Effects of metformin on spermatogenesis and paternal epigenetic inheritance of acquired metabolic disorders

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Somewhat controversial to the classical theory of genetic inheritance, human epidemiological data combined with experimental animal models have shown that along with genetic information, acquired conditions and traits can be transferred from parents to offspring. For example, parental traumatic stress and nutritional challenges have been shown to induce altered, sometimes disadvantageous phenotypes in the offspring. This phenomenon is today explained by epigenetic germline inheritance, and data regarding transmittable phenotypes and underlying causal mechanisms is building up. During recent years, a growing body of research has focused on paternal epigenetic inheritance. Epigenetic modifications in paternal gametes can be induced by different environmental factors, such as toxins, nutrition, and stress-related conditions. How the health status of the father is then passed on to the offspring has not yet been fully clarified, however, certain mechanistic vectors being responsible for transferring epigenetic information to the offspring have been identified, the most well-established ones being DNA methylation, histone modifications and non-coding RNAs (ncRNAs).

In this study, male mice were put on either high-fat diet (HFD) or low-fat diet (LFD), after which a metformin intervention was carried out. The main objective of this study was to control whether metformin caused significant disturbances in testis histology of studied mice. This study acted as a preliminary experiment for a greater study aspiring to investigate whether disadvantageous paternal epigenetic inheritance of metabolic disorders could be prevented using different interventions. To estimate the potential of metformin as a method to prevent unwanted paternal epigenetic inheritance of metabolic disorders, it is crucial that metformin itself will not cause defects in spermatogenesis. The second main objective of this study was to isolate germ cells for further ncRNA analysis included in the bigger study. Testis histology was studied in periodic-acid Schiff (PAS) as well as immunofluorescence-stained testicular tubule cross-sections. No critical defects in testicular histology of metformin-treated mice came across in comparison to other mice included in the study, therefore, metformin could be a potential intervention method in study settings aspiring to prevent disadvantageous paternal epigenetic inheritance. In addition, germ cell samples containing adequate amounts of wanted germ cell types (round spermatids) were isolated and can be used for further down-stream analysis.

Key words: paternal epigenetic inheritance, metformin, testis histology, spermatogenesis

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1. Introduction

1.1 Spermatogenesis in mouse

Spermatogenesis is a complex cellular differentiation process during which haploid spermatozoa are produced from diploid spermatogonial stem cells inside the seminiferous epithelium of the testis. The duration of spermatogenesis is specific for every species, lasting 35 days in mice (Oakberg, 1956). Generally, three subprocesses of mammalian spermatogenesis can be recognized: spermatogonial proliferation by mitosis and differentiation into spermatocytes, a meiotic phase that produces haploid spermatids from spermatocytes, and a spermiogenic phase, during which haploid spermatids undergo various morphological changes as they develop into compact spermatozoa (Leblond and Clermont, 1952). Spermatogenesis is regulated by various intrinsic and extrinsic factors, two of the most important ones being testosterone secretion by testicular Leydig cells, and the cell-cell interactions provided for the developing spermatids by Sertoli cells inside the seminiferous tubules of the testes. Leydig cells are somatic cells, and they locate in the testicular stroma, connective tissue that holds seminiferous tubules together. Their main function is to secrete testosterone under the influence of luteinizing hormone (LH) (Zhou *et al.*, 2019). Sertoli cells are somatic cells that maintain the structural integrity of the seminiferous epithelium with tight junctions as they form the blood-testis barrier, secrete diverse signaling molecules, discard cellular debris, and account for the openness of the seminiferous tubule lumen by fluid secretion (França *et al.*, 2016). In addition, they mediate the effects of follicle stimulating hormone (FSH) and testosterone to the germ cells.

In many mammalians, spermatogenesis is organized in a precise way so that certain differentiating germ cell types are present at the same tubular cross-section in different layers of the seminiferous epithelium. In mouse, there are 4 to 5 subsequent generations of germ cells differentiating synchronously at a given cross-section. When seminiferous tubule cross-sections are examined, these germ cell types appear inside the seminiferous epithelium adjacent to each other, i.e., certain spermatid types are always found with certain spermatocytes and spermatogonia. To simplify the cyclic functionality of spermatogenesis and aid the recognition of the various germ cell types inside seminiferous epithelium, a staging system has been established. Seminiferous tubule cross-sections can be staged according to changes in the morphology of developing germ cells and their associations to Sertoli cells, and every stage marks the presence of certain combination of spermatogonia, spermatocyte and spermatid types (Leblond and Clermont, 1952). In mouse, 12 stages (denoted by Roman numerals I-XII) can be defined (Oakberg, 1956) (**Figure 1**). However, staging the different phases of seminiferous epithelial cycle is a somewhat artificial method; the stages are not absolute, check-point-like steps of spermatogenesis, as spermatogenesis is an ongoing, cyclic process and germ

cells develop further in a constantly continuing manner. Staging is, nevertheless, a good tool for distinguish the various spermatid and spermatocyte types.

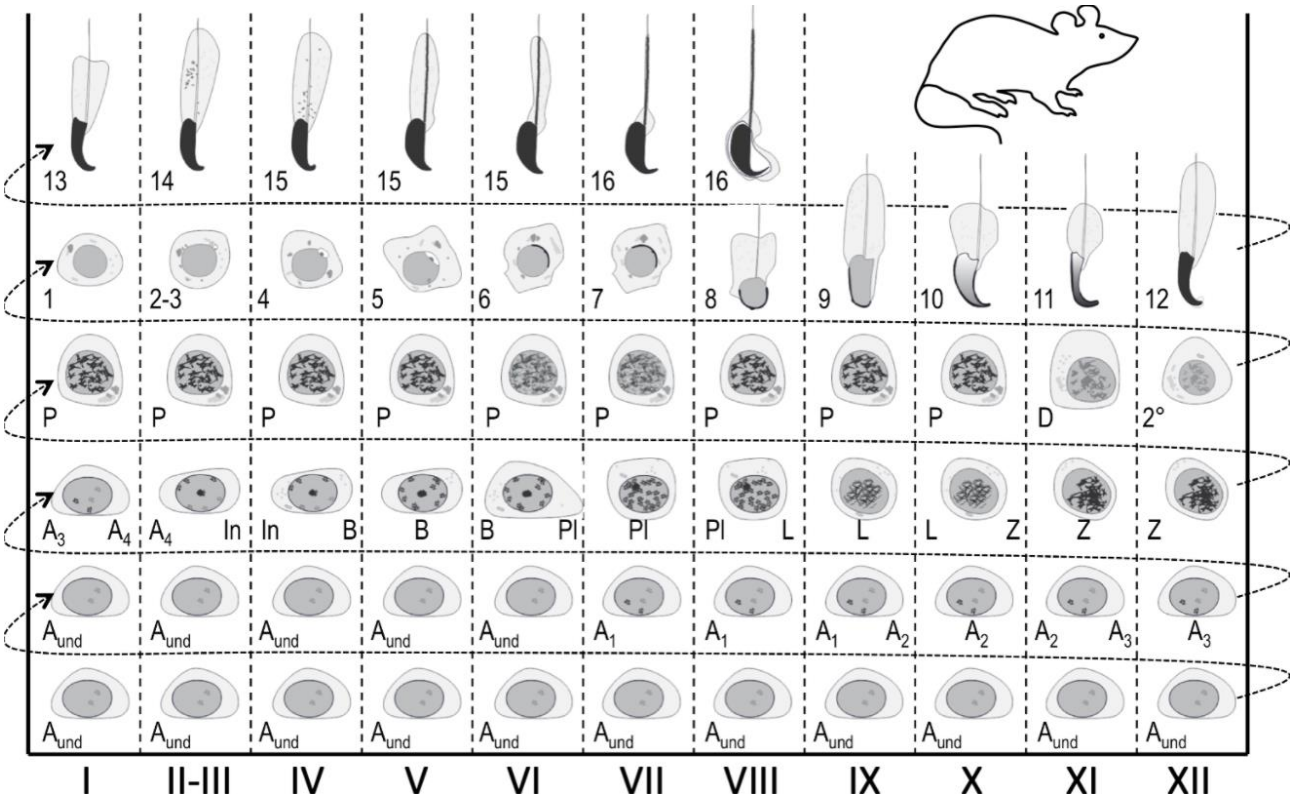

Figure 1. Spermatogenic cell differentiation and map for staging seminiferous tubule cross-sections in mouse (from Mäkelä *et al.*, 2020). Vertical columns represent the twelve stages (**I-XII**). To become released into the seminiferous tubule lumen as a mature elongated spermatid, a male gamete must pass through all depicted developmental phases (progressing from left to right and bottom to top) as well as 6 mitotic and 2 meiotic cell divisions. The cell associations in seminiferous tubule cross-sections appear in the same order as in vertical columns, hence, the most immature germ cells are at the bottom of the seminiferous epithelium. First, spermatogonial stem cells (SSC, not pictured) divide to form undifferentiated type A spermatogonia (**A_{und}**) that enter spermatogonial differentiation producing $A_{1–4}$, intermediate and B spermatogonia (**$A_{1–4}$, In, B**) that differentiate further into preleptotene spermatocytes (**Pl**), leptotone spermatocytes (**L**), zygotene spermatocytes (Z) and pachytene spermatocytes (**P**). After the diplotene phase (**D**), the two rapid meiotic cell divisions occur (**2°** marking the secondary meiotic cell division), producing haploid spermatids. The spermatids differentiate and mature into elongated spermatids in a process called spermiogenesis (**1-16**).

1.2 Paternal epigenetic inheritance

Controversial to the classical Mendelian theory of inheritance, experimental data using animal models combined with human epidemiological data have shown that ancestral environment affects the offspring's phenotype. The idea of environmental exposures being able to alter the phenotype inheritably was long met with skepticism, but today, data suggest that acquired yet heritable traits exist. For example, parental traumatic stress or nutritional challenges (overfeeding or famine) have shown to induce altered, sometimes disadvantageous phenotypes in the offspring, in both mice and humans (Kaati, Bygren and Edvinsson, 2002; Painter *et al.*, 2008; Fullston *et al.*, 2013; Veenendaal *et al.*, 2013; Gapp *et al.*, 2014; Cropley *et al.*, 2016). This phenomenon is today explained by (transgenerational) epigenetic inheritance, and the exact principles and mechanisms behind it are targets for intensive research.

As epigenetic germline inheritance is quite a recent finding, the terminology around it has not been fully established yet. Terms 'epigenetic germline inheritance', 'intergenerational/transgenerational epigenetic inheritance' or 'germline-dependent non-Mendelian inheritance' refer to the same concept: certain environmentally induced factors and processes that occur in germ cells can modify genome activity and regulation in the offspring, and therefore contribute to the phenotype of the progeny. It is crucial that these modifications take place in germ cells, otherwise they could not be inherited, and different environmental exposures and triggers induce them to appear. The word 'transgenerational' implies that these epigenetic modifications, or at least the phenotypes they induce, can be transferred all the way from parents to grandchildren, and potentially to further generations as well, even though the direct environmental trigger would vanish. The mechanisms underlying epigenetic germline inheritance are called epigenetic modifications, sometimes also epimodifications or epimutations. (Bohacek and Mansuy, 2015; Gapp and Bohacek, 2018; King and Skinner, 2020).

To date, research on mammalian epigenetic germline inheritance has mostly concentrated on epigenetic transmission via sperm cells, and exposure of fathers to various environmental factors, such as different diets, stress, or environmental toxins, has been a frequently used study design in animal studies. This is mainly because of practical reasons, as male germ cells are produced in abundance compared to oocytes and offer therefore more material for experimenting. Moreover, it is demanding to rule out the possible intrauterine or postnatal exposures contributing to the phenotype of the offspring when studying maternal line transmission. However, thanks to improved technologies, transmission of environmentally induced traits via maternal line has also become a more approachable study subject during the last few years. (Bohacek and Mansuy, 2017).

As many new concepts in research, germline epigenetic inheritance has faced some controversy. Questions have been proposed regarding the true transgenerationality of epigenetic inheritance. In most mammalian studies, acquired traits have been demonstrated to appear in F1 generation, which does not implicate that their transmission would be truly transgenerational, but rather intergenerational. What comes to studying paternal line transmission of acquired traits, only F2 and subsequent generations can be considered as evidence for truly transgenerational epigenetic inheritance (**Figure 2**). When F0 fathers are exposed to an environmental insult, their germline cells that eventually become the F1 generation are also directly predisposed. Hence, it is not possible to rule out the effect of direct exposure when examining the F1 progeny. Acknowledging the importance of this distinction is crucial, and data from epigenetic inheritance studies must be interpreted cautiously before generalizing on transgenerational effects. (Heard and Martienssen, 2014; Gapp and Bohacek, 2018; Fitz-James and Cavalli, 2022).

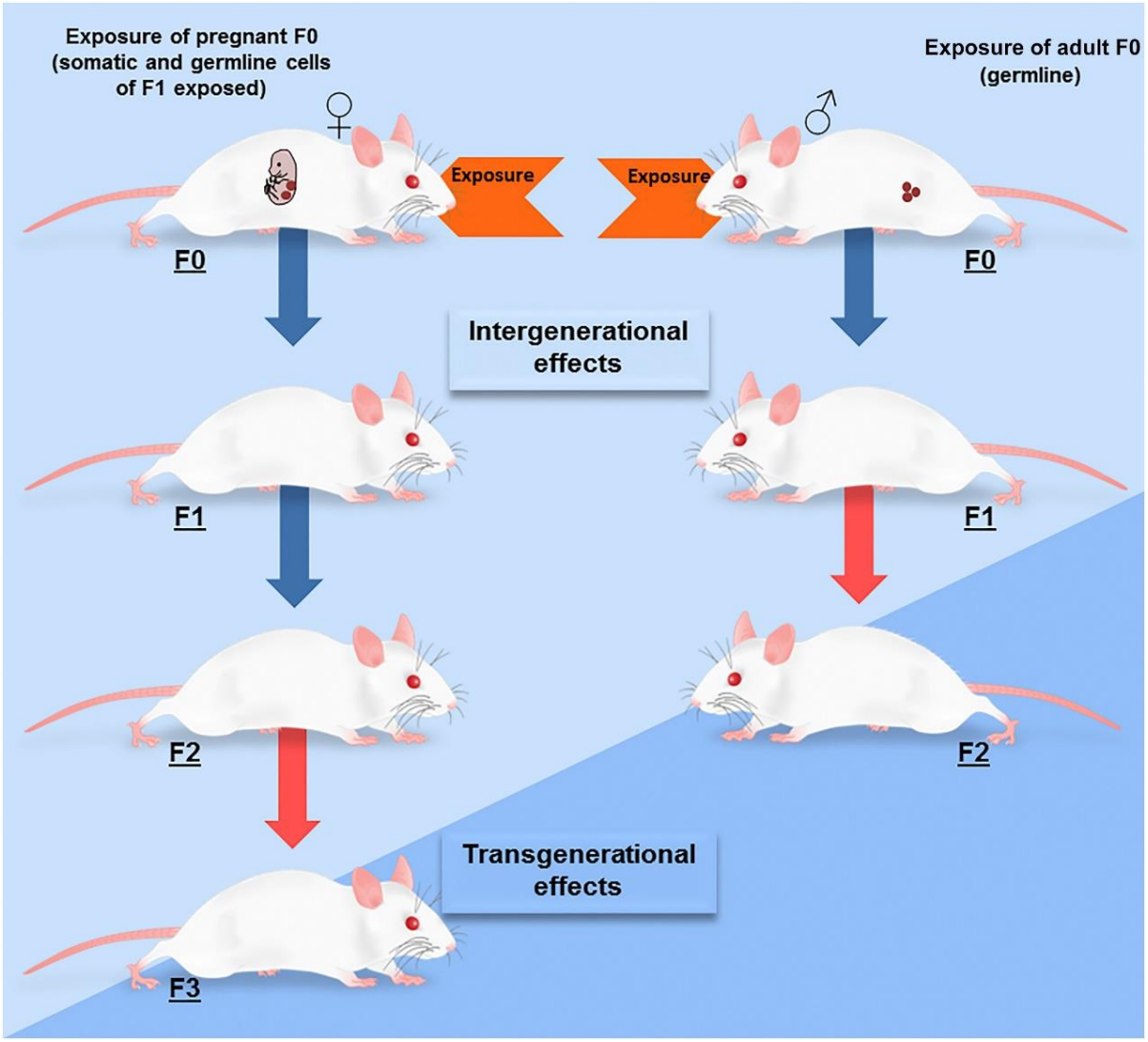

Figure 2. Intergenerational vs. transgenerational epigenetic germline inheritance in maternal (right) and paternal (left) germline (from Sales *et al*., 2017). When a mother is exposed to an environmental factor during pregnancy, the germ cells of the developing fetus that later form the F2 generation are also directly exposed. Therefore, only the effects on F3 generation are considered transgenerational when maternal epigenetic inheritance is studied. Paternal exposure leads to exposure of the sperm cells forming the future F1 generation, and therefore, only effects seen in F2 and subsequent generations are actually transgenerational.

1.2.1 Mechanisms of paternal epigenetic inheritance

At fertilization, what a father delivers to the oocyte is the spermatozoa. Traditionally, it was thought that sperm only affects the upcoming offspring by its haploid genome, as it is a transcriptionally inactive cell. However, today is known that sperm cells contain a unique epigenetic landscape composed of a distinctive DNA methylation profile, post-translational histone modifications and different subtypes of small RNAs, and that these epigenetic factors can contribute to the following descendant's phenotype (Casas and Vavouri, 2014; Bohacek and Mansuy, 2015).

Originally, the hunt for key mechanisms responsible for the transmission of acquired traits was focused on DNA methylation, a classic epigenetic mark and one way to transcriptionally silence a gene by covalently bonding methyl groups to DNA molecule. In vertebrates, methylation occurs primarily at the 5-position of cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides of the DNA sequence, and methylation of multiple CpG sites in a CpG island near the gene promoter results in gene silencing (Schübeler, 2015). It was already known that DNA methylation plays a role in genetic imprinting and X chromosome inactivation, but when evidence on heritability of environmentally induced traits began to accumulate, DNA methylation became the center of attention as a possible transgenerational epigenetic mechanism (Gapp and Bohacek, 2018). In sperm, DNA methylation is dynamically regulated and goes through phases of erasure and rearrangement during the development of the individual, however, there are certain areas in the chromatin where DNA methylation can escape these erasures (Bohacek and Mansuy, 2013), which makes the transmission of paternal methylation patterns possible. However, gathering proof of direct causal relations between certain DNA methylation patterns in sperm and certain phenotypes in the offspring is somewhat challenging, as sperm DNA methylation does not necessarily change dramatically in response to environmental exposures. Moreover, associations between DNA methylation and gene expression in sperm can be difficult to establish, as sperm cells are not very active transcriptionally (Bohacek and Mansuy, 2015).

However, a recent study managed to demonstrate causal transgenerational inheritance of DNA methylation in both maternal and paternal germ line (Takahashi *et al.*, 2023). Different DNA-methylation edited mouse models were generated in which either one of two metabolism-related genes, *Ankrd26* or *Ldlr*, was silenced by *de novo* DNA methylation in their promoter CpG islands. This led to either obesity or hypercholesterolemia in the studied mice, respectively. The methylation and phenotype status of the parent was found to be inherited up to F3 generation. The methylation status was transmitted to subsequent generations even though DNA methylation could become erased in primordial germ cell stage and re-established in embryonal stage. This study offers novel causal evidence of acquired DNA methylation as a vector for transgenerational epigenetic inheritance in mammals, and at the same time, suggests that yet unknown mechanisms of DNA methylation memory exist and need to be studied more.

In addition to DNA methylation, histone modifications are a well-established mechanism of epigenetic gene regulation. Histone proteins function as DNA packaging elements during different phases of cell cycle and control chromatin structure and function together with DNA methylation (Rothbart and Strahl, 2015). A key mechanism in their ability to control chromatin function is through post-translational modifications (PTMs). PTMs in histones cover for example acetylation, methylation, and ubiquitination, usually found in the N- or C-terminal of the protein. PTMs both alter the physical shape of chromatin and recruit various effector proteins, which can then lead to different downstream effects. However, sperm cells differ from somatic cells, as histones are mostly replaced by protamines during spermatogenesis, which indicates that the PTMs on replaced histones are lost (Bohacek and Mansuy, 2013). It is estimated that in mice, only 1-2% of histones are retained, whereas in humans 4-15% of histones remain in sperm cells. The PTMs of maintained histones can, however, be transferred to offspring, where they can contribute to embryonic development and gene expression of the progeny, possibly leading to inheritance of paternally acquired traits.

After discovering that mature murine sperm contains RNA in the early 1970s, the specific role of these RNA molecules has been a subject of great interest, as sperm was thought to be transcriptionally inactive. However, the presence of RNA in human sperm was fully and unquestionably established only in the late 1990s, as several laboratories published data from experiments using microarrays and RT-PCR, for example (Krawetz, 2005). To date, different subpopulations of the smallest, less than 40 nucleotides long RNAs, also called small non-coding RNAs (sncRNAs) have been recognized in both mice and human sperm (Grivna *et al.*, 2006; Krawetz *et al.*, 2011), microRNAs and tRNA

fragments being the most frequent ones associated with paternal epigenetic inheritance (Gapp and Bohacek, 2018).

First direct evidence for sperm RNAs being possible carriers for heritable phenotypes in the offspring was demonstrated already in 2006, in a mouse study in which a microinjection of certain sperm RNA fragments from transgenic mice (heterozygous for tyrosine kinase receptor Kit) into one-cell embryos induced a heritable white-tail phenotype in nearly 50% of the F1 offspring (Rassoulzadegan *et al.*, 2006). Moreover, this phenotype was efficiently transmitted to following generations in crosses with wild type partners. However, the true significance of this phenomenon was not fully understood until quite recent years, as more studies have now supported the role of sperm RNAs as operators in paternal epigenetic inheritance. One of the most notable studies demonstrated that alone sperm RNAs can transfer acquired phenotypes from parents to offspring, and these phenotypes can be inherited across generations (Gapp *et al.*, 2014). In this study, male mice were exposed to unpredictable maternal separation and stress during early life, leading to behavioral and metabolic alterations in the traumatized mice as well as their F1 and F2 progeny. Several microRNAs were found to be upregulated in the sperm of the early traumatized male mice, and microinjection of these recognized and purified sperm RNAs into wild type fertilized oocytes led to similar behavioral and metabolic alterations in the offspring, which provides evidence that sperm microRNAs can act as causal vectors for paternal epigenetic inheritance. After this, other laboratories have demonstrated the role of sperm RNAs in paternal epigenetic inheritance (Grandjean *et al.*, 2015; Rodgers *et al.*, 2015; Chen *et al.*, 2016). Direct causality has not yet been demonstrated with other mechanisms involved in epigenetic germline inheritance (Gapp and Bohacek, 2018).

Although the role of sperm RNAs in paternal epigenetic inheritance has been validated to some extent, there are some open questions remaining. It is not fully known how these RNAs may interact with other epimodifications during spermatogenesis to cause heritable changes in the germline. Many studies using zygotic RNA microinjections have not been able to induce all the aspects of a complex paternal phenotype in the offspring, which could indicate that sperm RNAs are just one link in a greater epigenetic process occurring during spermatogenesis and fetal development (Gapp and Bohacek, 2018). Therefore, it needs to be assessed in detail how sperm RNAs possibly crosstalk with other epimodifications.

Furthermore, another question is how the RNA profile of germ cells is generated and how it can adapt and adjust when the environment changes. There is research on the concept of extracellular vesicles

being a possible delivery mechanism for sncRNAs between somatic tissues and the spermatozoa. In a recent review, it is summarized that during their maturing in the epididymis, spermatozoa are in close interaction with different extracellular vesicles, both from epididymis and more distant somatic tissues (Sciamanna *et al.*, 2019). As a result, the RNA content of the mature sperm may become altered, because spermatozoa can take up these exogenous vesicles containing different types of RNA, further process the information they bring and, eventually, possibly transfer the information to offspring at fertilization. Considering the complexity of sperm RNAs, several unanswered questions remain regarding their function, nature, and involvement in paternal epigenetic inheritance, which is why more research on those themes must be conducted.

1.2.2 Epigenetic inheritance of acquired metabolic disorders

During previous decades, the worldwide prevalence of a major metabolic disorder, obesity, has increased critically in adults and adolescents, and children are also increasingly affected. Recently, the Finnish Institute for Health and Welfare (THL) published new statistics demonstrating that of Finnish adults over 30, 63% of women and 72% of men are overweight or obese (body mass index (BMI) over 25 kg/m² or more; THL, 2020). Overweight and obesity are associated with several additional pathological conditions, such as type 2 diabetes (T2DM), coronary heart disease and nonalcoholic fatty liver disease (Obesity in children, adolescents, and adults, Current Care Guidelines Abstract, 2023). Traditionally, energy-dense foods and massively reduced physical exercise have been accused of being the most consequential factors leading to the obesity pandemic. However, in the U.S. during 1988-2006, the predicted BMI increased by 2.3 kg/m² although at the same time, the frequency of leisure time physical activity more than doubled (Brown *et al.*, 2016), which indicates that other mechanisms than just inadequate exercise are likely involved. Genetic variants have been speculated to have a role in the pathophysiology of obesity, however, based on a meta-analysis of large genome-wide association studies, it has been estimated that genetic variants associated with obesity account only for approximately 2.7% of BMI variation between individuals (Locke *et al.*, 2015). These findings indicate that some aspects of the etiology of obesity and its associated metabolic disorders remain undiscovered.

What comes to the etiology of another acquired metabolic disorder, T2DM, obesity and ageing are the single most important risk factors, but in addition, intrauterine environment has been speculated to have a significant role. For instance, both high (due to maternal hyperglycemia) and low (due to undernourishment) birth weight are associated with increased risk for T2DM later in life. In addition, certain genetic variants have been associated with increased susceptibility to T2DM, however, they

do not anticipate the incidence of T2DM any better than clinical risk factors or family history (Chen *et al.* 2012). Altogether, obesity and T2DM are complex, multifactorial diseases and their pathogenesis is contributed by both genetic and environmental factors, and it is likely that epigenetic inheritance contributes somehow to the etiology and origin of these metabolic disorders. Deepening our knowledge on the transmission of acquired traits via epigenetic pathways could offer tools to cut down the increasing numbers of obesity and diabetes and reduce the global disease burden.

In animal studies, modifications in offspring behavior, stress reactions and metabolic processes are among the most studied examples of paternal epigenetic inheritance (Fullston *et al.*, 2013; Gapp *et al.*, 2014; Grandjean *et al.*, 2015; Rodgers *et al.*, 2015; de Castro Barbosa *et al.*, 2016). To induce these kinds of phenotypes in offspring, study designs use various models to mimic common environmental exposures (diet, toxins, stressful experiences, for example) that affect the epigenetic status of paternal gametes. What comes to studying acquired metabolic disorders, such as obesity and impaired glucose tolerance, the most frequently used study designs include high fat diet (HFD) to induce a potentially epigenetically inheritable phenotype in F0 fathers (Fullston *et al.*, 2013; Grandjean *et al.*, 2015; Cropley *et al.*, 2016).

To date, several rodent studies have demonstrated the significance of paternal high fat or western-like diet in relation to changes in adipose tissue mass, glucose tolerance or insulin secretion in the offspring, and that the effects of paternal HFD can be transgenerational. For instance, paternal HFD induced obesity in adult female F1 offspring throughout their life, with increased adiposity up to the age of 39 weeks and increased levels of circulating plasma lipids, despite of the offspring being maintained on a control diet. F1 male offspring had impaired glucose tolerance from 8 weeks up to 26 weeks of age, as did the F1 females that also had impaired insulin sensitivity from 16 weeks of age. In F2 generation, females sired by F1 males had insulin resistance and increased adiposity, whereas F2 males did not. F2 males born from F1 mothers had increased body weight from 14 days of age and represented impaired glucose tolerance at 8 and 14 weeks of age (Fullston *et al.*, 2013). In a rat study, feeding a HFD to F0 fathers led to F1 and F2 female progeny showing signs of impaired metabolic functions in response to a metabolic challenge which was given in a form of HFD during adulthood. Both F1 and F2 females from HFD fathers were resistant to HFD-induced weight gain compared to their respective controls, and F2 females showed impairments in glucose tolerance, as well as decreased insulin levels (de Gastro Barbosa *et al.*, 2016). In a mouse study, neonatal overgrowth was induced by decreasing the number of pups to 4 per mother during lactation in F0 generation. By the age of 4 months, F0 mice had developed signs of metabolic syndrome, including

obesity and insulin resistance. Male offspring sired by F0 fathers also showed signs of metabolic syndrome, although the phenotype was more moderate; males in F2 generation developed only fasting hyperglycemia and glucose intolerance (Pentinat *et al.*, 2010). These results indicate that high fat diet induces metabolic alterations in the progeny, and that some of the effects could be transgenerational.

Besides the data on potentially inheritable acquired phenotypes, evidence for possible mechanisms behind paternal epigenetic inheritance of acquired metabolic disorders has emerged during recent years. In a study aspiring to investigate how HFD-induced traits get transferred from fathers to offspring, male mice were fed a western-like diet and then, their sperm and testis RNAs were collected and microinjected into wild-type one-cell embryos (Grandjean *et al.*, 2015). This led to the establishment of the high-fat phenotype (higher body weight, altered glucose tolerance, impaired insulin sensitivity) in the offspring, whereas these features were not evident in offspring born after microinjection of RNAs extracted from healthy controls. What was also found in this study is that there were differences between western-like diet mice and control mice in testis and sperm sncRNA composition, and that injection of just one of the deregulated microRNAs (miR-19b) into one-cell embryos induced obesity in the offspring. Moreover, in the same rat study mentioned earlier, HFD-fathers and their F1 male progeny had similar spermatozoal sncRNA signatures and DNA methylation patterns (de Castro Barbosa *et al.*, 2016). These results therefore strongly suggest that sperm epigenome becomes deregulated when paternal diet consists of high percentages of fat, and that the profile of sncRNAs in sperm changes with the nutritional environment. Furthermore, sperm sncRNAs may act as a vector for paternal epigenetic inheritance of acquired metabolic disorders.

1.2.3 Paternal epigenetic inheritance in humans

In humans, one of the best-known examples of parental environment contributing to the phenotype and later morbidity of the offspring is the Dutch famine in 1944-45, during which a great number of individuals suffered from poor nutrition and undernourishment. Many epidemiological studies have utilized a cohort of people born during the famine to investigate the later health of prenatally undernourished individuals and their children. Malnutrition during fetal life has been shown to have far reaching consequences on offspring health. For example, children of parents born during the famine had increased neonatal adiposity and poorer health in later life than their unexposed controls (Painter *et al.*, 2008), and adult offspring of males that had been exposed to the famine prenatally had higher BMIs and body weights than the offspring of unexposed fathers (Veenendaal *et al.*, 2013). Other often used cohort of people originate from Överkalix, Northern Sweden, where the availability of food was strongly depending on yearly harvests. Using regional registers, studies have been able

to investigate the effect of poor or inadequate diet on later health and morbidity among the progeny of the exposed individuals. A study focusing on cardiovascular and diabetes related outcomes discovered that father's restrained access to food during slow growth period (years 9-12 of age, before prepubertal peak in growth) resulted in lowered cardiovascular death rates among their progeny (Kaati *et al.*, 2002). These are examples of environmental exposures affecting the health of the offspring (F1 generation) also in humans, not direct evidence on germline epigenetic inheritance. However, it is interesting to speculate whether there could be a nutrition-linked epigenetic transmission process occurring behind all this.

In 1992, Barker and Hales introduced a theory considering the etiology of T2DM in humans, suggesting that malnutrition in fetal and early infant life could cause underdevelopment and dysfunction of beta cells of the pancreas, possibly leading to diabetes later in life (Hales and Barker, 2013, reprint). This theory is also known as the 'thrifty phenotype hypothesis', and although it was not supported by direct data on the possible mechanistic process behind it, epigenetic modifications are one plausible explanation. However, it remains undetermined what kinds of changes undernourishment or food oversupply could induce in the gametes of the prenatally exposed individuals, and whether these epimodifications are transferred to the offspring leading to establishment of metabolic disorders. The Dutch famine and Överkalix cohorts of people are extraordinary, and therefore it is not likely that these findings could be replicated, nor could their mechanistic background be confirmed in independent studies. In addition, demonstrating causal relations between human sperm epigenome and offspring phenotype is difficult, as other forms of transmission (genetic, cultural, ecological) are likely involved and not easily excluded. Thus, transgenerational epigenetic inheritance in humans remains an interesting and debated idea with no direct mechanistic proof, for now at least.

Although there is no certainty whether the acquired paternal metabolic status could be delivered to offspring via sperm epigenetic modifications in humans, it has been shown that as in mice, the environmental exposures can alter the epigenome of human sperm as well. When the effect of weight loss on the epigenetic status of human spermatozoa was studied, it was found that the expression level of certain specific sncRNA fragments was altered and an impressive set of genes (9 081 in total) were differentially methylated in the sperm of lean and obese men (Donkin *et al.*, 2016). In the same paper, studying the spermatozoal DNA methylation in obese men before and after bariatric surgery (Roux-en-Y gastric bypass) revealed that one year after the procedure, 3 910 unique genes were differentially methylated compared to controls. The mean BMI of the subjects had shrunken from 42.6 to 33.9 one

year after the operation. Differences in DNA methylation and sncRNA profiles indicate that weight loss certainly influences the epigenetic marks in human sperm. Even though establishing transgenerational epigenetic inheritance in humans is difficult, the fact that human sperm epigenome is prone to environmental exposures might indicate that the transmission of those epigenetic modifications to offspring could be possible. Animal studies and new technologies can offer novel approaches to this intriguing question in the future.

1.3 Metformin

Today, metformin is the most common initial drug for patients with T2DM. It is preferred as a first-line treatment by several guideline committees, including the Finnish Current Care Guideline committee that recommends metformin treatment to be started instantly when a patient is diagnosed with T2DM (Type 2 diabetes, Current Care Guidelines Abstract, 2020). Several things support the role of metformin as first-line antidiabetic treatment: it has low costs and good effectivity also in monotherapy. In addition, metformin is a somewhat safe drug, as it does not cause hypoglycemia as an adverse effect, and per os administration makes it quite effortless for the patient. Metformin can be combined with other most common antidiabetic drugs, since it does not increase the risk of hypoglycemia, and has its very own mechanism of action (Sanchez-Rangel and Inzucchi, 2017.) Moreover, it has been demonstrated to lower both basal and postprandial plasma glucose (Gong *et al.*, 2012).

Metformin is not metabolized in humans, but excreted unchanged in the urine, and this active secretion in renal tubules is its main elimination route. The molecular basis behind metformin's pharmacodynamical effects have not yet been unquestionably validated. What is agreed on is that metformin induces the phosphorylation and therefore activation of AMP-activated protein kinase (AMPK) in the liver, although AMPK activation apparently is not crucial for metformin to function. Nevertheless, metformin's main pharmacological effect is that it suppresses gluconeogenesis and lipid synthesis in the liver, which leads to lower plasma glucose levels. There is evidence for other potential mechanisms of action, too, as metformin has been speculated to increase glucose uptake by skeletal muscle cells, increase insulin sensitivity and fatty acid β-oxidation, and possibly even reduce food intake and glucose absorption in the intestine. Moreover, there is discussion on whether metformin may have tumor suppressive abilities. (Gong *et al.*, 2012).

In addition to metformin working as a plasma glucose lowering agent, it has been speculated to have cardiovascular benefits. In a systematic meta-analysis of 13 studies including a total of 2079 subjects,

metformin did not statistically significantly decrease all-cause mortality or cardiovascular outcomes (myocardial infarction, peripheral vascular disease, stroke, cardiovascular death) in patients with type 2 diabetes, however, all outcomes except stroke favored metformin (Griffin *et al.*, 2017). Another matter of speculation is whether metformin has any impact on weight loss among patients with obesity predisposing them to T2DM. In a quite recent systematic review and network meta-analysis including 34 trials and 8461 subjects, it was concluded that metformin significantly decreased body mass index percentile (Hui *et al.*, 2019), however, some previous reviews do not fully establish metformin's ability to reduce obesity (Levri *et al.*, 2005; Min *et al.*, 2009). In conclusion, metformin has potential to act as weight loss promoting agent, and if anything, it is a weight neutral drug.

The most common adverse effects of metformin are gastrointestinal (GI) tract related, such as nausea, diarrhea, or mild abdominal pain. These effects are dose-related and titrating the dose may reduce them. However, as much as 50% of patients on metformin medication have been shown to possibly have these GI tract-related side effects, and approximately 5% of patients cannot tolerate metformin because of them. Another possible adverse effect of metformin is B12-vitamin malabsorption, which can be prevented by oral supplements during metformin treatment (Sanchez-Rangel and Inzucchi, 2017.)

What comes to severe adverse effects of metformin, it has the potential to cause lactic acidosis, especially in patients with impaired renal function. This is due to metformin's elimination happening mainly via renal clearance, and therefore reduced glomerular filtration rate (GFR) can lead to accumulation of metformin in the body and subsequent acidosis (Sanchez-Rangel and Inzucchi, 2017). Lactic acidosis is a potentially lethal, but very rare side effect, however, it must be considered, and therefore in patients with estimated GFR (eGFR) in the range of 30–60 ml/min/1,73 m², the dosage of metformin should be reduced (Type 2 diabetes: Current Care Guidelines Abstract, 2020).

1.3.1 Metformin and male reproductive health

As described above, metformin is used to manage hyperglycemia in patients with T2DM that itself has been associated with reduced fertility in males. However, there is some controversy regarding the effects of metformin on male reproductive health. As summarized by Faure and colleagues, prenatal exposure to metformin in mice has been associated with decreased fetal testicular size and less Sertoli cells (Faure *et al.*, 2018). Yet, in humans, in utero exposure to metformin has not been described as acutely harmful to the fetus (Gestational diabetes, Current Care Guidelines Abstract, 2013). In addition, a follow-up study of prepubertal male offspring born to mothers with metformin-treated

gestational diabetes did not find any difference in testis volume compared with male offspring of mothers treated with insulin (Tertti *et al.*, 2016). All in all, data from both human and animal studies suggest that metformin may have sperm and testis health improving abilities, especially among obese individuals, as metformin can restore some of the decrease in spermatozoa concentration and motility that obesity itself causes (Faure *et al.*, 2018). In humans, metformin may enhance the pulsatile secretion of LH from the pituitary gland and regulate steroidogenesis. In conclusion, metformin can be viewed as a potentially fertility improving agent, depending on the dose and species used.

Of special note is one study investigating the possible fertility protective role of metformin in rats fed with HFD (Yan *et al.*, 2015). In this study, it was found that treatment with metformin was protective against the HFD-induced weight gain and decrease in the number of spermatogonia, Sertoli cells and Leydig cells. In addition, metformin treatment improved semen parameters (concentration, viability, motility, morphology) in HFD rats, as well as restrained the increase in serum glucose, insulin, and leptin. These effects have possibly transmitted through metformin-induced weight loss, but metformin may have direct testicular effects as well. However, metformin's effect on epigenetic modifications in sperm has not been significantly studied yet. It is interesting to speculate whether metformin could reverse potentially adverse epigenetic changes in the sperm of fathers with a poor diet, for example, and thus prevent the transmission of disadvantageous paternal traits to offspring.

2. Aim of the experimental part

The main objective of this study was to find out whether metformin caused significant disturbances or harmful effects on testicular histology and spermatogenesis in mice. In addition, the second objective of this study was to isolate round spermatid populations from mouse testes in a process called modified density gradient for round spermatids (MDR) (Da Ros *et al.*, 2019).

This study acted as a preliminary experiment for a greater study aiming at discovering whether paternal epigenetic inheritance of disadvantageous metabolic disorders (obesity and impaired glucose tolerance, for example) could be prevented using different interventions. The studied interventions (healthy diet, exercise, metformin) were carried out to examine whether the adverse changes that high-fat, western-like diet causes to mouse sperm epigenome could be reversed. In addition, the effect of the intervention on offspring's metabolic phenotype and sperm epigenetic signature was explored, and the possible inter- and/or transgenerational inheritance of metabolic disorders was investigated.

To explore metformin as an intervention method in a study setting aspiring to clarify whether disadvantageous paternal epigenetic inheritance could be prevented, one must first be certain of its testicular safety. Therefore, this study aimed at ruling out metformin's possible effects on seminiferous tubule histology in studied mice. Testis histology was examined in periodic-acid-Schiff (PAS) and hematoxylin-stained sections of the testes, and the testicular histology findings were further confirmed with immunofluorescence (IF). The second objective, isolation of round spermatids using the MDR protocol, was carried out to ensure experimental material for further analysis of sncRNAs and other epigenetic modifications in the F0 father's germ cells.

3. Materials and methods

3.1 Animal experiments

8-week-old male C57B1/6N mice were divided into high-fat diet group (HFD, n = 8) feeding on diet high in fat, and low-fat diet group (LFD, n = 10) feeding on low fat diet (**Figure 3**). HFD (Research Diets) contained 60 % of energy in fat, whereas LFD (Research Diets) contained 10 % energy in fat but had otherwise same nutritional values as high fat diet. Diet started when mice were 8 weeks old, lasting 8 weeks (**Figure 3**). At this point, metabolic parameters (body weight, body composition, glucose tolerance, insulin levels) were measured. Then, metformin intervention started (HFD-Met, n = 4; LFD-Met, n = 4) and rest of the mice stayed on HFD (n = 4) or LFD (n = 6). Metformin was given p.o. for 6 weeks, with aimed dosage of 300 mg/kg/day. After the metformin intervention, the same metabolic parameters were measured again. Before collecting tissue samples for analysis, F0 mice mated with C57Bl/6N females to produce the F1 and later F2 progeny. Finally, the founder male mice were euthanized, and their testes were collected. In addition, several other samples were collected, including serum, epididymal fat and liver, as they were used for metabolic and RNA analysis included in the bigger study. In this study, only the testes were utilized.

Animal experiments were carried out at the Central Animal Laboratory of University of Turku, Turku, Finland. Mice were housed under 12 h light / 12 h dark cycle and maintained at 21±3°C in specific pathogen-free conditions. Animal husbandry and use were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, were following Finnish laws, and incorporated the 3Rs principle. All experiments were authorized by the National Animal Experiment Board.

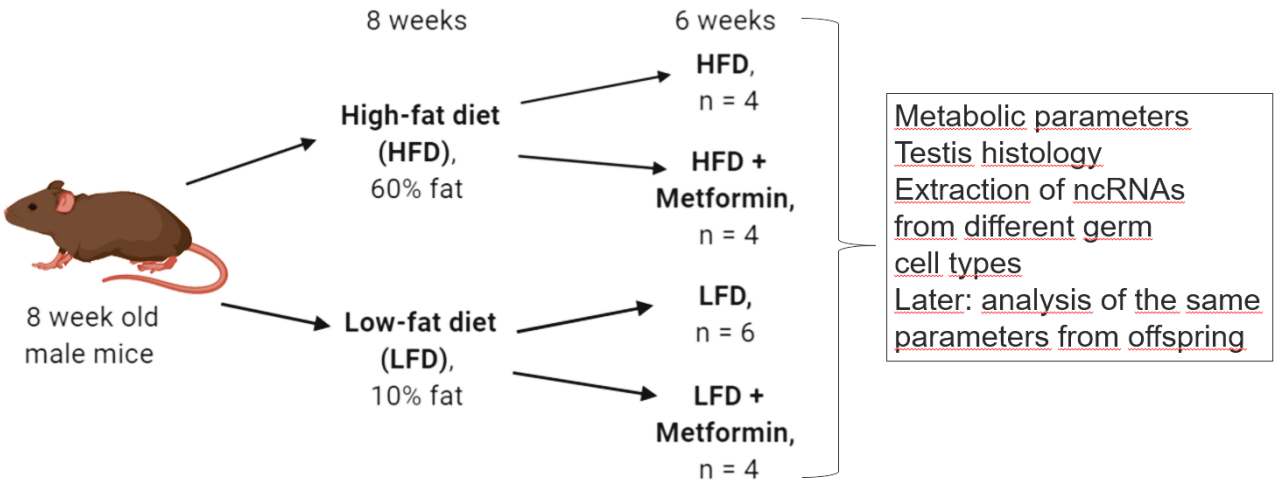

Figure 3. An illustration of the mouse experiment (produced with BioRender, 2020). Male mice fed either HFD or LFD for 8 weeks, followed by 6-week-long metformin intervention (HFD-Met, n = 4 and LFD-Met, n = 4). Rest of the mice from both groups remained on HFD or LFD (HFD, n = 4 and LFD, n = 6). Before tissue collection, male mice mated with C57Bl/6N females producing F1 generation, and the F0 metabolic parameters (body composition, glucose tolerance, insulin levels, for example) were determined. In this study, the collected testes were used to investigate possible differences in testicular histology and to isolate different germ cell types for downstream analysis.

3.2 Histological staining of the testis cross-sections

To evaluate whether metformin caused significant defects or changes in the testicular morphology of the studied mice, testicular tissue sections were stained using PAS and hematoxylin stain. Testes were fixed with Bouin's solution (Sigma-Aldrich) overnight at room temperature (RT) in gentle rocking and embedded in paraffin blocks. Then, testes were cut into 4 µm thick histological sections with a microtome (Leica) and overlaid on microscopy slides. Slides were stored at 4 ºC and allowed to warm up in room temperature before staining. Testicular sections were deparaffinized and rehydrated by passing them through xylene, 100%, 90% and 70% alcohols for 4 minutes each, followed by washing with mQ-H_2O 3 times for 2 minutes. After this, slides were soaked in 1% periodic acid for 10 minutes. After rinsing with tap water for 10 minutes and washing with mQ-H_2O for 3 minutes, slides were soaked in Schiff's reagent (Merck KGaA) for 45 minutes, rinsed with warm tap water for 10 minutes, washed with mQ-H_2O for 3 minutes, and counterstained with Mayer's hematoxylin (Merck KGaA) for 5 minutes. After rinsing with tap water and washing with mQ-H_2O for 2 minutes, the sections were dehydrated by passing them through increasing alcohol gradient (70%, 90%, 100% alcohols) and xylene, 4 minutes each. Finally, testis samples were mounted with Pertex mounting medium (Histolab) and a coverslip was placed on them. The stained microscopy slides were scanned using

Pannoramic 250 digital slide scanner (3DHistech). Files were converted to TIFF file format using a dedicated software (https://www.3dhistech.com/caseviewer). Images were processed with Photoshop (Adobe).

3.3 Immunofluorescence staining of the testis cross-sections

Testes used for IF stains were fixed with 4% paraformaldehyde (PFA) overnight, embedded in paraffin blocks and cut with a microtome (Leica) into 4 µm thick histological sections that were overlaid on microscopy slides. For paraffin removal and rehydration, the samples were passed through xylene and decreasing alcohol gradient (100%, 90% and 70% alcohol), followed by washing with mQ-H_2O. For antigen retrieval solution, 0.1 M citrate buffer (pH 6.0) was used, and the retrieval was performed in a pressure cooker. Antigen blocking was performed with blocking solution (10% BSA in 0.1% PBS-Tween), for 1 hour at RT, before incubating the samples in primary antibody against Espin (1:500, BD Biosciences, 611656) at 4 ºC overnight. After washing 3 times with 0.1% PBS-Tween, the samples were incubated in secondary antibody (goat anti-mouse, 1:1000, Alexa Fluor 488, Life Technologies, A32723) and rhodamine-conjugated peanut agglutinin (PNA, 1:2000, Vector Laboratories, RL-1072-5) in dark for 1 hour at RT. The samples were washed 3 times with 0.1% PBS-Tween and once with PBS and then stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:20 000 in PBS, Sigma-Aldrich, D9542) for 5 minutes at RT and washed with PBS and mQ-H_2O for 5 minutes each. Finally, the samples were mounted with mounting medium containing DAPI (Prolong Diamond Antifade Mountant, Life Technologies, P36971), a coverslip was placed on them, and the slides were imaged with Pannoramic MIDI digital slide scanner (3DHistech). Files were converted to TIFF using a dedicated software (see above) and images were processed with Photoshop (Adobe).

3.4 MDR

Some of the collected testes were utilized to isolate different germ cell types in a protocol called modified density gradient for round spermatids (MDR) (Da Ros *et al.*, 2019). MDR protocol enables the enrichment of the three most abundant cell types in mouse testis: pachytene spermatocytes, round spermatids, and elongating spermatids, using only standard laboratory equipment. Studying testicular cell populations separately is challenging, as the adult mouse testis consists of a complex set of germ cells accompanied by somatic Sertoli cells, and the isolation techniques for these cell types have required quite a lot of expertise. However, the MDR protocol makes the cell population-based approach more achievable. The basis of the protocol is a discontinuous bovine serum albumin (BSA) density gradient through which the germ cells are allowed to sediment. The velocity by which cells sediment depends on the size of cells. Larger cells (pachytene spermatocytes) move through the BSA

gradient faster than smaller cell types (round and elongating spermatids). Therefore, enriched amounts of round spermatids can be collected from the top of the BSA gradient.

To start the MDR protocol, dissected testes were digested into a germ cell suspension. This digestion phase was optimized for two testes. The dissected testes were stored in 1X Krebs solution (bicarbonate buffer) until decapsulation. After decapsulation, seminiferous tubules were treated with collagenase IV solution (1 mg/ml in 1X Krebs buffer, Sigma) in a 37 ºC water bath for 3 minutes. The purpose of collagenase IV was to separate seminiferous tubules by removing interstitial cells. Then, released tubules were washed twice with warm (34 ºC) 1X Krebs buffer, and the specimen was allowed to sediment for 1 minute at RT. The supernatant was discarded, and trypsin (0,6 mg/ml, Worthington) and DNAse I (>3,2 ku/ml, Worthington) solution (in 1X Krebs buffer) was added onto the cells. The specimen was incubated in the trypsin/DNAse I solution for 15-20 minutes at 34 ºC on a rotator to release cells from the tubules, and placed onto ice once the solution became cloudy. The cell solution was then filtrated through a 40 µm cell strainer, added up to 50 ml with ice cold Krebs buffer, and centrifuged 600 x g for 5 minutes at 4 ºC. The supernatant was discarded, and the same washing step with cold Krebs buffer was repeated. After the second wash, 1 ml of 0,5% BSA in 1X Krebs buffer was added, into which the cell pellet was resuspended. Finally, the solution was added up to 4 ml of volume with 0,5% BSA in 1X Krebs and filtrated through a 40 µm cell strainer before loading it onto the BSA gradient.

To prepare the discontinuous BSA gradient, BSA solutions with different weight per volume concentrations (1%, 2%, 3%, 4%, 5% in 1X Krebs buffer) were prepared. The BSA gradient was built into a 50 ml Falcon tube at 4 ºC, using a 1 ml pipette tip. To moderate the pipetting velocity, the tip of the pipette was cut at about 5-10 mm from the tip. The discontinuous BSA gradient was built by slowly pipetting a total volume of 5 ml of each BSA solution on top of the previous layer, starting with 5% BSA in 1X Krebs and proceeding towards smaller concentrations. Finally, the single-cell suspension in 0,5 % BSA in 1X Krebs was loaded on top of the gradient. The cells were allowed to sediment through the gradient for 1,5 hours at 4 ºC.

To collect the fractions containing enriched amounts of different germ cells, a 1 ml pipette tip was cut at about 5-10mm from its tip. The cut tip was placed right on top of the gradient surface, and a fraction was collected by slowly suctioning it into the pipette, at the same time being careful not to disturb the gradient. Fractions of approximately 1 ml were collected into separate 1,5 ml tubes and numbered from 1 to 25, in the same order as they were collected. The collected germ cell fractions were centrifuged at 600 x g for 8 minutes at 4 ºC. The supernatant was mostly discarded, and the cell pellet was resuspended. 900 µl of ice cold 1X Krebs buffer was added into each tube, and the centrifugation was repeated. After the washing, most of the supernatant was discarded, and the cell

pellet was resuspended carefully into the remaining solution (approximately 100 µl). All cell fractions were kept on ice to decrease the cell damage and breakage.

To ensure the purity of cell fractions, a sample slide of each fraction was prepared for microscopy. Using a grease pen, a ring was drawn on numbered microscopy slides for each fraction, and 2 µl of the corresponding cell solution was added with 4% PFA for fixation. The slides were dried overnight at RT. The next day, slides were washed once with 1X PBS and mounted with mounting medium containing DAPI (Vectashield Antifade Mounting Medium with DAPI, H-1200-10). The nuclear morphology of germ cells from each fraction was analyzed (Leica DMRB microscope with Olympus DP72 digital color camera and cellSens Entry 1.5 digital imaging software). Using 40x objective lens, cells in 4-5 visual fields were calculated to estimate the enriched germ cell type in the fraction. 80% or more of round spermatids was considered as adequate purity percent for downstream analysis.

After taking 2 µl sample from each fraction for purity analysis, 1 ml of ice cold 1X Krebs buffer was added into each tube, and the cell fractions were centrifuged at 13 000 x g for 10 minutes at 4 ºC. The supernatant was removed, and the cell pellets were snap frozen in liquid nitrogen and stored at -70 ºC for later downstream analysis.

3.5 Statistical analysis

Statistical analysis was performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) using two-way ANOVA, and $p < 0.05$ was considered statistically significant.

3. Results

3.1 HFD mice gained more weight than LFD mice, but the weight gain was smaller in HFD-Met group

To monitor the weight change of mice during the diet period and metformin intervention, the body weight was measured weekly from beginning of the diet until the end of metformin treatment. A two-way ANOVA was performed to analyze the effect of diet and metformin treatment on body weight change (**Figure 4**). Diet had a significant effect on body weight change, as mice on HFD gained statistically significantly more weight than LFD mice ($p < 0.0001$). Metformin also had a significant effect on body weight change, as the 6-week-long metformin intervention statistically significantly reduced the weight gain ($p = 0.0229$). Therefore, metformin treatment during HFD prevented further weight gain.

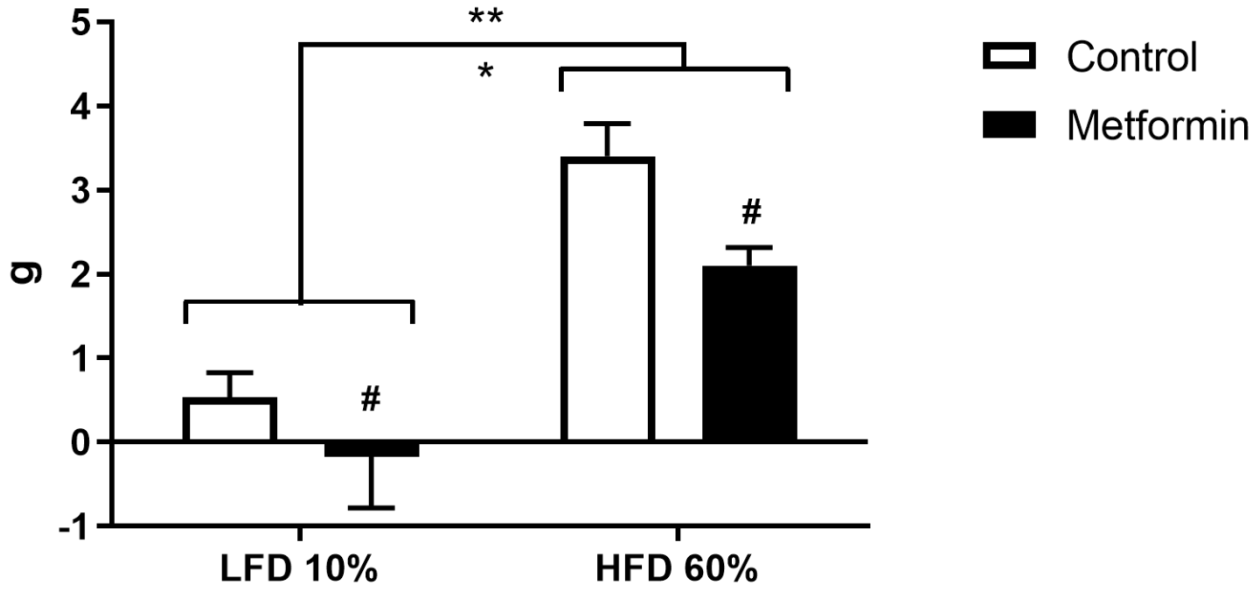

Figure 4. Two-way ANOVA of the effects of diet and metformin on body weight change during the mouse experiment (8-week diet period and 6-week metformin intervention). Y-axis = body weight change (g). Data shown as means. White columns represent the body weight change of control mice on LFD (10 % energy in fat, right) and HFD (60 % energy in fat, left). Dark columns represent the body weight change of metformin treated mice in both groups, respectively (LFD-Met and HFD-Met). Diet had a statistically significant effect on body weight change (**, $p < 0.0001$), as the body weight increased more if the mouse was on HFD than LFD. Metformin treatment also had a statistically significant effect on body weight change (*, $p = 0.0229$), as the increase of body weight was smaller in HFD-Met group than in HFD group. The interaction of diet and metformin treatment

was not statistically significant (#, p = 0.4642). This indicates that metformin had weight gain neutralizing impacts during high fat diet.

3.2 Metformin did not cause critical defects in testis histology

To examine if metformin had critical adverse effects on spermatogenesis, testis histology was examined in PAS and hematoxylin counter-stained seminiferous tubule cross-sections. PAS stain was selected, because it stains the acrosomes in developing sperm cells, which helps distinguishing different spermatid types and the stages of the seminiferous epithelium. A representative image from every group and relevant seminiferous epithelial stage (II-V, VII-VIII, IX-XI) was selected to evaluate possible defects in seminiferous tubule formation (**Figure 5**). Comparing the four groups, no major differences in testicular histology came across. In all groups, relevant stages of the seminiferous epithelial cycle were identified, and no crucial morphological defects were observed.

Some inter-individual variation occurred, as some HFD mice presented more defects in testicular histology than others (**Figure 6**). However, in all four groups, spermatogenesis was active and during the seminiferous epithelial cycle, spermatozoa were seen to gather in the middle of the seminiferous tubule before their release into the lumen (stage VII-VIII; **Figure 5**). Therefore, metformin did not cause critical disruptions in spermatogenesis, but HFD might have had some disadvantageous effects on seminiferous tubule morphology and sperm production.

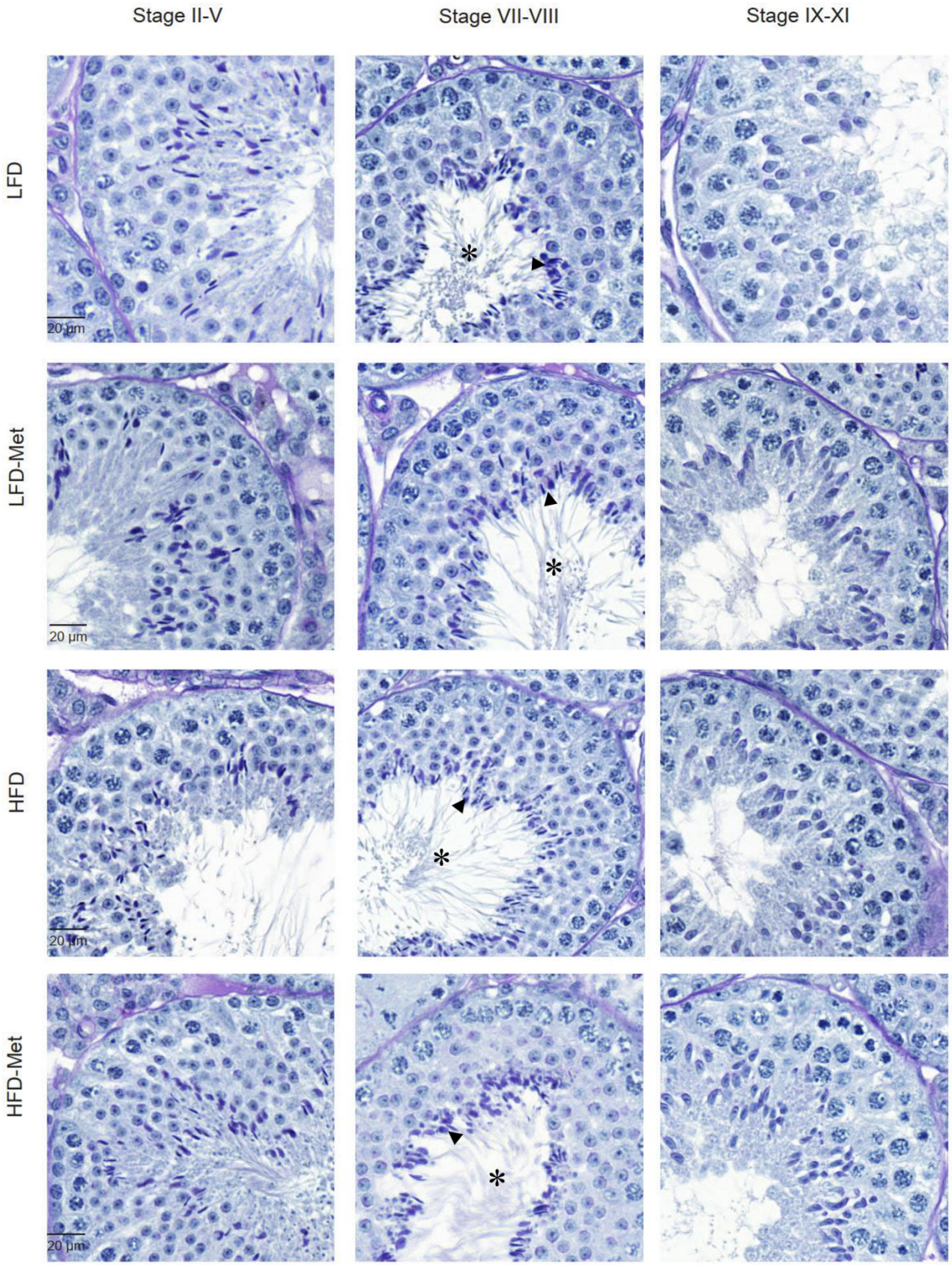

Figure 5. A representative image of PAS and hematoxylin-stained testicular tubule cross-sections from every group, classified according to stages of seminiferous epithelium (every vertical column represents different stage; II-V, VII-VIII, IX-XI). In every group, there is ongoing spermatogenesis,

as in stage VII-VIII spermatozoa (black arrowhead) have gathered in the middle of seminiferous tubules before spermiation, with their tails reaching out into the tubule lumen (*). The structure and organization of the seminiferous epithelium is consistent in every group, and no major differences or defects come across. Scale bar = 20 μm.

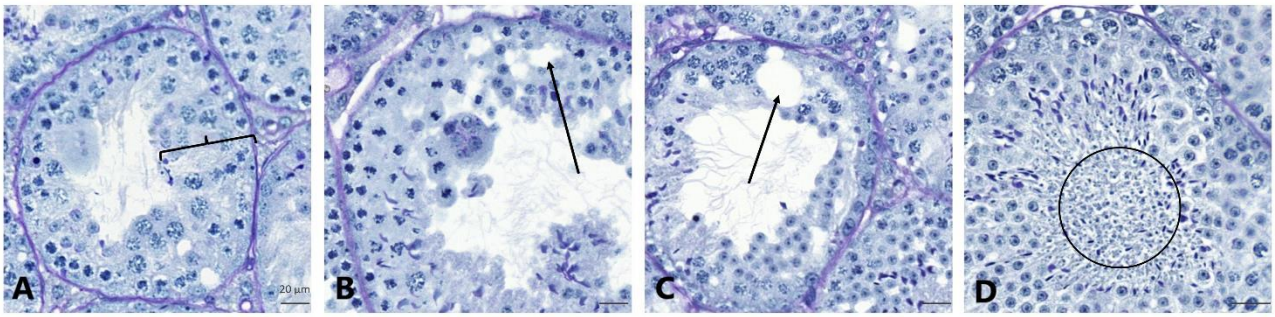

Figure 6. Examples of disrupted seminiferous epithelium organization in HFD mice. **A:** the structural organization of the seminiferous epithelium (square bracket) is lost, and only a few spermatids are visible. **B** and **C:** there are empty areas (arrows) inside the seminiferous tubules where germ cells have underwent cell death. **D:** the seminiferous tubule lumen is full of cellular debris (circle). Scale bar = 20 μm.

To further confirm the testicular histology findings and the normal organization of the seminiferous epithelium, IF staining of the testis tissue sections was done using PNA and Espin antibody DAPI for nuclear stain. PNA binds to the acrosome, which helps the further visualization of developing spermatid types. Espin stains the cell−cell junctions between Sertoli cells, as well as between Sertoli cells and elongating spermatids, and therefore allows better recognition of the seminiferous epithelium organization and blood-testis barrier. Espin and PNA were chosen because they allow the visualization of seminiferous epithelium in an almost histology-like manner while offering more detailed structural information. Inspecting the Espin, PNA and DAPI stained IF testicular tubule sections, no major differences or defects came across in seminiferous epithelium organization or acrosome formation (**Figure 7**). This finding further validated that metformin did not critically disrupt with spermatogenesis or seminiferous tubule organization.

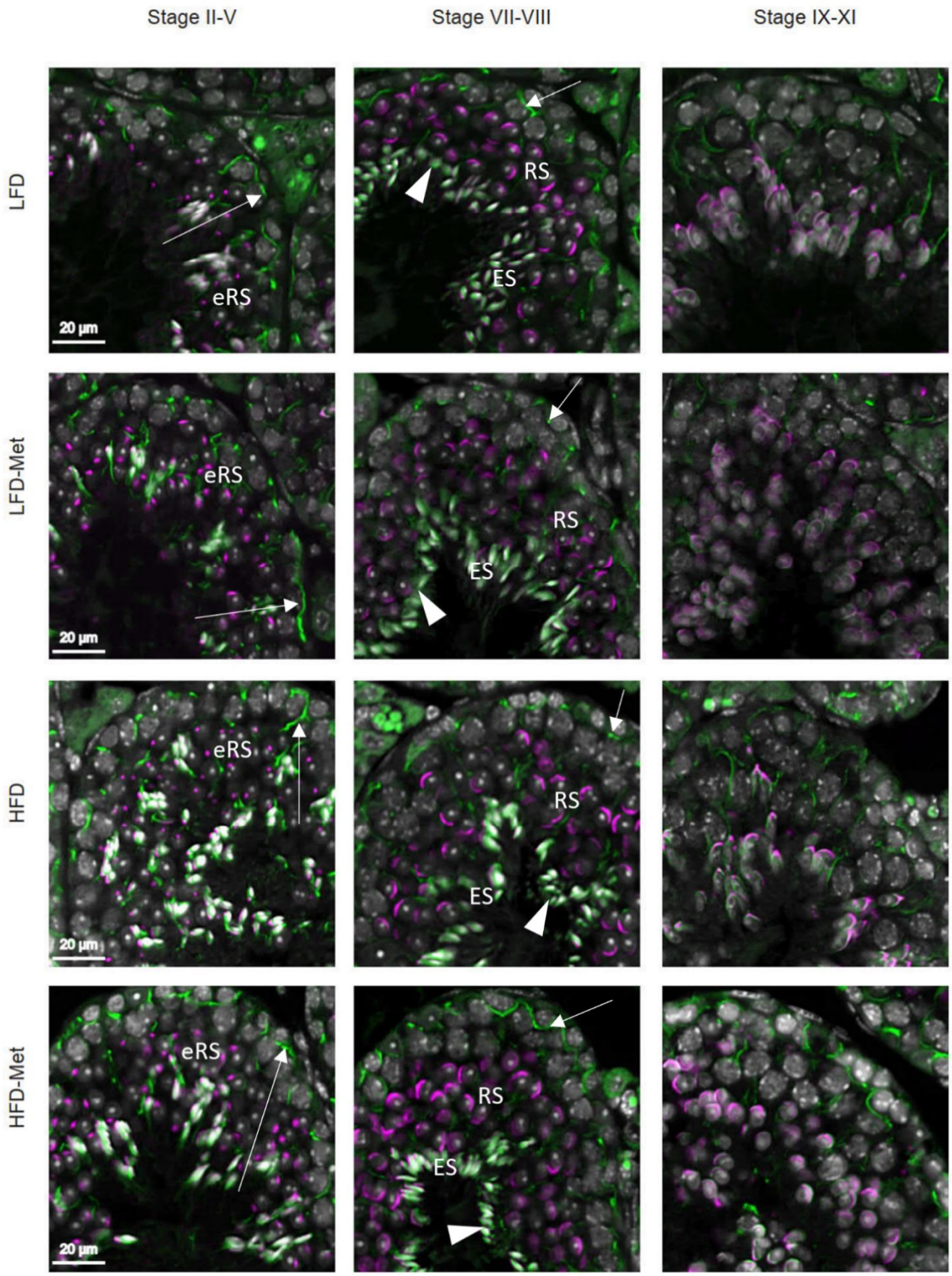

Figure 7. IF stain of testicular tubule cross-sections with PNA (pink; acrosome) and Espin (green; cell-cell junctions) and DAPI (gray) for nuclear staining. From every group, a representative image

was selected for comparison and classified according to early (II-V), middle (VII-VIII), or late (IX-XI) stage of seminiferous epithelium. Pink represents the acrosome which is small and roundly formed in early-stage round spermatids (stage II-V, eRS), and grows and flattens when the round spermatids develop further (stage VII-VIII, RS). Green areas represent the cell-cell junctions between Sertoli cells, forming the blood-testis barrier (arrows), and between Sertoli cells and elongating spermatids (arrowheads), which is necessary for the spermiogenesis. Inspecting the four groups, no major defects or differences come across, and elongated spermatids (ES) can be seen gathering in the middle of seminiferous tubules (stage VII-VIII). Scale bar = 20 μm.

3.3 MDR protocol allowed the extraction of round spermatids

The second main objective of this study was to isolate round spermatids using the MDR protocol. Isolated round spermatids could later be used to analyze the sncRNA content and other epigenetic modifications in the germ cells of studied mice, which is relevant for the objectives of the greater study. The success of the MDR protocol was validated by taking a sample of each isolated germ cell fraction for DAPI staining, and the enriched cell type in each fraction was determined by studying the nuclear morphology of the cells. Purity of the fractions collected from the top of the discontinuous BSA gradient was of special interest, as those fractions were most likely to contain highest concentrations of round spermatids. Adequate sample quality for later analysis was considered 80% or more of round spermatids. The cell percentage estimate was calculated by counting the cells in 4 or 5 visual fields with 40x lens (150-200 cells). In every group, cell fractions containing over 80% round spermatids were collected (**Figure 8**). MDR protocol was therefore successful.

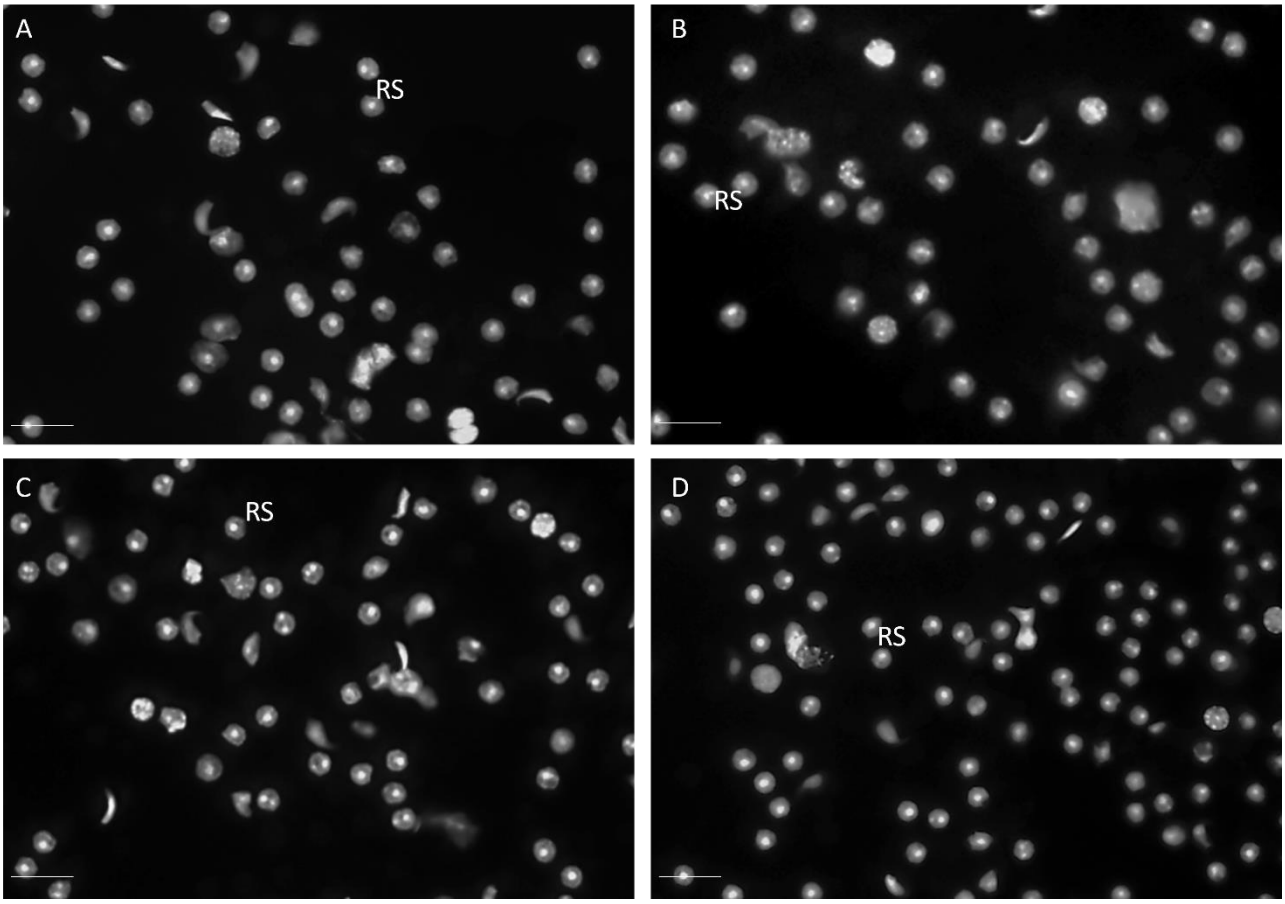

Figure 8. DAPI stained samples of cell fractions collected in the MDR protocol. In every group, cell fractions in which the most abundant (80% cells or more) cell type was round spermatid were collected. Using the DAPI stain, round spermatids (RS) appear as small and evenly round nuclei, with a brighter round center inside the nucleus (the chromocenter). A = HFD, B = HFD-Met, C = LFD, D = LFD-Met. Scale bar = 10 μm.

4. Discussion

The initial objective of this study was to investigate if metformin had any critical adverse effects on spermatogenesis in studied mice. In the bigger picture, metformin was studied as a possible intervention method to prevent paternal epigenetic inheritance of disadvantageous metabolic disorders. Therefore, metformin's possible adverse effects on spermatogenesis had to be excluded. In every mouse group, seminiferous tubule composition was normal in PAS and hematoxylin-stained testis samples, and no major disturbances came across. This finding was further validated with IF staining. These results indicate that metformin does not act as a critical spermatogenesis disrupting agent and, accordingly, is a potential intervention in study settings investigating how paternal epigenetic inheritance of metabolic disorders could be prevented. However, these results focus mainly on seminiferous tubule histology, which does not exclude the possibility of sperm motility

malfunctions, for example. If one wanted to have a deeper insight into possible impairments in spermatogenesis during metformin treatment, more detailed methods and statistical analyses would be required. In addition, bigger data would be necessary to ensure statistical power.

Secondly, another main objective of this study was to isolate round spermatids from diet and metformin-exposed male mice for further downstream analysis of epigenetic markers, such as sncRNAs. Isolation was done using the MDR protocol. The success of the protocol was controlled with DAPI stained cellular samples of collected cell fractions. In every group, fractions containing 80 % or more of round spermatids were collected. Therefore, the MDR protocol was productive, and samples with 80 % or more round spermatids can be used in further analyses.

An interesting side note in this study was that HFD mice seemed to have slightly more defects in seminiferous epithelium organization compared to other mice. Thus, it is not likely that metformin disrupted the spermatogenesis critically but, rather, it is HFD that may have disadvantageous effects on sperm production. Moreover, metformin seemed to decrease the amount of these defects, as HFD-fed mice treated with metformin did not present quite as many disturbances in seminiferous epithelium organization as HFD mice. However, this observation was made only by looking through the prepared microscopy slides and was not quantified. This observation is consistent with previous research on obese mice having more testicular defects and disturbances in testicular cell formation than lean mice (Oliveira *et al.*, 2017). As summarized by Oliveira and colleagues, obese mice have been shown to have more morphological changes in testicular cells in comparison with lean mice, possibly due to increased permeability of the blood-testis barrier. In addition, it has been previously described that male mice on HFD successfully mate with fewer females and therefore produce less progeny than male mice on control diet, and that F1 male progeny of these founder HFD mice have reduced sperm motility and increased sperm DNA damage, compared to offspring of mice on control diet (Fullston et al., 2013). Moreover, HFD may have far-reaching consequences, as high fat diet has been described to significantly alter the metabolic status of testes and affect the viability and motility of mouse sperm, and that these changes persevered even when diet was switched back to normal (Crisóstomo *et al.*, 2019).

All in all, little is still known about paternal epigenetic inheritance and the mechanisms behind it, but data is building up. Novel discoveries in the field of sperm epigenetics will possibly widen our knowledge on paternal epigenetic inheritance of acquired metabolic disorders, which could further lead to discovering new prevention strategies for T2DM, prediabetes and obesity, for example. In

addition, studying epigenetic inheritance and its mechanisms could offer explanation for the globally increasing incidence of diabetes and obesity.

In the future, study of sperm epigenetics can change our outlook on parental counseling before impregnation, as the health of paternal gametes at the time of conception has previously been quite a neglected subject, and the guidance has been mostly fixated on maternal health. It is interesting to speculate whether epigenetic signatures in paternal gametes could one day act as a predictive marker to assess the risk of metabolic diseases in the offspring.

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