

All procedures in this study were approved by the ethical committee of the University of Antwerp and performed accordingly (ECD nr. 2018-05), and all methods were performed in accordance with the relevant guidelines and regulations.

3.2. ANIMALS, DIET AND EXPERIMENTAL DESIGN

Five-week-old female outbred Rj:Orl Swiss, hereafter referred to as "Swiss" mice (Janvier labs) were used. At the start of the experiment, mice were randomly divided into two groups with ad libitum access to either a control (CTRL) or a high fat/high sugar diet (HF/HS) for a period of seven weeks.

Afterwards, based on their weight, some of the HF/HS-mice were switched to two different preconception care interventions for two or four weeks, while the remaining HF/HS and the control mice remained on their corresponding diet for comparison. This resulted in four different treatment groups of equal size: 1) control diet for up to 11 weeks (CTRL_CTRL), 2) HF/HS diet for up to 11 weeks (HF_HF), 3) HF/HS diet for seven weeks then a switch to an *ad libitum* control diet for two or four weeks (HF_CTRL) and 4) HF/HS diet for seven weeks then a switch to a 30% caloric restriction diet for two or four weeks (HF_CR). Mice were put on the diet in subgroups of 8 animals per treatment per time point with an interval of few weeks between replicates to facilitate handling and sample collection procedures. An experimental timeline is shown in figure 1.

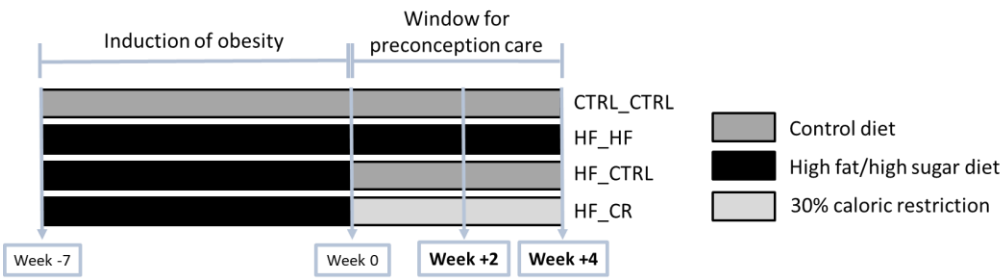


Figure 1: Overview of the experimental design

The HF diet was composed of 60 kJ% fat from beef tallow, 20 kJ% carbohydrate and 20 kJ% protein (E15741-34, Sniff diets). In addition 20% (w/v) fructose (Merck, 102109450) was added to the drinking water of mice on the HF diet. The matched, purified control diet contained 10 kJ% fat, 70 kJ% carbohydrate and 20 kJ% protein (E157453-04; Sniff Diets). Access to water was provided ad libitum. The 30% caloric restriction diet had the same composition as the control diet, however, the HF_CR mice received only 70% of the amount of food consumed by the CTRL_CTRL mice (paired feeding regimen, calculated on the basis of the daily consumption).

Mice were weighed weekly before the start of the PCCI and every four days during the PCCI period to closely follow-up weight changes.

An overview of all outcome parameters at specific time points (week 2 and 4 after start of the PCCI) are presented in table 1. The final numbers of animals and oocytes used to collect data for each outcome parameter are described in the figure legends.

Table 1. Overview of the outcome parameters assessed at specific time points during the preconception care intervention period (PCCI).

		PCCI week 2	PCCI week 4
Metabolic health	Insulin sensitivity test* (n=8 mice/treatment group/time point)	✓	✓
	Glucose tolerance test* (n=8 mice/treatment group/time point)	✓	✓
	Serum° - Glucose - Insulin - Cholesterol - Triglycerides - ALT (n=8 mice/treatment group/time point)	✓	✓
Oocyte quality	Lipid droplet volume in oocytes° (n=8 mice/treatment group/time point)	✓	✓
	Mitochondrial activity in oocytes° ((n=8 mice/treatment group/time point)	✓	✓
	Localisation of active mitochondria in mature oocytes° (n=8 mice/treatment group/time point)	✓	✓
	Mitchondrial ultrastructural abnormalities (TEM)° in cumulus cells and oocytes (n=3-5 mice/treatment group)		✓
	Gene expression analysis in cumulus cells° (n=4 mice/treatment group/time point)	✓	✓
	Oocyte developmental competence (n=6 mice/treatment group/time point)	✓	✓

ALT = alanine aminotransferase; TEM = transmission electron microscopy

*Same mice were used to determine the selected outcome parameters over all PCCI time points

° Same mice were used per PCCI to analyse the selected outcome parameters

3.3. SERUM COLLECTION AND ANALYSIS

At week 2 and week 4 of the PCCI period, mice (n=8 per treatment per time point) were fasted overnight and were sacrificed by decapitation to allow blood collection. Blood was centrifuged 30 min after collection for 2 min at 6000g at 4°C, and serum was stored at −80 °C until analysis. Insulin concentrations were measured using an ultrasensitive mouse insulin ELISA kit (90080, CrystalChem, The Netherlands). In addition, the following serum analyses were performed in a commercial laboratory (Algemeen Medisch Labo, Antwerp, Belgium): Triglycerides, Cholesterol and Alanine aminotransferase (ALT, an overall indicator of liver damage), were measured on an Abbott Architect c16000 (Abbott, Illinois, U.S.A).

3.4. INSULIN AND GLUCOSE TOLERANCE TEST

At PCCI week 2 and 4, mice (n=8 per treatment per time point) were fasted for six hours prior to the glucose (GTT) and insulin tolerance test (ITT). As reported by Smits et al. (2021) in detail, basal glycaemia was measured using a glucose meter (OneTouch Verio; ADC, Belgium) and immediately afterwards the mouse was intraperitoneally injected with 20% glucose solution (2g glucose/kg body weight; Thermo Fisher Scientific, Belgium) for the GTT or with 0,075 IU insulin/kg body weight (Novorapid (Novo Nordisk), ADC, Belgium) for the ITT. Glycaemia was measured again 15, 30, 60, 90 and 120 minutes after the injection. Afterwards, the area under the curve (AUC) and elimination rate (ER) of glucose were calculated for both tests.

The same mice were used to perform GTT and ITT with an interval of 2 days. The analysis was repeated using the same mice at two and four weeks of PCCI (n=8 per treatment group).

3.5. OOCYTE AND CUMULUS CELL COLLECTION AND PREPARATION FOR SUBSEQUENT ANALYSES.

In order to avoid any bias due to the glucose and insulin injections, different mice than those used for the GTT and ITT were selected to determine oocyte quality (n=8 mice per treatment per time point). At PCCI week 2 and 4, mice received intraperitoneal injections of 10 IU equine chorionic gonadotropin (eCG, Synchrostim; Ceva Santé Animale) followed by 10 IU human chorionic gonadotropin (hCG, Pregnyl; Organon) 48 h later to induce and synchronize ovulations. Mice were sacrificed 13–14 h after hCG injection. In vivo matured oocytes were obtained from the oviducts immediately after euthanasia. Each oviduct was dissected together with the ovary and a part of the uterine horn and transferred to a collection tube containing L15 medium (Thermo Fisher Scientific, Belgium) supplemented with 50 IU/mL penicillin G sodium salt (Merck, Belgium), and 10% Fetal Bovine Serum (Greiner Bio-One, Belgium).

The cumulus oocyte complexes (COCs) collected from both oviducts of the same animal were pooled. Only COCs that met the following selection criteria were used for analysis:

oocytes surrounded by an expanded cumulus cell mass with a perfect spherical shape, a regular zona and a translucent, homogeneously colored cytoplasm without inclusions. Morphologically good quality COCs from each mouse were distributed for downstream analysis, according to the total number of COCs available, as follows: one whole COC per mouse was fixed in glutaraldehyde solution for transmission electron microscopy (TEM) (only at PCCI week 4). The remaining COCs were completely denuded by repeated pipetting through 125 µm Stripper tips fitted on EZ-grip (Origio, The Netherlands) in a droplet of L15 medium containing 0.3 mg/mL hyaluronidase (Merck, Belgium). Denuded oocytes were transferred to a fresh drop of L15 medium. One or two denuded oocytes (per mouse) were immediately transferred to JC-1 staining to determine localisation of active mitochondria and mitochondrial activity. One to two oocytes per mouse were fixed in paraformaldehyde 4% for 30 minutes then stored in PBS-PVP (1 mg/mL) and used for the determination of lipid droplet content. Meanwhile, the droplets containing the detached cumulus cells from all animals in each subgroup were also immediately pooled, centrifuged, and the cell pellet was washed in PBS-PVP, centrifuged again and snap frozen. All frozen samples were stored at −80 °C until further analyses.

Another batch of mice (n=6 mice per treatment per time point) were used to collect COCs for in vitro fertilization and culture to follow up oocyte developmental competence.

3.6. ASSESSMENT OF OOCYTE LIPID DROPLET VOLUME

Intracellular lipid droplets in the fixed denuded oocytes were examined using BODIPY staining according to Marei et al. (2020b). Briefly, oocytes were first permeabilized in PBS containing 0.1 % (w/v) saponin (Fiers, Kuurne, Belgium) and 0.1 M glycine. Next, oocytes were incubated in 20 µg/ml BODIPY 493/503 (Thermo Fisher Scientific, Belgium) in PBS for 1h. Oocytes were washed twice in PBS containing 3 mg/mL PVP after each step in the staining procedure. Finally, the oocytes were transferred to droplets of PBS-PVP on glass-bottom dishes and immediately examined under a confocal microscope. High resolution images were obtained using a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Zaventem, Belgium) equipped with 488 nm diode lasers for excitation of green fluorophores. For each oocyte, a z-stack of 40 µm (with steps of 1 µm) was acquired in the lower half of the oocyte (closest to the objective lens where the image is sharpest). Images were analyzed using Volocity 6.0.1 software (PerkinElmer) to quantify the total volume of the lipid droplet content in each oocyte. To exclude background, only particles ≥0.5 μm^3 in size were considered as lipid droplets and included in the analysis.

3.7. ASSESSMENT OF MITOCHONDRIAL ACTIVITY AND LOCALISATION OF ACTIVE MITOCHONDRIA

Oocyte mitochondrial activity was assessed using a fluorescence staining technique using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Invitrogen) as described by Komatsu et al. (2014). Freshly collected oocytes were incubated for 30 min in L15 medium containing JC1 (5 µg/mL) (from 1000X stock solutions in DMSO) at 37°C. They were then washed and transferred to L15 medium droplets under mineral oil on a 35 mm dish with a glass bottom. Stained oocytes were immediately examined under a Leica SP8 confocal microscope enclosed in a warm chamber (37°C) and equipped with white laser source (Leica WLL) lasers. Mitochondrial activity was measured as the mean grey scale intensity of the J-aggregates (excitation/emission 561/590 nm, which is dependent on mitochondrial inner membrane potential). Four optical sections were examined for each oocyte at four different sections. The grey scale intensity was measured using Leica Application Suite X (LAS X) software.

The regional distribution of active mitochondria was examined, as described by Smits et al. (2021). To determine pericortical localisation of active mitochondria, one optical section was taken (excitation/emission 561/590 nm) at the maximum oocyte diameter. Based on its radius, the oocyte was divided into 5 equal circular zones (Wakefield et al. (2008)), in which the most central zone was labelled as zone 1 and the most peripheral zone was labelled as zone 5. If mitochondria were localised in zone 5 of the oocyte, they were categorised as "Pericortical". If not, they were labelled as "Diffuse".

3.8. TRANSMISSION ELECTRON MICROSCOPY (TEM)

The ultrastructure of mitochondria in cumulus-oocyte-complexes was only assessed at four weeks of PCCI, according to Marei, et al. (2020). Briefly, freshly collected whole COCs were immediately fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5 % glutaraldehyde solution at 4°C for a maximum of 1 month. Individual COCs were then embedded in 2% agar blocks to enable handling. Afterwards, blocks were washed three times in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5 % saccharose solution. Post-fixation was performed by incubating the blocks for 2 h with 1 % OsO_4 solution. After dehydration in an ethanol gradient, samples were embedded in EM-bed812. Ultrathin sections were stained with lead citrate, and examined in a Tecnai G2 Spirit Bio TWIN microscope (Fei, Europe BV, Zaventem, Belgium) at 120 kV. For each COC, images of at least 5 cumulus cells and at least 10 random fields in the oocyte (covering most of the oocyte area), were acquired at 16500–25000×. Mitochondria in the acquired images were morphologically evaluated by an expert blind to the corresponding treatment group and were classified based on their morphology, according to Marei et al. (2020).

3.9. QUANTIFICATION OF GENE EXPRESSION BY QPCR

RNA extraction

Total RNA was isolated from pools of cumulus cells of 4 mice/treatment group at week 2 and 4 using the PicoPure™ RNA Isolation Kit (Thermo Scientific) corresponding to the manufacturer's guidelines.

The concentration and purity of the collected RNA from each sample was assessed using a Nanodrop (Thermo Scientific). The integrity of the RNA was evaluated based on the RIN (RNA integrity number) value. The RNA concentration of each sample was used to calculate the volume of RNA necessary in the cDNA synthesis reaction.

cDNA synthesis

Before cDNA synthesis, the extracted RNA was treated with DNase I (ThermoFisher) to eliminate genomic DNA. cDNA was synthetized via reverse transcription of 150ng RNA/sample using the Omniscript RT kit (QIAGEN).

Quantification of gene expression using qPCR

Quantification of the transcripts of interest was performed using quantitative Polymerase Chain Reaction (qPCR) (CFX connect, BioRad) with SYBR Green (SsoAdvanced Universal SYBR Green supermix, Bio-Rad). No template control samples (lacking cDNA template) and RT-negative control samples were included. Data was normalized against 3 housekeeping genes: *YWHAZ, 18S* and *PPIA* calculated by NormFinder software (Camberley, UK). The relative abundance of each gene was calculated using the comparative quantification cycle (Cq) method, $2^{-\Delta\Delta Cq}$, as described by Livak and Schmittgen (2001a). Genes of interest are involved in following pathways: insulin signaling pathway (*IRS1* and *MTOR*), glucose metabolism (*SLC2A1*, *GAPDH*, *PFKp*, *LDHA, G6PD*), cumulus cell expansion and oocyte quality (*HAS2* and *PTX3*), lipid metabolism (*ACSL1* and *ACADM*) and oxidative stress (*SOD2*, *PRDX6*, *CAT* and *SIRT3*). Primers for the genes of interest were designed using the Primer-BLAST tool (Table 2).

Table 2. Overview primer design

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product size (bp)	Gene Bank accession number
YWHAZ	GGCCCTCAACTTCTCTGTGT	AAAGGTTGGAAGGCCGGTTA	246	NM_001253807.1
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC	219	NR_003278.3
PPIA	ATGGCAAGCATGTGGTCTTTGG	GGGTAGGGACGCTCTCCTGA	198	NM_008907.2
SOD2	CTGGACAAACCTGAGCCCTA	GCAGCAATCTGTAAGCGACCT	186	NM_013671.3
PRDX6	GTTGACTGGAAGAAGGGAGAGA	GCCACGATCTTTCTACGGAC	246	NM_007453.4
CAT	ACCAGATACTCCAAGGCAAAGG	GGGTCACGAACTGTGTCAGC	113	NM_009804.2
SIRT3	GACGGGCTTGAGAGAGCATC	AATGTCGGGTTTCACAACGC	183	NM_022433.2
ACSL1	AGTGGAACTACAGGCAACCC	TCTGTGGAAGCGATGAATGC	110	NM_001302163.1
ACADM	ACCGAAGAGTTGGCGTATG	GTCACACAGTAAGCACACATC	158	NM_007382.5
SLC2A1	GAGTGACGATCTGAGCTACGG	CTCCCACAGCCAACATGAGG	182	NM_011400.3
GAPDH	GGAGAGTGTTTCCTCGTCCC	ACTGTGCCGTTGAATTTGCC	202	NM_001289726.1
PFKp	TCAACGTGGTGGGTATGGTG	CACCAAGGCCAAGTAACCAC	188	NM_019703.4
LDHA	GGAGTGGTGTGAATGTTGCC	ATCACCTCGTAGGCACTGTC	124	NM_010699.2
G6PD	CGCAAAGCTGAAGTGAGACT	CAGGGAGCTTCACATTCTTGT	211	NM_008062.3
HAS2	GAAACTTCCTTCCACGACCCT	TGATTCCGAGGAGGAGAGACA	153	NM_008216.3
PTX3	GAAGCGTGCATCCTGTGAGA	ACCAACACTAGGGACTGGGA	163	NM_008987.3
IRS1	GGACATCACAGCAGAATGAAGA	CTGAAGAGGAAGACGTGAGGT	119	NM_010570.4
mTOR	GAGGCTGATGGACACAAATACC	CTGCTTTCTTATGGGCTGGTTC	128	NM_020009.2

3.10. OOCYTE DEVELOPMENTAL COMPETENCE - *IN VITRO* FERTILIZATION AND CULTURE OF *IN VIVO* MATURED MURINE CUMULUS-OOCYTE COMPLEXES

Oocyte in vitro fertilization and culture were performed as described in detail by Valckx et al. (2014b). Briefly, spermatozoa were isolated out of the caudae epididymis of two male Swiss mice into M16 fertilization medium (Merck, M7292) supplemented with 3% BSA (Merck, A6003). A final concentration of 10 000 spermatozoa/µl was made. Expanded cumulus oocyte complexes (COCs) were collected 13-14 hours post-hCG stimulation from female mice and transferred to 30 µl fertilization droplets in a fertilization plate covered with sterile mineral oil (Irvine) with a maximum of 12 oocytes/fertilization droplet. COCs from different mice were kept separately as each individual mouse was considered an experimental unit. Finally, 10 µl of the sperm suspension was added to the COCs in the fertilization droplets and incubated at 37°C, 6% CO2 and maximal humidity. After three hours of fertilization, presumptive zygotes were manually denuded with a stripper tip in denudation droplets of 100 µl filtered M16 medium (Merck, M7292) under oil. The denuded zygotes were cultivated in 75 µl culture medium (M16 medium (Merck, M7292)) at 37°C, 6% CO2, 5% O_2 and a maximal humidity for five days. Only oocytes that met the selection criteria (as mentioned

above), were selected for IVF. Cleavage rates of those selected oocytes were assessed at day 1 post insemination (p.i.) while blastocyst rates were assessed at day 5 p.i.

3.11. STATISTICAL ANALYSIS.

Statistical analysis was performed with IBM SPSS Statistics 26 (for Windows, Chicago, IL, USA).

Numerical data, e.g. weight, blood parameters, mitochondrial activity and ROS, were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Within each time point, numerical data were analysed using One-way ANOVA. Post-hoc LSD was performed in a sequential manner for predefined comparisons based on the null hypothesis for each of the following conditional research questions: 1) did exposure to a HF_HF diet induce a change compared to the CTRL_CTRL, if yes: 2) where the PCCIs effective in achieving any IMPROVEMENT compared to the HF_HF group, and if yes: 3) were the measurements in the PCCI groups RECOVERED to the level of the CTRL_CTRL group. To test questions 1 and 2, a post-hoc test was performed using the HF_HF group as a reference group. If a significant difference (and thus an improvement) was detected in the PCCI group(s) compared to the HF_HF group, a second post-hoc test was performed using the CTRL_CTRL group as a reference group to check for potential recovery (i.e. considered as no significant difference anymore between a PCCI group and the CTRL_CTRL group). Non-homogenous data were analysed using non-parametric independent sample Kruskal Wallis and a series of Mann-Whitney t-tests using the same sequential approach. On the other hand, categorical data, e.g. cleavage rates, were analysed using a binary logistic regression model also using the same strategy for comparisons.

The number of mice and oocytes used to generate the data are described in the results section for each parameter. Differences with *P*-values ≤ 0.05 were considered statistically significant. Differences with *P* values > 0.05 and ≤ 0.1 were reported as tendencies. Data are expressed as means ± S.E.M unless otherwise stated.

4. RESULTS

4.1. WEIGHT GAIN AND LOSS

Feeding a HF/HS diet already resulted in a significantly higher weight in HF_HF mice after only one week when compared with mice fed the control diet (CTRL_CTRL). HF-fed mice kept increasing in weight resulting in 24.19% more weight than the control group after seven weeks on the HF/HS-diet (Fig.2).

As soon as the PCCI period started, mice that switched from a HF/HS to an ad libitum control (HF_CTRL) or a 30% caloric restricted control diet (HF_CR) started to lose weight (Fig. 2). After only 8 days of PCCI, the HF_CR group showed a mean weight loss of 14.17%, and reached similar weights as the CTRL_CTRL group (P>0.1). As the PCCI continued, HF_CR mice kept losing weight, up to 23.32% at week 4. In the HF_CTRL group, complete weight normalization was achieved after 16 days of PCCI with a weight loss of 13.14%. Afterwards, weight of HF_CTRL mice stayed stable.

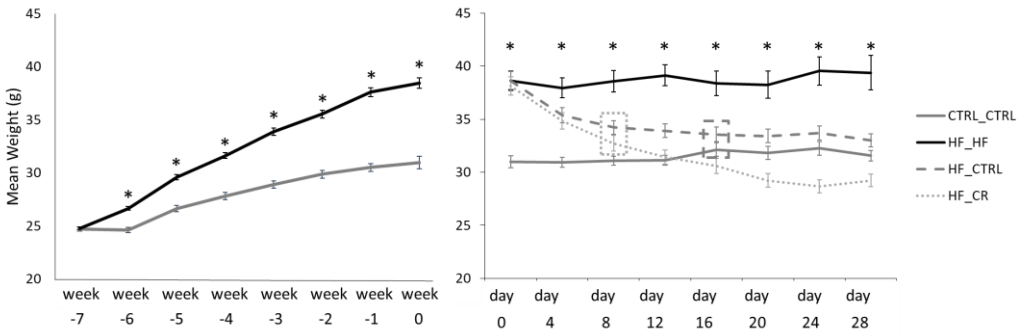


Figure 2. The effect of high fat/high sugar diet on **body weight gain** during the first seven weeks (induction of obesity); and **body weight changes after the start of the preconception care intervention** (**PCCI) period**. Data of weight gain are shown as means ± SEM from 192 mice in total. Significant difference ($P < 0.05$) between HF_HF and CTRL_CTRL group are indicated by an asterisk (*). Time point at which HF_CR or HF_CTRL group showed no significant difference anymore with the CTRL_CTRL group are indicated by a rectangle with the same lay-out.

4.2. METABOLIC HEALTH

4.2.1. BLOOD SERUM PROFILE

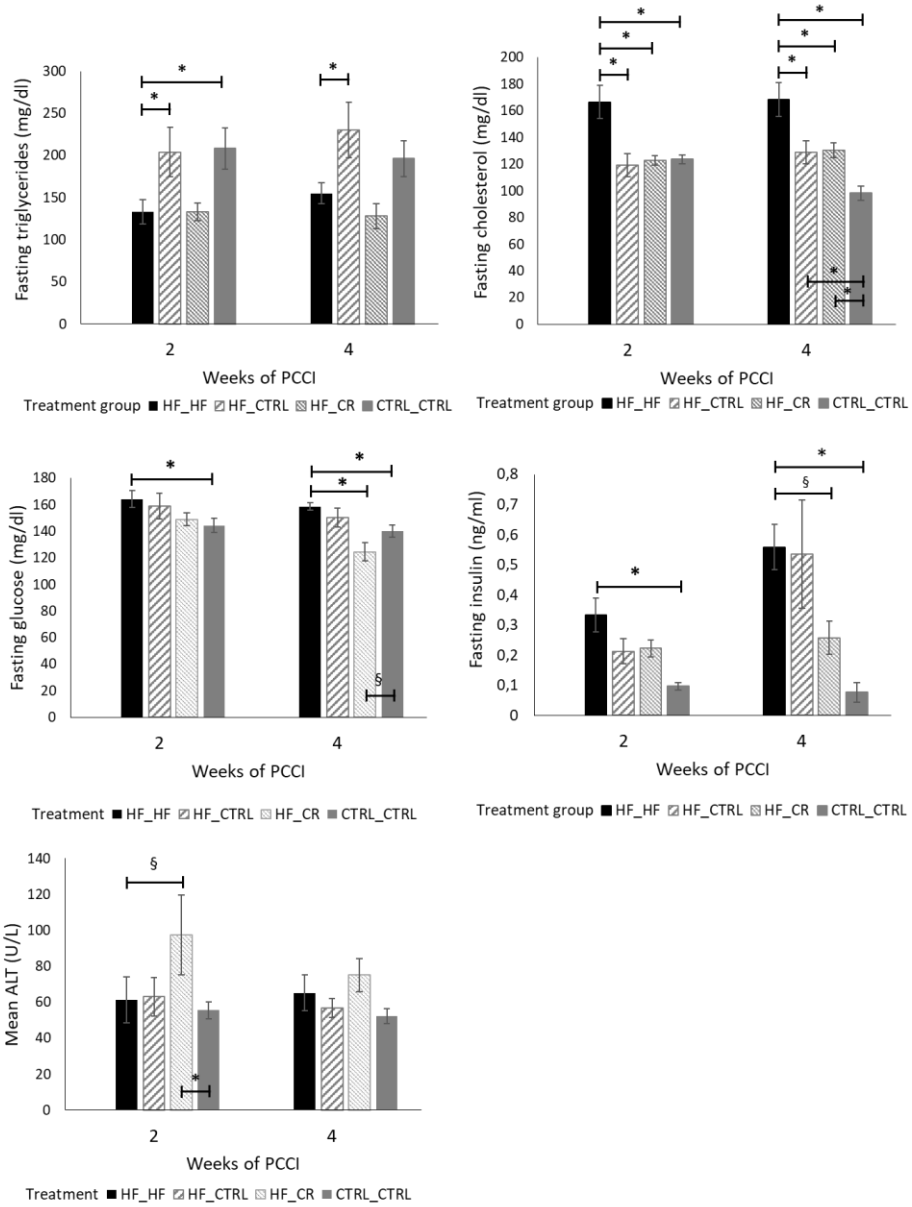


Figure 3. **Fasting serum** concentrations of glucose, insulin, cholesterol, triglycerides and alanine aminotransferase (ALT) among all treatment groups at different time points after starting the preconception care intervention (PCCI). Data are shown as means ± SEM from 6-8 mice per group per time point. Asterisks (*) indicate significant differences between the indicated treatment groups within the same PCCI period (P<0.05). Values labelled with "§" tend to be different from each other at 0.05 < P <0.1.

Feeding a HF/HS diet significantly increased fasting blood glucose concentrations compared with the CTRL_CTRL group at each time point (PCCI week 2 and 4 (P<0.05)) (Fig.3). This was associated with a marked increase in serum fasting insulin concentrations at both time points. Switching from a HF/HS to an ad libitum control diet (HF_CTRL) did not improve serum glucose and insulin concentrations when compared with the HF_HF group at both time points.
In contrast, both glucose and insulin concentrations in the HF_CR group were restored when compared with the HF_HF group, but only after four weeks of PCCI (glucose P<0.05, insulin P<0.1).

When focusing on the blood lipid profile, HF_HF mice displayed significantly higher fasting cholesterol concentrations when compared with the CTRL_CTRL group at both time points (P<0.05); Fig 3). Mice that underwent diet normalization (HF_CTRL) or a caloric restriction diet (HF_CR) showed restored cholesterol concentrations both at PCCI week 2 and 4 (P<0.05). Interestingly, triglyceride concentrations in the HF_HF group were lower at two weeks of PCCI when compared with the CTRL_CTRL group (P<0.05). Feeding a control diet to obese mice as a PCCI (HF_CTRL) significantly increased triglyceride concentrations again at two weeks of intervention (P<0.05). A 30% calorie restricted diet (HF_CR) had no impact at all on the lowered triglyceride concentrations seen in the HF_HF group.

Feeding a HF/HS diet had no effect on ALT concentrations, which is an indicator of liver damage. HF_CR mice, however, showed higher ALT concentrations than the control (CTRL_CTRL; P<0.05) and the high fat (HF_HF; P<0.1) group after two weeks of PCCI.

4.2.2. GLUCOSE AND INSULIN TOLERANCE TESTS

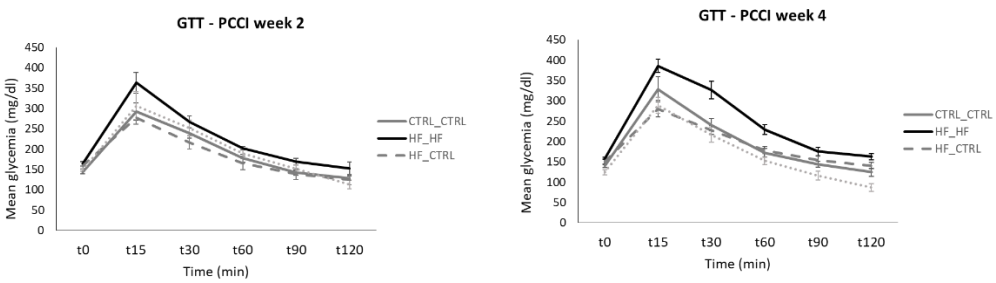


Figure 4. Serum glucose concentrations during a **glucose tolerance test (GTT)** at different time points after starting the preconception care intervention (PCCI) (week 2 and 4). Data are shown as means ± SEM. Per treatment, the same 8 mice were used at each PCCI.

Glucose tolerance tests (GTT) were performed at both time points on the same mice per treatment group.

Mice fed the HF/HS diet displayed impaired glucose tolerance compared with the CTRL_CTRL mice throughout the experiment (Fig. 4). More specifically, HF_HF mice

showed higher area under the curve (AUC) (PCCI week 2 *P*<0.1; PCCI week 4 *P*<0.05) and glucose peak (*P*<0.1) concentrations compared with the CTRL_CTRL group at both time points (Supplementary Fig. 1). Despite the increased AUC for glucose in the HF_HF mice, no significant differences in elimination rate (ER) were present.

The curves in figure 4 indicate an overall improvement in glucose tolerance in the HF_CTRL mice already after two weeks of PCCI. HF_CTRL values for AUC and peak glucose concentrations were significantly decreased when compared with the HF_HF mice (*P*<0.05) and even restored to the level of the control group.

Similarly, HF_CR mice showed a partially improved overall glucose tolerance after only two weeks of PCCI and a complete recovery after four weeks, substantiated by a significant decreased peak glucose concentration and AUC (*P*<0.05). In addition, ER was higher than the HF_HF group (*P*<0.1).

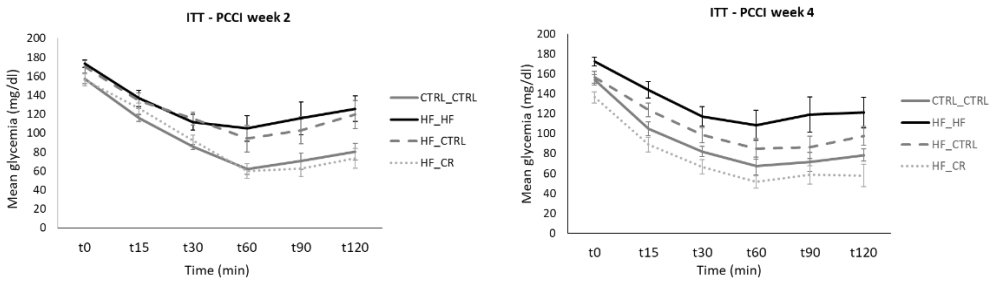


Figure 5. Serum glucose concentrations during an **insulin tolerance test (ITT)** at different time points after starting the preconception care intervention (PCCI) (week 2 and 4). Data are shown as means ± SEM. Per treatment, the same 8 mice were used at each PCCI.

Insulin tolerance tests (ITT) were performed on the same mice at both time points.
HF_HF mice displayed an overall impaired insulin sensitivity compared with CTRL_CTRL mice throughout the experiment (Fig. 5). This was confirmed by a significantly higher AUC at both time points (*P*<0.05) and a lower ER for glucose than the CTRL_CTRL group at PCCI week 4 (*P*<0.05) (Supplementary Fig. 2).
The curve in figure 5 showed no improved insulin sensitivity in HF_CTRL mice at PCCI week 2, confirmed by the similar AUC and ER for glucose compared to the HF_HF group (Supplementary Fig. 2). At PCCI week 4, a partial improvement in insulin sensitivity was seen in figure 5, however AUC and ER were still aberrant compared to CTRL_CTRL mice.

In contrast, a caloric restriction diet (HF_CR) was able to significantly improve insulin sensitivity after only two weeks of PCCI, substantiated by a significantly lower AUC for glucose (*P*<0.05). In addition, ER was completely restored to the level of the CTRL_CTRL mice at PCCI week 4.

4.3. OOCYTE QUALITY - FERTILITY

4.3.1. MITOCHONDRIAL ULTRASTRUCTURE IN CUMULUS CELLS AND OOCYTES

Table 3. Proportions of mitochondria with normal or abnormal ultrastructure (using TEM) in cumulus cells and oocytes from all treatment groups at PCCI week 4

	Total Mitochondria evaluated	**Abnormal mitochondria evaluated**
Oocytes		
HF_HF	279	*79 (28.3%)[b]*
HF_CTRL	503	*110 (21.9%)[a]*
HF_CR	504	*164 (32.5%)[b]*
CTRL_CTRL	581	*112 (19.3%)[a]*
Cumulus cells		
HF_HF	*252*	*11 (4.37%)*
HF_CTRL	*429*	*15 (3.50%)*
HF_CR	*381*	*27 (7.09%)*
CTRL_CTRL	*347*	*11 (3.17%)*

Data are presented as proportions from total number of mitochondria evaluated, from 3 to 4 COCs per treatment group (from 3 to 4 mice per treatment group). Significant differences between the indicated treatment groups are indicated with letters a, b and c ($P < 0.05$)

In the CTRL_CTRL oocytes, 19.3% of the evaluated mitochondria were categorized as structurally abnormal. Exposure to a HF/HS diet (HF_HF group) significantly increased that percentage to 28.3% ($P<0.001$) (Table 3). Only oocytes from HF_CTRL mice displayed a significant improvement of mitochondrial ultrastructural abnormalities to only 21.9% at PCCI week 4. Switching to a CR diet for four weeks did not improve mitochondrial ultrastructural abnormalities compared with the HF_HF group. No significant differences in the ultrastructure of cumulus cell mitochondria could be observed (Table 3).

4.3.2. MITOCHONDRIAL ACTIVITY AND LOCALIZATION OF ACTIVE MITOCHONDRIA IN OOCYTES

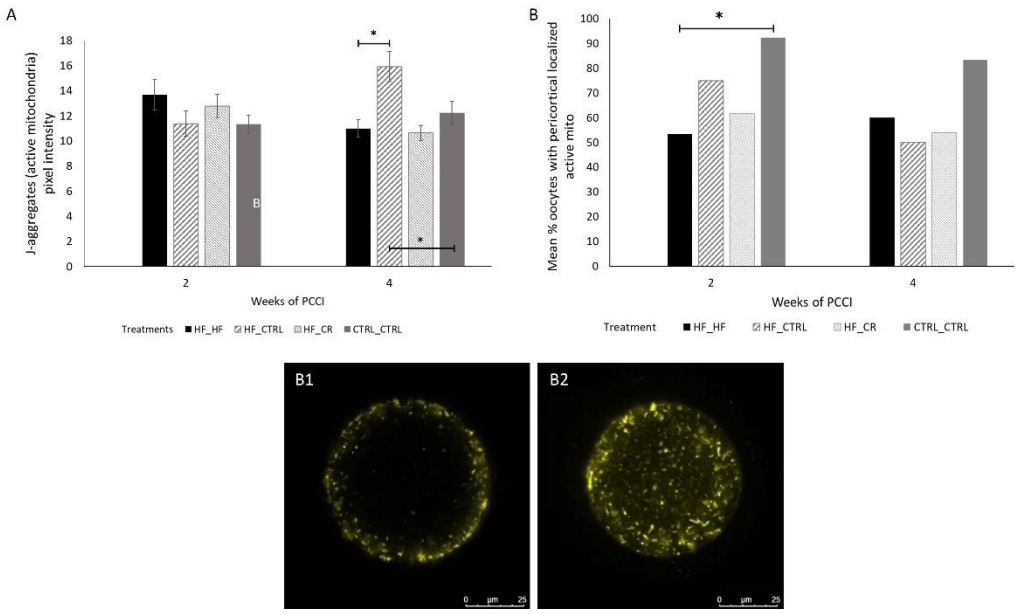


Figure 6. Quantification of **mitochondrial activity** (A; JC1- staining) and percentage of oocytes with **pericortical distribution of active mitochondria** (B; pericortical (B1) or diffuse (B2)), at different time points after starting the preconception care intervention (PCCI). Data are shown from 1-3 oocytes per mouse and 5-6 mice per group per time point. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period ($P<0.05$). Values labelled with "§" tend to be different from each other at $0.05 < P < 0.1$.

A HF/HS diet (HF_HF) did not alter oocyte mitochondrial activity compared with CTRL_CTRL oocytes at both timepoints (Fig. 6). At PCCI week 4, oocytes from HF_CTRL mice showed a significantly higher mitochondrial activity compared with both the HF_HF and CTRL_CTRL group. HF_CR mice showed no difference with the HF_HF and CTRL_CTRL groups at both time points. However, the proportion of oocytes with a pericortical distribution of active mitochondria was markedly lower in HF_HF mice compared with CTRL_CTRL mice at both time points but this was only significantly different at PCCI week 2 ($P<0.05$, Fig. 6). This was not improved in both PCCI groups.

4.3.3. LIPID DROPLET VOLUME

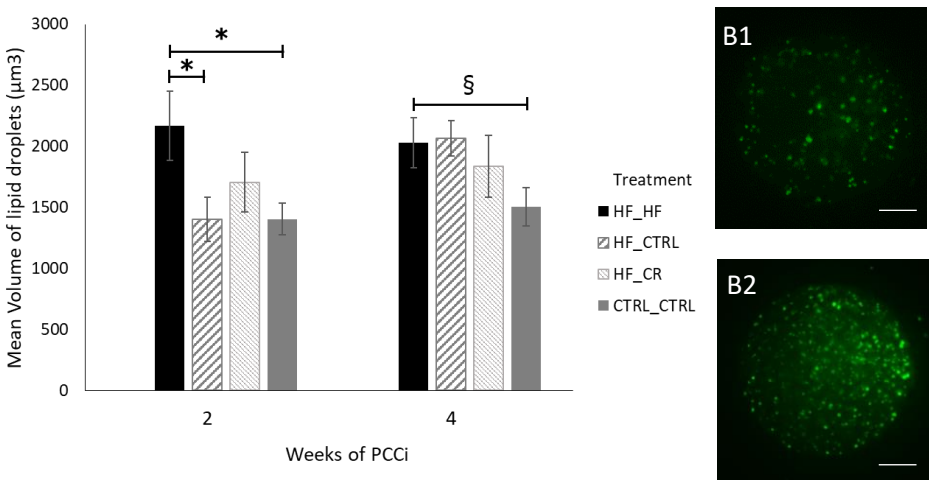


Fig. 7 BODIPY 493/503 staining assessing lipid droplet volume in oocytes collected from mice of all treatment groups, after two or four weeks of preconception care intervention (PCCI). Each image is a z-stack projection of 40 ×1 µm steps. Data are presented as means ± SEM from 10-12 oocytes per group, collected from 7-8 mice per group per time point (panel A). Representative confocal microscope images are presented in panel B1 and B2. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period ($P<0.05$). Values labelled with "§" tend to be different from each other at $0.05 < P < 0.1$.

Oocytes were examined for total lipid droplet volume using BODIPY 493/503 staining. Oocytes collected from HF_HF mice displayed a higher lipid droplet volume than the CTRL_CTRL mice at PCCI week 2 ($P<0.05$) and week 4 ($P<0.1$) (Fig. 7). Oocytes collected from HF_CTRL mice showed a significantly lower lipid droplet volume than mice on the high fat diet (HF_HF) after two weeks of PCCI ($P<0.05$). However, at PCCI week 4, this was not the case anymore. Switching to a caloric restriction diet (HF_CR) did not improve the elevated lipid content in the oocytes.

4.3.4. OOCYTE DEVELOPMENTAL COMPETENCE

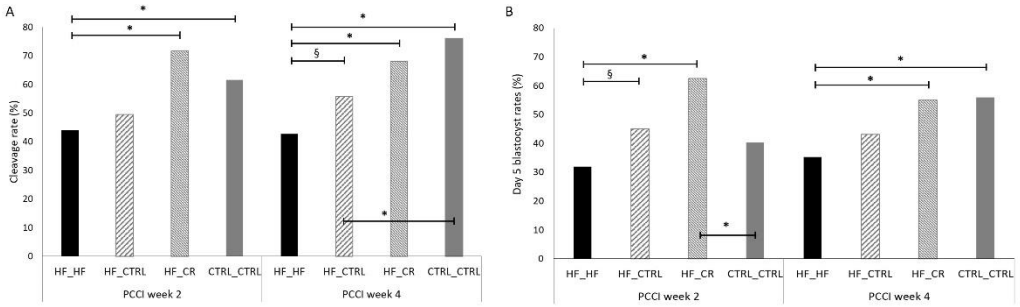


Figure 8. **Developmental competence** (cleavage (A) and blastocyst rates (B)) of oocytes selected for IVF at different time points after starting the preconception care intervention (PCCI). Data are shown from pools of oocytes collected from 5-6 mice per group per time point. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period (P<0.05). Values labelled with "§" tend to be different from each other at 0.05 < P <0.1.

Oocytes from HF_HF mice that were selected for IVF (see supplementary table S2) showed significantly lower cleavage rates compared with CTRL_CTRL oocytes at both time points (P<0.05; Fig. 8A). Day 5 blastocyst rates of the HF_HF group were only significantly lower at PCCI week 4 (Fig. 8B). However, at PCCI week 2, the blastocyst rate of the CTRL_CTRL group was surprisingly low, explaining the lack of significant difference with the HF_HF group. Oocytes collected from mice that underwent diet normalization (HF_CTRL) showed no significantly improved cleavage or day 5 blastocyst rates at both timepoints. However, oocytes collected from HF_CR mice displayed restored cleavage rates and day 5 blastocyst rates already after two weeks of PCCI.

Once a fertilized oocyte cleaved, the capacity to develop until the blastocyst stage was the same in all groups at both timepoints (Suppl. Fig 3). Only at PCCI week 2, the HF_CR group displayed higher day 5 blastocyst/cleaved ratios than both the HF_HF (P<0.1) and CTRL_CTRL groups (P<0.05).

4.3.5. CUMULUS CELL GENE EXPRESSION

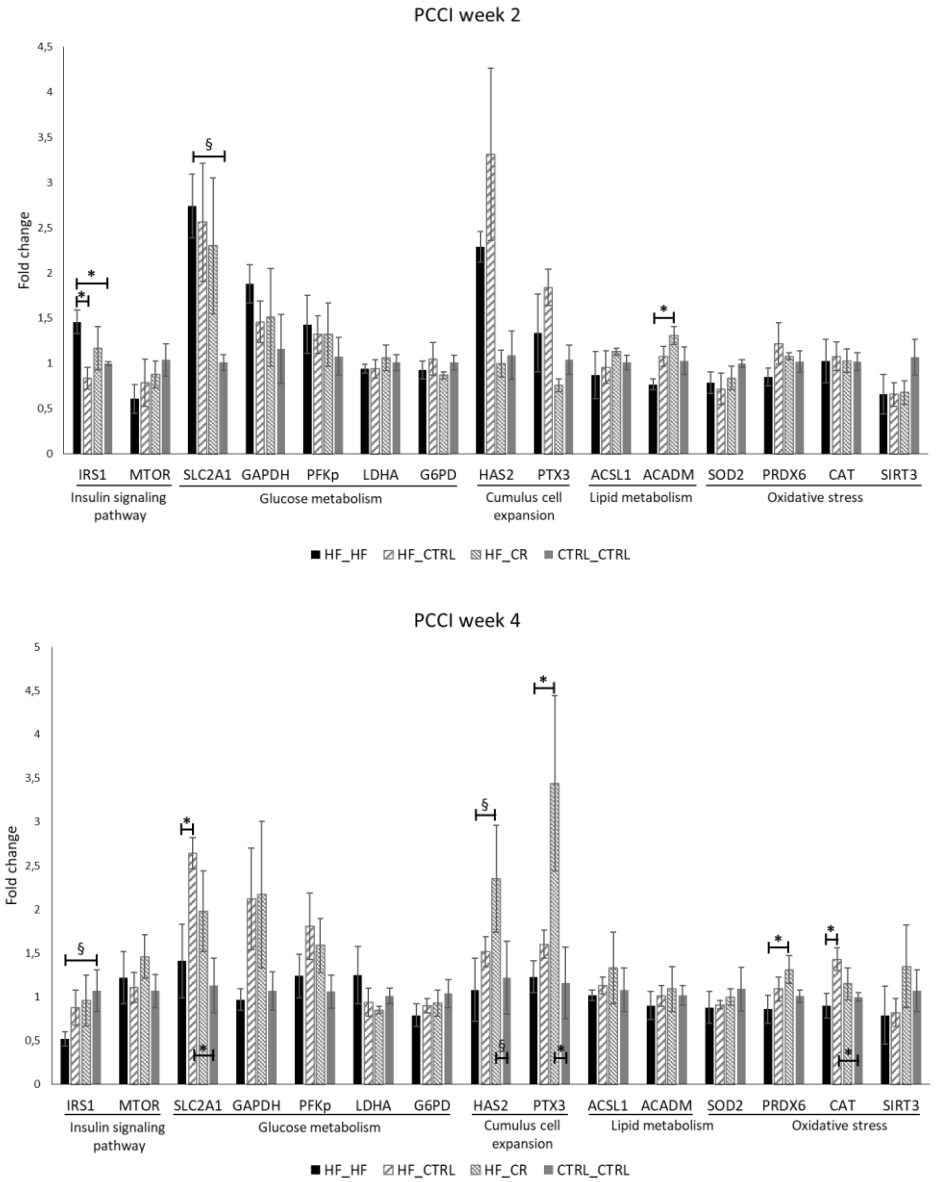


Figure 10. Relative **transcript abundance of genes** related to insulin signaling pathway (*IRS1, MTOR*), glucose metabolism (*SLC2A1, GAPDH, PFKp, LDHA, G6PD*), cumulus cell expansion/oocyte quality (*HAS2, PTX3*), lipid metabolism (*ACSL1, ACADM*) and oxidative stress (*SOD2, PRDX6, CAT, SIRT3*) in cumulus cells of all treatment groups at both time points. Data are shown as means ± S.E.M from pools of cumulus cells from 4 mice/treatment group/time point. Significant differences (P<0,05) are indicated by an asterisk (*). Tendencies (P<0,1) are indicated by a dollar sign ($).

When comparing mRNA transcript abundance of genes in cumulus cells from HF_HF mice with those from CTRL_CTRL mice, only significant differences could be found in genes involved in the insulin signaling pathway and genes related to glucose metabolism. *IRS1* and *SLC2A1* expression was upregulated in cumulus cells of HF_HF mice compared with the CTRL_CTRL group at two weeks of PCCI (P<0.05 and P<0.1 respectively; Fig. 10). At PCCI week 4, the opposite effect was present as *IRS1* expression was lower in HF_HF group compared with the CTRL_CTRL group (P<0.1; Fig. 10) and the expression of *SLC2A1* was reduced in HF_HF cumulus cells to a similar expression as CTRL_CTRL cumulus cells.

In CC's from HF_CTRL mice, expression level of *IRS1* was already restored at PCCI week 2 (Fig. 10). At the same timepoint, diet normalization failed to alleviate the increase in *SLC2A1* expression. At PCCI week 4, CC's from HF_CTRL mice still showed high expression of *SLC2A1*, in contrast to the reduced expression in HF_HF mice.

No significant differences could be detected in genes related to cumulus cell expansion and lipid metabolism in HF_CTRL mice. However, HF_CTRL mice showed a significantly upregulated expression of *CAT*, a gene encoding a key antioxidant enzyme, compared to both reference groups but only at PCCI week 4 (P<0,05).

Cumulus cells collected from mice that underwent a caloric restriction diet (HF_CR) showed almost no differences in gene expression levels at PCCI week 2. The HF_CR group only displayed a significantly higher expression of *ACADM,* a gene involved in lipid metabolism*,* compared to the HF_HF group at this time point (P<0,05). However, at PCCI week 4, a caloric restriction diet (HF_CR) increased the expression of *HAS2* and *PTX3,* genes related to cumulus expansion and oocyte quality, in cumulus cells compared to both the HF_HF (P<0.1; P<0.05 respectively) and CTRL_CTRL group (P<0.1; P<0.05 respectively). In addition, caloric restriction diet (HF_CR) significantly increased the expression of *PRDX6,* a gene involved in oxidative stress, in cumulus cells compared to the HF_HF group (P<0,05) at PCCI week 4.

5. DISCUSSION

The aim of this study was to investigate whether a preconception care intervention through diet normalization or a calorie restricted diet for two or four weeks, resulted in an improved metabolic health and oocyte quality in obese outbred mice. We confirmed that a HF/HS diet exposure resulted in an obese phenotype characterized by a significantly deteriorated metabolic health and reduced oocyte quality.
Metabolic health significantly improved in both PCCIs after two weeks but only completely recovered in the calorie restricted group after a four week intervention period. Calorie restricted mice showed significantly improved oocyte developmental capacity already after two weeks of intervention while mitochondrial morphology and function remained aberrant. Oocytes from HF_CTRL mice displayed significantly less mitochondrial ultrastructural abnormalities and higher mitochondrial activity compared with the HF_HF group at PCCI week 4 but still had an abnormal cellular mitochondrial distribution after maturation. Their in vitro developmental capacity was only partly improved.

Impact of a HF/HS diet and PCCI on metabolic health

We could confirm that HF/HS diet feeding significantly increased body weight of mice, (Marei et al., 2020; Smits et al., 2021), resulting in an obese phenotype. Excessive long-term intake of a diet rich in saturated fat and sugar is known to induce a state of reduced insulin sensitivity (Holm, 2003; Karpe et al., 2011) and thus decreased responsiveness to insulin (Shanik et al., 2008). To compensate for this insulin resistant state, the pancreatic β-cells increase insulin secretion, resulting in hyperinsulinemia (Turner and Robker, 2015). Accordingly, next to a significantly decreased insulin sensitivity at both time points, HF_HF mice also showed significantly higher fasted serum insulin concentrations when compared with the CTRL_CTRL group. In addition to the reduced glucose tolerance and insulin sensitivity, HF_HF mice also had an aberrant serum lipid profile, indicated by hypercholesterolemia as commonly seen in diet induced obese individuals (Marei et al., 2020; Podrini et al., 2013; Smits et al., 2021) and hypotriglyceridemia as seen in other studies (Biddinger et al., 2005; Guo et al., 2009; Meugnier et al., 2007; Podrini et al., 2013) (Smits et al., 2021).
Earlier research described a more severe impact of the HF/HS diet on metabolic health compared to a HF-only diet (Della Vedova et al., 2016; Moreno-Fernandez et al., 2018). However, in this study, the impact of this HF/HS diet on the metabolic health is very similar to what we described in our previous study using a high fat only diet induced obese mouse model (Smits et al., 2021). Both diets resulted in a weight gain of 24-25% and the metabolic health impact was very similar. However, it is not known if fructose might have accelerated the development of obesity and the insulin resistance phenotype as we only measured this after a 7 week feeding period.

HF/HS-fed obese mice that underwent diet normalization (HF_CTRL) lost 13% of their weight after two weeks of PCCI, reaching similar weights as the CTRL_CTRL mice. In addition, they already showed a complete recovery of the aberrant serum lipid profile (triglyceride and cholesterol concentrations). Although insulin sensitivity has been reported to improve in proportion to the degree in weight loss (Ferrannini and Camastra, 1998), no significant improvements in insulin sensitivity were present at PCCI week 2 while glucose tolerance was already restored at this timepoint. This indicates a higher insulin release by the pancreatic β-cells in HF_CTRL mice and not yet an improved insulin sensing in the peripheral tissue. Even a longer period of diet normalization (four weeks) was still not able to significantly improve insulin sensitivity. In contrast, earlier research investigating the impact of diet normalization in high fat-diet only induced obese mice, did show a partially improved insulin sensitivity, including restored serum insulin concentrations after fasting (Smits et al., 2021). These data clearly confirm that the addition of high fructose concentrations to the diet reduced insulin sensitivity and made it more difficult to improve after diet normalization..

Interestingly, insulin sensitivity in HF_CR mice was completely restored at PCCI week 2, indicating that the caloric restriction diet seemed a more promising PCCI method to improve insulin sensitivity in HF/HS-fed mice compared to those that underwent diet normalization. However, after four weeks of PCCI, both serum glucose concentration and the GTT and ITT data indicated a state of hypoglycemia suggesting that the CR diet for four weeks and the extreme weight loss might be a too extreme intervention. These signs of fasting hypoglycemia were not seen after four weeks of PCCI in our previous study using a high fat only obese mouse model (Smits et al., 2021). Therefore, two weeks of a 30% calorie restriction is better to improve metabolic health.
After two weeks of caloric restriction diet, mice already showed restored cholesterol serum concentrations. However, serum contained higher ALT concentrations than both HF_HF and CTRL_CTRL groups. This has been reported in other studies (Friis et al., 1987; Gasteyger et al., 2008; Hoy et al., 1994; Kreitzman et al., 1984; Smits et al., 2021). The damage of the hepatocytes can cause an inadequate export of triglycerides from the liver which explains the low serum triglyceride concentrations seen in the HF_CR mice at both time points (Smits et al., 2021).

Impact of a HF/HS diet on oocyte quality and cumulus cell gene expression

We could confirm that oocytes collected from HF_HF mice contained a significantly higher lipid droplet volume than oocytes from CTRL_CTRL mice. Lipids are an important source of energy in the oocyte. However, obesity leads to hyperlipidemia (Sutton-McDowall et al., 2012), which is reflected in the composition of the follicular environment (Valckx et al., 2012). Oocytes actively take up and metabolize these lipids. The accumulation of intracellular lipids results in lipotoxicity, causing damage to cellular organelles like mitochondria (Suzuki, 2017; Wu et al., 2011). We confirmed an elevated proportion of mitochondrial ultrastructural abnormalities in oocytes from HF_HF mice

(Marei et al., 2020; Smits et al., 2021). In addition, oocytes collected from HF_HF mice showed an improper distribution of mitochondria after final maturation. Correct pericortical localization of active mitochondria supports ATP levels during embryo cleavage (Van Blerkom et al., 2003), is required for successful sperm penetration and exocytosis of cortical granules (Van Blerkom and Davis, 2007) and guarantees the even distribution of the maternally inherited mitochondria over de blastomeres upon first cleavages (Van Blerkom et al., 2000). Therefore, this reduction in the percentage of pericortical localized active mitochondria might result in decreased fertilization and cleavage rates.

Earlier research has demonstrated both decreases and increases in mitochondrial membrane potential (MMP) in metabolically-compromised oocytes or oocytes collected from obese mice (Marei et al., 2017) (Igosheva et al., 2010; Marei et al., 2020) compared to control. Both high and low MMP have been linked to reduced oocyte developmental competence (Acton et al., 2004; Wilding et al., 2001). This could be explained by an uncoupled oxidative phosphorylation which occurs when oxidative stress levels reach a threshold, resulting in a reduction in MMP. The aim of this uncoupling is to reduce further production of reactive oxygen species to avoid further cell damage (Brand, 2000). This results in a heterogeneous population of hyperactive and inactive mitochondria within the same oocyte or even between different oocytes from the same treatment group (Marei et al., 2020). The lack of significant differences in mean mitochondrial activity between the HF_HF and CTRL_CTRL group in this study might be a result of this non-uniform uncoupling process as 66.67% and 44.44% of oocytes collected from HF_HF mice at PCCI week 2 and 4, respectively, showed extreme values of mitochondrial activity (located in Q1 (inactive) or Q4 (hyperactive) intervals) compared to only 18.18% and 22.22% in CTRL_CTRL oocytes.

As reported in other murine studies, a reduced developmental capacity was present in oocytes derived from HF_HF mice (Finger et al., 2015; Igosheva et al., 2010; Sohrabi et al., 2015). However, once an oocyte was able to cleave for the first time, the chances to develop until the blastocyst stage were not different between the CTRL_CTRL and the HF_HF group. In our previous study, pregnancy rates in high fat induced obese mice were 87.5% reduced compared with the control mice further indicating the importance of blastocyst quality probably more than blastocyst rates (Smits et al., 2021).

Gene expression analysis of cumulus cells (CC's) from HF_HF mice only showed significant differences in genes related to insulin signalling and glucose transport, *IRS1* and *SLC2A4* respectively. As the oocyte's capacity to metabolize glucose is very poor, it almost completely relies on its surrounding CC's for the uptake of glucose (Richani et al., 2021a; Sutton-McDowall et al., 2010). *IRS1* plays an important role in the insulin-stimulated glucose uptake of the cumulus cells. It has been reported that expression of *IRS1* is remarkably reduced in insulin resistant liver and muscle of both humans and mice (Goodyear et al., 1995; Saad et al., 1992). Assessment of metabolic health in HF_HF mice clearly confirmed the presence of insulin resistance in these mice. At PCCI

week 2, *IRS1* and *SLC2A1* expression in CC's was significantly increased when compared with the CTRL_CTRL group, indicating a possible compensatory effect to sustain normal insulin signalling and glucose transport. However, it seems that this compensatory effect was only temporary as both *IRS1* and *SLC2A1* expression was reduced in CC's of HF_HF mice at PCCI week 4. A significantly reduced expression of these genes might result in a further reduced responsiveness of CC's to insulin and glucose, possibly affecting oocyte quality.
However, several other factors that were not evaluated in the present study control the expression of *SLC2A1* such as estrogen and growth factor concentrations (Simmons, 2017). More research is needed to better understand the impact and the importance of a disturbed insulin signaling cascade and glucose uptake in COCs in the context of obesity and a HF/HS diet.

Do caloric restriction or diet normalization have the potential to improve or restore oocyte quality?

During folliculogenesis, mitochondrial biogenesis and replication of mtDNA takes place, which is crucial to support mitochondrial functions in the oocyte (May-Panloup et al., 2016). Switching to a caloric restriction diet had no beneficial effects on oocyte mitochondrial morphology (at PCCI week 4), mitochondrial activity or localization of active mitochondria in the mature oocyte, indicating that a caloric restriction diet might not be able to improve mitochondrial quality in oocytes, regardless of the PCCI covering the whole folliculogenesis (PCCI week 4) or not (PCCI week 2). This confirms the findings of earlier research (Smits et al., 2021). As follicular activation and development in a high fat/high sugar-free environment (PCCI week 4) was not able to restore oocyte mitochondrial quality, our results indicate that the pool of primordial follicles seems to be sensitive to this obesogenic metabolic environment. Of course, remnant effects of a slowly recovering metabolic health after the start of the PCCI may also directly impact on oocyte mitochondria during folliculogenesis. Much more research on this concept is needed.

Oocytes from HF_CR mice still had a high oocyte lipid content at both PCCI time points. This can be a result of efficient fatty acid storage as a method to store energy for preimplantation development (Ferguson and Leese, 2006; Hillman and Flynn, 1980; Sturmey et al., 2009). In accordance, the elevated expression of *ACADM* (a gene that catalyzes the first step in fatty acid β-oxidation (FAO)) in cumulus cells of HF_CR mice may be indicative of the increased oocyte developmental competence at this time point as a strong role of FAO during oocyte maturation has been recognized in several studies (Downs et al., 2009; Dunning et al., 2010; Paczkowski et al., 2014a; Valsangkar and Downs, 2013). Cumulus cells play a significant role in this process as it has been reported that the majority of FAO occurs in the cumulus cells of the murine COC (Dunning et al., 2010).

Interestingly, at PCCI week 4, *HAS2* and *PTX3*, genes related to cumulus cell expansion and oocyte development were significantly upregulated in CC's , in accordance with *PRDX6*, involved in antioxidant defense (Wood et al., 2003). Several studies (human, murine and bovine) have shown that higher *HAS2* expression in CC is associated with increased oocyte developmental competence (Assidi et al., 2008; Cillo et al., 2007; McKenzie et al., 2004; Vigone et al., 2013). Earlier research in patients undergoing IVF demonstrated that a 3-fold to 12-fold increase in the expression of *PTX3* was present in cumulus cells from fertilized oocytes compared to CC from oocytes that failed to fertilize (Zhang et al., 2005). In support of our findings, oocyte developmental competence of HF_CR mice was significantly restored to the level of the CTRL_CTRL group, as indicated by increased cleavage and blastocyst rates. In contrast, expression levels of *HAS2* and *PTX3* in CC's from HF_CTRL mice were not upregulated which coincided with no significant improvement of their oocyte developmental competence.

Similarly to the HF_CR group, mature oocytes collected from mice that only underwent diet normalization (HF_CTRL) for two weeks still showed an aberrant mitochondrial localization. Interestingly, at the same time point, lipid droplet volume was restored. This was in accordance with normalized weight and serum cholesterol concentrations, as reported before (Smits et al., 2021). However, the reduced lipid droplet volume could not be related to upregulated FAO in the CC's as the lipid metabolism genes were not significantly different from both the HF_HF and CTRL_CTRL groups.

Two weeks later, at PCCI week 4 when body weight was stabilized for two weeks, oocyte lipid droplet volume was elevated again. This coincided with an already stabilized body weight for two weeks. In contrast with oocytes from HF_CR mice, mitochondrial morphology was restored at this timepoint in oocytes of HF_CTRL mice and mitochondrial activity was significantly higher than both the HF_HF and CTRL_CTRL group. In accordance with the high *SLC2A1* gene expression, this increase in mitochondrial activity can possibly be a result of improved uptake and metabolism of glucose in the cumulus cells after four weeks of diet normalization.

These data show that switching to an ad libitum control diet for four weeks, longer than the timeframe of folliculogenesis, was able to restore mitochondrial ultrastructural abnormalities to the level of the CTRL_CTRL group and to improve mitochondrial activity. This might indicate that, in contrast to extreme weight loss strategies, the access to an ad libitum control diet might be the better option to improve oocyte mitochondrial characteristics. In addition, at PCCI week 4, there was a significantly increased expression of *Catalase* compared to both the HF_HF and CTRL_CTRL group, indicating an increased level of antioxidant defense. Although some HF_CTRL oocyte quality parameters showed promising results at PCCI week 4, this was only reflected in a partially improved oocyte developmental competence. This is surprising as in vitro oocyte developmental competence was completely restored in the HF_CR group, despite worse mitochondrial quality parameters.

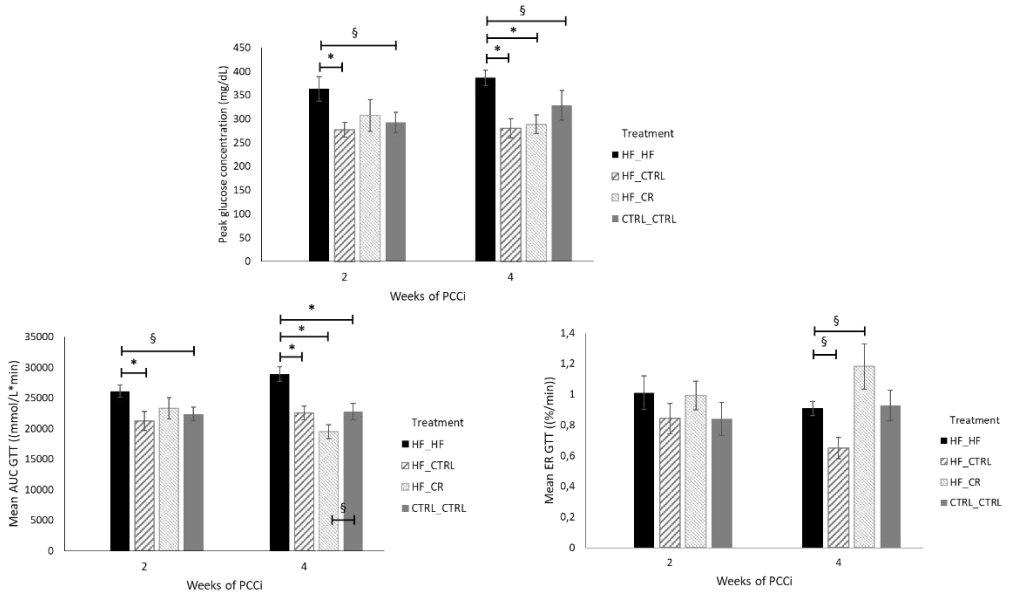
Conclusions

This strategically designed research provides further fundamental insights regarding the potential of preconception care interventions using diet normalization or a calorie restricted diet for two or four weeks to improve metabolic health and oocyte quality in Western type diet fed obese outbred mice. We confirmed that obesity, induced by a fat and sugar rich diet, has a detrimental effect on both metabolic health and oocyte quality, resulting in drastically altered oocyte lipid content, mitochondrial morphology, subcellular localization and functionality, cumulus gene expression patterns and subsequent in vitro developmental capacity.

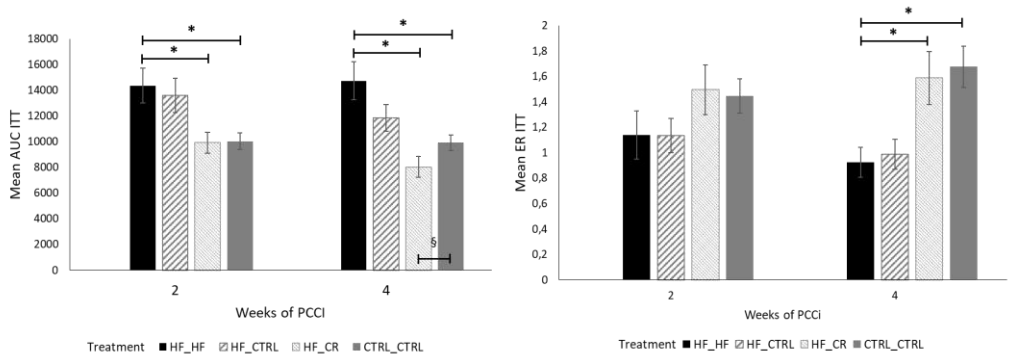
Metabolic health significantly improved in both PCCIs after two weeks but only completely recovered in the calorie restricted group after a four week intervention period. Calorie restricted mice showed significantly improved oocyte developmental capacity already after two weeks of intervention while mitochondrial morphology and function remained aberrant. Obese mice that underwent diet normalization only showed improved oocyte quality after four weeks of PCCI. Full oocyte recovery in terms of mitochondrial distribution and lipid content could not be observed in both intervention groups suggesting that oocytes from the dormant primordial follicle pool may be affected by an obesogenic environment.

Translating these results to the human setting should be done with caution. However, the in depth study and follow up of the metabolic health changes together with the strategic implementation of specific PCCI intervals (2 and 4 weeks) related to the duration of the mouse folliculogenesis (three weeks), should aid in the extrapolation of our findings to the human setting. Our study results, with a specific focus on oocyte quality, provide important fundamental insights to be considered when developing preconception guidelines for obese metabolically compromised women planning for pregnancy.

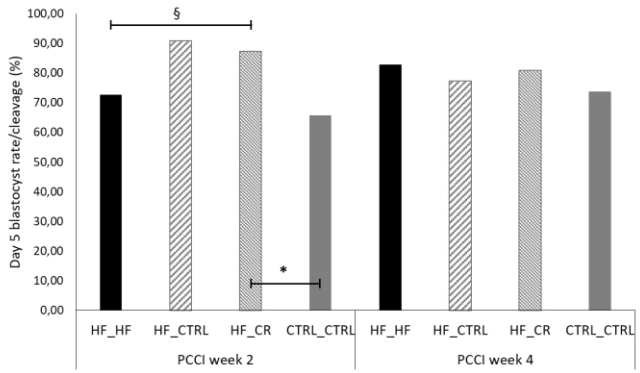
6. SUPPLEMENTARY FIGURES



Supplementary figure 1. **Peak glucose concentration, area under the curve (AUC) and elimination rate (ER)** of the **glucose tolerance test** of all treatment groups at different time points after starting the preconception care intervention (PCCI) (week 2 and 4). Data are shown as means ± SEM from 8 mice per group per time point. Asterisks (*) indicate significant differences between the indicated treatment groups within the same PCCI period (P<0.05). Values labelled with "§" tend to be different from each other at 0.05 < P <0.1.



Supplementary figure 2. Glucose **area under the curve (AUC) and elimination rate (ER)** of the **insulin tolerance test** (ITT) of all treatment groups at different time points after starting the preconception care intervention (PCCI). Data are shown as means ± SEM from 8 mice per group per time point. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period (P<0.05). Values labelled with "§" tend to be different from each other at 0.05 < P <0.1.



Supplementary figure 3. Developmental competence (**Day 5 blastocyst/cleavage rates**) of oocytes selected for IVF at different time points after starting the preconception care intervention (PCCI). Data are shown from pools of oocytes collected from 5-6 mice per group per time point. Asterisks (*) indicate significant differences between indicated treatment groups within the same PCCI period ($P<0.05$). Values labelled with "§" tend to be different from each other at $0.05 < P < 0.1$.

Table S1: Detailed quantification of different categories of observed normal and abnormal mitochondrial ultrastructure in oocytes at PCCI week 4. Data are presented as proportions of 279 - 581 mitochondria counted in 3-4 oocytes per treatment group.

	CTRL_CTRL	HF_HF	HF_CTRL	HF_CR
Total N mitochondria evaluated (%)	581	279	503	504
Normal mitochondria	***469** (80,7%)[a]*	***200** (71,7%)[b]*	***393** (78,1%)[a]*	***340** (67,5%)[b]*
Abnormal mitochondria	***112** (19,3%)[a]*	***79** (28,3%)[b]*	***110** (21,9%)[a]*	***164** (32,5%)[b]*
a. Membranous vacuoles	26 (4,5%)	12 (4,3%)	27 (5,4%)	30 (6,0%)
b. Electron dense foci	2 (0,3%)	6 (2,2%)	2 (0,4%)	28 (5,6%)
c. Dumbell-shaped	1 (0,2%)	1 (0,4%)	3 (0,6%)	2 (0,4%)
d. Dumbell-shaped with vacuoles	3 (0,3%)	0 (0,0%)	2 (0,4%)	2 (0,4%)
e. Rose-petal shaped	13 (2,2%)	0 (0,0%)	4 (0,8%)	10 (2,0%)
f. Degenerated	9 (1,5%)	1 (0,4%)	2 (0,4%)	10 (2,0%)
g. Ruptured	1 (0,2%)	0 (0,0%)	0 (0,0%)	0 (0,0%)
h. Double membrane	17 (2,9%)	17 (6,1%)	25 (5,0%)	21 (4,2%)
i. Double membrane and vacuolated	25 (4,3%)	25 (9,0%)	29 (5,8%)	34 (6,7%)
j. Double membrane and membranous vacuoles	6 (1,0%)	9 (3,2%)	4 (0,8%)	20 (4,0%)
k. Other abnormalities	9 (1,5%)	8 (2,9%)	12 (2,4%)	7 (1,4%)

Mitochondria were considered normal when spherical or spherical with regular vacuoles. Mitochondrial abnormalities include vacuolation with loose inner membrane structures (c), electron dense foci (d), dumbbell shapes with vacuolation (e), dumbbell shapes (f), rose petal appearance (g) or degeneration (h).

Table S2: Proportion of good quality oocytes selected for IVF from all treatment groups at both time points (PCCI week 2 and 4)

	Treatment	Total collected oocytes	Oocytes selected for IVF
PCCI week 2	**HF_HF**	91	75 (82.42%)[a]
	HF_CTRL	112	111 (99.1%)[b]
	HF_CR	92	88 (95.65%)[b]
	CTRL_CTRL	53	52 (98.11%)[b]
PCCI week 4	**HF_HF**	83	82 (98.80%)[a]
	HF_CTRL	115	95 (82.61%)[b]
	HF_CR	73	69 (94.52%)[a]
	CTRL_CTRL	84	75 (89.29%)[b]

7. REFERENCES

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PROTEOMIC CHANGES IN CUMULUS CELLS FROM HIGH FAT/HIGH SUGAR-FED OBESE OUTBRED MICE AND POSSIBLE IMPACT OF PRECONCEPTION DIETARY INTERVENTIONS ON THIS PROTEOMIC PROFILE

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In preparation

1. INTRODUCTION

Maternal metabolic disorders like obesity are significantly increasing worldwide and have become a global health problem (WHO, 2018). Western type diets composed of high saturated fatty acids and carbohydrates have been indicated to play a major role in the development of obesity and the significant reduction in metabolic health (Taskinen et al., 2019). Next to increases in weight, dyslipidemia, reduced glucose tolerance and insulin sensitivity have all been reported in obese women (Grundy, 2000). In addition, there is a significant impact of obesity on fertility. It is shown that the microenvironment in which the oocyte develops and matures, is altered in obese women compared to non-obese women (Valckx et al., 2012). These changes in the composition of the ovarian follicular fluid have a direct impact on oocyte maturation and development but also on the metabolism of the cells that support the developing oocyte, such as cumulus cells (Jungheim and Moley, 2010; Valckx et al., 2012). Cumulus cells (CCs) are important predictors of oocyte quality (Dumesic et al., 2015). They play a crucial role in proper oocyte maturation by keeping the oocyte under meiotic arrest, by participating in the induction of meiotic resumption and by supporting cytoplasmic maturation (Tanghe et al., 2002) (Russell et al., 2016). The oocyte relies on these cumulus cells for their metabolizing capacities and for the transport of important metabolites, antioxidants, amino acids etc. via gap junctions (Da Broi et al., 2018). In the absence of cumulus cells during maturation, oocyte developmental competence was severely compromised (Luciano et al., 2005). In addition, cumulus cells support successful fertilization by 1) attracting, trapping and selecting spermatozoa, 2) creating an optimal environment to facilitate sperm capacitation, acrosome reaction and penetration and by 3) preventing precocious hardening of the zona pellucida (Tanghe et al., 2002). Removal of the cumulus cells before fertilization resulted in a marked decrease in fertilization rates (Suzuki et al., 2000; Tanghe et al., 2002; Zhang et al., 1995).

Many human and animal studies expanded on the potential of the CC transcriptome to serve as a predictor of oocyte quality. Several marker genes in CC were linked with oocyte developmental capacity and pregnancy success. Recently, human research has indicated that CC proteomics can also be a useful approach to predict pregnancy success (Braga et al., 2016).

More and more research emphasizes the important role that reduced oocyte quality plays in the pathogenesis of subfertility in obese, metabolically compromised women. We (see chapter 7A) and other researchers investigated the impact of HF/HS diet-induced obesity on oocyte quality and reported a significant impact on mitochondrial quality, intracellular lipid droplet volume and oocyte developmental competence (Boots et al., 2016; Saben et al., 2016b). In addition, genes related to insulin signaling and glucose metabolism showed an aberrant expression in cumulus cells from HF/HS-fed obese mice compared with control mice.

Although the impact of obesity on oocyte and cumulus cell quality is clear, very little information is known regarding the underlying mechanisms. Recent technological advances have enabled the accurate identification and quantification of proteins in a small sample size (Zapalska-Sozoniuk et al., 2019) and it is only since recently that the impact of obesity on the reproductive tissue has been explored at this more functional level.

Ovulated oocytes collected from high fat diet-fed mice showed a reduced expression of the TIGAR (TP53-induced glycolysis and apoptosis regulator) protein (Wang et al., 2018). Specific depletion of TIGAR in mouse oocytes results in the marked elevation of reactive oxygen species (ROS) levels and the failure of the meiotic apparatus assembly. The study of Marei et al. (2019) analyzed cellular stress responses at the proteome level in bovine oocytes and cumulus cells after short-term *in vitro* exposure to lipotoxic conditions. The results showed a reduced metabolic activity in cumulus cells indicated by the differentially expressed proteins involved in carbohydrate metabolism and fatty acid β-oxidation. In addition, proteins involved in the formation of mitochondrial cristae were downregulated in cumulus cells. Also a dysregulation of several endoplasmic reticulum (ER) and ribosomal proteins was observed in cumulus cells as a consequence of oxidative stress. However, most of these changes were pro-apoptotic. In contrast to the observations in cumulus cells, the proteomic changes in oocytes were predominantly directing towards an upregulation of pro-survival pathways, highlighting the importance of compensatory mechanisms. This included pro-survival ER and mitochondrial unfolded protein responses (UPRs), redox regulatory and compensatory metabolic mechanisms (Marei et al., 2019). These insights show that the proteome of oocytes as well as cumulus cells are already responsive to short-term lipotoxic exposure, at least in vitro.

However, there is still a clear lack of information regarding the response mechanisms at the protein level of cumulus cells following long-term exposure to lipotoxic conditions, as present in the obese patient. This would provide more in-depth insights into the pathways in the cumulus cell affected by obesity and may contribute to further clarify the fundamental mechanistic basis of the reduced oocyte and cumulus cell quality as observed in obese women. Understanding these mechanisms at the level of the oocyte and cumulus cells is important as it might significantly increase our knowledge on how to treat these issues.

Up until now, obese women who are confronted with subfertility are often advised to lose weight before pregnancy to increase their chance of a healthy offspring (Jungheim and Moley, 2010; Lassi et al., 2014; Sim et al., 2014). Nowadays, lifestyle modifications such as dietary treatments are considered the first line of therapy (Best et al., 2017; Legro, 2017). Earlier research in our lab (Chapters 6 and 7A) indicated that diet normalization or a 30% caloric restriction diet for a period of four weeks showed to be the most optimal time frame to improve metabolic health. However, very little information is known regarding the effect of these dietary preconception care

interventions on oocyte quality itself. Therefore, we investigated the impact of diet normalization or caloric restriction as preconception care intervention on oocyte quality of obese outbred mice (Chapter 7A). Based on the outcome parameters assessed, diet normalization resulted in a significant reduction of mitochondrial ultrastructural abnormalities and improved mitochondrial activity at PCCI week 4. However, oocyte developmental competence was not significantly improved. In contrast, switching to a caloric restriction diet did significantly improve oocyte developmental competence, probably due to the improved cumulus cell functions. However, mitochondrial quality was still aberrant. To understand the complete potential of the tested PCCI, it is important to understand the cellular functionality of the cumulus cells. This might increase our knowledge on how the oocyte's micro environment changes and the response of the cumulus-oocyte-complex to this change.

Based on this information, we hypothesized that HF/HS-induced obesity has a significant impact on the proteomic profile of the cumulus cells. In addition, we aimed to investigate the possible impact of dietary preconception care interventions on the proteome of these cumulus cells.
To investigate this, 5-week-old female outbred Swiss mice were fed a HF/HS diet for a period of seven weeks to obtain an obese phenotype. Afterwards, HF/HS-fed mice were switched to different PCCIs; 1) an *ad libitum* control diet or 2) a 30% caloric restriction control diet, for a total period of four weeks. At the end of the PCCI period, we aimed to investigate the proteomic profile of the cumulus cells from all treatment groups.

2. MATERIALS AND METHODS

2.1. ETHICAL APPROVAL

All procedures in this study were approved by the ethical committee of the University of Antwerp and performed accordingly (ECD nr. 2018-05), and all methods were performed in accordance with the relevant guidelines and regulations.

2.2. ANIMALS, DIET AND EXPERIMENTAL DESIGN

Five-week-old female outbred Rj:Orl Swiss, hereafter referred to as "Swiss") mice (Janvier labs) were used. At the start of the experiment, mice were randomly divided into two groups with ad libitum access to either a control (CTRL) or a high fat/high sugar diet (HF/HS) for a period of seven weeks.

Afterwards, some of the HF/HS-mice were switched to two different preconception care interventions for four weeks, while the remaining HF/HS and the control mice remained on their corresponding diet for comparison. This resulted in four different treatment groups of equal size: 1) control diet for 11 weeks (CTRL_CTRL), 2) HF/HS diet for 11 weeks (HF_HF), 3) high fat diet for seven weeks then a switch to an *ad libitum* control diet for four weeks (HF_CTRL) and 4) high fat diet for seven weeks then a switch to a 30% caloric restriction diet for four weeks (HF_CR). An experimental timeline is shown in figure 1.

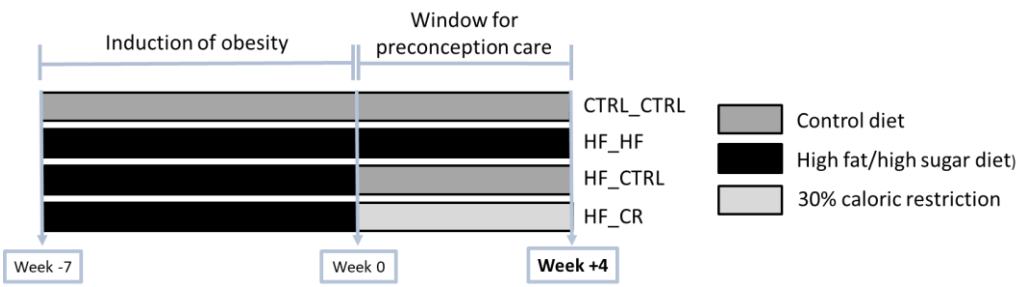

Figure 1: Overview of the experimental design

The HF diet was composed of 60 kJ% fat from beef tallow, 20 kJ% carbohydrate and 20 kJ% protein (E15741-34, Sniff diets). In addition 20% fructose (Merck, 102109450) was added to the drinking water of mice on the HF diet, resulting in a high fat-high sugar (HF/HS) group. The matched, purified control diet contained 10 kJ% fat, 70 kJ% carbohydrate and 20 kJ% protein (E157453-04; Sniff Diets). Access to water was provided ad libitum. The 30% caloric restriction diet had the same composition as the control diet, however, the HF_CR mice received only 70% of the amount of food consumed by the CTRL_CTRL mice (paired feeding regimen, calculated on the basis of the daily consumption).

2.3. CUMULUS CELL COLLECTION AND PREPARATION FOR SUBSEQUENT ANALYSIS

All mice (6 mice/ treatment group, one replicate) received intraperitoneal injections of 10 IU equine chorionic gonadotropin (eCG, Synchrostim; Ceva Santé Animale) followed, 48 h later, by 10 IU human chorionic gonadotropin (hCG, Pregnyl; Organon) to induce and synchronize ovulations. Mice were sacrificed 13–14 h after hCG injection. *In vivo* matured oocytes were obtained from the oviducts immediately after euthanasia. Each oviduct was dissected together with the ovary and a part of the uterine horn and transferred to a collection tube containing L15 medium (Thermo Fisher Scientific, Belgium) supplemented with 50 IU/mL penicillin G sodium salt (Merck, Belgium), and 10% Fetal Bovine Serum (Greiner Bio-One, Belgium).

The cumulus oocyte complexes (COCs) collected from both oviducts of the same animal were pooled. Only COCs that met the following selection criteria were used for analysis: oocytes surrounded by an expanded cumulus cell mass with a perfect spherical shape, a regular zona and a translucent, homogeneously colored cytoplasm without inclusions. The remaining COCs were completely denuded by repeated pipetting through 150 µm Stripper tips fitted on EZ-grip (Origio, The Netherlands) in a droplet of L15 medium supplemented with 0.3 mg/mL hyaluronidase (Merck, Belgium). The droplets containing the detached cumulus cells from all animals in each subgroup were immediately pooled, centrifuged, washed in PBS-PVP and snap frozen. Vials containing the droplets with the cumulus cells were centrifuged at 600g for 10 min. Afterwards, supernatant was taken off and 900µL RNAse free PBS-PVP was added. Samples were centrifuged again at 600g for 10 min, supernatant was removed and samples were snap frozen. All frozen samples were stored at −80 °C until further analysis.

2.4. SHOT-GUN PROTEOMIC ANALYSIS OF CUMULUS CELLS

The proteomic analysis of cumulus cells was adapted from Marei et al. (2019).

Sample preparation: homogenization

Cell lysis of the samples (pools of cumulus cells from 16-46 oocytes per mouse, total of 6 mice/treatment group, one replicate) occurred by adding 50 µL RIPA buffer (Thermo Scientific, Rockford, IL), 0,5 µL HALT phosphatase inhibitor (Thermo Scientific) and 0,5 µL HALT protease inhibitor (Thermo Scientific), after which a 3 x 10s sonication (Branson Sonifier SLPe ultrasonic homogenizer, Labequip, Ontario, Canada) was performed on ice. The samples were centrifuged for 10 min at 16,000 x g at 4°C whereafter the supernatant was collected.

Sample preparation: Reduction and alkylation for in-solution digestion

To acquire an optimal solubility and efficient digestion of the proteins, 5 µL of 1% Rapigest SF surfactant (Waters, Milford, MA) was added to the supernatant whereafter it was incubated at 100°C for 5 minutes. The proteins in the samples were reduced by adding 45 µl of 200 mM TEAB and 5 µl of 200 mM TCEP, followed by an incubation step for 1 hour at 55°C. Subsequently, the samples were alkylated with 5 µl of iodoacetamide and incubated for 30 minutes in dark at room temperature. 1ml of pre-chilled (-20°C) acetone was added and the samples were vortexed and frozen overnight

at -20°C. The next day, the samples were centrifuged at 8,000 x g for 10 minutes at 4°C and the pellet was collected.

Sample preparation: In-solution digestion and C18 clean-up

The precipitated protein pellet was re-suspended by adding 10 µl of 100 mM TEAB. The samples were digested overnight at 37°C with trypsin (enzyme:protein ratio = 1:40). Next, Pierce C18 spin columns (Thermo Scientific) were used to desalt the tryptic digests following manufacturer's guidelines.

Liquid chromatography and mass spectrometry

The eluted sample were dried in a speedvac vacuum concentrator and kept at -20°C until Shotgun analysis. Liquid chromatography was followed by mass spectrometry (LC-MS/MS) and was performed by the Centre for Proteomics.

Data analysis

Raw files were analyzed by Maxquant (1.6.7). Spectra were analyzed against Mus musculus database, generated from Uniprot, with a false discovery rate of 1%
based on a decoy approach. A peptide tolerance of 10 ppm was applied. Trypsin was specified as digesting enzyme and 2 missed cleavages were allowed. Perseus (1.6.7) was used to select differentially regulated proteins (P<0.05) between the different treatment groups and to establish heat maps of the proteomics data. A two-sample t-test (P<0.05) was performed in Perseus between two relevant treatment groups in a stepwise approach: 1) HF_HF *vs.* CTRL_CTRL, 2) HF_CTRL *vs.* HF_HF and 3) HF_CR *vs.* HF_HF. GO functional annotation analysis was carried out using the DAVID functional annotation chart tool (https://david.ncifcrf.gov) and by manual extraction of the subcellular location data and biological processes from the UniProt database (https://www.uniprot.org). Venn diagrams were constructed using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/).

3. RESULTS

3.1. IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS

A total of 2552 proteins were detected in cumulus cells. Exposure to a HF/HS diet (HF_HF) resulted in a significant change (P<0.05) in the relative abundance of 42 proteins compared to the CTRL_CTRL group, of which 34 were downregulated and 8 were upregulated. Interestingly, more differentially regulated proteins (DRPs) (P<0.05) were detected when comparing the PCCIs to the HF_HF group. Compared to the HF_HF group, the HF_CTRL group displayed differential expression of 78 proteins, of which 39 were downregulated and 39 were upregulated, while the HF_CR group showed differential expression of 138 proteins, of which 79 were downregulated and 59 were upregulated. An overview of the localization of the DRPs can be found in table 1. The majority of DRPs in cumulus cells were located in mitochondria and nuclei.

Table 1. Cellular localization of differentially regulated proteins in all pairwise comparisons in cumulus cells. Proteins were divided in up- and down-regulated in the HF_HF *vs.* the CTRL_CTRL group and in the HF_CTRL and HF_CR *vs.* the HF_HF group. Reference group is underlined.

Differentially regulated proteins in cumulus cells	**HF_HF *vs.* CTRL_CTRL**			**HF_CTRL *vs.* HF_HF**			**HF_CR *vs.* HF_HF**		
	Total	**Up**	**Down**	**Total**	**Up**	**Down**	**Total**	**Up**	**Down**
Total in different cellular compartments	**42**	**8**	**34**	**78**	**39**	**39**	**138**	**59**	**79**
Nucleus (GO:0005634)	18	0	18	31	11	20	68	34	34
Endoplasmic reticulum (GO:0005783)	8	0	8	7	7	0	0	0	0
Mitochondrion (GO:0005739)	0	0	0	16	9	7	25	11	14
Golgi apparatus (GO:0005794)	6	0	6	0	0	0	0	0	0
Lipid particle (GO:0005811)	0	0	0	0	0	0	3	3	0

3.2. WHICH PROTEINS ARE DIFFERENTIALLY REGULATED IN HF_HF *VS.* CTRL_CTRL

To view the pattern of expression of these 42 DRPs in the HF_HF *vs.* CTRL_CTRL group across all the treatment groups, a heatmap of these 42 proteins was constructed (Fig. 2). As expected, the 42 DRPs nicely showed an opposite expression pattern when

comparing the HF_HF to the CTRL_CTRL group. The expression of these 42 DRPs in the samples of the HF_CTRL and HF_CR group was highly variable, where some samples showed similar expression to the HF_HF group while others were more similar to the CTRL_CTRL group.

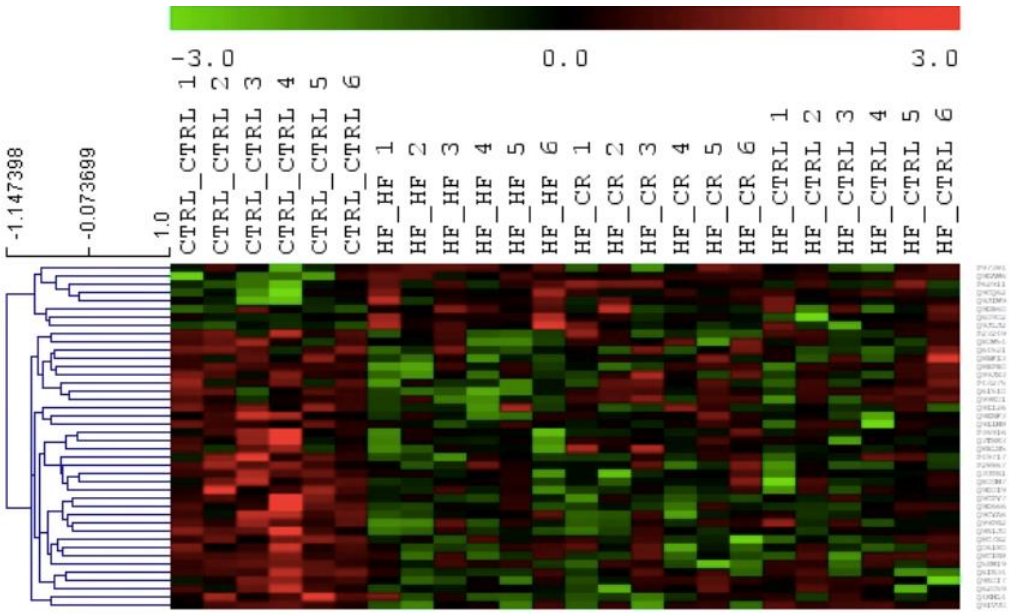

Figure 2. **Heatmap of 42 differentially regulated proteins in the HF_HF *vs.* CTRL_CTRL group.** Red and green represent up- and downregulated expression, respectively. Black indicates no significant change in expression.

3.2.1. GO FUNCTIONAL ANNOTATION ANALYSIS

To identify functional annotations (molecular function, biological process, pathways) of DRPs, gene ontology (GO) enrichment analysis using DAVID software was performed on the 42 significantly up and downregulated proteins in the HF_HF *vs.* CTRL_CTRL group. Figures 3 and 4 show the significantly enriched GO-terms associated with up- and downregulated proteins in cumulus cells of HF_HF mice *vs.* CTRL_CTRL mice. To facilitate clear interpretation, a selection was made of the molecular functions, biological processes and pathways that were most relevant for our research. Interestingly, upregulated proteins were found to be related to oxidoreductase activity and oxidation-reduction process. In addition, proteins that were significantly downregulated due to the HF/HS diet were found to be involved in cholesterol and steroid biosynthetic processes.

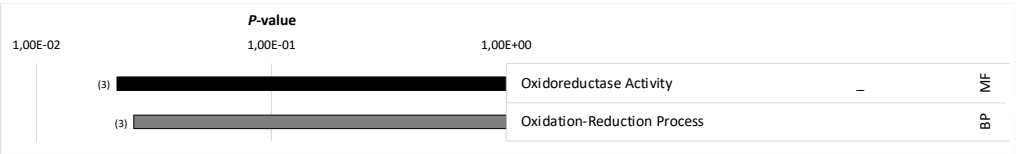

Figure 3. **Significantly enriched GO-terms (*P*<0.05) of upregulated proteins in the HF_HF *vs*. CTRL_CTRL group.** Number of proteins involved in each GO-term is displayed between brackets. MF, molecular function; BP, biological process.

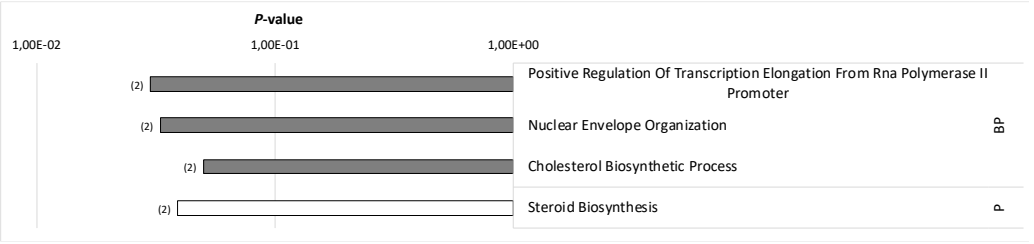

Figure 4. **Significantly enriched GO-terms (*P*<0.05) of downregulated proteins in the HF_HF *vs.* CTRL_CTRL group.** Number of proteins involved in each GO-term is displayed between brackets. BP, biological process; P, pathway.

3.2.2. IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS IN HF_HF *VS.* CTRL_CTRL THAT SHOW A SIGNIFICANTLY IMPROVED EXPRESSION IN HF_CTRL *VS.* HF_HF

From the 42 DRPs in HF_HF *vs.* CTRL_CTRL, only 3 were found to be also differentially expressed in the HF_CTRL *vs.* HF_HF comparison. This was to be expected due to the high variability in the expression pattern of these proteins in the PCCI groups shown in the heatmap above (Fig. 2). When comparing both conditions, these 3 DRPs displayed an opposite expression pattern (downregulated in HF_HF *vs.* CTRL_CTRL and upregulated in HF_CTRL *vs.* HF_HF, or vice versa), indicating that these proteins are significantly improved in the HF_CTRL group compared to the HF_HF group (Fig. 5). The 3 DRPs that displayed a significantly improved expression in HF_CTRL group included E3 ubiquitin/ISG15 ligase (TRIM25), protein FAM3C (FAM3C) and annexin A11 (ANXA11). Functions and subcellular locations of these proteins can be found in table 2.

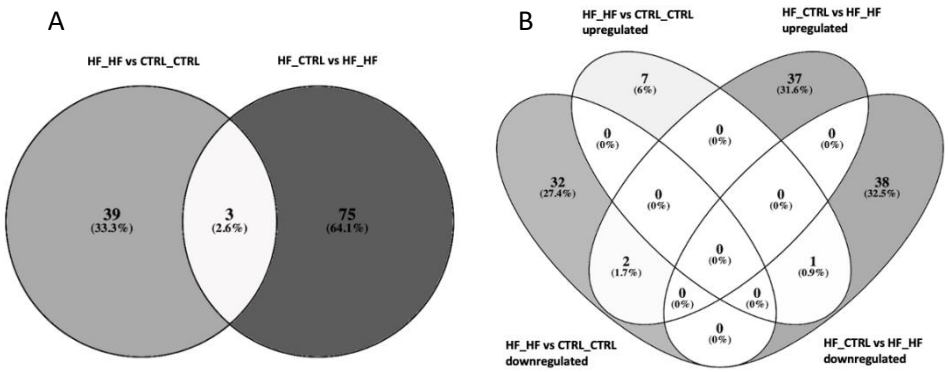

Figure 5. Venn diagrams showing (A) the number of (common) differentially regulated proteins in the HF_HF *vs*. CTRL_CTRL group and the HF_CTRL *vs*. HF_HF group, (B) the direction of expression of these differentially regulated proteins in the HF_HF *vs.* CTRL_CTRL group and the HF_CTRL *vs.* HF_HF group.

Table 2. GO biological process and subcellular location of differentially regulated proteins in the HF_HF *vs.* CTRL_CTRL group that displayed a significantly improved expression in the HF_CTRL *vs.* the HF_HF group. FC, fold change; GO, gene ontology; CTRL_CTRL, control-control; HF_HF, high fat-high fat; HF_CTRL, high fat-control.

Protein	**Log FC in HF_HF vs CTRL_CTRL**	**Log FC in HF_CR vs HF_HF**	**Subcellular location**	**GO biological process**
TRIM25: E3 ubiquitin/ISG15 ligase (Q61510)	-0,134	0,135	• cytosol nucleus	• positive regulation of DNA-binding transcription factor activity positive regulation of I-kappaB kinase/NF-kappaB signaling regulation of protein localization response to estrogen response to vitamin D ubiquitin-dependent ERAD pathway ubiquitin-dependent protein catabolic process
FAM3C: protein FAM3C (Q91VU0)	-0,120	0,121	• golgi extracellular space	multicellular organism development
ANXA11: annexin A11 (P97384)	0,024	-0,022	• cytoskeleton: spindle	• cytokinetic process

3.2.3. IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS IN HF_HF *VS.* CTRL_CTRL THAT SHOW A SIGNIFICANTLY IMPROVED EXPRESSION IN HF_CR *VS.* HF_HF

Similarly, only 5 proteins were common between the HF_HF *vs.* CTRL_CTRL (42 DRPs) and HF_CR *vs.* HF_HF (138 DRPs) comparisons. These 5 DRPs were downregulated in the HF_HF *vs.* CTRL_CTRL but upregulated in the HF_CR *vs.* HF_HF, and therefore considered as improved (Fig. 6). These 5 DRPs are E3 ubiquitin/ISG15 ligase (TRIM25), pyridoxal-dependent decarboxylase domain-containing protein 1 (PDXDC1), beta-actin-like protein 2 (ACTBL2), general transcription factor IIF subunit 1 (GTF2F1) and lanosterol synthase (LSS). Functions and subcellular location of these proteins are displayed in table 3.

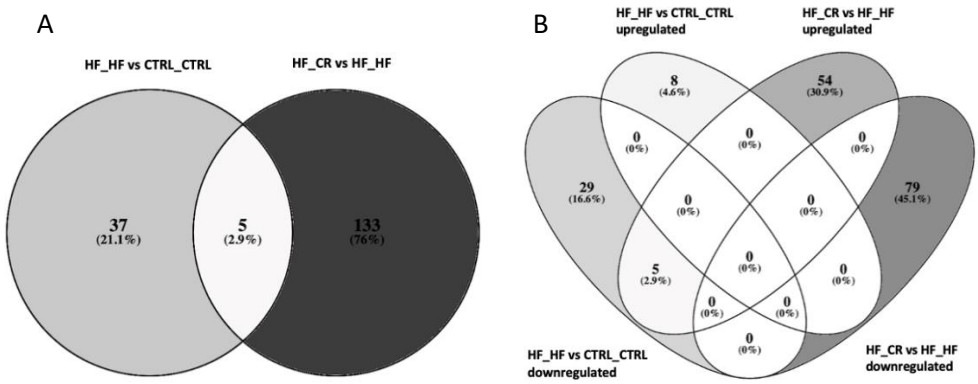

Figure 6. Venn diagrams showing (A) the number of (common) differentially regulated proteins in the HF_HF *vs.* CTRL_CTRL group and the HF_CR *vs.* HF_HF group, (B) the direction of expression of these differentially regulated proteins between the HF_HF *vs.* CTRL_CTRL group and the HF_CR *vs*. HF_HF group.

Table 3. GO biological process and subcellular location of differentially regulated proteins in the HF_HF group *vs.* CTRL_CTRL group that displayed a significantly improved expression in the HF_CR *vs.* the HF_HF group. FC, fold change; GO, gene ontology; CTRL_CTRL, control-control; HF_HF, high fat-high fat; HF_CTRL, high fat-control.

Protein	Log FC in HF_HF vs CTRL_CTRL	Log FC in HF_CR vs HF_HF	Subcellular location	GO biological process
TRIM25: E3 ubiquitin/ISG15 ligase (Q61510)	-0,134	0,136	• cytosol nucleus	• positive regulation of DNA-binding transcription factor activity positive regulation of I-kappaB kinase/NF-kappaB signaling regulation of protein localization response to estrogen response to vitamin D ubiquitin-dependent ERAD pathway ubiquitin-dependent protein catabolic process
PDXDC1: pyridoxal-dependent decarboxylase domain-containing protein 1 (Q99K01)	-0,090	0,115	• ER golgi	• sphingolipid catabolic process carboxylic acid metabolic process
ACTBL2: beta-actin-like protein 2 (Q8BFZ3)	-0,088	0,066	• cytoskeleton	• cell motility
GTF2F1: general transcription factor IIF subunit 1 (Q3THK3)	-0,051	0,033	• nucleus	• negative regulation of protein binding positive regulation of transcription by RNA polymerase II transcription elongation from RNA polymerase II promoter

LSS: lanosterol synthase (Q8BLN5)	-0,031	0,037	• ER	• cholesterol biosynthetic process steroid biosynthetic process sterol metabolic process

3.3. WHICH PROTEINS ARE DIFFERENTIALLY REGULATED IN HF_CTRL *VS.* HF_HF A heatmap based on the protein abundance of the 78 DRPs in the HF_CTRL *vs.* HF_HF group was constructed to visualize the expression of these proteins in the CTRL_CTRL group. As shown in figure 7, the heatmap revealed a clear distinction in the expression patterns of HF_CTRL and HF_HF samples. The expression of these 78 DRPs in the samples of the CTRL_CTRL group was highly variable. Several proteins in the HF_CTRL group displayed similar expression patterns as those in the CTRL_CTRL group, suggesting a trend towards normalization in the HF_CTRL samples. However, some proteins showed similar expression patterns in both the HF_HF and CTRL_CTRL group, whereas the HF_CTRL group showed an opposite expression pattern, suggesting protein responses specifically caused by the PCCI.

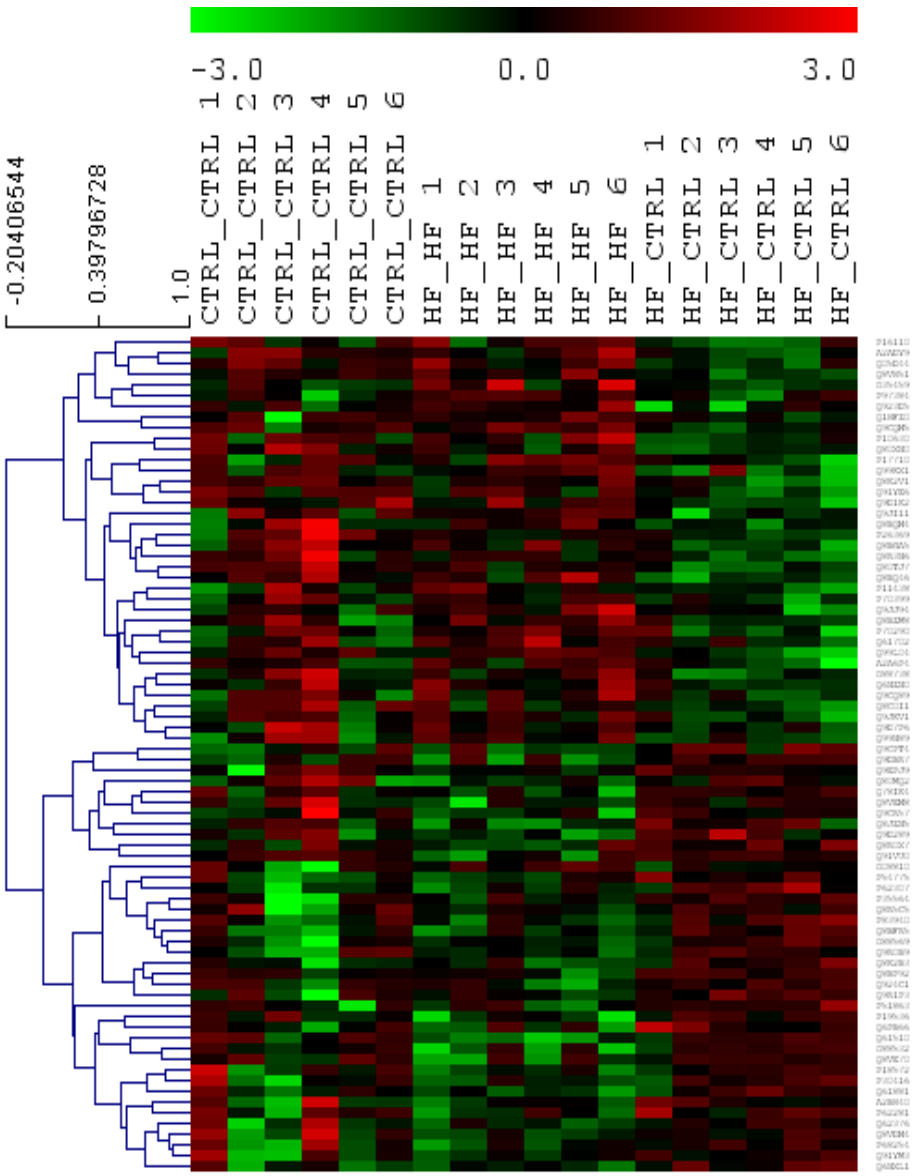

Figure 7. **Heatmap of 78 differentially regulated proteins in the HF_CTRL *vs.* HF_HF group.** Red and green represent up- and downregulated expression, respectively. Black indicates no significant change in expression.

3.3.1. GO FUNCTIONAL ANNOTATION ANALYSIS Figures 8 and 9 show the significantly enriched GO-terms associated with up- and downregulated proteins in cumulus cells of HF_CTRL mice *vs.* HF_HF mice. Interestingly, upregulated proteins were found to be involved in the androgen receptor signaling pathway and embryo implantation. Downregulated proteins were found to be related to regulation of mitophagy.

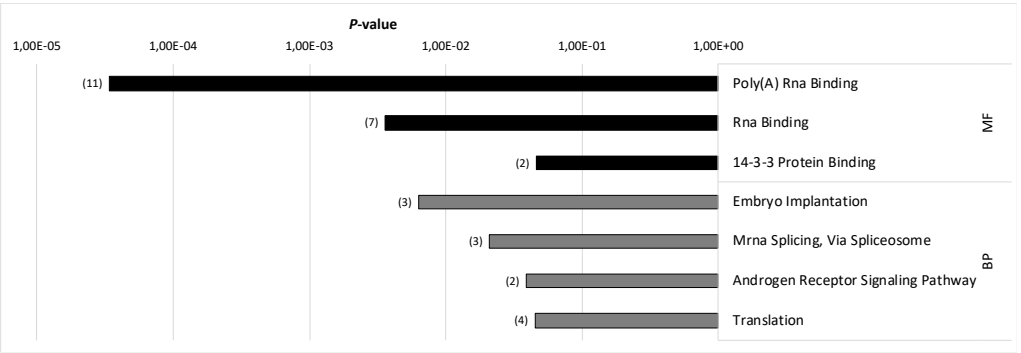

Figure 8. **Significantly enriched GO-terms (*P*<0.05) of upregulated proteins in the HF_CTRL *vs.* HF_HF group.** Number of proteins involved in each GO-term is displayed between brackets. MF, molecular function; BP, biological process; P, pathway

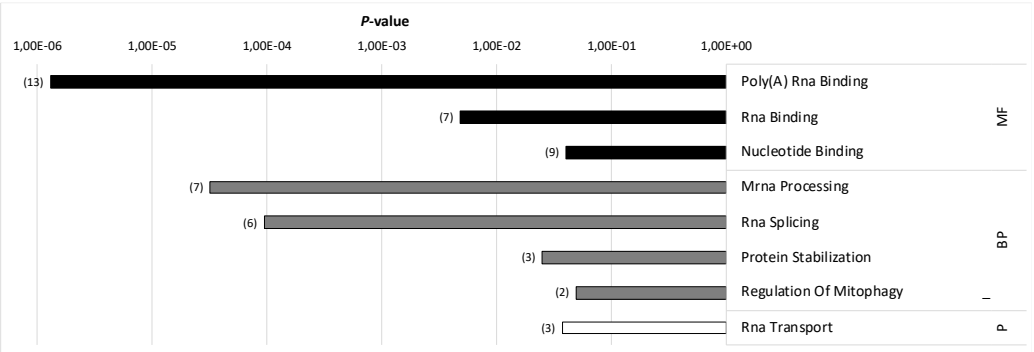

Figure 9. **Significantly enriched GO-terms (*P*<0.05) of downregulated proteins in the HF_CTRL *vs.* HF_HF group.** Number of proteins involved in each GO-term is displayed between brackets. MF, molecular function; BP, biological process; P, pathway.

3.4. WHICH PROTEINS ARE DIFFERENTIALLY REGULATED IN HF_CR *VS.* HF_HF

A heatmap based on the protein abundance of the 138 DRPs in the HF_CR *vs.* HF_HF group was constructed to visualize the expression of these proteins in the CTRL_CTRL group. When comparing the HF_CR samples to the HF_HF samples, a clearly opposite expression pattern could be seen. Compared with the previous comparison, less variation between the individual CTRL_CTRL samples was present. In this case, only several proteins in the HF_CR group showed similar expression patterns as those in the CTRL_CTRL group. More proteins showed similar expression patterns in both the HF_HF and CTRL_CTRL group, and an opposite expression in the HF_CR group (Fig. 10).

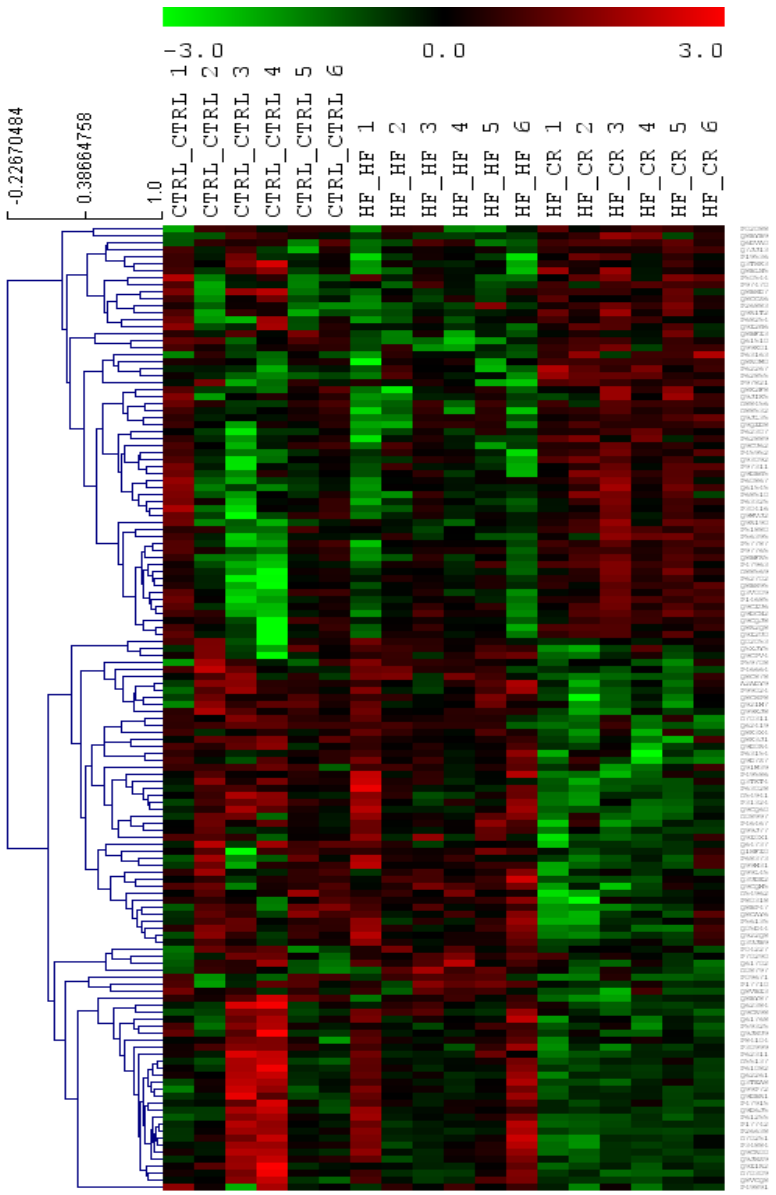

Figure 10. **Heatmap of 138 differentially regulated proteins in the HF_CR *vs.* HF_HF group.** Red and green represent up- and downregulated expression, respectively. Black indicates no significant change in expression.

3.4.1. GO FUNCTIONAL ANNOTATION ANALYSIS

Fig. 11 and 12 show the significantly enriched GO-terms associated with up- and downregulated proteins in cumulus cells of HF_CR mice compared to HF_HF mice.

Upregulated proteins in HF_CR were significantly enriched with GO terms such as fatty acid β-oxidation and endoplasmic reticulum-associated degradation (ERAD) pathway, while downregulated proteins were involved in the response to oxidative stress and the regulation of apoptotic processes.

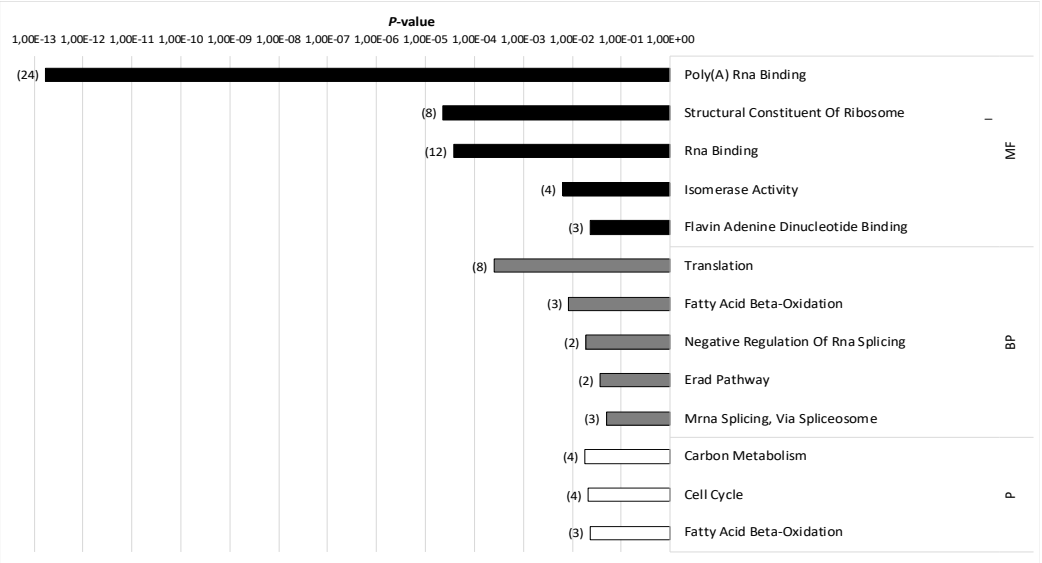

Figure 11. **Significantly enriched GO-terms (*P*<0.05) of upregulated proteins in the HF_CR *vs.* HF_HF group.** Number of proteins enriched in each GO-term is displayed between brackets. MF, molecular function; BP, biological process; P, pathway.

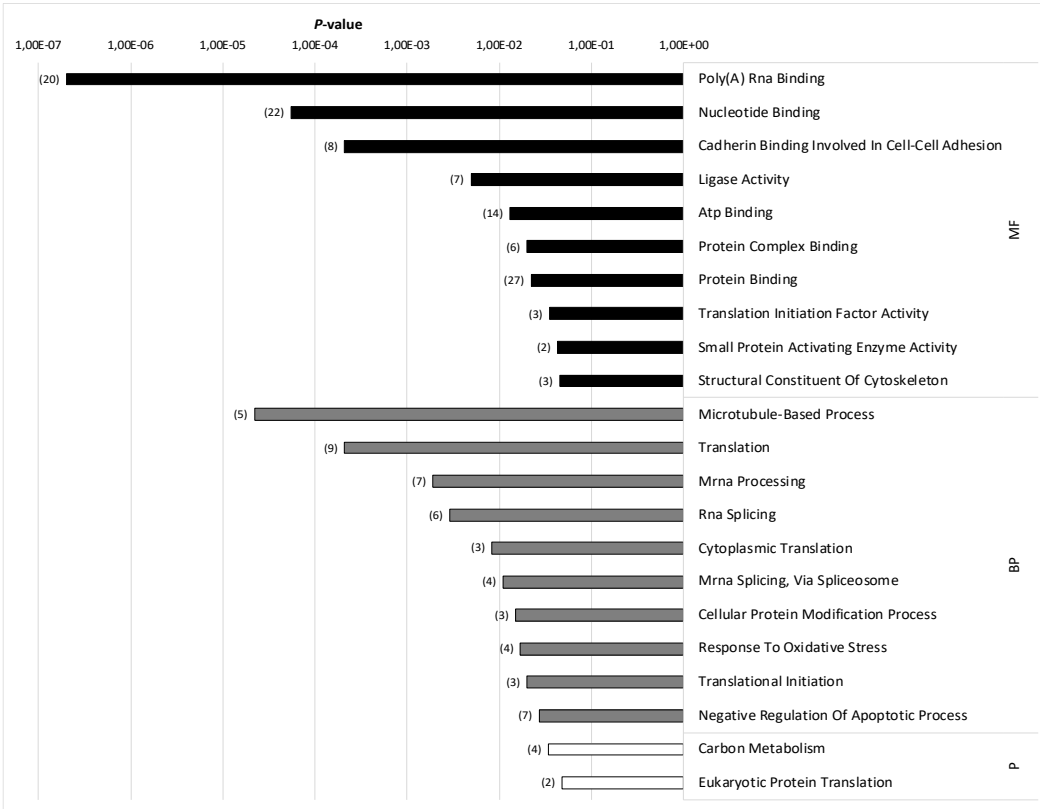

Figure 12. **Significantly enriched GO-terms (*P*<0.05) of downregulated proteins in the HF_CR *vs.* HF_HF group.** Number of proteins enriched in each GO-term is displayed between brackets. MF, molecular function; BP, biological process; P, pathway.

4. DISCUSSION

Bearing in mind the complexity of the proteomics data, we focus in this discussion on the top pathways and biological processes that are in line with our research and make hypotheses based on the functional annotations of the involved proteins. More detailed information concerning the DRPs and their functional annotations can be found in the results section.

In the first step, we focused on the DRPs between cumulus cells collected from HF_HF mice and cumulus cells collected from CTRL_CTRL mice. From the 2552 proteins that were detected in cumulus cells, the relative abundance of 42 proteins was significantly changed due to exposure to a HF/HS diet. More specifically, 8 proteins were significantly upregulated and 34 proteins were significantly downregulated in the HF_HF group compared to the CTRL_CTRL group. Performing GO functional enrichment analysis on these set of up- and downregulated proteins allowed us to determine the overrepresented GO-terms. Several upregulated proteins (DECR1, ALDH9A1 and FAM213) in cumulus cells of mice on a HF/HS diet were found to be associated with oxidoreductase-activity and to be involved in the protection against oxidative stress. It has been demonstrated that the earlier stages of obesity are associated with an initial elevation of antioxidant activity to counteract the increased levels of ROS, which could explain the upregulation of these proteins in cumulus cells from HF_HF mice. However, over time the antioxidant capacity gets overwhelmed by the increased production of ROS, resulting in oxidative stress and associated cellular damage (Alcala et al., 2018).

Interestingly, proteins involved in cholesterol biosynthesis (LSS, NSDHL) were downregulated in cumulus cells of HF_HF mice. This is of major concern as oocytes rely on cumulus cells to provide them with cholesterol, indicated by the low expression of enzymes of the cholesterol synthesis pathway in oocytes compared to cumulus cells. As a compensation for the oocyte deficiencies in cholesterol production, the oocyte promotes cholesterol biosynthesis in cumulus cells via the secretion of oocyte derived paracrine factors (OSF), particularly BMP15 and GDF9 (Su et al., 2008). Studies have proven that the expression levels of OSF are reduced in oocytes from patients with polycystic ovary syndrome, a disorder frequently associated with obesity (Wei et al., 2011). Perhaps the reduced expression of OSF can be a potential explanation for the observed downregulation of cholesterol biosynthesis in cumulus cells of HF_HF mice. However, more research is required to confirm this hypothesis. Interestingly, cumulus cells of mice on caloric restriction diet were able to reverse the downregulated expression of LSS, indicating a repair of the reduced cholesterol biosynthesis.

From the 42 DRPs between the HF_HF and CTRL_CTRL group, 1 protein, TRIM25, was found to be significantly improved in both the HF_CTRL and HF_CR group compared to the HF_HF group. While this protein showed a significant downregulation in the HF_HF

group compared to the CTRL_CTRL group, it was significantly upregulated in both PCCIs compared to the HF_HF group. Previous studies have shown that TRIM25 mainly acts as an E3 ligase and mediates protein ubiquitination. Upon ER stress, TRIM25 is significantly induced in order to promote degradation of misfolded proteins in the ER via the ER-associated protein degradation (ERAD) pathway and finally restores ER homeostasis (Zhang et al., 2020). Studies have shown that depletion of TRIM25 in cancer cells resulted in an increase of ER stress (Liu et al., 2020b). Besides its function in the ERAD pathway, TRIM25 also plays a major role in the response to estrogen as it functions as a co-factor for the transcription of the estrogen receptor (ER)-α (Nakajima et al., 2007). Earlier research has demonstrated that estradiol and its nuclear receptors in granulosa cells have an important role in governing oocyte meiotic resumption as knock-out of the ER-α in mice COCs remarkably compromised oocyte meiotic arrest (Liu et al., 2017). Overall, this suggests that downregulation of TRIM25, as observed in cumulus cells of HF_HF mice, impairs the response to estrogen and the functioning of the ERAD pathway, potentially hampering cumulus cell function. However, cumulus cells of HF_CTRL and HF_CR mice were able to normalize this aberrant expression of TRIM25 at four weeks of PCCI, indicating a recovered estrogen response and functioning of the ERAD pathway. The detailed description of other proteins that were significantly improved in the HF_CTRL or HF_CR group compared to the HF_HF group was beyond the scope of this thesis.

In the next step, we focused on the DRPs between the PCCIs and the HF_HF group. When comparing the relative abundances of proteins, 78 proteins were found to be differentially expressed between cumulus cells collected from HF_CTRL mice and cumulus cells collected from HF_HF mice. Diet normalization for a period of 4 weeks resulted in a significant upregulation of proteins involved in embryo implantation (BSG, ARID1A, FKBP4) and androgen receptor signaling pathway (ARID1A, FKBP4) compared to the HF_HF group. BSG is a highly glycosylated transmembrane protein that is found to play an important role in female reproduction (Li and Nowak, 2020). Kuno, et al. demonstrated that targeted disruption of the BSG gene in mice hampered female reproductive processes, including fertilization and implantation. In addition, this study demonstrated a high expression of BSG in cumulus cells compared to other regions in the ovary, suggesting that specifically the absence of BSG in cumulus cells is most likely the cause of fertilization failure in BSG knock-out mice (Kuno et al., 1998). In addition, FKBP4, also known as FKBP52, is an important modulator in the androgen receptor-mediated transcription (Cheung-Flynn et al., 2005) and plays an important role in increasing the affinity of the androgen receptor for hormone binding (De Leon et al., 2011). Androgens exert their effects through the androgen receptor and are known to regulate pre-antral follicle development, prevent follicular atresia and are required for antral follicle formation. In addition, androgens are thought to be involved in primordial follicle recruitment as well as the ovulation process (Prizant et al., 2014). Knock-out of the androgen receptors specifically in granulosa cells of mice, resulted in premature

ovarian failure, fewer antral follicles and ovulated oocytes, longer estrous cycles,… indicating that specifically androgen receptor expression in granulosa cells is essential for normal follicular development and fertility (Sen and Hammes, 2010). Based on these data, it is suggested that diet normalization for a period of four weeks is associated with beneficial effects on reproduction.

U2AF2 and WBP11, proteins involved in the stimulation of mitophagy (Ivatt et al., 2014), were significantly downregulated in cumulus cells of HF_CTRL mice compared to HF_HF mice. Mitophagy or mitochondrial degradation is the cellular process where damaged mitochondria are selectively removed through autophagy and plays a pivotal role in maintaining cellular homeostasis (Ma et al., 2020). The downregulation of mitophagy-related proteins in cumulus cells of HF_CTRL mice may be suggestive of a reduced presence of damaged mitochondria. However, our results did not demonstrate a difference in mitochondrial abnormalities between cumulus cells of HF_CTRL mice compared to cumulus cells of HF_HF mice. Therefore, it is possible that the higher rate of mitophagy in HF_HF cumulus cells succeeded to remove the majority of damaged mitochondria, resulting in no difference in the percentage of mitochondrial abnormalities between HF_CTRL and HF_HF cumulus cells.

The application of a caloric restriction diet for a period of four weeks resulted in a significant change in the relative abundance of 138 proteins in cumulus cells of HF_CR mice compared to cumulus cells of HF_HF mice. Our results demonstrated that proteins involved in the endoplasmic reticulum-associated degradation (ERAD) pathway were upregulated in the HF_CR group, indicating a possible recovery of the aberrant functioning of the ERAD pathway caused by the HF/HS diet, as previously described. In addition, upregulated proteins in cumulus cells of HF_CR mice were significantly enriched in fatty acid β-oxidation (ACADM, ACADVL, CYB5A, ECHS1). The latter finding is in agreement with the elevated expression of the *ACADM* gene as observed in cumulus cells of HF_CR mice and may be suggestive of an enhanced oocyte developmental competence, as mentioned before (chapter 7A). Surprisingly, proteins related to the response of oxidative stress (NDUFS8, ATOX1, AIFM1, SOD2) were downregulated in cumulus cells of HF_CR mice. Since ATOX1 and SOD2 function as antioxidant enzymes, these results do not correspond with the earlier reported elevated expression of the *PRDX6* gene and thus increased antioxidative capacity in the HF_CR group (chapter 7A).

In addition, several downregulated proteins were found to be associated with negative regulation of apoptotic processes (BAG6, SMARCA4, HK1, MIF, SERPINB9, SOD2, USP47), indicating a reduced inhibition of pathways leading to programmed cell death and thus a higher rate of apoptosis in cumulus cells of HF_CR mice. Apoptotic cell death, opposed to necrotic cell death, is a critical process to selectively eliminate damaged cells that could negatively affect the normal cell functioning (Elmore, 2007). In

accordance with this finding, it has been reported that dietary restriction increased spontaneous apoptotic rates in hepatocytes of mice known to develop spontaneous liver tumors, resulting in a marked reduction in subsequent development of hepatoma and an increase in disease-free life span. In addition, proteins involved in the cell cycle pathway (MCM6, SMC1A, YWHAH, YWHAQ) were significantly upregulated, indicating an increased proliferative capacity of HF_CR cumulus cells, probably to balance the increased cell loss caused by apoptosis. Based on these data, it is proposed that cumulus cells of HF_CR mice show an adaptive mechanism in order to maintain normal cumulus cell functioning and rescue the oocyte quality.

CONCLUSIONS

The proteomic profile of cumulus cells collected from HF_HF mice suggested a failure in cholesterol biosynthesis and a disturbed oxidoreductase activity. In addition, the downregulation of TRIM25 potentially impairs the response to estrogen and functioning of the ERAD pathway. Administration of an *ad libitum* control diet or caloric restriction diet for a period of four weeks resulted in a normalized expression of TRIM25, suggesting a recovered estrogen response and ERAD pathway of cumulus cells. Cumulus cells of HF_CR mice were also able to reverse the reduced cholesterol biosynthesis. Furthermore, HF_CTRL and HF_CR cumulus cells showed a differential expression of several proteins compared to HF_HF cumulus cells that mediated beneficial effects. While diet normalization showed positive effects on embryo implantation and androgen receptor signaling pathway, cumulus cells of HF_CR mice showed higher rates of apoptosis and increased proliferative capacity, possibly indicating an adaptive mechanism to maintain normal cumulus cell functioning and oocyte quality. In accordance, upregulated proteins in HF_CR cumulus cells were involved in fatty acid oxidation, suggestive of an increased oocyte developmental competence.

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CHAPTER 8:

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

The main scope of this PhD thesis was to study the impact of pre- and/or post-conception care intervention strategies on metabolic health and fertility, and more specifically on oocyte and embryo quality in obese, metabolically compromised women. As well controlled studies are necessary to approach this aim and as working with human biological material is difficult from an ethical and logistical point of view, we used strategically designed in vitro and in vivo animal models.

It becomes more and more clear that metabolic health disorders like obesity have a significant impact on female fertility (Gesink Law et al., 2007; Loy et al., 2018; Wise et al., 2010). More specifically, the important role of the oocyte and embryo in this subfertility problem is becoming increasingly important and has been studied in detail the past years (Jungheim et al., 2010; Leroy et al., 2017; Luzzo et al., 2012; Robker, 2008). It has been shown that the aberrant metabolic health status of the individual is reflected in the composition of the follicular fluid, hereby directly affecting folliculogenesis, oocyte and embryo quality (Leroy et al., 2005; Leroy et al., 2017; Valckx et al., 2012), ultimately resulting in decreased pregnancy rates. As a result, there is more and more interest towards research investigating possible interventions to improve fertility in metabolically compromised women. But is it possible to improve oocyte quality of metabolically compromised, obese women who are having issues with getting pregnant? How long does it take to improve this quality? And which approach is the best to do this? Obese women often undergo assisted reproductive treatment (ART) to increase pregnancy chances. Can we rescue lower quality oocytes during in vitro culture in the lab before implantation? Or is a preventive approach the better option to improve fertility? Is losing weight before pregnancy the way to go?

To provide further, necessary insights regarding this topic, the following research questions were answered:

1) Are supportive in vitro embryo culture conditions, supplemented with anti-apoptotic, antioxidant and mitogenic factors, able to rescue the developmental capacity and resulting blastocyst quality of metabolically compromised oocytes after maturation?
2) The impact of dietary preconception care interventions:
    a. Can metabolic health of a diet-induced obese mouse model be improved or restored by dietary preconception care interventions (PCCI)? And how long does it take to see such improvements?
    b. Can we prevent the detrimental impact of obesity and the obesogenic diet on the oocyte by implementing a dietary PCCI before the window of folliculogenesis?
    c. Can oocyte quality be improved by taking away the obesogenic environment during the process of folliculogenesis? Is the developing

oocyte already damaged and can any further damage be prevented? Or can the existing damage be repaired by a dietary PCCI during the window of folliculogenesis?

d. Which preconception dietary intervention is the most suited to restore oocyte quality? Is it necessary for obese individuals to undergo a severe calorie restricted diet or is diet normalization already enough?

3) High fat versus high fat/high sugar diets to induce obesity in a mouse model – what is the most suited model to mimic human obesity and do they have a similar impact on metabolic health, oocyte quality and the tested PCCI?
4) When investigating the impact of obesity and PCCI on metabolic health and oocyte quality in an obese mouse model, which one is the most suited? Is the often used inbred C57LB/6 strain the best choice or is the outbred Swiss strain the better option as they mimic the genetic diversity in the human population?

1. CAN SUPPLEMENTATION OF THE EMBRYO CULTURE MEDIA RESCUE DEVELOPMENTAL COMPETENCE AND QUALITY OF EMBRYOS DERIVED FROM METABOLICALLY COMPROMISED OOCYTES?

There is sufficient data illustrating the negative impact of exposure to lipotoxic conditions during folliculogenesis and oocyte maturation on early embryo development and quality (Leroy et al., 2017). On the other hand, the microenvironment in which early embryos grow also has a significant impact on its developmental rate and quality. Rizos et al. (2002) stated that the in vitro culture system is a major determinant of blastocyst quality, whereas the developmental capacity itself is mainly determined during maturation. However, earlier research in our lab showed a positive impact of MitoQ, a mitochondria targeted antioxidant, supplementation during in vitro embryo culture of metabolically-compromised oocytes, resulting in both an increased oocyte developmental competence and resulting blastocyst quality (Marei et al., 2019). These results were very promising, indicating a possible important role of in vitro culture supplements to rescue metabolically-compromised oocytes. Results obtained in chapter 4 showed that supplementation of mitogenic, anti-apoptotic and antioxidative factors (insulin-transferrin-selenium (ITS)) also resulted in an increased oocyte developmental competence. However, the resulting blastocysts were of low quality (Figure 4). Although earlier results were promising, these data indicate that not all supplements might be suited to rescue oocytes from developmentally compromised mothers. It seems that an antioxidant treatment that specifically targets mitochondria might have a bigger rescue potential than those that do not target mitochondria.

After ITS treatment, resulting blastocysts showed a good morphology. However, in depth assessment indicated that these resulting blastocysts were of low quality. It seems that ITS might be able to support the development of lower quality oocytes that otherwise would not have developed further. As blastocyst quality cannot be checked in a human setting, this might result in the transfer of a low quality embryo to the human uterus, resulting in long term consequences on establishment of pregnancy or on offspring health. In a recent study, day 7 bovine blastocysts, produced in vitro from metabolically-compromised oocytes, were transferred to a healthy in vivo uterine environment. Although transferred blastocyst were of morphologically good quality, resulting day 14 blastocysts showed a significant retarded growth and quality (Desmet et al., 2020). This also illustrates the long lasting carry-over effect of the oocyte's maturation environment.

Therefore, additional research should be performed to further investigate the impact of post-conception care interventions before approving new applications of any in vitro supportive treatments in IVF.

The pre-implantation embryo has shown a very high plasticity during its development, adapting to changes in its environmental conditions (in vitro culture conditions, aberrant maternal in vivo environment etc.) (Fleming et al., 2011). During the pre-implantation period, the embryo undergoes numerous important transitions: 1)

Depending on the species, the embryo switches from the maternal genome to its own, newly formed embryonic genome (2-cell stage in mice, 8-16-cell stage in bovine, 4-8 cell stage in humans) (Figure 1); 2) Changes in its metabolic and nutrient needs; 3) Morphological specification into the inner cell mas and trophectoderm; 4) Preparing for implantation by signaling at the embryo-maternal interface (Fleming et al., 2004; Van Winkle et al., 2006). Alle these transitions can be influenced by the environment in which the embryo develops.

In human settings, embryos are cultured in media with different compositions. Up until now, it is not known which media composition is the most optimal to obtain good quality embryos (Mantikou et al., 2013). Furthermore, the exact composition of the media used is often not accessible as companies do not provide such detailed information (Bolton et al., 2014). Some studies report a significant impact of in vitro culture media on offspring health while others did not (Bouillon et al., 2016; Zandstra et al., 2015). Differences in birth weight and altered growth patterns have been reported. The Developmental Origins of Health and Disease (DOHaD) hypothesis indicated that changes in the early developmental environment can have long-lasting effects (Fleming et al., 2015), further emphasizing the need to increase knowledge regarding possible impacts of embryo culture media.

Although some in vitro culture supplements might improve developmental competence of metabolically-compromised oocytes and resulting blastocyst quality, it is important to keep in mind that the resulting embryos are transferred back into the aberrant, obese maternal micro-environment. Obesity has been associated with endometrial changes (Metwally 2007) and dysregulation of endometrial gene expression during the window of implantation (Bellver 2011).
Even if the transferred blastocysts are of good quality, it is possible that they might not implant and subsequently not result in a successful pregnancy. However, literature on that is not consistent. Luke et al. (2011) reported that increasing obesity was indeed associated with decreased clinical pregnancy rates from autologous oocytes. However, this was not the case when healthy donor oocytes were transferred, suggesting that BMI does not affect uterine receptivity. In contrast, others reported a negative impact of obesity on implantation rate (30.9% *vs*. 40% in the control group), clinical pregnancy (45.3% *vs.* 55.9-56.9%) and live birth rate (27.7% *vs*. 37.9-38.6%) after oocyte donation from normal weight donors (Bellver et al., 2013).

Furthermore, obese women are often denied access to assisted reproductive treatment (ART) by their fertility specialist until they reach a certain amount of weight loss or a set target BMI. Some guidelines suggest that a BMI of more than 35 kg/m^2 (cut-off for extreme obesity) should be an absolute contraindication to ART (RANZCOG guidelines and Legro (2016)). In some countries, insurance coverage for ART is limited to people with a BMI < 32 kg/m² (Ethics et al., 2010; Gillett et al., 2006). However, over

the past years, more and more ethical and scientific concerns are rising concerning this prohibition. Recently, an opinion on this topic was published by Tremellen et al. (2017). Although they support the overall principle of restricted access of obese patients to ART, they believe that the situation is not always black and white. Next to BMI, other important parameters, like metabolic health and maternal age, should be considered when allowing obese women access to ART. They suggest a treatment protocol for obese women seeking fertility assistance in which preconception weight loss plays a significant role.

The question arises if, from an ethical point of view, a preconception, instead of a postconception lifestyle intervention might overall be a better approach for obese women. Do we want to interfere with the natural process of oocyte development by adding 'goodies' to the in vitro culture medium if there is a possible window for improvement before conception. Public health programs and reports by the WHO emphasize that health is a fundamental good that governments have the responsibility to protect and promote (Kass et al., 2014). Instead of only trying to rescue the metabolically compromised oocyte without improving the mothers health, improving all aspects of fertility and overall metabolic health of the obese patient might be a far more promising approach, resulting in a win-win situation for both the health of the mother and any (future) babies. Maternal obesity has shown to significantly impair offspring health (Andreas et al., 2019). The impact on the oocyte even has a long-term transgenerational effect up to the third generation (Stephenson et al., 2018), suggesting a significant epigenetic impact. Children of obese mothers have a 35% higher mortality rate in adulthood irrespective of potential confounders in the postnatal environment (Reynolds et al., 2013). These higher mortality rates have been linked to a higher incidence of metabolic syndrome, including offspring obesity, diabetes type II and cardiovascular diseases. These observations confirm Barker's "thrifty phenotype hypothesis" (Hales and Barker, 2001). Especially the early gestation period showed to be a vulnerable period with regards to offspring health (Ravelli et al., 1976). So even if the embryo was rescued during in vitro culture, the complete folliculogenesis and further embryo development still takes place in the aberrant maternal environment, possibly impacting offspring health. Earlier research has demonstrated that both folliculogenesis and early embryogenesis are crucial time frames for epigenetic programming and during these periods, the follicle and early embryo are very sensitive to changes (figure 1). DNA methylation and histone modification are acquired during oocyte growth and become established in the mature oocyte (Obata and Kono, 2002). Early embryos undergo extensive DNA demethylation from the zygote to the blastocyst stage before re-establishment of methylation during gastrulation (Eckersley-Maslin et al., 2018). With regards to imprinted genes, folliculogenesis is even more crucial as their epigenetic pattern is established during this folliculogenesis and cannot be altered afterwards.

For these reasons, preconception care intervention might be the preferential way to improve fertility.

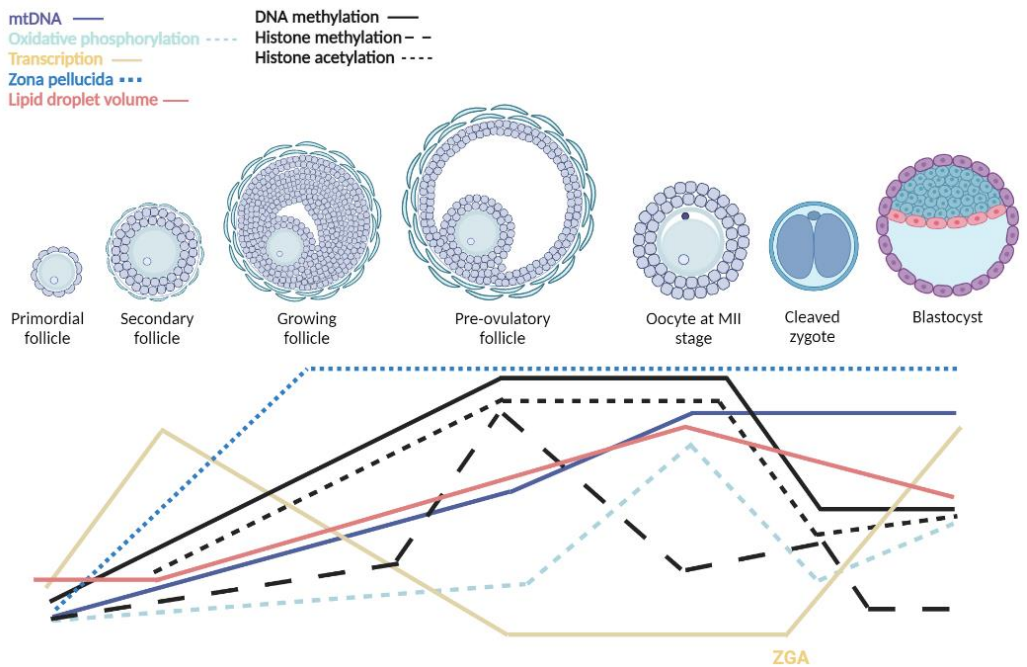

Figure 1. Dynamic changes during follicle development and early embryogenesis (Made with Biorender)

2. THE IMPORTANCE OF THE CORRECT CHOICE OF MOUSE STRAIN FOR YOUR RESEARCH

To study the impact of obesity on metabolic health and fertility, a diet-induced obese mouse model is often used (Wong et al., 2016). A mouse model is suitable to study the human situation as both mice and humans have a lot of physiological similarities (Perlman, 2016). They progressively gain weight, develop hypercholesterolemia, glucose intolerance and insulin resistance in response to a high fat or high fat/high sugar diet (Boudoures et al., 2016; Reynolds et al., 2015). Furthermore, the reproductive tract is similar to humans with a comparable oogenesis, oocyte maturation and preimplantation embryo development (Jamsai and O'Bryan, 2011). However, compared to humans, the period of oocyte recruitment in mice is a lot shorter, as is their gestation period (Clarke, 2017). In addition, they are poly-ovulatory animals. There is increasing evidence that their genetic background might influence the outcome of diet-induced obese murine studies (Kleinert et al., 2018). Inbred strains often undergo genetic drift and mutations, resulting in different substrains with

different susceptibility to diet-induced obesity. For example, the C57BL/6J strain rarely develops hyperglycemia when fed an obesogenic diet (Attie and Keller, 2010; Leiter, 2002) while the closely related C57BL/6N strain (only 5 SNP differences) develops hepatosteatosis, hyperglycaemia and hyperinsulinaemia after only three weeks on a high fat diet (Kahle et al., 2013). When measuring insulin resistance in more than 100 inbred mouse strains after eight weeks on a high fat/high sugar diet, a 37-fold variation in the degree of insulin resistance was reported across the strains (Parks et al., 2015).

Up until now, most research focusing on the impact of diet-induced obesity on metabolic health and fertility use the well-established inbred C57BL/6 mouse strain (Champy et al., 2008). The inbred strain has several advantages. These mice are genetically homogenous and one of the main reasons inbred strains are preferred over outbred strains is the reduced experimental variability as often more animals are needed when using an outbred strain to reach statistical significance. C57/BL6 mice progressively gain weight and develop hyperlipidemia when fed a high fat diet (Nicholson et al., 2010; Spearow and Barkley, 1999; Wu et al., 2015). However, this strain is characterized by low fertility, small litter size and pup cannibalism (Guo et al., 2015), which makes it less reliable for studies focusing on fertility outcome. Using an outbred strain might improve translation to human (outbred) physiology as they are more reminiscent of the genetic heterogeneity of the human condition (Chia et al., 2005). Furthermore, outbred strains are better breeders since they have a higher reproductive performance, lower neonatal mortality and better nurturing behavior.
Therefore, we investigated if high fat diet-induced obesity has a similar effect on metabolic health but mostly oocyte quality of an outbred Swiss mouse, compared to the more often used, inbred C57BL/6 mouse (chapter 5).
Earlier research investigating the impact of a high fat diet on metabolic health of inbred C57BL/6 mice reported an aberrant serum lipid profile (Boudoures et al., 2016; Williams et al., 2014). Outcome parameters assessed in this PhD research confirmed this and also showed a similar impact of the high fat diet on weight gain and serum lipid profile in the outbred Swiss mouse. This suggests a similar metabolic response to the high fat diet, regardless of the strain used. Earlier research performed in only Swiss mice also reported significant weight gain, next to increased serum NEFA and cholesterol concentrations after high fat diet feeding for four weeks (Simino et al., 2021).
Also in chapter 6, Swiss mice were exposed to a high fat diet after which metabolic health was assessed. Although both statistically significant, total cholesterol concentrations after 13 weeks of high fat feeding were ±180 mg/dL in Swiss mice of chapter 5 while this was only ±145 mg/dL in chapter 6. Furthermore, opposite effects on serum triglyceride concentrations were reported. While feeding a high fat diet tended to increase serum triglyceride concentrations in Swiss mice of chapter 5, a reduction was reported in serum collected from Swiss mice of chapter 6. Although most research on diet-induced obesity in mice reports a significant increase in serum triglyceride concentrations, some articles report a decreased after high fat diet feeding

(Biddinger et al., 2005; Guo et al., 2009; Podrini et al., 2013). As discussed in chapter 6, this can possibly explained by several mechanisms but more in depth research is needed to confirm this.
Although not analyzed in detail, variation of all outcome parameters assessed was similar in both strains tested. This confirms earlier research where phenotypic variability in metabolic health parameters was comparable between inbred and outbred strains of mice (Jensen et al., 2016).
One of the biggest disadvantages of the C57BL/6 mouse is their reduced fertility, possibly due to many generations of inbreeding. More specifically, inbreeding results in an increased homozygosity of deleterious recessive alleles and as a direct result, fertility is decreased (Bittles et al., 2002; Ober et al., 1992). Inbreeding has shown to increase the risk of spontaneous abortions due to complications in embryo development (Diamond, 1987). Furthermore, inbred mothers who were confronted with increased still births and early offspring death, have an increased chance to face the same issues in future pregnancies (Stoltenberg et al., 1999).

However, if this inbreeding has a significant impact on oocyte quality itself, was not known. Earlier research investigating the impact of the genetic background and inbreeding on reproduction, already confirmed a strain-dependent impact (inbred *vs.* outbred) on meiotic maturation rate (Ibanez et al., 2005), oocyte developmental competence (Kaleta, 1977; Luckett and Mukherjee, 1986), parthenogenetic activation (Gao et al., 2004) and preimplantation development (Suzuki et al., 1996). To our knowledge, we reported for the first time differences in lipid droplet distribution between oocytes from the inbred C57BL/6 and the outbred Swiss strain, regardless of their diet. This process can be explained by several mechanisms, as discussed in chapter 5, but further research is needed to investigate this. Next to lipid droplet distribution, a significant strain-dependent impact of diet-induced obesity on oocyte mitochondrial quality (alterations in mitochondrial membrane potential, mitochondrial ultrastructural abnormalities and mtDNA copy numbers) was present. Inbreeding seems to result in significantly decreased oocyte mitochondrial quality, which could be a possible explanation for the reported low fertility and small litter sizes. As mitochondria are only maternally inherited, this further suggests a significant impact of inbreeding on fertility, and more specifically oocyte quality, of future generations of inbred mice.

Shortly summarized, not only did we report significant differences between control groups of inbred *vs.* outbred mice, we also showed a differential impact of diet-induced obesity on oocyte quality of inbred *vs.* outbred mice. These results corroborate that the correct choice of mouse strain is very important and depending on the research hypothesis that needs to be investigated. Furthermore, it confirms that effects of diet-induced obesity on oocyte quality are strain dependent and that strain differences

should be considered when interpreting reproductive experiments, especially when aiming for translation to the human physiology (Ibanez et al., 2005; Kaleta, 1977).
Of course, these results do not diminish the value of earlier published obesity research using the inbred C57BL/6 strain. Depending on the aim of the study, the inbred mouse strain might still be a good choice. With regards to metabolic health, similar effects of the high fat diet were reported regarding weight gain and serum lipid profile in both strains, making them both suitable for research on this topic. However, when investigating the impact of obesity on oocyte quality, and more specifically mitochondria, the C57BL/6 mice might not be the best option. When choosing for this inbred strain, important effects of diet-induced obesity on oocyte mitochondria can be missed due to the already high abnormalities in the control strain.

3. PRECONCEPTION CARE INTERVENTIONS: THE IMPACT OF DIETARY INTERVENTIONS ON FEMALE METABOLIC HEALTH AND FERTILITY

There is increasing evidence confirming a significant association between lifestyle and infertility. Therefore, more and more obese women who are having issues with getting pregnant are advised to lose weight before conception to increase pregnancy chances. However, so far there are no univocal guidelines, resulting in a wide range of advices over the different Belgian clinics. Recently, a study was published, highlighting that staff at Belgian fertility clinics clearly recognize the importance of promoting a healthy lifestyle in people with infertility (Boedt et al., 2021). Interestingly, according to this staff, weight was the most important lifestyle factor that needed to be addressed in infertile patients (Figure 2). 98.20% of Belgian fertility staff indicated that weight was routinely discussed in relation to lifestyle modification. Less than half of this staff indicated to discuss diet and exercise and only 2.70% of participants indicated that a structured lifestyle modification program is offered in their clinic. Such program is not available due to a clear lack of guidelines or expertise available to offer such a program. This study clearly again confirms the need for more fundamental research, investigating the impact of preconception interventions on fertility in order to obtain crucial insights towards clear preconception guidelines.

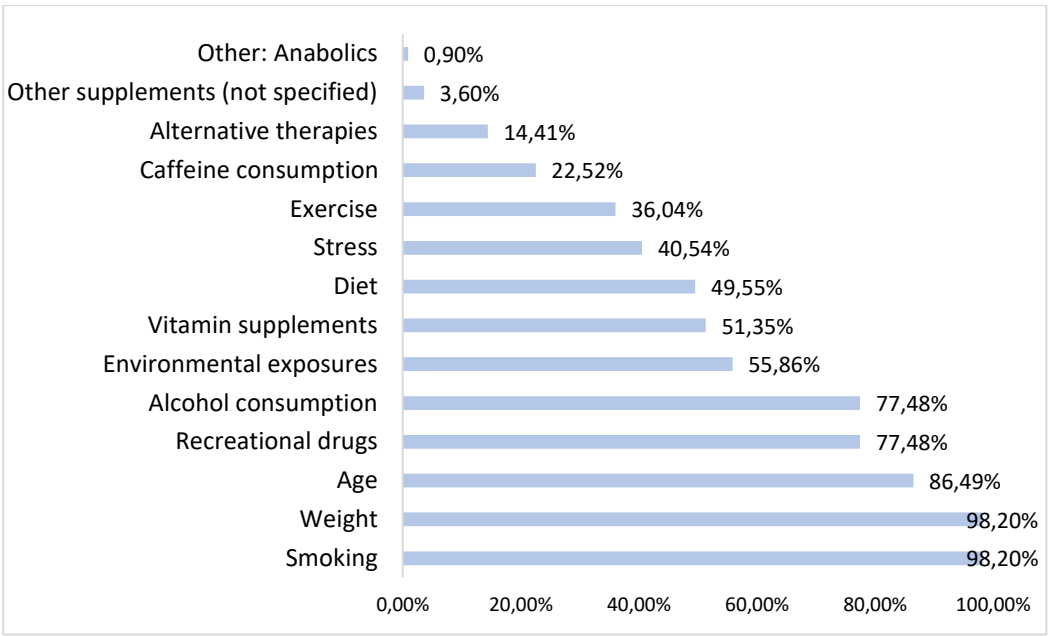

Figure 2. List of lifestyle factors that needs to be addressed in infertile patients (adapted from Boedt T. et al, 2021).

Lifestyle interventions are considered as the first step regarding preconception care as they are easy accessible for all people and not expensive (Best et al., 2017; Legro, 2017). Lifestyle interventions range from increased exercise to changes in diet composition and amount/calories of food consumed or a combination of those (Boudoures et al., 2016; Marei et al., 2017; Reynolds et al., 2015). In this project, the impact of dietary preconception care interventions alone on both metabolic health and fertility was investigated using an obese mouse model. To investigate this, obese mice were switched to two different diets: 1) an ad libitum control diet where only diet composition was changed or 2) a severe calorie restricted control diet where both dietary composition was changed and calorie intake was significantly reduced (by 30%) compared to the intake of the control group. Although a calorie restricted diet has shown to improve metabolic health (Aksungar et al., 2017; Andersen and Fernandez, 2013; Cui et al., 2013; Vangoitsenhoven et al., 2018), extreme weight loss leads to increased lipid mobilization, resulting in high free fatty acid (FFA) concentrations in the bloodstream. These high FFA concentrations are reflected in the follicular fluid and directly impact oocyte quality through lipotoxicity associated pathways (Leroy et al., 2005; Valckx et al., 2012). Therefore, less severe diet change might be a better option.

Although many researchers have shown a positive impact of a calorie restricted diet on metabolic health, we and other researchers, report increased levels of markers associated with liver damage (ALT) and high serum cholesterol concentrations (Friis et

al., 1987; Gasteyger et al., 2008; Hoy et al., 1994; Phinney et al., 1991). In addition, Claude Bernard described the development of a diabetes-like state, so-called starvation pseudo-diabetes after severe weight loss (Lundbaek, 1948). The associated symptoms were also present in our study in obese mice that were on the calorie restricted diet, indicating that this diet may be a too extreme intervention. However, both high cholesterol and liver parameters were reported to be transient increases that restored when weight stabilized (Gasteyger et al., 2008; Phinney et al., 1991). In addition, other researchers also reported an ultimate long term positive impact of the pseudo-diabetes state on metabolic health (Bravo-San Pedro et al., 2019). Both these transient increases in cholesterol and liver parameters and the long-term positive impact of the pseudo-diabetes state might indicate that the best option to improve metabolic health is to switch to a normal ad libitum control diet after following a severe calorie restricted diet. However, diet normalization itself, resulting in a more gradual weight loss, also showed a promising impact on metabolic health. Results obtained showed a restoration of almost all metabolic health parameters assessed (serum lipid profile and glucose tolerance) after four weeks on the diet. Only insulin sensitivity showed to be improved but not restored.

But what was the impact of dietary changes in obese mice on their oocyte quality? Reynolds et al. (2015) reported no improvements in oocyte quality in obese mice that underwent a diet normalization for eight weeks. Based on the outcome parameters assessed in this thesis, dietary changes were able to improve some oocyte quality parameters while others were still aberrant. It seems that oocytes were able to activate some repair mechanisms, ultimately resulting in an improved oocyte quality or developmental competence (discussed in more detail below). Although some improvements were present, it is clear that dietary interventions do not result in complete restoration of the oocyte quality. Perhaps other preconception interventions might be more promising. Earlier research in obese mice showed some positive results regarding the impact of exercise on oocyte quality (Boudoures et al., 2016). Furthermore, human lifestyle intervention studies often combine dietary interventions with exercise to maximally improve fertility in obese individuals (Sim et al., 2014). However, intervention programs tested in obese women differ substantially from one study to another. Not only are there differences in experimental set-up, obese women who enroll in these studies come from a different background (clinical history, societal and lifestyle background). Besides diet normalization or caloric restriction, other methods of diet interventions are often used. In addition to the contradictory results on fertility, more fundamental knowledge is necessary. As far as we know, very limited research has been conducted investigating the impact of exercise with/without dietary interventions on oocyte quality of obese mice. Exercise itself has shown to improve oocyte metabolism (increased β-oxidation and lower lipid accumulation) and embryo quality in an obese mouse model (Boudoures et al., 2016). At the molecular level, exercise is known to stimulate the nutrient sensing AMPK pathway in muscle cells,

which stimulates mitophagy and improve insulin sensitivity (Heo et al., 2021). In addition, no information is known regarding the most optimal time period of preconception care intervention.

4. DIFFERENT METHODS OF DIETARY INTERVENTIONS

The dietary interventions methods tested in this PhD thesis consisted of 1) diet normalization or 2) a 30% caloric restriction diet. As stated before, earlier research has indicated that severe weight loss results in significantly increased lipid mobilization with a significant impact on fertility (Jensen et al., 2014; Legro, 2017). Therefore, severe weight loss right before conception has been discouraged in clinical settings (Legro, 2016). Based on this information, diet normalization was chosen as the first intervention method for this thesis. Diet normalization was expected to result in a less severe weight loss, improving metabolic health without drastically increasing lipid mobilization, hereby avoiding the negative impact on oocyte quality. Results obtained in this thesis confirm a positive impact of diet normalization on metabolic health, although complete recovery to the control group was not present in all parameters (Chapters 6 and 7A). With regards to oocyte quality, a positive impact on oocyte mitochondrial parameters could be reported when the dietary intervention lasted longer than the timeframe of folliculogenesis (four or six weeks). However, again, no complete recovery was present, indicating that the primordial follicle pool might be affected. Diet normalization as such is not prescribed in human as weight reduction will be limited over time when diet intake is tuned to the basal metabolism and the subsequent calorie intake. However, in our mouse model, diet normalization led to a weight reduction of 13% . A weight reduction of +/- 10% is generally advised to improve fertility potential in anovulatory women (Clark et al., 1995).

There are many dietary intervention methods that result in severe weight loss and that have shown beneficial effects on metabolic health. One of the most known and applied PCCI methods is a caloric restriction diet. As clearly summarized before (Chapters 2, 6 and 7A), a caloric restriction (CR) diet has shown a beneficial impact on metabolic health by improving body composition, plasma lipids, insulin sensitivity etc. (Aksungar et al., 2017; Andersen and Fernandez, 2013; Cui et al., 2013; Vangoitsenhoven et al., 2018). However, results obtained in this thesis also indicated some negative results of a CR diet on metabolic health that may or may not be transient when weight stabilizes (by switching e.g. to an ad libitum control diet).
Up until now, very limited information was available on the impact of such a CR diet on oocyte quality. Unfortunately, switching to a short term CR diet for two or four weeks only showed to be beneficial on oocyte developmental competence while oocyte quality stayed aberrant. Switching to a CR diet for six weeks, however, did show beneficial results on oocyte quality while such a long-term intervention was detrimental for metabolic health. These results indicate that a severe calorie restricted diet might not be the best option to improve metabolic health and oocyte quality.

Based on the results obtained in this study, it might be better to let obese patients undergo a short-term calorie restricted diet followed by switching to an ad libitum control diet. Or is another dietary intervention a better choice to improve oocyte quality?
It is important to keep in mind that the composition of a caloric restriction diet in mice is not the same as in humans. Mice receive 70% of the food that is consumed by the control group without changing the composition of the diet. This might result in decreased uptake of essential nutrients and antioxidants with a possible impact on general health of the mice. We should consider this as a limitation of our study. However, mice on the CR diet did show restored pregnancy rates, similar to mice on the ad libitum control diet. Obese women that undergo a calorie restricted diet are often consulted by a dietician to guarantee optimal intake of essential (micro)nutrients and antioxidants.

The past years, intermittent fasting has gotten more and more attention. Intermittent fasting (IF) is the term used for diets that restrict food intake to a certain time window. IF has shown beneficial effects on metabolic health, resulting in reduced serum glucose, insulin and cholesterol concentrations (Tinsley and La Bounty, 2015). These results indicate that, next to a CR diet, IF might also be a good approach to improve metabolic health in obese individuals. Other studies, however, reported no impact of IF on food intake and weight loss in a mouse model (Anson et al., 2003). Studies comparing the impact of intermittent fasting *vs.* caloric restriction in type 2 diabetic individuals reported similar positive effects on weight change, fasting glucose and lipid levels (Carter et al., 2018). Another study also reported beneficial effects of both IF and a CR diet on metabolic health in obese individuals (Aksungar et al., 2017). They suggested that the beneficial effects were attributed to different signaling pathways. Many non-scientific websites debate on whether IF fasting is good for fertility but to the best of our knowledge, scientific research on the impact of IF in obese individuals on oocyte quality has not been performed yet. Clearly, more research is needed regarding the impact of IF on fertility, and more specifically, on oocyte quality.

5. THE IMPORTANT ROLE OF THE MOST OPTIMAL TIME PERIOD OF PRECONCEPTION CARE INTERVENTIONS

Human studies performed so far all used different time periods to investigate the impact of lifestyle interventions on female fertility. Some studies last a few weeks while others last several months (Sim et al., 2014; Tsagareli et al., 2006). To our knowledge, any scientific motivation regarding these time periods is lacking. However, the time period of a PCCI cannot be ignored as long-term interventions have shown to conflict with advancing maternal age and are often not feasible to maintain for obese women. Therefore, we believe that, next to the most optimal dietary intervention, the most optimal time period to improve fertility is also important. It is not known if a short dietary intervention might already be able to improve metabolic health and oocyte

quality or if a longer PCCI period is needed to restore aberrant parameters. Therefore, we included different time periods of PCCI exposure in our experimental set-up, based on the duration of the folliculogenesis which lasts three weeks in mice (Clarke, 2017). During this process, the growing follicle and the enclosed oocyte are very sensitive to changes in its micro-environment which impacts the quality of the oocyte at the moment of ovulation (Valckx et al., 2014b). As clearly depicted in figure 1, a lot of important processes take place during folliculogenesis, going from important epigenetic mechanisms to an increase in mtDNA copy numbers. As folliculogenesis lasts 3-4 months in humans, using this process as an important reference point for the chosen PCCI time periods might improve translation of results obtained with outbred mice to the human setting. In addition, metabolic health parameters were assessed at the same time points as oocyte quality, further improving translation to the human setting.
With regards to metabolic health, obese mice that underwent diet normalization already showed an improved metabolic profile after only two weeks of PCCI. Extending the PCCI period to four weeks further ameliorated their metabolic health, as depicted in figure 3. Obese mice that switched to a caloric restriction diet showed the most promising improvements after 2-4 weeks of PCCI. Extending the PCCI period seemed to be a too extreme intervention.

The first time period of two weeks does not cover one complete cycle of folliculogenesis and was chosen to test if a preconception dietary intervention was still able to restore oocyte quality when it only started during the folliculogenesis (Figure 3). Based on the outcome parameters assessed, switching to a PCCI treatment for this short time period was not sufficient to restore oocyte quality but did show some improvements. Oocyte lipid droplet volume was already significantly restored, coinciding with weight loss and restoration of serum cholesterol concentrations, but only in obese mice that underwent diet normalization. However, oocyte parameters associated with mitochondrial quality were still aberrant in both PCCI groups. There is growing evidence that mitochondria are key players in the negative impact of obesity or metabolic syndrome on oocyte quality and developmental competence (Saben et al., 2016b; Zhao et al., 2017). During folliculogenesis, mitochondrial biogenesis and replication of mtDNA increase mitochondria and mtDNA copies to more than 100.000 copies, which is crucial to support mitochondrial functions (Figure 1) (May-Panloup et al., 2016). The total number of mtDNA does not further increase during early embryogenesis and is distributed among the blastomeres upon cleavage. Furthermore, as described before, important epigenetic processes take place during folliculogenesis. This significantly emphasizes the importance of folliculogenesis and the possible impact of any dietary change during this period. Furthermore, during the final stage of oocyte maturation, mitochondrial activity increases significantly and the mitochondria redistribute to support successful cleavage and fertilization (Van Blerkom, 2011). High fat(/high sugar) diet-induced obesity has shown a significant impact on oocyte mitochondrial quality,

indicated by an aberrant mitochondrial ultrastructure, distribution and function (Boots et al., 2016; Saben et al., 2016b). As mitochondrial quality was clearly still aberrant after two weeks of PCCI, the HF/(HS) diet seemed to significantly impair the oocyte during the first week of folliculogenesis to the extent that its quality could not be restored by switching to a healthy, control diet. However, switching to a severe calorie restricted diet for only two weeks did improve oocyte in vitro developmental competence, possibly due to improved cumulus cell functions. However, it is important to know that the quality of resulting blastocysts was not checked.

A time period of four or six weeks does cover one (or more) complete cycle(s) of folliculogenesis and gives the opportunity to investigate if the pool of dormant follicles was affected by the long term high fat(/high sugar) diet exposure, as the complete cycle of folliculogenesis took place in a non-high fat/(high sugar) environment (Figure 3). Results obtained indicated some improvements in oocyte quality, suggesting that the dormant pool of primordial follicles was not completely affected by the dietary insult or was able to (partially) restore due to the activation of recovery or repair mechanisms. However, no complete restoration of all aberrant oocyte quality parameters were reported in both intervention groups. These data might indicate for the first time that the long term high fat(/high sugar) diet exposure does have an negative impact on the dormant pool of primordial follicles which leads to remnant defects in the mature oocytes even if folliculogenesis took place under non-obesogenic conditions. However, as not all metabolic health parameters were restored after four or six weeks of intervention, any remaining negative impact may still exert effects during follicle growth and maturation. Of course, more in depth research is needed to further elaborate on this concept.

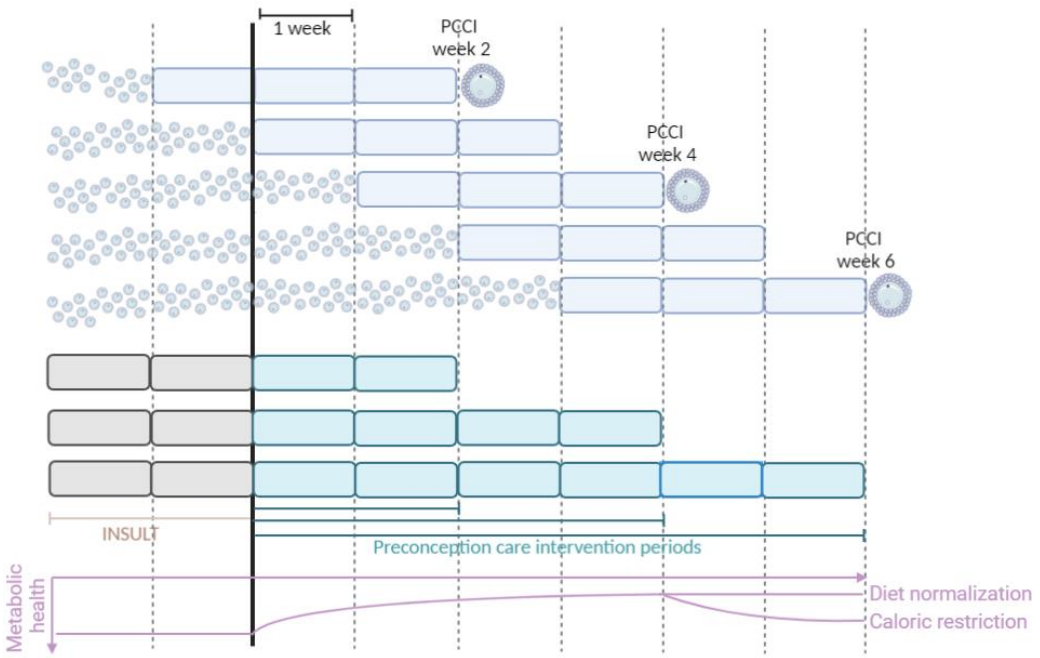

Figure 3. Overview of the time periods of the tested preconception care intervention, linked to the timeframe of folliculogenesis.(Made with Biorender)

6. THE CONCEPTS OF 'RESCUE', 'PREVENTION', 'IMPROVEMENT' AND 'RESTORATION'

During the course of this written PhD work, different concepts were addressed, depending on the method of intervention applied. It has clearly been demonstrated that high NEFA exposure during in vitro maturation significantly decreased oocyte developmental competence (Leroy et al., 2017). Therefore, in chapter 4, post-conception care interventions during in vitro embryo culture were tested to investigate their RESCUE potential (Figure 4). But what does rescue mean? The Cambridge Dictionary defines rescue as 'to help someone or something out of a dangerous, harmful, or unpleasant situation'. Other researchers investigating the impact of supplementation during in vitro embryo culture also use the concept of 'rescue' (De Bie et al., 2021; Marei et al., 2019). Other concepts like 'repair' are, however, also suggested (De Bie et al., 2021). Repair is defined as 'to put something that is damaged, broken, or not working correctly, back into good condition or make it work again'. Depending on the hypothesis and aims of the proposed research, both concepts are valid options. However, with regards to the in vitro research described in Chapter 4 of this PhD thesis, we cannot talk about 'repair'. Thanks to the supplements provided during in vitro culture and the high plasticity of the embryo during its development, the

low quality oocyte was able to keep growing. As a result, they showed a significantly higher developmental competence than the metabolically-compromised oocytes that were cultured under normal, non-supplemented conditions. It has been shown that the embryo is able to adapt to its environment until a certain threshold is reached. When that threshold is reached, adaptation cannot longer rescue the embryo and it dies. Therefore, we believe that the supplements were able to rescue the lower quality oocytes as they were able to adapt, due to its supplemented environment, and develop further without reaching this threshold. However, as the resulting blastocyst were of low quality, it is clear that in vitro culture supplementation with ITS was not able to repair the oocyte.

The second part of this thesis focused on the impact of preconception dietary interventions on metabolic health and fertility in an obese mouse model. Here, a clear distinction was made between 'IMPROVEMENT' and 'RESTORATION' (Figure 4). Outcome parameters were improved when a significant difference (mostly decrease) was present between the PCCI groups and the high fat(/high sugar) group, but there was still a significant difference when compared with the control group. The term 'Restoration' was only used when outcome parameters assessed were completely restored to the level of the control group. These concepts were used consistently throughout chapters 6 and 7.

Preconception care interventions are labelled as preventive methods. But is prevention the most suited concept to use? Prevention is defined as 'the act of stopping something from happening or of stopping someone from doing something'. If a preconception care intervention was only applied for two weeks, the process of folliculogenesis was already ongoing (Figure 3), so any damaging effect of the HF(/HS) diet could not be prevented anymore but only improved or restored. Switching to a PCCI for four or six weeks meant that the follicles were able to develop in a fat- and sugar-free environment. Furthermore, the time periods of four and six weeks do cover at least one complete cycle of folliculogenesis, opening a possible window of prevention but only if the primordial follicle pool was not damaged (figure 3). Based on the data collected in this thesis, it seemed, however, that the primordial pool was affected by the obesogenic diet. Therefore, again only the concepts of improvement and restorations were applicable.

7. HIGH FAT VERSUS HIGH FAT/HIGH SUGAR DIETS TO INDUCE OBESITY IN A MOUSE MODEL – WHAT IS THE MOST SUITED MODEL TO MIMIC HUMAN OBESITY AND DO THEY HAVE A SIMILAR IMPACT?

Up until now, both a high fat and a high fat/high sugar diet are used to induce obesity in a mouse model. Both diets have elicited significant effects on metabolic health that are similar to the impact described in obese women. Both diets have shown to increase serum lipid concentrations next to hyperinsulinemia, insulin resistance, impaired

glucose tolerance and hepatic steatosis (Panchal and Brown, 2011). However, as stated by Panchal and Brown (2011): 'Although a high fat diet induces most of the symptoms of human metabolic syndrome in rodents, it does not resemble the diet causing metabolic syndrome and associated complications as the human diet is more complex than a high fat diet alone.' 'The high fat/high sugar diet is more similar to the Western type human diets. Therefore, this model is probably the best to study the human metabolic syndrome.'

Although no direct comparison was made in this PhD thesis, we performed two separate experiments with an identical set-up, in which mice were exposed to a high fat (HF)-only or a high fat/high sugar (HF/HS) diet to induce obesity and metabolic syndrome (Table 1, columns 3 and 6).

Table 1. Overview of the impact of a high fat versus high fat/high sugar diet on metabolic health and fertility

		High fat diet induced obesity	Dietary normalization	Caloric restriction	**High fat/high sugar diet induced obesity**	Dietary normalization	Caloric restriction
METABOLIC HEALTH	**Weight**	High weight gain	Weight normalization	Continuous decline in weight	High weight gain	Weight normalization	Continuous decline in weight
	Serum lipid profile	Hyper-cholesterolemia Hypo-triglyceridemia	Restored Restored	Not restored Not restored	Hyper-cholesterolemia Hypo-triglyceridemia	Restored Restored	Restored Not restored
	Glucose tolerance	Hyperglycemia Reduced glucose tolerance	Restored	Restored (too extreme?)	Hyperglycemia Reduced glucose tolerance	Restored	Restored
	Insulin sensitivity	Hyper-insulinemia Reduced insulin sensitivity	Improved	Restored	Hyper-insulinemia Reduced insulin sensitivity	Not improved	Restored (too extreme?)
FERTILITY – OOCYTE QUALITY	**Lipid droplet volume**	Increased	Temporarily restored	Still high (not improved)	Increased	Temporarily restored	Still high (not improved)
	Mitochondrial activity	Not increased (only increased after 13w of diet)	Increased	Not increased	Not increased – mitochondrial uncoupling	Increased	Not increased

	Oxidative stress	Increased	High at PCCI w2, followed by a decline	Restored at PCCI w6	/	/	/
	Localization of active mitochondria	Decreased pericortical localization	Not improved	Not improved	Decreased pericortical localization	Not improved	Not improved
	Mitochondrial ultrastructure abnormalities	Increased	Improved at PCCI w6	Improved at PCCI w6	Increased	Restored at PCCI w4	Not improved
	Oocyte developmental competence	/	/	/	Reduced oocyte development	Partially restored	Restored
	Gene expression analysis in cumulus cells	/	/	/	Aberrant expression of genes related to insulin signaling and glucose metabolism	IRS1 restored SLC2A1 still high	No differences
	Pregnancy rates	Reduced pregnancy rates	Restored	Restored	/	/	/

Both HF- and HF/HS-fed mice not only significantly increased their absolute weight compared to the control group, their weight gain was also very similar after seven weeks on their respective diet: 24-25%. With regards to metabolic health, both groups developed hypercholesterolemia, hyperinsulinemia and hyperglycemia next to reduced glucose tolerance and insulin sensitivity, regardless of their diet (Table 1). Although obesity is mostly associated with hypertriglyceridemia, mice from both diets showed low serum triglyceride levels compared to the control group. Potential mechanisms that could explain this were suggested before: an inadequate export of triglycerides from the liver, a suppressed triglyceride production and/or an increased triglyceride clearance from the blood stream. Regardless, additional research is needed to fully comprehend the mechanisms causing reduced serum triglyceride concentrations during HF or HF/HS diet feeding.
A high fat intake is known to be associated with reduced glucose tolerance and insulin sensitivity (first described by Himsworth and Marshall (1935)). However, fructose itself can lead to increased fat deposition in the liver and skeletal muscle with subsequent insulin resistance (Stanhope, 2016). Therefore, a combination of both a high fat and high fructose diet is believed to more significantly impair insulin resistance than a high fat-only diet. Research directly comparing the impact of high fat versus high fat/high sugar diets on metabolic health, showed that although both diets elicit symptoms linked to metabolic syndrome (high plasma glucose and insulin, cholesterol and triglyceride levels), they were more severe in rodents on the HF/HS diets (Della Vedova et al., 2016; Moreno-Fernandez et al., 2018). When comparing absolute concentrations of the assessed metabolic health outcome parameters in this thesis, these were found to be very similar in both obese groups, not indicating an additional impact of fructose itself. Of course, it is important to keep in mind that after seven weeks on their respective diet, both HF- and HF/HS-fed mice had obtained the obese phenotype. It is not known if fructose might have accelerated the development of obesity and metabolic syndrome. As direct comparison between the impact of the HF *vs*. HF/HS diet was not the scope of this thesis, additional research should be performed to investigate this.
Based on these results, our conclusion is very similar to the one made by Panchal and Brown 10 years ago (Panchal and Brown, 2011).

To our knowledge, research directly comparing the effects of different diets on fertility and more specifically oocyte quality, is non-existing. As stated before, Fabozzi et al. (2021) summarized the impact of a HF or HF/HS diet on oocyte mitochondrial function but did not compare or discussed possible differential effects elicited by these two diet exposure models. They concluded that both a HF and a HF/HS diet have a significant impact on oocyte mitochondrial quality with fertilization failure, implantation failure, miscarriages etc as long-term consequences. Although not exactly the same outcome parameters were assessed in both studies performed in this thesis, those that were analyzed showed very similar results, regardless of the diet used (Table 1). The impact

on oocyte quality was very clear. Lipid droplet volume was significantly increased with 73.24% in the HF-diet fed group and 54.33% in the HF/HS-diet fed group (at PCCI week 2) compared to controls. Furthermore, a significant impact on mitochondrial quality was present. Mitochondrial ultrastructural abnormalities were increased and there was a significant decrease in the proportion of oocytes with pericortical localized active mitochondria. These results have been reported before in other studies that used a HF or HF/HS diet to induce obesity (Reynolds et al., 2015; Wu et al., 2010). Overall, both diets elicited a significant very similar impact on oocyte quality.

8. DOES THE ADDITION OF SUGAR TO THE HIGH FAT DIET HAS A DIFFERENTIAL IMPACT ON THE TESTED PRECONCEPTION CARE INTERVENTIONS?

Next to the comparison between HF and HF/HS diet induced obesity, we do not know if the addition of sugar to the high fat diet has a differential impact on the tested preconception care interventions (for an overview of the data: see Table 1). One of the aims of this PhD was to gain more fundamental insights regarding the impact of a PCCI on metabolic health and fertility, to be considered in developing substantiated preconception guidelines for obese metabolically compromised women planning for pregnancy. As it is stated that the HF/HS diet might mimic the human Western type diet more closely, it is interesting to evaluate if differential effects are seen on metabolic health and oocyte quality after a PCCI, depending on the diet that was used to induce obesity.

To obtain complete recovery of their weight, mice that underwent diet normalization (HF_CTRL) lost on average 13% of their weight, regardless of the diet used to induce obesity (Table 1). When assessing the serum lipid profile, HF_CTRL mice showed fully restored cholesterol and triglyceride concentrations, again regardless of the type of diet that was used to induce obesity. Interestingly, mice that underwent a caloric restriction diet (HF_CR) only showed restored cholesterol concentrations when obesity was induced by a HF/HS diet. This is surprising. As the addition of fructose is believed to have an additional impact on dyslipidemia (Adiels et al., 2008), restoration of this serum parameters would rather be expected after a HF-only diet exposure.

Switching from a HF diet to an ad libitum control diet (HF_CTRL mice) was able to restore glucose tolerance and improve insulin sensitivity after four weeks. In contrast, HF/HS-fed mice that switched to a similar diet (HF_CTRL) did only restore glucose tolerance while insulin sensitivity remained aberrant. These data confirm that the addition of high fructose concentrations to the diet made it more difficult to improve insulin sensitivity after diet normalization. Mice that underwent a more severe calorie restricted diet (HF_CR) for four weeks showed a restored glucose tolerance and insulin sensitivity. In addition, results from the insulin tolerance test suggested that switching to a 30% caloric restriction diet for this time period might be a too extreme intervention, regardless of the diet that was used to induce obesity.

With regards to oocyte quality, impact of both PCCI's on lipid droplet volume was exactly the same, regardless of obesity induction by a HF or HF/HS diet. These results might indicate that the fat component of the diet was the biggest cause of the increased oocyte lipid uptake.
Diet normalization resulted in a gradual decrease in weight with a temporary reduction in lipid droplet volume. However, as suggested before, extreme weight loss due to a caloric restriction diet did seem to result in an increased lipid mobilization, leading to high free fatty acids in the follicular fluid. This might lead to increased fatty acid uptake in the oocyte, resulting in a high lipid droplet volume. When assessing oocyte mitochondrial parameters, mitochondrial activity was increased in HF_CTRL mice in both experiments (Chapter 6 and 7A). We suggested in chapter 6 that the increased mitochondrial activity, together with assessed values on ROS and ROS: active mitochondria ratio, might be an indication of a more metabolically efficient oocyte with a higher antioxidant capacity. Although ROS levels were not measured, increased mitochondrial activity was also reported in HF_CTRL oocytes from HF/HS-fed obese mice (Chapter 7A). Furthermore, mitochondrial morphology was restored in both experiments while localization of active mitochondria was not. These data again indicate a similar impact of diet normalization on oocyte mitochondrial quality, regardless of the obesogenic diet that was used to induce obesity.

HF_CR mice from HF/HS-diet induced obesity showed no improvements in any of the mitochondrial quality parameters assessed, again confirming a long lasting impact of the HF/HS diet. Similar effects were present in HF-induced obese mice that underwent a caloric restriction diet for four weeks as ratio of ROS: active mitochondria was very high and the percentage of oocytes with a pericortical localization of active mitochondria was even lower than the control group. Interestingly, mitochondrial quality parameters assessed after six weeks of PCCI did show an improvement, indicating that a long term intervention might be needed to have a positive impact on oocyte mitochondria. Unfortunately, this time point was only analyzed in the HF-induced obese group so comparison with the HF/HS-fed mice was not possible. It is clear that more research is needed, investigating whether the impact of dietary preconception intervention on mitochondrial quality is indeed dependent on the diet and time period used to induce obesity.

9. A FIRST PEAK INTO THE PROTEOMIC PROFILE OF CUMULUS CELLS FROM OBESE MICE AND MICE THAT UNDERWENT A PRECONCEPTION CARE INTERVENTION – WHAT DID WE LEARN AND WHERE ARE THE GAPS?

Up until now, very little information is known regarding the underlying mechanisms of the impact of obesity on cumulus cell (CC) and oocyte quality. Therefore, in chapter 7B, we opted to further explore this by investigating the proteomic profile of CC's collected

from obese mice. In addition, the possible impact of dietary preconception care interventions (PCCI) on the proteome of these CCs was also investigated. Based on the results assessed in chapters 6 and 7A, it seems that the impact of the tested PCCI and the resulting improved metabolic profile are sensed by the cumulus oocyte complex. As a next step, we wondered if this resulted in an altered physiology of the cumulus cells. As earlier research in our lab indicated that the time period of four weeks PCCI is the most promising, it was decided to focus on this timeframe.

After thorough investigation, we opted to analyze the data in a stepwise approach, very similar to the statistical method that was applied in chapters 6 and 7A. This allowed to focus on relevant comparisons between two treatment groups of interest. Furthermore, we mainly focused on the top pathways, molecular functions and biological processes that are in line with our research question to improve the most accurate and valuable interpretation of these complex proteomic data. To further improve these insights, functional annotations of the involved proteins was performed. Of course, other approaches can be used to analyze these complex and rich data.

Comparison between CC collected from mice on the HF/HS diet versus mice on the control diet confirmed the negative impact of obesity on quality of the cumulus oocyte complex. 42 significant differentially regulated proteins (DRP) were found, associated with upregulation of proteins linked to redox processes and downregulation of proteins linked to cholesterol and steroid biosynthesis. To our knowledge, this was the first in vivo murine experiment, investigating the impact of obesity on the proteomic profile of CC. These data increased insights regarding the underlying mechanisms of the impact of obesity on the cumulus oocyte complex. Unfortunately, as literature on this subject is very limited, direct comparisons between these results and already published results was not possible. Therefore, much more research on this topic is needed.

In the next step, the impact of possible PCCI on the cumulus cell proteomic profile was investigated. Interestingly, only three DRP were common between the HF/HS-fed *vs*. control-fed comparison and the diet normalization *vs.* HF/HS comparison, indicating only a partial improvement of the aberrant proteomic profile reported in the HF/HS group. The same pattern was present in mice that underwent a caloric restriction diet for four weeks, as they only displayed 5 common DRP. Both interventions groups indicated an improvement of proteins involved in the estrogen response and ERAD pathway. Cumulus cells from mice on the caloric restriction diet were also able to reverse the reduced cholesterol biosynthesis. Earlier research in this thesis (chapter 6 and 7A) did not focus on functional outcome parameters associated with these functions, making direct comparisons with these results rather difficult. Interestingly, 75 other DRP were found when comparing CC from mice that underwent diet

normalization compared to the HF/HS-fed group. The same pattern was present in mice that underwent a caloric restriction diet for four weeks, with 133 DRP. These results indicated that instead of improvement or restoration of the affected proteins, the cumulus cells rather activate adaptive mechanisms in both PCCI groups at different levels.

These results provide a first glimpse into the underlying mechanisms of dietary PCCI on cumulus cells from obese individuals. As literature on this topic is non-existing, more research is definitely needed. These data might indicate interesting avenues through which the quality of the cumulus oocyte complex can be improved. As a next step, the same analysis should be performed on oocytes. It will provide additional insights into this topic. Earlier research in an in vitro exposure model has indicated opposite effects of lipotoxic conditions on cumulus cells versus oocytes (Marei et al., 2019).

10. LIMITATIONS OF THE STUDY AND RECOMMENDATIONS FOR FUTURE RESEARCH

The **in vitro** study performed in this thesis used a model in which oocytes are exposed to elevated NEFA concentrations for only 24 hours, during final oocyte maturation. Earlier research in our lab has shown a significant impact of this short term exposure window on oocyte and embryo quality (Leroy et al., 2017), making this a suited in vitro model to test the short term impact of in vitro culture supplementation on developmental competence and quality of metabolically compromised oocytes. However, the growing oocyte has spent several weeks, months or years in an obese individual before it will ovulate (Evans, 2003). As stated in the introduction, Britt (1992) suggested that the developmental competence of the oocyte is determined by its biochemical microenvironment during a long period of follicular growth before ovulation. Therefore, the aberrant metabolic condition during follicular growth may have an impact on the oocyte's ability to develop properly. Therefore, to better mimic the in vivo situation, follicles should be cultivated in lipotoxic conditions for a much longer period, as performed in a murine in vitro system in our lab (Valckx et al., 2014). In this experimental set-up, murine follicles were exposed to high NEFA concentrations for a period of 12 days. However, the most relevant model would be an in vivo model. As human oocytes for research are very scarce, the in vivo mouse model can be a valid alternative in which mature oocytes will be collected from obese mice.
Research performed during this PhD has shown a significant impact of in vitro culture (IVC) supplementation of metabolically compromised oocytes on oocyte developmental competence and resulting embryo quality. However, no information is known regarding the long-term impact of these IVC conditions on further post implantation development. The ultimate long-term goal of IVC supplementation is to rescue the metabolically compromised oocyte so it can result in the birth of a healthy offspring. Therefore, future research should investigate further embryo and fetal

development. In addition, possible impacts on offspring health should also be assessed. Other research in our lab is currently focusing on the impact of changing maternal metabolic conditions on offspring health and fertility.

As stated before in this PhD thesis (chapter 1, subtitle 3.3), the **quality of the oocyte** plays a key role in proper embryo development and is often the central point of reduced fertility (Fedorcsak et al., 2004; Lashen et al., 2004; Robker, 2008). Therefore, the oocyte quality was chosen as main outcome target for this PhD research, in chapters 5, 6 and 7. Research performed in this thesis has significantly increased knowledge on the impact of preconception care interventions (PCCI) on the reduced oocyte quality in an obesogenic environment. Of course, the oocyte continues to develop after a successful fertilization and detailed information beyond that point of development is currently lacking. What happens when the oocyte reaches the oviduct and uterus? What is the impact of the tested preconception care interventions on the oviductal and uterine environment and on the embryo-maternal communication? There are still a lot of questions to be answered. As very limited literature is available regarding the impact of a high fat diet and obesity on the oviduct, research in our lab is trying to further bridge that gap.

As stated before, **age** has a significant impact on the reproductive success of a woman. Women show a progressive decline in fertility as they pass through their reproductive years (Rowe, 2006). The primordial follicle pool that is present at birth reduces in number and quality with age. Starting from the age of 30, fertility begins to decline and by the age of 50, most women have reached menopause (Klein and Sauer, 2001). The reproductive life span of a mouse is a lot shorter than in human, starting from the age of 4-7 weeks until 6-8 months (University, 2019). As the complete time span of our experimental set-up was 13 weeks, the oldest mice have reached the age of 18 weeks (±4.5 months) at the time of oocyte collection. Therefore, a possible impact of increasing maternal age on oocyte quality cannot be ignored. To avoid this possible impact as much as possible, mice started their control or obesogenic diet as young as possible. In addition, based on the outcome parameters assessed, no significant impact of age on oocyte quality or pregnancy rates was present in this experimental set-up. Other research in our lab confirmed this.

One of the disadvantages or working with a murine model is limited **availability of certain research material**, like blood. To assess a sufficient amount of metabolic serum parameters at the same time point, the mouse should be sacrificed to collect a sufficient volume of blood. In other, larger animal models like the cow, this is not an issue and blood could have been collected from the same animals over the whole preconception period. In addition, oocyte collection in mice is only possible when the

mouse is sacrificed. This resulted in the sacrification of different mice at each PCCI time point for most outcome parameters, making a linear comparison over the different time points not possible. Due to this limitation, statistical analysis was only performed within the same time point and this was also taken into consideration when drawing conclusions.

In this study, we only took into account female obesity and the possible impact of PCCI in obese women on oocyte quality and fertility. We did not consider the role of the **male factor**. More and more research confirms a negative impact of male overweight or obesity on sperm quality. Male obesity affects sperm concentration, motility and morphology (Braga et al., 2012; Jensen et al., 2004; Koloszar et al., 2005). In couples who have fertility issues, often not only the woman is obese but also the male. Earlier research in our lab has demonstrated a significant impact of both male and female BMI and waist circumference on embryo quality and pregnancy outcomes (Desmet, 2019). In another study, couples in which the male had a BMI of >28 exhibited a significantly lower fertilization rate, good-quality embryo rate and clinical pregnancy rate compared to their normal BMI counterparts (Yang et al., 2016). These results indicate that the male factor cannot be ignored. As stated by Best et al. (2017), there is a lack of studies investigating the impact of PCCI in obese men. They reported only two studies investigating the impact of diet and exercise on semen quality (Faure et al., 2014; Hakonsen et al., 2011), making it very difficult to draw clear conclusions. In the future, research focusing on PCCI should also focus on the male obese parent.

As already discussed earlier in this chapter (subsection 4.), the most optimal **duration of the PCCI** plays an important role. The maximum time period tested in this PhD research was a timeframe of six weeks. With regards to fertility, six weeks translates to the human setting with a PCCI period of six to eight months. As advancing maternal age has a significant negative impact on fertility, we opted to not include a longer PCCI period. Do we still agree with this decision now that we know a lot more? Although some promising improvements were reported in oocyte quality of obese mice that underwent a caloric restriction diet for six weeks, it was clear from the results in chapter 6 that this time period was too detrimental for the metabolic health of the mice and will therefore never be advised in a clinical human setting. Following the caloric restriction for a period longer than six weeks, may even result in mice that become anorexic with an even more detrimental impact on oocyte quality. Obese mice that underwent diet normalization showed some promising improvements on their metabolic health and oocyte quality, starting from four weeks of PCCI. This coincided with their weight stabilization (chapter 6). With regards to metabolic health, no further improvements were present after a PCCI period of six weeks while some improvements were present at the level of oocyte mitochondrial activity and ROS.

Reynolds et al. tested the impact of diet normalization in obese mice for a period of eight weeks. They still reported higher rates of meiotic spindle, lipid and mitochondrial defects (Reynolds et al., 2015). Based on these results, it seems that even a longer time period of diet normalization might not result in a completely restored oocyte quality. These data, together with the importance of maternal age in mind, led to the conclusion to focus on a PCCI period of four weeks in chapter 7 as it seemed the most promising preconception time frame.

A range of different **outcome parameters** were analyzed to get a clear picture on the quality of oocyte. As described in detail in the introduction (chapter 1, sub section 6.3.), mitochondria are essential for successful oocyte development (Van Blerkom, 2004). Fluorescent dyes for measuring the mitochondrial membrane potential have become commonly used tools for monitoring changes in this important mitochondrial parameter as it relates to the capacity of the cell to generate ATP. Oocyte mitochondrial activity and localization were measured in this PhD thesis with a fluorescent JC1 staining. At low concentrations, it is monomeric and emits green fluorescence, while at high concentrations, there is a spectral shift because of the 'J-aggregate' formation. As such, the emission ratio gives a relative measure of mitochondrial membrane potential in the cell (Al-Zubaidi et al., 2019). Furthermore, JC1 staining also has a big advantage that it can be combined with CellRox staining to asses both oxidative stress and mitochondrial membrane potential in the same oocyte. This is not the case with other mitochondrial dyes.

Mitochondrial ultrastructure was assessed using transmission electron microscopy (TEM), based on a protocol described by Boudoures et al. (2016). Using this technique, a cohort of mitochondria are categorized as normal or abnormal to get a representative overview of the percentage of structurally abnormal mitochondria in the oocyte. Unfortunately, with this technique it is not possible to assess the total number of normal and abnormal mitochondria in the oocyte. To our knowledge, this is not possible yet. When assessing this outcome parameter, we did not aim to focus on the total number of mitochondria in the oocyte but on the proportion of structurally abnormal mitochondria.

Due to a limited number of oocytes that could be collected per mouse, the number of possible assessed outcome parameters was limited. If the oocyte numbers would be higher, another interesting mitochondrial outcome parameter would be the use of the Seahorse to measure the Oxygen Consumption Rate (OCR) of the oocytes, an established measure of mitochondrial function. It will provide us with more detailed information. The Seahorse is based on extracellular flux analysis, which is non-invasive and allows real-time determination of the impact of compounds on OCR (Muller et al.,

2019). It detects extracellular changes in the cellular consumption of oxygen and the efflux of protons to measures rates of cellular mitochondrial respiration, glycolysis and ATP production. Very recently, a Seahorse Analyzer was purchased by our laboratory and will be used extensively in the future.

Research has indicated that interventions that inhibit mitochondrial function cause defects in meiotic and mitotic spindles and inhibit oocyte maturation and embryo development (Dalton et al., 2014; Wu et al., 2015; Zhang et al., 2006). Therefore, another potential interesting outcome parameter would be the assessment of oocyte spindle defects.

11. FUTURE PERSPECTIVES – POSSIBLE IMPLICATION FOR THE HUMAN SETTING

Using a mouse model to investigate preconception care interventions has several advantages and gave us the opportunity to obtain necessary, fundamental insights regarding the impact of such dietary preconception care interventions on oocyte quality. Translating the results from this mouse study to the human setting should, of course, be done with caution. However, the use of an outbred mouse strain, the specific implementation of preconception time periods related to the duration of mouse folliculogenesis together with the assessment of weight changes and metabolic health, should help in understanding the potential consequences of our study's fundamental information for the human fertility setting.

While some studies report a positive impact of in vitro culture supplementation on developmental competence and blastocyst quality of metabolically-compromised oocytes, research performed in this thesis only reported improved oocyte developmental competence. The effect seemed to be dependent on the kind of supplement that was used. Therefore, more (fundamental) insights need to be obtained regarding the impact of in vitro culture supplementation on oocyte and embryo developmental competence and quality. As literature on non-supplemented human culture media already describes a significant impact of the media used on offspring health, the possible impact of in vitro culture "goodies" cannot be ignored and should be further investigated.

Research performed in this thesis significantly increased fundamental insights regarding the impact of dietary preconception care interventions on metabolic health and oocyte quality in an obese mouse model. However, this is only the tip of the iceberg. Although effects on metabolic health seemed promising, oocyte quality parameters were not always improved. The remaining impact on the oocyte at PCCI week 4 and 6 indicates that the primordial follicle pool might be affected by the obesogenic diet exposure. A dietary preconception care intervention seemed not to be able to completely restore this damaging effect. To our knowledge, we are the first to

report this. However, further research is needed to further elaborate on this concept. In addition, other PCCI like exercise and dietary intake of supplements are often advised. Although some fundamental knowledge is available regarding the impact of these PCCI on oocyte quality, no information is known regarding the best time periods for these interventions. Therefore, we believe that additional mouse experiments might further increase insights regarding this topic.

Based on the results obtained during this PhD thesis, we propose that undergoing diet normalization for a period of at least four weeks seemed the most promising approach to improve metabolic health and oocyte quality. When translating this to the human setting, we propose a weight loss of ±13% which should result in restored serum cholesterol and triglyceride concentrations and significantly improved glucose tolerance and insulin sensitivity. When taking into account the timeframe of folliculogenesis (three weeks in mice and 3-4 months in humans), an intervention period of four weeks in mice can be translated to at least 4 months in humans when only focusing on the improvement of oocyte quality.

As discussed by Simon (2019), more and more evidence is emerging that environmental and genetic factors during the preconception period affect the development of the embryo, potentially impairing the health of future offspring. Therefore, the fundamental data generated in the present studies should also be regarded in a wider setting as the preconception period is more and more seen as a ‘‘window of opportunity’’ to improve offspring health. Preconception care should become a key component of reproductive medicine not only to improve implantation and pregnancy rates, but also to further optimize the health of both mothers and children and to set the stage for the child's adult life.

12. CONCLUSIONS

This thesis investigated the potential of post- and preconception care interventions on metabolic health and fertility, using an in vitro bovine or in vivo mouse model. A summary of the results can be found in figure 4.

- Research performed the past years in our laboratory was able to confirm a significant impact of an aberrant maternal micro-environment (mimicked by high NEFA exposure during in vitro bovine oocyte maturation) on oocyte developmental competence and resulting embryo quality. Using this well-established in vitro bovine model, it was shown that supplementation of the in vitro culture medium with mitogenic, anti-apoptotic and antioxidative factors might improve oocyte developmental competence but not resulting embryo quality with possible consequences on post-implantation development and offspring health.

- The outbred Swiss mouse model seems the best choice when performing research on the impact of diet-induced obesity on oocyte quality as inbreeding seemed to reduce mitochondrial quality.
- Although no direct comparison was made, both high fat and high fat/high sugar diets were able to significantly reduce metabolic health and oocyte quality. However, impact of the tested preconception care interventions seemed to partially depend on the diet that was used to induce obesity. More research should be performed to increase knowledge regarding this topic.
- Investigating the impact of dietary preconception care interventions on metabolic health and oocyte quality in an obese mouse model has significantly increased fundamental insights regarding this topic. With regards to metabolic health, we suggest that undergoing diet normalization for four weeks or a severe calorie restricted diet for two or four weeks, followed by diet normalization seemed the most promising approaches, however this last notion needs to be tested. Although some improvements on oocyte quality were present, a complete restoration could not be seen. Based on the results obtained during this PhD thesis, undergoing diet normalization for a period of at least four weeks seemed to be the most promising approach to improve both metabolic health and oocyte quality.

Results in this thesis gained significantly more fundamental insights regarding the impact of pre- or postconception care interventions on maternal metabolic health and fertility (oocyte and embryo quality). This work increased awareness regarding the possible impact of in vitro culture supplementation on oocyte developmental competence, resulting embryo quality and possible future implications. Furthermore, the results obtained regarding the impact of dietary preconception interventions on an obese mouse model, can further bridge the gap towards clear preconception guidelines for obese women planning for pregnancy.

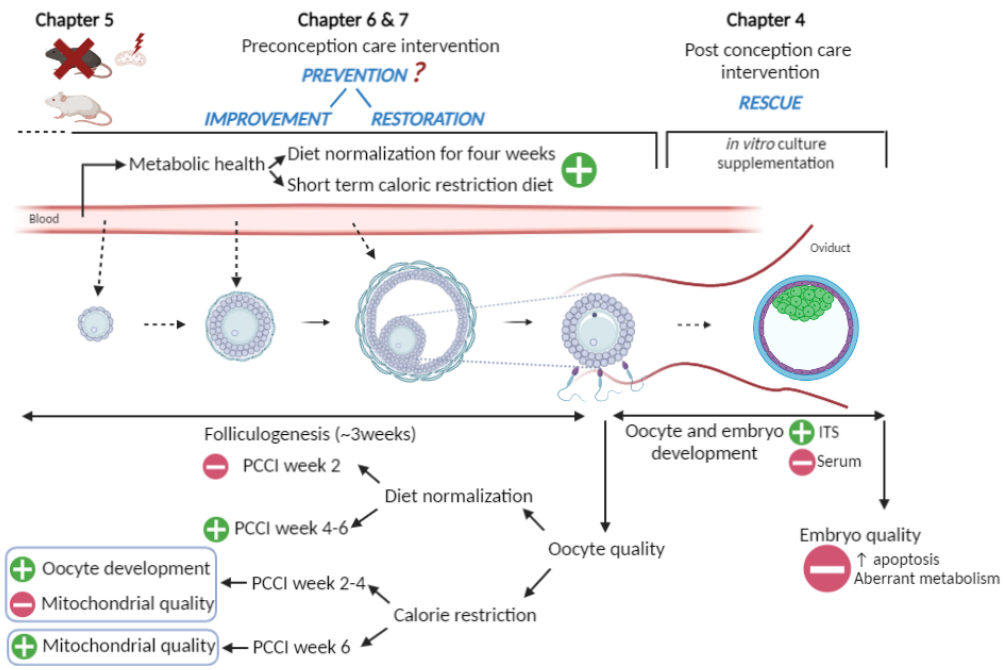

Figure 4. Overview of the results obtained during this PhD thesis, regarding the impact of pre- or postconception care interventions on metabolic health and fertility of obese individuals (Made with Biorender)

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SUMMARY

Maternal metabolic disorders like obesity are associated with a significant reduction in metabolic health and oocyte and embryo quality. In **CHAPTER 1**, we provide a state-of-the-art on the impact of obesity on metabolic health with a detailed discussion on the roles of dyslipidemia and insulin resistance. Furthermore, the effect of obesity on female fertility is discussed in detail, including an overview of the well-orchestrated processes that take place during folliculo- and oogenesis, fertilization and embryo development. In addition, the impact of obesity on the oocyte's micro-environment with subsequent impact on important metabolic processes and the importance of mitochondria and oxidative stress are overviewed.
In **CHAPTER 2**, we explore the different possibilities of improving metabolic health and fertility in obese, metabolically-compromised individuals, starting from the possible impact of 'post' conception interventions during in vitro embryo culture to the application of preconception dietary interventions. Furthermore, we discuss the importance of the most optimal model and diet for our research.

In **CHAPTER 3**, the specific aims of the thesis are overviewed. We first investigated the impact of in vitro culture supplementation as post-conception care intervention on the development and embryo quality of metabolically-compromised oocytes (CHAPTER 4). Before exploring the impact of different dietary preconception care interventions on metabolic health and oocyte quality in an obese mouse (CHAPTERS 6 and 7), the correct mouse model for this research was investigated (CHAPTER 5).

The consequences of maternal metabolic disorders on oocyte quality are clear. However, it is not known if this metabolically-compromised oocyte can be rescued. In **CHAPTER 4**, a well-established in vitro bovine exposure model was used to obtain metabolically-compromised oocytes. To investigate the impact of post-conception care interventions, the in vitro culture (IVC) medium was supplemented with anti-apoptotic, antioxidative and mitogenic factors (insulin-transferrin-selenium (ITS) or serum). Supplementation with ITS significantly improved developmental competence of metabolically compromised oocytes. However, resulting blastocysts showed to be of inferior quality. Supplementation of serum during IVC did not restore developmental competence nor resulting blastocyst quality.

Research investigating the impact of obesity on metabolic health (and fertility) often uses the inbred C57BL/6 strain. As inbred strains are characterized by reduced fertility and might limit translation to human outbred physiology, the use of an outbred strain might be more relevant for our research. In **CHAPTER 5** we investigated if high fat diet-induced obesity has a differential impact on oocyte quality in the outbred Swiss mouse, compared to the routinely used inbred C57BL/6 strain. Results indicated that the Swiss mouse seems more metabolically responsive to a high fat diet with a bigger impact on oocyte quality than the C57BL/6 strain. Oocytes from C57BL/6 control mice display already increased cellular stress and significant mitochondrial ultrastructural abnormalities.

Nowadays, more and more attention is going towards the development of preconception care interventions (PCCI) in obese women to improve their fertility. However, so far there are no clear preconception guidelines. Information regarding the impact of dietary PCCI on oocyte quality and the most optimal time period for this PCCI is severely lacking. Therefore in **CHAPTER 6**, the impact of diet normalization or caloric restriction for different time periods on metabolic health and oocyte quality of high fat diet-induced obese mice was tested. Results indicated that both switching to an ad libitum control or a 30% caloric restriction (CR) diet for four weeks was the best approach to improve metabolic health, of which the ad libitum control diet showed the most promising. Longer exposure to the CR diet was detrimental. In contrast, most promising improvements in oocyte quality were reported after six weeks of PCCI in both groups.

Evidence is emerging that in our Western society, not only diets high in saturated fat but also a high sugar content has a detrimental effect on metabolic health and fertility. In **CHAPTER 7**, we aimed to investigate if there are any additional or differential effects of a high fat/high sugar diet and the tested preconception care interventions on metabolic health and oocyte quality compared to the findings in chapter 6. Results confirmed that switching to an ad libitum control or 30% caloric restriction diet for four weeks seemed the most promising approach. Oocyte quality was improved, but not restored, in obese mice that underwent diet normalization for four weeks. Although switching to a 30% CR for only two weeks already showed restored oocyte developmental competence, oocyte quality stayed aberrant.

Finally, in **CHAPTER 8**, all findings are summarized and collectively discussed in light of the existing literature. From this, the following conclusions were drawn:

- Supportive embryo culture conditions might improve oocyte developmental competence but therefore not resulting embryo quality with possible consequences on post-implantation development and offspring health.
- The outbred Swiss mouse showed to be the best in vivo murine model to investigate the impact of obesity on oocyte quality.
- Both high fat and high fat/high sugar diets significantly reduced metabolic health and oocyte quality while impact of the tested PCCI seemed to depend partially on the diet used to induce obesity.
- Diet normalization for four weeks showed to be the most promising approach to improve both metabolic health and oocyte quality in an obese mouse model.

With this research, we increased awareness on the possible impact of in vitro culture supplementation on oocyte developmental competence, resulting embryo quality and possible implication for the future. Furthermore, the increased fundamental insights regarding the impact of dietary preconception care interventions on metabolic health

and fertility can further bridge the gap towards clear preconception guidelines for obese women planning for pregnancy.

SAMENVATTING

Maternaal metabole stoornissen zoals obesitas worden geassocieerd met een significante vermindering van de metabole gezondheid en eicel en embryo kwaliteit. In **HOOFDSTUK 1** voorzien we een state-of-the-art over de impact van obesitas op de metabole gezondheid, met een gedetailleerde weergave van de rol van dyslipidemie en insuline resistentie. Verder wordt het effect van obesitas op de vrouwelijke vruchtbaarheid in detail besproken. Dit houdt een overzicht in van belangrijke processen die plaatsvinden tijdens folliculo- en oogenese, fertilisatie en embryo ontwikkeling. Verder wordt de impact van obesitas op de omgeving van de eicel aangehaald met o.a. bespreking van belangrijke metabole processen en het belang van mitochondria en oxidatieve stress in de eicel.
In **HOOFDSTUK 2** halen we de verschillende mogelijkheden aan om metabole gezondheid en vruchtbaarheid te verbeteren in obese, metabool gecompromitteerde vrouwen, startend van mogelijke impact van 'post' conceptie interventies tijdens in vitro embryo cultuur tot het toepassen van diëtaire preconceptie interventies. Verder bespreken we ook het belang van het juiste model en dieet voor ons onderzoek.

In **HOOFDSTUK 3** kan je een overzicht terugvinden van de specifieke doelstellingen van deze doctoraatsthesis. Eerst onderzochten we de impact van supplementatie tijdens in vitro cultuur als post-conceptie interventie op de ontwikkeling en embryo kwaliteit van metabool gecompromitteerde eicellen (HOOFDSTUK 4). Voor we de impact van verschillende diëtaire preconceptie interventies op metabole gezondheid en eicelkwaliteit in een obees muizenmodel onderzochten (HOOFDSTUK 6 en 7), voerden we eerst onderzoek uit naar het juiste muizenmodel hiervoor (HOOFDSTUK 5).

De gevolgen van maternale metabole stoornissen op de eicelkwaliteit zijn duidelijk. Het is echter nog niet geweten of deze metabool gecompromitteerde eicellen gered kunnen worden. In **HOOFDSTUK 4** werd een gevestigd in vitro bovien model gebruikt voor het verkrijgen van deze metabool gecompromitteerde eicellen. Om de impact van post-conceptie interventies te onderzoeken, werd het in vitro cultuurmedium gesupplementeerd met anti-apoptotische, antioxidatieve en mitogene factoren (insuline-transferrine-selenium (ITS) of serum). Het toevoegen van ITS verbeterde de ontwikkelingscompetentie van metabool gecompromitteerde eicellen. Kwaliteit van de resulterende blastocysten was echter inferieur. Supplementatie van serum tijdens IVC was niet in staat om zowel de ontwikkelingscompetentie als kwaliteit van de blastocysten te herstellen.

Onderzoek naar de impact van obesitas op metabole gezondheid (en vruchtbaarheid) maakt vaak gebruikt van de inteelt C57BL/6 muizenstam. Inteelt stammen worden echter gekarakteriseerd door afgenomen vruchtbaarheid en beperken translatie naar de humane, outbred fysiologie. Daarom is het gebruik van een outbred muizenstam mogelijks meer relevant voor ons onderzoek. In **HOOFDSTUK 5** hebben we onderzocht of obesitas, veroorzaakt door een vetrijk dieet, een verschillende impact heeft op eicelkwaliteit in de outbred Swiss muis wanneer deze wordt vergeleken met de

routinematig gebruikte inteelt C57BL/6 muis. Resultaten toonden aan dat de Swiss muis meer metabool gevoelig is aan een vetrijk dieet met een grotere impact op de eicelkwaliteit. Eicellen van C57BL/6 controle muizen vertoonden reeds toegenomen cellulaire stress en significant hoge ultra structurele mitochondriale abnormaliteiten.

De laatste jaren gaat er meer en meer aandacht naar de ontwikkeling van preconceptie interventies (PCCI) in obese vrouwen met als doel hun vruchtbaarheid te verbeteren. Tot op heden zijn er echter geen duidelijke preconceptie richtlijnen. Informatie rond de impact van diëtaire PCCI op eicelkwaliteit en de meest optimale tijdsspanne hiervoor is zeer beperkt. Daarom werd in **HOOFDSTUK 6** onderzoek gedaan naar de impact van dieet normalisatie of een calorie restrictie dieet voor verschillende tijdspannes op metabole gezondheid en eicelkwaliteit in een vetrijk dieet-geïnduceerd muizenmodel. Resultaten toonden aan dat de overgang naar een ad libitum controle of 30% calorie restrictie (CR) dieet voor vier weken de beste benadering was om metabole gezondheid te verbeteren. Hiervan was het ad libitum controle dieet het meest belovend. Langere blootstelling aan het CR dieet was zeer nadelig. In tegenstelling hiermee, vonden de meest veelbelovende verbeteringen in eicelkwaliteit plaats na zes weken PCCI in beide groepen.

Er is meer en meer bewijs dat in onze Westerse omgeving niet enkel diëten hoog in gesatureerde vetzuren maar ook een hoge suikerinhoud een zeer negatief effect heeft op metabole gezondheid en vruchtbaarheid. In **HOOFDSTUK 7** onderzochten we of er verschillende effecten zij van een vet- en suikerrijk dieet én de geteste PCCI op metabole gezondheid en eicelkwaliteit, vergeleken met de bevinden van hoofdstuk 6. Resultaten bevestigden dat wisselen naar een ad libitum controle of 30% CR dieet voor vier weken een goede benadering is. Eicelkwaliteit was verbeterd, maar niet hersteld, in obese muizen die dieet normalisatie ondergingen voor een periode van vier weken. Overschakelen naar een 30% CR dieet voor slechts twee weken toonde reeds een herstelde eicel ontwikkelingscompetentie maar eicelkwaliteit bleef afwijkend.

Tot slot werden in **HOOFDSTUK 8** alle bevinden samengevat en bediscussieerd in het licht van de bestaande, wetenschappelijke literatuur. Hieruit werden volgende conclusies getrokken:

- Ondersteunde embryo cultuur condities kunnen eicel ontwikkelingscompetentie verbeteren maar daarvoor niet resulterende embryokwaliteit, resulterend in mogelijke gevolgen op postimplantatie ontwikkeling en gezondheid van de nakomelingen.
- De outbred Swiss muis was het beste in vivo muizenmodel om de impact van obesitas op eicelkwaliteit te onderzoeken
- Zowel vetrijke als vet- én suikerrijke diëten veroorzaakten een sterke afname aan metabole gezondheid en eicelkwaliteit terwijl de impact van de geteste

PCCI gedeeltelijk afhankelijk lijkt te zijn van het dieet dat gebruikt wordt om obesitas te induceren.

- Dieet normalisatie voor vier weken was de meest belovende aanpak om zowel metabole gezondheid als eicelkwaliteit te verbeteren in een obees muizenmodel.

Met dit onderzoek hebben we meer bewustzijn gecreëerd rond de mogelijke impact van in vitro cultuur supplementatie op eicel ontwikkelingscompetentie, resulterende embryokwaliteit en mogelijke implicaties voor de toekomst. Verder kunnen de toegenomen fundamentele inzichten rond de impact van diëtaire preconceptie interventies op metabole gezondheid en vruchtbaarheid verder de kloof overbruggen richting de ontwikkeling van duidelijke preconceptie richtlijnen voor obese vrouwen.

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EDUCATION

Laboratory Animal Science FELASA type C – University of Antwerp 2016

Master Molecular and Cellular Biomedical Sciences, minor Research 2013-2016
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Scriptie: Gentherapie met T-cel-receptor gemodificeerde T-lymfocyten in kanker

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Thesis: Effect of embryo culture conditions on developmental potential of bovine oocytes matured under metabolic stress

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EDUCATIONAL ACTIVITIES

Coach master thesis and internship Yana Stevens 2020-2021
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Subject: Preconception care strategies in obese mice and effects on oocyte quality and cumulus cell function

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Coach master thesis and internship Jolien De Schrijver 2018-2019
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Subject: Preconception care strategies in metabolically compromised mothers and the impact on oocyte quality

Coach bachelor thesis Karolien Ceulemans 2018-2019
Bachelor Biomedical Sciences – University of Antwerp
Subject: Maternale metabole stress en het effect ervan op eicel en embryo kwaliteit en hoe inteelt een rol speelt in het onderzoek hiernaar

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