Paternal programming of offspring kidney and metabolism disease

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

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1 Introduction

1.1 Conception of fetal programming

“Fetal programming”, also known as “Developmental Origins of Health and Disease (DOHaD)”, is a concept in medical and health research that explores how environmental factors during prenatal and early postnatal development can exert profound and enduring effects on an individual's long-term health and disease risk throughout their life (1, 2). It suggests that the conditions and experiences a fetus is exposed to during gestation can shape its development and predispose it to certain health outcomes in adulthood. The early evidence supporting developmental programming emerged from epidemiological studies in humans, notably the research conducted by Barker and Clark in 1997 (3), which focused on pregnant women experiencing severe malnutrition during gestation due to wartime food supply shortages.

Fetal programming is based on the idea that the prenatal period is a pivotal timeframe characterized by vulnerability and adaptation. During this time, the maturing fetus displays heightened sensitivity to various environmental elements, including maternal nutrition, stress, toxins, hormones, and more. These factors can influence the fetus's development, altering its physiology, metabolism, and organ structure (4, 5). Fetal programming recognizes the plasticity of fetal development, meaning that the fetus can adapt to the conditions it experiences in utero. This adaptability allows it to optimize its chances of survival in the specific environment it expects to encounter after birth (6, 7). The fetus may make adaptive responses to adverse conditions, which can involve altering gene expression, hormone levels, and organ development (8). These adaptations are beneficial for immediate survival but may have long-term consequences.

Maternal diet during pregnancy is a significant factor. Inadequate nutrition (malnutrition) or excessive nutrition (overnutrition) can lead to fetal programming effects (9). For example, maternal undernutrition can increase the risk of metabolic disorders like obesity and diabetes in the offspring(10). In addition, maternal stress during pregnancy, whether psychological or physiological, can affect the fetus. Stress hormones and other factors can impact brain development and the stress response system in the offspring (11). Moreover, exposure to environmental toxins, such as smoking, alcohol, and certain pollutants, can disrupt fetal development and lead to health problems later in life (12). Last but not the least, hormonal

imbalances during pregnancy, including gestational diabetes, can contribute to fetal programming effects.

Fetal programming can result in a wide range of health outcomes, and these outcomes are often linked to the specific environmental exposures and conditions experienced by the developing fetus during pregnancy. The potential health outcomes associated with fetal programming are listed as follows: **(1) Metabolic Disorders**: a. Obesity: Fetal exposure to maternal overnutrition, especially high-calorie and high-fat diets, can program the developing fetus to have an increased number of fat cells and altered metabolism (13-15). This can lead to a higher risk of obesity in childhood and adulthood. b. Type 2 Diabetes: Fetal programming can be a contributing factor in the development of insulin resistance and compromised glucose metabolism, heightening the risk of later-life onset of type 2 diabetes. c. metabolic syndrome: Fetal programming may be linked to the emergence of metabolic syndrome, encompassing a constellation of conditions such as abdominal obesity, elevated blood pressure, heightened blood sugar level, and abnormal lipid profiles (11, 16, 17). **(2) Cardiovascular Conditions**: a. Hypertension (High Blood Pressure): Fetal exposure to maternal factors like poor nutrition or maternal stress can lead to altered blood vessel structure and function, increasing the risk of hypertension in adulthood (6, 18, 19). b. heart disease: Fetal programming may contribute to the development of atherosclerosis (hardening of the arteries) and an increased susceptibility to heart disease. c. stroke: Individuals exposed to adverse conditions during fetal development may have a higher risk of stroke later in life due to changes in blood vessel health(20). **(3) Neurological and Behavioral Issues**: a. Cognitive Deficits: Fetal programming can impact brain development and neural connectivity, potentially leading to cognitive deficits and learning difficulties (21, 22). b. Behavioral Problems: Some studies suggest that fetal exposure to maternal stress or malnutrition might be connected to an elevated likelihood of behavioral problems and mental health disorders such as anxiety and depression (23, 24). **(4) Immune System Dysfunction**: Altered Immune Responses: Fetal programming can influence the development of the immune system, potentially leading to an altered immune response and increased susceptibility to infections or autoimmune diseases. **(5) Endocrine Disorders**: Hormonal Imbalances: Fetal exposure to certain factors can disrupt the endocrine system, leading to hormonal imbalances that affect growth, puberty, and reproductive health (25-28). **(6) Epigenetic Changes**: Altered Gene Expression: Fetal programming often involves epigenetic changes, such as histone modifications, DNA methylation and non-coding RNA regulation, that can permanently alter gene expression patterns. These

changes can influence various aspects of health and disease risk (7, 29, 30). **(7) Renal (Kidney) Dysfunction**: Kidney Disease: Fetal programming can affect kidney development and function, potentially increasing the risk of kidney disease later in life (31). It's important to note that the specific health outcomes associated with fetal programming can vary based on the nature and timing of the prenatal exposures, genetic factors, and other individual and environmental factors (32, 33). Additionally, while fetal programming may increase the risk of certain health conditions, it doesn't guarantee that an individual will develop these conditions; it simply raises the susceptibility. Preventive measures, including a healthy maternal lifestyle during pregnancy, can help mitigate these risks and promote better lifelong health for the offspring.

In summary, fetal programming is a compelling concept that underscores the significance of the early environment in shaping an individual's lifelong health. It emphasizes the need for a holistic approach to maternal and child health, aiming to provide the best possible conditions for fetal development and reduce the risk of developing chronic diseases in later life.

While a significant portion of scientific research has traditionally focused on understanding how the maternal environment during pregnancy impacts the future health of offspring, recent investigations have expanded their scope to consider the experiences of the father in the lead-up to conception and during the early stages of reproduction (1, 2, 34, 35). Specifically, researchers are looking at three critical phases: spermatogenesis (the process of sperm production), the period when sperm reside in the epididymis (a duct where sperm mature), and the formation of seminal fluids in the accessory reproductive glands (36). These aspects collectively contribute to what is now termed "paternal programming."

“Paternal programming” represents a groundbreaking concept in the field of developmental biology. It acknowledges that factors affecting the male parent, such as his nutrition, exposure to stressors, and overall health, can exert a significant influence on the health and well-being of his future offspring (1). This paradigm shift underscores the idea that the health of offspring isn't solely determined by maternal factors but is also intricately linked to the paternal side.

In summary, paternal programming is a cutting-edge area of research that delves into the intricate processes occurring within the male reproductive system and their potential impacts on the next generation. It represents a paradigm shift, expanding our understanding of the factors that shape

offspring outcomes and highlighting the importance of considering both maternal and paternal influences on development.

1.2 Paternal risk factors on offspring health

1.2.1 Paternal nutrition and diet

Paternal nutritional status wields a profound influence that extends well beyond its impact on early embryonic development. Extensive research in animal models has illuminated the far-reaching consequences of both paternal overnutrition and undernutrition for the overall well-being of their offspring.

High fat diet

Ng et al. (37) delved into the effects of inducing a high-fat diet on the F1 generation. Their hypothesis centered on the potential for fathers to initiate an intergenerational transmission of metabolic disorders as well as obesity via exposure to a high-fat diet. In their study, male rats were divided into two groups, one on a high-fat diet and the other on a control diet and were subsequently paired with females on a control diet. The male rats on the high-fat diet exhibited a constellation of adverse outcomes, including heightened body weight, elevated adiposity, increased energy intake, augmented liver mass, higher levels of plasma leptin, in addition to glucose intolerance and insulin resistance when compared to their counterparts on the control diet. Strikingly, the female offspring mirrored these adverse effects and displayed elevated blood glucose levels, diminished insulin secretion, and notable reductions in β-cell functionality and islet area, reflecting a striking impact stemming from their fathers' dietary choices (37). Masuyama et al. provided compelling evidence that the offspring born to male rats with a high-fat diet demonstrated a metabolic syndrome-like profile. This included characteristics such as increased body weight, heightened fat accumulation, glucose intolerance, increased total triglyceride levels, elevated leptin levels, and reduced adiponectin concentrations. Astonishingly, this phenomenon persisted across two successive generations, underscoring its enduring impact (38). As supported by Ng et al. (39) and elucidated by McPherson et al. (40), male mice that were exposed to a high-fat diet prior to conception, resulted in their female offspring displaying increased insulin resistance and greater accumulation of adipose tissue. Notably, dietary interventions and exercise for fathers proved effective in ameliorating the metabolic health of their female progeny.

Furthermore, Fullston et al. (41) demonstrated that when male mice were exposed to a high-fat diet, leading to obesity, it induced a unique transgenerational phenotypic pattern marked by impaired glucose tolerance and insulin resistance in their male and female descendants alike.

In a previous research endeavor, we subjected male rats to a diet mirroring the unhealthiness of fast-food, characterized by high levels of fats, carbohydrates, and salt—typically consumed by young men. Our investigation aimed to explore whether the detrimental consequences of a paternal unhealthy diet before reproduction on the offspring could be mitigated through folate treatment administered either to the mothers or the fathers before conception. Our findings unveiled a noteworthy outcome: when dams received folate treatment, it effectively alleviated the adverse impacts on the metabolic health of their female offspring. This improvement can be partly credited to the protection of beta cells induced by folate, along with the restoration of normal hepatic connective tissue density in the female offspring. Additionally, folate treatment acted as a corrective agent, reversing the dysregulation observed in Ppara, Tmcc2 and Lcn2 gene expression, and restoring normal levels of liver total DNA methylation (42).

Low protein diet

A paternal low protein diet can have a range of consequences for the health of offspring, both during fetal development and into postnatal life. In a study conducted by Watkins and Sinclair (43), it was found that adult offspring of male mice fed low protein diets experienced substantial impairments in their cardiovascular and metabolic health. These effects included impaired glucose tolerance, vascular dysfunction, and increased fat accumulation in adulthood. Similarly, the impact of a paternal low protein diet is particularly notable in its effects on lipid metabolism signaling pathways, as revealed by Carone et al. in their 2010 study (44). In addition, A specific study has provided evidence that a paternal low protein diet can lead to the downregulation of multiple genes related to lipogenesis in the white adipose tissue of offspring. This down-regulation of genes involved in fat synthesis may result in reduced lipogenesis and potentially elevate the risk of metabolic disorders in the offspring (45). Importantly, these deviations in growth patterns and metabolic profiles observed during fetal development continue to manifest in postnatal life, underscoring the enduring consequences of paternal dietary choices.

Undernutrition

In a mouse model, subjecting the fathers to a 70% caloric restriction, which is intended to replicate the nutritional conditions often seen in developing countries, had notable effects on their male offspring. Specifically, this undernutrition resulted in several notable outcomes, including dyslipidemia (abnormal lipid levels), accumulation of body fat, changes in the expression of pancreatic genes, and a decrease in body weight in the offspring (46). Likewise, a study involving rats found that a 25% reduction in paternal caloric intake had significant effects on the adult offspring. These effects included an increase in anxiety-like behaviors, a decrease in food intake, a lower levels of serum leptin and reduced weight gain (47). Consistently, experiments in which male mice underwent food deprivation prior to conception resulted in decreased blood glucose levels. Additionally, this paternal food deprivation triggered alterations in corticosterone (a stress hormone) and insulin-like growth factor 1 (IGF-1) levels, affecting both male and female offspring (48). What's particularly intriguing is that the researchers found that when undernourished fathers were provided with vitamin and antioxidant supplements, it had a normalizing effect on the weight and growth of their offspring (46). This highlights the potential for dietary interventions in fathers to mitigate some of the negative consequences of undernutrition on the well-being of their offspring.

1.2.2 Obesity

The influence of obesity on male reproduction, particularly concerning sperm and semen parameters, remains an area of ongoing research. Obesity in men has been associated with several factors that can adversely affect fertility. One significant effect of obesity in males is a notable decrease in testosterone levels. This decline is partially linked to increased levels of circulating hormones, including leptin, estrogen, and insulin, which have the potential to disrupt gonadotropin secretion and hinder the spermatogenesis process (2, 49). Additionally, excess scrotal adiposity, which is common in obese individuals, has been linked to reduced sperm motility and an elevation in DNA damage and oxidative stress (50). Furthermore, studies have shown alterations in the metabolomics and proteomics of seminal fluid in men with obesity. These alterations could potentially contribute to the observed irregularities in sperm characteristics, such as reduced sperm concentration, motility, semen volume, and an increased incidence of abnormal sperm morphology (51, 52). Obesity in men also appears to affect the integrity of sperm membranes and DNA because of increased levels of reactive oxygen species (ROS) in suboptimal-functioning mitochondria.

These ROS have been linked to a greater DNA fragmentation index, aberrant methylation patterns in sperm and abnormal chromatin condensation, all of which are known to compromise sperm quality (52-54). Importantly, the relationship between obesity and sperm quality has implications for fertilization rates and the development of embryo. Obesity in men has been linked to extended time to achieve conception, reduced fertilization rates, impaired sperm capacitation, and decreased sperm binding to the oocyte. When fertilization does occur, studies in both animals and humans have shown a slowdown in the embryo development, often accompanied by abnormalities in the activation of the paternal genome (55, 56).

Obesity in men has been linked to a range of concerning effects on offspring, particularly when it comes to their weight, abdominal measurements, and distribution of adipose tissue. Interestingly, these effects tend to manifest more prominently during the early stages of childhood (57). One intriguing finding is that offspring born to obese fathers frequently display hypomethylation in specific imprinted loci, which are linked to serious conditions like cardiovascular diseases, neurological disorders, and cancer (58). In animal studies, particularly in mice, it has been observed that offspring from obese fathers can inherit certain health issues across generations. These issues encompass glucose intolerance, insulin resistance, and heightened adiposity, enduring for as long as two generations. This indicates the engagement of both genetic and epigenetic mechanisms in the inheritance across generations (41). One critical aspect of this transgenerational impact lies in the sperm itself. Sperm isolated from obese mice has demonstrated distinct histone H3 occupancy patterns at genes associated with the regulation of the formation and development of embryos (59). More recently, research has unveiled another layer of complexity in this phenomenon. When specific non-coding RNAs extracted from the sperm of obese males are introduced into normal zygotes, they have been observed to impact the metabolic well-being of the offspring during adulthood, which further emphasizes the role of epigenetic mechanisms in transmitting health-related traits to the next generation (60, 61). In essence, obesity in males appears to have far-reaching implications for the health of their offspring, involving both genetic and epigenetic mechanisms. These findings underscore the importance of understanding how lifestyle factors, such as obesity, can impact not only individual health but also the health and well-being of future generations.

1.2.3 Diabetes mellitus

Penesova et al. in their 2010 study (62) showed that children born to fathers who had diabetes before 35 of age tended to be leaner. Additionally, these children exhibited reduced early insulin secretion. This suggests a potential link between paternal diabetes onset at a younger age and certain metabolic characteristics in their offspring. Furthermore, Myklestad et al.(63) in their study involving a substantial sample of 14,000 families identified a connection between low birth weight in children and adverse metabolic indicators in fathers, such as elevated BMI, blood pressure, and blood glucose levels. Some other studies also confirmed that paternal diabetes was linked to an elevated risk of low birth weight in their children (64-67).

Wang et al. (68) established a noteworthy positive correlation between parental diabetes and the occurrence of type 2 diabetes (T2DM) in their descendants. Notably, this association was particularly pronounced in overweight individuals. Interestingly, the study revealed that the risk of T2DM was more strongly linked to paternal diabetes than maternal diabetes in this context. In the study from Praveen et al. (69), offspring born to a family with T2DM exhibited notably higher BMI (Body Mass Index) compared to those without such a family history. Additionally, these offspring displayed elevated levels of plasma insulin, proinsulin, and C-peptide, indicative of altered insulin dynamics. Importantly, they also demonstrated lower β-cell compensation and reduced insulin sensitivity. Interestingly, the study did not find significant distinctions between the offspring born to diabetic mothers and those born to diabetic fathers. What's more, according to the findings of Linares Segovia et al.(70), the offspring born to parents with diabetes displayed the highest BMI values. In contrast, the lowest levels of total cholesterol and glucose were observed in the offspring born to parents without diabetes and were considered healthy. The study from Shields et al.(71) showed that paternal insulin resistance had a significant influence on the insulin concentrations found in the umbilical cord. Importantly, this influence appeared to contribute to the development of fetal insulin resistance. What's noteworthy is that this effect was observed independently of maternal factors, indicating that paternal insulin resistance plays a distinct role in shaping fetal metabolic outcomes, separate from the influence of maternal factors.

1.2.4 Smoking

Pembrey et al. (72) conducted a noteworthy study exploring the enduring consequences of paternal smoking during mid-childhood on the growth of their offspring. Their research unveiled a

remarkable transgenerational influence of paternal smoking on the BMI of offspring at the age of 9, with this impact conspicuously confined to male children. Through their findings, Pembrey et al. unveiled the presence of a sex-specific effect in humans, which primarily operates along the male lineage and is likely orchestrated by the intricate interplay of sex chromosomes. Moreover, they put forth a thought-provoking hypothesis suggesting that this unique male transgenerational response might be transmitted via the sperm's genetic material, possibly involving factors such as prions, viruses, RNA molecule. A study from De Jonge et al. (73) showed that when fathers smoked 15 or more cigarettes daily, it was linked to a higher likelihood of hypertension in their adult offspring. Additionally, in their study, Dior et al. (74) uncovered a positive correlation between maternal/paternal smoking and diverse indicators of offspring growth, encompassing height, weight, and BMI, when assessed at the age of 17. Intriguingly, they also noted an inverse relationship with pulse rates. These patterns remained consistent when studying individuals at the age of 32, as long as at least one parent had a history of smoking. Golding et al. (75) provided compelling evidence showcasing a robust connection between paternal smoking prior to the age of 11 and an increased fat mass observed in the adult offspring. Nonetheless, it's noteworthy to mention that these conclusions diverge from the research conducted by Carslake et al.(76) whose study did not uncover a definitive link between early-onset paternal smoking (prior to the age of 11) and heightened BMI in their offspring. On the contrary, Dougan et al. (77) presented an intriguing study that revealed a connection between grand-paternal smoking during the grandmother's pregnancy and an increased likelihood of their granddaughters being overweight or obese by the age of 12. However, no such association was established between grand-paternal smoking and the BMI of grandsons. In separate investigations, Deng et al. (78) and Cresci et al. (79) both reported significant associations between paternal smoking and the occurrence of conotruncal heart defects. One study has provided compelling evidence linking paternal tobacco smoke with the development of childhood asthma by the age of six(80).

1.2.5 Paternal exposure to environmental toxins

Paternal exposure to environmental toxins refers to the concept that environmental exposures experienced by fathers can potentially affect the health and development of their offspring (2). While much of the focus on the impact of environmental toxins has traditionally centered on

maternal exposure (i.e., exposures experienced by the mother during pregnancy), emerging research suggests that paternal exposures may also play a role in the health of future generations. Environmental toxins can potentially damage sperm and affect their quality. This damage may include genetic mutations or changes in the DNA structure of sperm. These altered sperm may contribute to developmental issues or genetic abnormalities in the offspring. Environmental exposure can also lead to epigenetic changes in sperm. These alterations have the potential to be inherited by the subsequent generation, exerting an impact on the health and susceptibility to diseases of their offspring. Studies in animals have suggested that exposure of the father to specific toxins, such as heavy metals, pesticides, or endocrine-disrupting chemicals, can lead to transgenerational effects. This means that the effects of paternal exposure are not limited to the immediate offspring but can also manifest in subsequent generations. The timing of paternal exposure is important. Exposures that occur before conception and during spermatogenesis (the process of sperm development) may have a more significant impact on the genetic and epigenetic quality of sperm. While research in this area is ongoing, it is still not fully understood to what extent paternal exposure to environmental toxins affects human health. Some studies have suggested links between paternal exposures and adverse health outcomes in offspring.

Through a comprehensive population meta-analysis, a groundbreaking study unveiled paternal exposure to a combination of nicotine, ethanol, and caffeine (referred to as PME) as a significant risk factor for adverse pregnancy outcomes. This study went a step further and established a novel rat model for PME, emulating unhealthy human lifestyles. These experiments confirmed that PME involving nicotine, ethanol, and caffeine could result in fetal dysplasia, with notable gender-specific effects. These effects appeared to be linked to the activation of the HPA axis, contributing to paternal reproductive dysfunction and adverse pregnancy outcomes. Additionally, PME seemed to influence the programming of the "GC-IGF1" axis, ultimately leading to multiple organ dysfunction in the developing fetus (81). In another investigation, it was demonstrated that paternal exposure to phthalates prior to conception had a significant impact on the epigenetic modifications of sperm, referred to as the sperm methylome. These alterations in sperm epigenetics had notable consequences on embryonic programming (82). One study underscored that paternal exposure to cyclophosphamide triggers abnormal epigenetic programming in early-stage embryos (83). In men, heightened exposure to bisphenol-A (BPA) was linked to a notable decline in various critical sperm parameters, including count, concentration, motility, vitality, morphology, and an increase in DNA

damage, as observed in studies (84, 85). On the other hand, even lower levels of BPA exposure appear to affect sperm motility exclusively. Furthermore, research has revealed that exposure to dioxins can significantly reduce the proportion of normally shaped sperm midsections and elevate the multiple abnormality index in men (86). However, it's important to note that more extensive research is necessary to firmly establish causality and gain deeper insights into the underlying mechanisms at play.

As a precautionary measure, individuals who are planning to embark on parenthood may wish to consider minimizing their exposure to well-known environmental toxins. This may involve avoiding habits such as smoking and excessive alcohol consumption, as well as limiting contact with hazardous chemicals. For those working in environments where potential toxin exposure is a concern, adhering to strict occupational safety measures becomes increasingly crucial.

1.2.6 Alcohol

Chronic paternal alcohol exposure prior to conception constitutes another form of paternal influence that imparts lasting effects on inflammation, glucose metabolism, and the behavior of offspring (87, 88). These effects become apparent early in the developmental process, with even minute alcohol concentrations exerting adverse impacts on the growth of the fetoplacental unit in offspring (89). Short-term outcomes in fetoplacental growth are intricately linked to the dosage. Elevated paternal alcohol exposure levels lead to the expansion of the placental labyrinthine layer while leaving fetal growth unaffected. Conversely, lower levels of paternal alcohol exposure result in enhanced fetal crown-rump length, especially in males, underscoring the sex-specific nature of these alterations (89). Emerging epidemiological research has begun to illuminate the significant role of preconception paternal alcohol exposure in shaping development and health of children (35, 89, 90). For instance, clinical data has established correlations between paternal alcoholism and adverse effects on the behavior of child (91, 92) and cognitive development (93, 94). Furthermore, clinical associations have established a connection between paternal alcohol consumption and elevated incidences of congenital abnormalities, smaller head circumferences, and reduced birth weight in infants (95). In animal models, preconception paternal alcohol exposures have been associated with cognitive impairment, elevated occurrences of congenital anomalies, and reduced birth weight in offspring (96-99). One study has even linked chronic paternal alcohol exposure before conception to prenatal and postnatal growth restriction, as well as sex-specific alterations

in long-term immune dysfunction, metabolic disorder, and hepatic fibrosis (100). The epigenetic mechanisms through which paternal alcohol consumption before conception influences the phenotype of offspring involve changes in non-coding RNAs (ncRNAs) within sperm. However, numerous questions in this intricate area of research remain unanswered.

1.3 Epigenetic mechanism involved in paternal programming

Epigenetics is a branch of biology that investigates alterations in gene expression or cellular traits that can be passed down through generations, all without any modifications to the fundamental DNA sequence (101, 102). In other words, it delves into how environmental factors and life experiences can impact the activation or deactivation of genes, resulting in alterations to an organism's traits. Epigenetic changes can be conceptualized as alterations that take place alongside the genetic code, exerting an influence on gene activity. Importantly, these modifications are reversible and have the potential to be inherited by subsequent generations (35). The environment in which a sire lives can lead to epigenetic changes of sperm, which includes histone modifications, DNA methylation, and alterations in various types of non-coding RNAs like tsRNA, piRNA, miRNA, lncRNA, and circRNA (103).

1.3.1 DNA methylation

DNA methylation is a foundational epigenetic mechanism pivotal in governing gene expression and maintaining the stability of the genome. This process involves the specific addition of a methyl group (CH3) to the DNA molecule, particularly at the cytosine base within a cytosine-guanine (CpG) dinucleotide pair. DNA methylation patterns can have a significant impact on gene activity, and abnormal DNA methylation is linked to a range of diseases, including developmental disorders and cancer. DNA methylation patterns can be inherited during cell division and, in certain instances, can be transmitted from one generation to the next. This epigenetic inheritance can play a role in developmental processes and disease susceptibility.

In mice, paternal low-protein diet has been linked to global sperm DNA hypomethylation, accompanied by a reduction in the expression of critical regulatory methyltransferase genes in testis, namely Dnmt1 and Dnmt3L(104). Similarly, altered DNA methylation patterns in sperm have been linked to caloric restriction (46) and nutritional deficiencies in essential minerals and vitamins, such as folate (105). Additionally, in male mice, obesity has been shown to cause alterations in microRNA (miRNA) populations and DNA methylation profiles in the sperm (41).

In studies involving men, distinct non-coding RNA (ncRNA) profiles and DNA methylation profiles in sperm have been noted between lean and obese individuals (106). Regarding paternal obesity's impact, research has unveiled hypomethylation of the Insulin-like Growth Factor 2 (IGF2) differentially methylated region in leukocytes of offspring at birth (107). Moreover, substantial hypomethylation has been detected in other imprinted genes, including PEG3, MEST, and NNAT, in the offspring born to obese fathers (107). Considering the roles of these genes in the regulation of growth and metabolism, their distinct DNA methylation patterns may serve as one mechanism linking paternal obesity to alterations in growth and metabolism of offspring.

1.3.2 Non-coding RNA

Non-coding RNAs (ncRNAs) are a diverse group of RNA molecules that do not code for proteins but instead play various regulatory roles within cells. Some ncRNAs have been found to have significant implications for offspring in the context of epigenetics, development, and disease (35). Some ncRNAs are involved in epigenetic regulation, which can influence the development and health of offspring (108). For example, small interfering RNAs (siRNAs) and microRNAs (miRNAs) are types of ncRNAs that can target and silence specific genes by interacting with messenger RNA (mRNA) molecules. Changes in the expression of these ncRNAs can lead to epigenetic modifications that affect the development and phenotype of offspring. ncRNAs, particularly long non-coding RNAs (lncRNAs), can play critical roles in embryonic development and tissue differentiation. They can help orchestrate the complex processes of development by regulating gene expression patterns. Dysregulation of these ncRNAs can lead to developmental abnormalities that may affect the offspring. Certain ncRNAs play a crucial role in the intricate process of parental imprinting, where specific genes are selectively silenced or activated based on their origin from either the mother or father. Imprinted ncRNAs have the capacity to exert a significant influence on the expression of imprinted genes, ultimately shaping the phenotypic traits of offspring. Emerging research suggests that ncRNAs may be involved in transgenerational epigenetic inheritance, where epigenetic changes are passed from one generation to the next. This inheritance can potentially affect the health and traits of offspring, even if they were not directly exposed to the initial environmental trigger.

Interestingly, mature sperm harbor diverse RNA populations, residing in both mitochondria and nucleus (109). These sperm-borne RNAs are not only present within fertilized oocytes but also

play a role at the early stage of embryonic development (110). The significance of sperm RNAs in molding the development of offspring is underscored by a striking observation: injecting specific sperm transfer RNA (tsRNA) fragments obtained from obese mice induced by diet into normal zygotes can exert a profound influence on the long-term metabolic health of the offspring (60).

1.3.3 Histone modifications

Histone modifications are pivotal in the intricate orchestration of gene expression regulation and can have important implications for offspring in the context of epigenetics (101). Histone modifications are one of the key mechanisms through which epigenetic information is transmitted from one generation of cells to the next, including from parents to offspring. During the formation of gametes (sperm and egg cells), epigenetic marks, including histone modifications, can be established, or modified in response to environmental factors or other influences (111). These epigenetic marks can be passed on to the next generation when the sperm and egg combine to form a zygote, which develops into an embryo. Histone modifications can influence the development and differentiation of cells in the developing embryo. Specific histone modifications at critical genes can determine the fate of cells, affecting which tissues and organs they will become part of. These early developmental decisions can have a lasting impact on the health and phenotype of the offspring (103). In some cases, histone modifications and other epigenetic marks can be inherited across multiple generations. This means that the effects of environmental exposures or other factors experienced by one generation can influence the health and traits of descendants in subsequent generations.

A recent study unveils an unprecedented function of the histone-modifying complex, PRC2, in mediating the paternal intergenerational transmission of epigenetic influences on offspring. This discovery holds significant implications for comprehending the inheritance of diseases (112). Perez-Cerezales et al. demonstrated that the removal of methylation marks from lysines 4 and 9 on histone 3 (H3K4 and H3K9) within spermatozoa brings about alterations in the phenotype of offspring (113). In a separate study, Terashima, M. et al. observed distinct histone H3 occupancy patterns at genes associated with embryogenesis and differential enrichment of H3K4me1 at genes responsible for transcription regulation in fathers exposed to high-fat diet compared to control mice (59). These findings strongly implicate that dietary exposure has an influence on the composition of histones at genes that play critical roles in developmental processes. Furthermore,

Cronican, A. A et al. reported that disturbances in histone methylation during sperm development have a transgenerational impact on offspring health (114). Meanwhile, Yao, X. H et al. demonstrated that prenatal ethanol exposure in rats led to enduring endoplasmic reticulum stress and oxidative stress. These stress responses elucidate the upregulation of gluconeogenic genes and histone deacetylases (HDAC) proteins. Through the deacetylation of foxo1 and PEPCK, these HDAC proteins play a role in increasing gluconeogenesis (115).

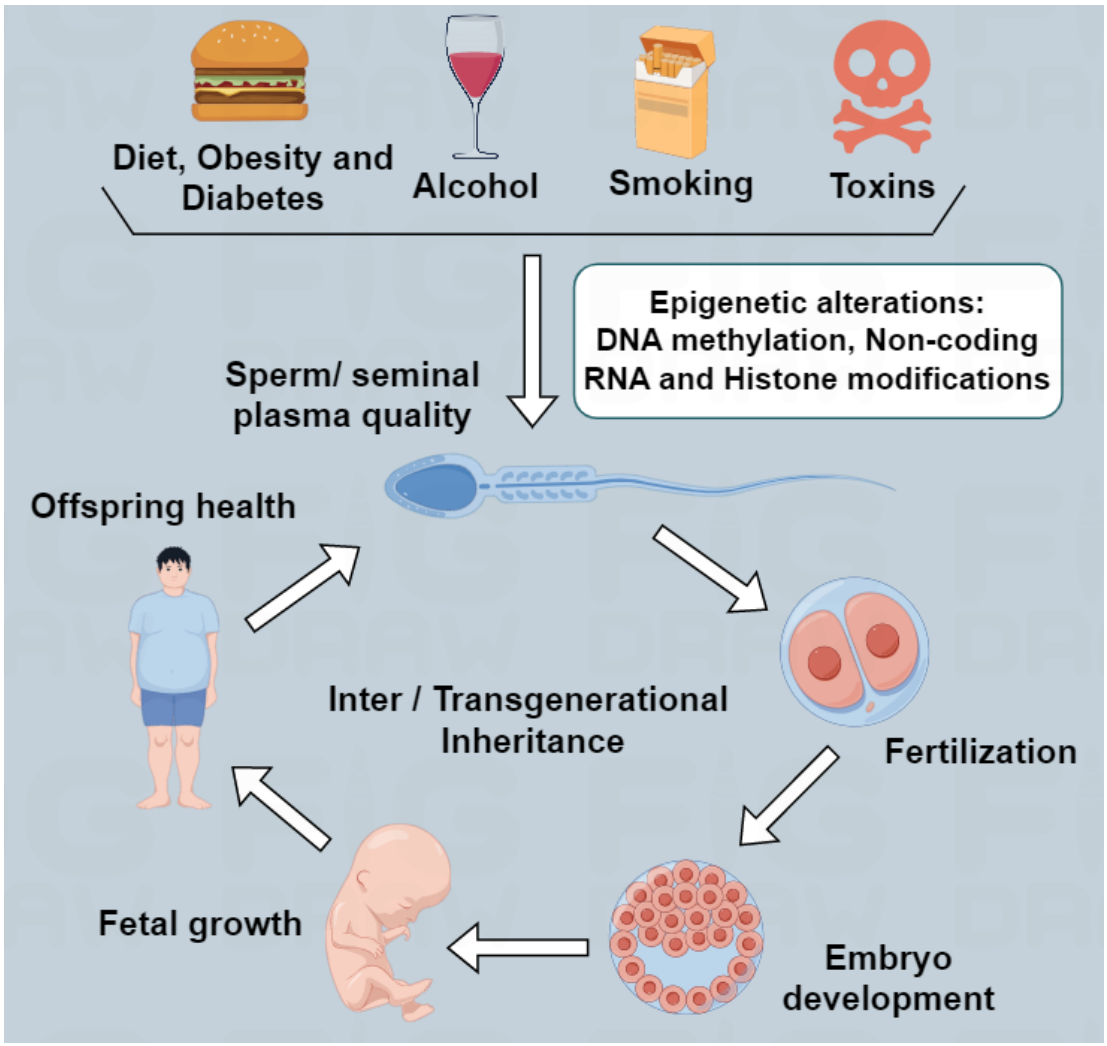

Figure 1. Effect of paternal adverse factors exposure on sperm quality, embryo development and offspring health.

The figure was created with https://www.figdraw.com.

1.4 Paternal programming of offspring kidney disease

1.4.1 Studies from others

The number of nephrons in the kidney of humans, assessed by stereological analysis of glomeruli from post-mortem kidneys, exhibits significant variability among individuals, with the count of nephrons in one kidney ranging from approximately 250,000 to as high as 2 million (31). Human metanephric kidney development commences around the sixth week of pregnancy, and by around the 36th week of gestation, all nephrons have been formed. However, it's important to note that tubular maturation and renal growth persist beyond the neonatal period. Therefore, no matter how much 'catch-up' growth occurs in kidney or body mass later in life, any factor that hampers or postpones nephron formation during fetal development cannot be rectified.

A mother's factors during pregnancy can profoundly influence her child's risk of developing kidney disease. Maternal dietary habits, particularly those characterized by a high-fat or high-salt diet, can exert a lasting influence on the development of the fetal kidneys (116-120). Maternal obesity can affect fetal kidneys and heighten the risk of kidney issues in the offspring (121, 122). Similarly, uncontrolled maternal diabetes poses a risk to fetal kidney development (123). Additionally, maternal behaviors such as smoking (32, 124-126), and toxic substance exposure(127-129) can have detrimental effects on the developing fetal kidneys.

While maternal factors during pregnancy play a significant role in influencing the risk of kidney disease in offspring, paternal factors can also have an impact, albeit to a lesser extent. These factors encompass lifestyle choices and environmental exposures, including smoking, alcohol consumption, toxin exposure, and dietary habits, which can indirectly impact the child's kidney health (130, 131). Harrison and colleagues' research, as outlined in their study (132), conducted on Wistar rats subjected to a low protein diet (with a protein content of 9% as opposed to 18% casein), indicates that the cardiovascular issues previously observed in the F2 generation offspring can be attributed, at least partially, to the inheritance of diminished nephron endowment along both the maternal and paternal lines. A single study uncovered that sustained paternal exposure to PM2.5 over an extended period induces hypertension in male offspring through the upregulation of renal AT1R (Angiotensin II type 1 receptor) expression and its enhanced functionality (133). Another study indicated that paternal obesity is linked to the accumulation of triglycerides in the kidneys and histological alterations in tubules, implying a subtle renal injury in offspring, potentially elevating their susceptibility to developing chronic kidney disease (CKD) (134).

Nonetheless, there remains a scarcity of information regarding epigenetic alterations within the kidney resulting from paternal influences. A particular study has illuminated that when father was exposed to synthetic glucocorticoids, DNA methylation patterns related to the glucocorticoid receptor (GR) in the kidney of their offspring were altered (135). This research revealed that sperm global non-CpG methylation was increased in the initial males (F0 generation) and significant demethylation was observed in the GR's regulatory regions in the kidney of offspring during their postnatal development.

1.4.2 Our previous work

In our previous study, male rats were exposed to a high-fat, high-sucrose, high-salt diet before mating, and it was discovered that this diet had adverse effects on the glucose metabolism of their female offspring (F1 generation) (42). These effects could be reversed through folate treatment during pregnancy. Additionally, the male rats' unhealthy diet predisposed their F2 generation offspring to mild liver functional issues and alterations in gut microbiota (136).

1.4.3 Purpose of the current study

In our current study, we utilized the same animal model to investigate the effects of feeding male rats the same unhealthy diet (high-fat, high-sucrose, high-salt) for two consecutive generations (F0 and F1) and focused on evaluating kidney function in the F2 generation. We defined abnormal kidney function as a combination of an elevated urinary-albumin-to-creatinine ratio (UACR) and reduced glomerular filtration rate (GFR).

1.5 Paternal programming of offspring metabolism disease

1.5.1 Studies from others

The initial accounts of paternal programming on the metabolic health of offspring emerged from epidemiological investigations, and a substantial body of evidence now supports the existence of such effects in the male lineage of humans. These studies have unveiled the intricate dynamics of paternal programming, underscoring the influence of deterministic factors, including the gender lineage and the timing of exposure, in eliciting distinct responses in the offspring.

The most prominent epidemiological evidence supporting male lineage metabolic effects is derived from the "Överkalix" studies, which link lifespan and disease risk to the nutritional habits

of paternal ancestors (137). These studies stand out not only for their capacity to identify paternal programming across multiple generations but also because the nutritional challenges were encountered well before reaching reproductive age. Additional epidemiological investigations have shed light on the influence of paternal metabolic health at the time of reproduction on the well-being of their children. A comprehensive study based on data from over 230,000 individuals in the UK Biobank revealed that paternal diabetes at the point of conception predicts the likelihood of diabetes in offspring (138). Furthermore, it was found that birthweight plays a role in mediating this association, with low birthweight being a known predictor of type 2 diabetes later in life (139). In addition, a longitudinal study demonstrated that increased paternal body fat serves as a robust predictor of long-term changes in body fat in prepubertal daughters (140). Importantly, this influence was independent of maternal body fat and the girls' own energy expenditure. A British birth cohort study suggested that paternal BMI correlated with childhood BMI at age 11 and continues to do so at 45 years (141). Our research team performed a birth cohort study in Guangzhou China uncovered positive correlations between paternal BMI and fetal growth in males, as well as cortisol levels in newborns (142).

Even though just over two decades have elapsed since the commencement of studies exploring paternal participation in the inter- and transgenerational transmission of metabolic disorders, a limited number of investigations explored the mechanism involved in the paternal programming on offspring's metabolic health. One study unveiled that paternal prediabetes induced alterations in sperm DNA methylation patterns, in conjunction with modifications in insulin gene methylation in the offspring (143). This cascade of events resulted in the downregulation of genes connected to insulin signaling pathways and glucose metabolism, ultimately contributing to the development of insulin resistance and glucose intolerance.

1.5.2 Endothelial Nitric Oxide Synthase (eNOS)

Endothelial Nitric Oxide Synthase (eNOS), also known as Nitric Oxide Synthase 3 (NOS3), is a crucial enzyme primarily located in the endothelial cells lining blood vessels(144). It plays a central role in the production of nitric oxide (NO), a vital signaling molecule in the body. eNOS catalyzes the conversion of L-arginine into NO, which acts as a vasodilator, regulating blood vessel tone, and promoting increased blood flow. This function is essential for maintaining vascular health and regulating blood pressure. Dysfunctional eNOS can lead to endothelial dysfunction and

conditions like hypertension(145). Besides its role in blood pressure regulation, eNOS contributes to cardiovascular health, neurotransmission in the nervous system, and immune responses(146, 147). Genetic variations in the eNOS gene can influence an individual's susceptibility to cardiovascular diseases, and it is a target for certain pharmacological interventions aimed at enhancing nitric oxide effects(148).

1.5.3 ENOS and intrauterine growth

eNOS plays a crucial role in intrauterine growth by ensuring the exchange of essential nutrients for fetal development through the placenta(149). The formation of a specific placental vascular bed is of paramount importance. In this process, NO appears to act as a vital paracrine mediator, as it serves several essential functions, including vasodilation of placental vessels, maintenance of low vascular resistance in the fetoplacental vascular unit, promotion of angiogenesis in the developing placenta, potential involvement in endovascular invasion and remodeling processes, influence on placental steroid synthesis and hormone secretion, and possible regulation of placental glucose transport(150, 151). Various NOS isoforms have been identified in placentas of rats, mice, sheep, humans, and rhesus monkeys. Among these, eNOS, through NO release, likely plays a primary role in regulating vascular adaptation processes during pregnancy. eNOS is expressed in trophoblasts, which are involved in the adhesion and penetration of uterine endometrium during implantation, as well as in extravillous trophoblasts(152). These findings highlight the critical role of eNOS in the process of intrauterine growth, ensuring that the fetus receives the necessary nutrients for normal development. Some observations support the assumption that late pregnancy is a crucial time window for the manifestation of fetal growth restriction and, ultimately, the induction of fetal programming.

In addition to classical gene knockout models, the treatment of rats with the eNOS inhibitor L-NAME has also led to fetal growth restriction (153). Interestingly, this effect could be reversed by administering the nitric oxide precursor molecule L-Arginine (154).

One possible explanation for intrauterine growth restriction (IUGR) involves alterations in eNOS activity influencing the remodeling of placental and uterine blood vessels. Here, "remodeling" refers to the process by which the vascular system adapts to changes in physiological conditions, including the migration and proliferation of smooth muscle and endothelial cells, as well as the formation of neointima.

In summary, NO produced by eNOS plays a crucial role in normal intrauterine growth.

1.5.4 ENOS and testicular vascular function

ENOS is a crucial enzyme found in the endothelial cells lining the blood vessels, including those within the testes, and it plays a pivotal role in maintaining testicular vascular function and male reproductive health(155). By producing NO, eNOS induces vasodilation, widening blood vessels, which is essential for enhancing blood flow to the testes(156, 157). This heightened blood flow is integral to supporting spermatogenesis by ensuring a continuous supply of oxygen and nutrients to developing sperm cells(158, 159). Furthermore, eNOS-mediated vasodilation aids in maintaining the health and function of Leydig cells, responsible for producing testosterone, a critical male sex hormone(155). Additionally, this vascular regulation supports efficient hormone delivery into the bloodstream and contributes to temperature regulation in the scrotum, where the testes are located, ensuring optimal conditions for sperm production. In summary, eNOS and testicular vascular function are intricately linked and are indispensable components of male reproductive health, vital for spermatogenesis, hormone production, and temperature regulation, all of which are essential for male fertility and overall reproductive well-being.

1.5.5 Our previous work

Another potential mechanism contributing to fetal programming might be linked to maternal genes that independently shape the fetal phenotype, separate from the fetal genome. An initial illustration of this phenomenon is demonstrated by the wimp mutation in fruit flies, which influences the lethal phenotype of offspring, even if the mutation is not transmitted (160). Our research team pioneered the extension of this concept to mammals and humans. We provided evidence that a maternal gene, likely responsible for regulating uterine blood supply, was significantly linked to a considerable reduction in birth weight of offspring, even though this genetic variation was not actually passed on to the offspring (161, 162).

To substantiate the idea that maternal genes can indeed impact the phenotype of offspring, as implied by the aforementioned studies, we conducted a study involving the mating of male wild-type (wt) mice with female heterozygous eNOS mice. We then compared the resulting wild-type offspring with the offspring born to wild-type parents, observing differences in their phenotypes. We found that maternal genetic alterations, such as a deficiency in the eNOS gene, can lead to the changes in the liver phenotype of wild-type offspring without passing on the inherent defect.

Notably, these effects manifest in a sex-specific manner, with more pronounced adverse consequences observed in female (163).

1.5.6 Purpose of the current study

Whether paternal gene defect, which is not directly transmitted to the offspring, can also have an impact on the offspring's phenotype remains an unanswered question. We postulated that such an influence could indeed impact the phenotype of offspring. To do so, we employed the same animal model used previously, involving the mating of female wild-type (wt) mice with male heterozygous eNOS（eNOS+/-）mice. Our study focused on assessing how paternal NO deficiency might result in epigenetic changes in sperm. Additionally, we examined the metabolic phenotype of the wild-type offspring resulting from father with a heterozygous eNOS genotype. Subsequently, we delved into the analysis of differentially expressed genes and any associated epigenetic modifications in these genes, aiming to uncover potential factors responsible for the observed phenotype. Furthermore, we conducted an in-depth metabolomics analysis to compare the impact of maternal and paternal eNOS deficiency on genetically normal offspring. This head-to-head comparison aimed to enhance our understanding of phenotypic variations and reveal underlying molecular mechanisms, with a special focus on discrepancies in glycemic control.

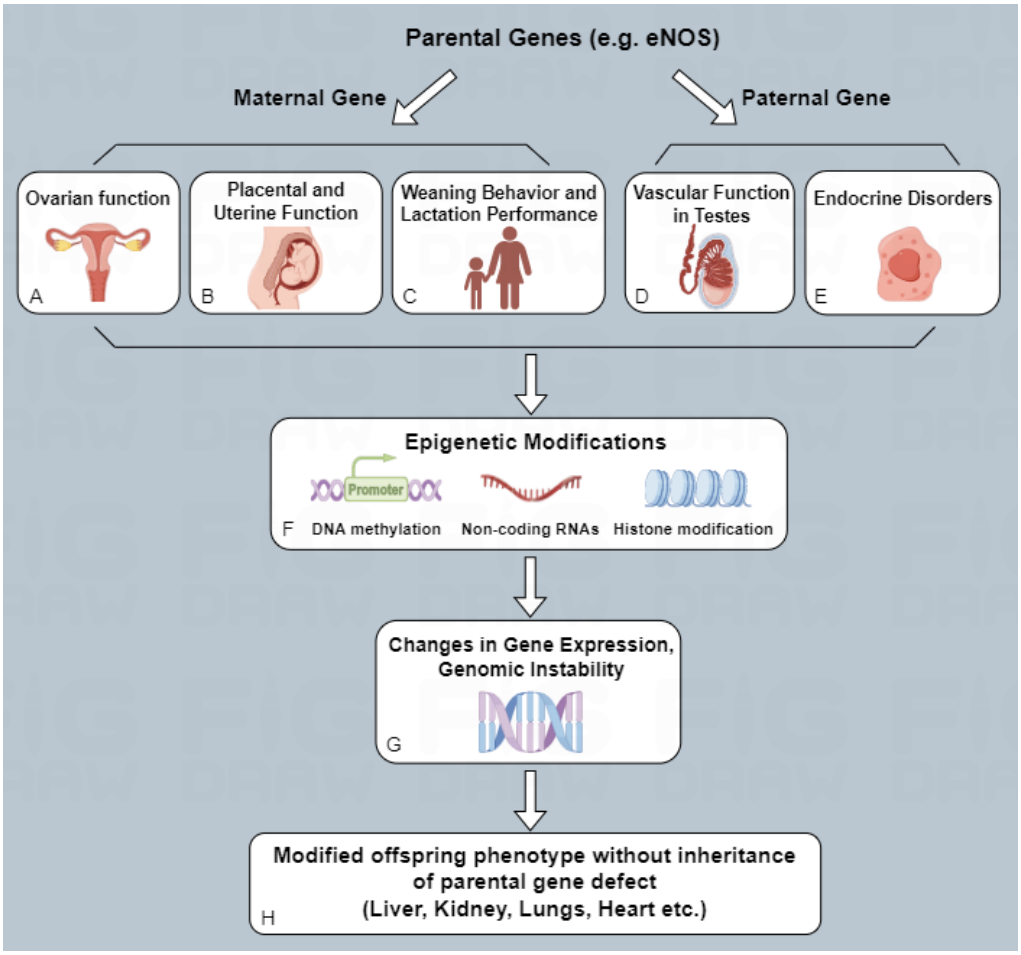

Figure 2. The advanced fetal programming hypothesis. (A) Dysfunction in maternal genes can disrupt ovarian function; (B) Maternal gene dysfunction can impact placental and uterine function; (C) Maternal gene dysfunction may influence weaning behavior and lactation performance; (D) Paternal gene dysfunction can impact vascular function in testes; (E) Paternal gene dysfunction may lead to endocrine disorders; (F) The consequences of parental gene dysfunction on embryonal, fetal, and neonatal environmental factors listed in A-E can potentially trigger enduring, epigenetic adaptations in the offspring. These epigenetic mechanisms encompass DNA methylation, non-coding RNAs, and chromatin modifications. (G) Epigenetic modifications, specifically DNA methylation and non-coding RNAs, can lead to lasting changes in gene expression within the offspring. (H) Changes in gene expression and genomic instability brought about by epigenetic maladaptation can influence the offspring's phenotype by permanently altering the structure and function of organs. The figure was created with https://www.figdraw.com.

2 Publication 1: High-fat, sucrose and salt-rich diet during rat spermatogenesis led to the development of chronic kidney disease in the female offspring of the F2 generation

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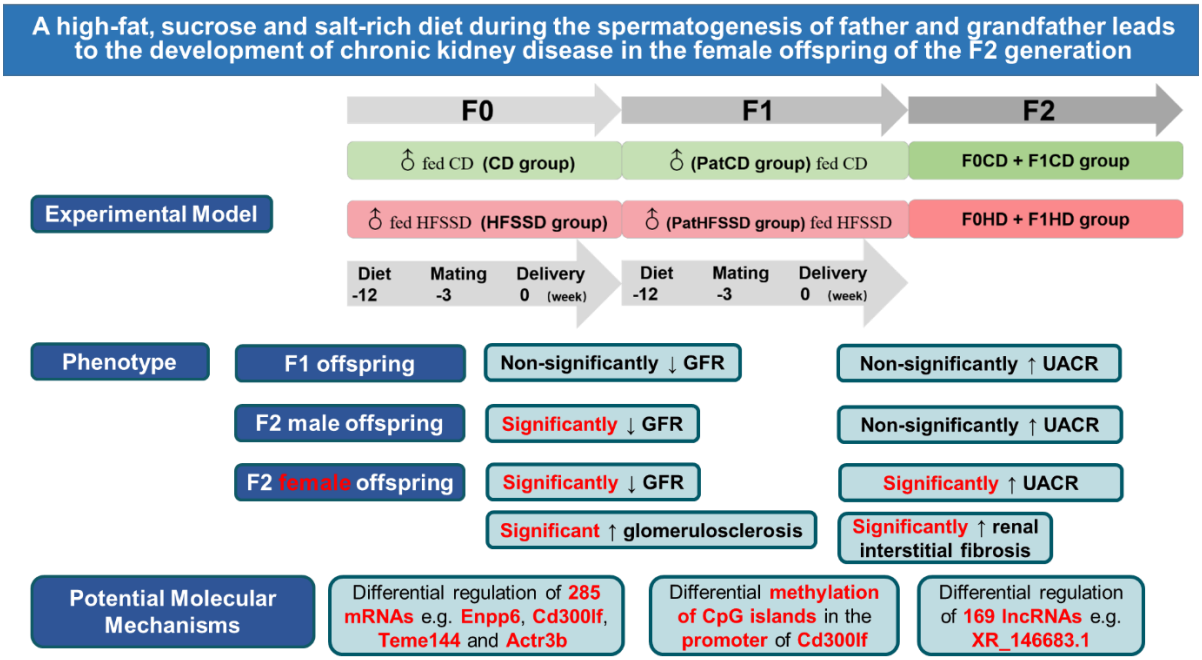
2.1 Graphic abstract



2.2 Author's contribution

In this publication, Xiaoli Zhang undertook a comprehensive range of responsibilities, including: 1) leading both animal and molecular biology experiments while conducting statistical analyses; 2) ensuring data integrity; 3) drafting the manuscript; 4) completing the submission process; 5) revising the manuscript and addressing reviewers' feedback.

2.3 Main content of the publication

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RESEARCH ARTICLE

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High-fat, sucrose and salt-rich diet during rat spermatogenesis lead to the development of chronic kidney disease in the female offspring of the F2 generation

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Abstract

Effects of feeding male rats during spermatogenesis a high-fat, high-sucrose and high-salt diet (HFSSD) over two generations (F0 and F1) on renal outcomes are unknown. Male F0 and F1 rats were fed either control diet (F0CD+F1CD) or HFSSD (F0HD+F1HD). The outcomes were glomerular filtration rate and urinary albumin excretion in F1 and F2 offspring. If both outcomes were altered a morphological and molecular assessment was done. F2 offspring of both sexes had a decreased GFR. However, increased urinary albumin excretion was only observed in female F2 F0HD+F1HD offspring compared with controls. F0HD+F1HD female F2 offspring developed glomerulosclerosis (+31%; $p < .01$) and increased renal interstitial fibrosis (+52%; $p < .05$). RNA sequencing

Abbreviations: ACTR3B, Actin Related Protein 3B gene; CD, control diet; CD300LF, CD300 Molecule Like Family Member F gene; CKD, chronic kidney disease; ENPP6, Ectonucleotide Pyrophosphatase/Phosphodiesterase 6 gene; HFSSD, high-fat, high-sucrose and high-salt diet; TMEM144, Transmembrane Protein 144 gene.

Xiaoli Zhang and Ahmed A. Hasan contributed equally to this work.

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followed by qRT-PCR validation showed that four genes (Enpp6, Tmem144, Cd300lf, and Actr3b) were differentially regulated in the kidneys of female F2 offspring. lncRNA XR-146683.1 expression decreased in female F0HD+F1HD F2 offspring and its expression was ($r = 0.44$, $p = .027$) correlated with the expression of Tmem144. Methylation of CpG islands in the promoter region of the Cd300lf gene was increased ($p = .001$) in female F2 F0HD+F1HD offspring compared to controls. Promoter CpG island methylation rate of Cd300lf was inversely correlated with Cd300lf mRNA expression in F2 female offspring ($r = -0.483$, $p = .012$). Cd300lf mRNA expression was inversely correlated with the urinary albumin-to-creatinine ratio in female F2 offspring ($r = -0.588$, $p = .005$). Paternal pre-conceptional unhealthy diet given for two generations predispose female F2 offspring to chronic kidney disease due to epigenetic alterations of renal gene expression. Particularly, Cd300lf gene promotor methylation was inversely associated with Cd300lf mRNA expression and Cd300lf mRNA expression itself was inversely associated with urinary albumin excretion in F2 female offspring whose fathers and grandfathers got a pre-conceptional unhealthy diet.

KEYWORDS

epigenetics, high-fat-sucrose-salt diet, kidney function, paternal programming

1 | INTRODUCTION

The 'fetal programming' hypothesis proposes that environmental factors before birth can alter the offspring phenotype through a range of mechanisms.[1,2] These factors are most well defined in mothers for example, maternal undernutrition during pregnancy, maternal high protein diet during pregnancy, and glucocorticoid exposure of the fetus. While alterations in epigenome and phenotype of offspring by maternal factors are widely reported, there are also reports in the past years suggesting that fetal programming can also be paternally initiated.[3–6] For example, paternal smoking, age, and occupational chemical exposure are associated with an increased risk of cancer and mental health disorders in the offspring.

Fetal programming of kidney diseases was likewise established. Independent research teams worldwide showed that maternal undernutrition, caloric restriction, protein restriction or maternal dietary fat excess all have a negative impact on nephrogenesis and kidney structure, contributing to offspring kidney disease in later life.[7–9] Moreover, there is also evidence from human studies that comparable maternal factors increase the risk of kidney disease in the offspring.[10] While there were several studies investigating the impact of maternal factors on the offspring renal phenotype, the literature on the later-life effects of paternal factors for example, pre-conceptional unhealthy diet on the renal phenotype of the offspring is limited. *Chowdhury* et al. reported that paternal obesity can lead to increased triglyceride content in offspring kidneys with signs of tubular damage, such as cell sloughing, absence of brush border which may be indicative of early signs of kidney damage.[11]

Previously, we fed a diet resembling an unhealthy diet (high-fat, high-sucrose and high-salt diet [HFSSD], often consumed by young men) to male rats prior to mating and analyzed the effect on healthy outcome in offspring. It was shown that paternal pre-conception unhealthy diet had detrimental effects on F1 female offspring's glucose metabolism, which can be reversed by folate treatment of pregnant dams.[12] Furthermore, we found that a paternal pre-conceptional unhealthy diet predisposed the F2 offspring to mild liver functional alterations and alterations of gut microbiota in later life.[13]

In the current study, we analyzed the effect of feeding male rats a high-fat, high-sucrose and high-salt diet (HFSSD) over two generations (F0 and F1) on their offspring's (F2) kidney function. We defined abnormal kidney function by two parameters: decreased glomerular filtration rate (GFR) combined with increased urinary-albumin-to-creatinine ratio (UACR). In case of developing significantly declined GFR and significantly elevated UACR after exposure of the fathers and/or grandfathers to an unhealthy diet, we analyzed the renal morphology as well as the underlying molecular mechanisms causing paternal programmed of chronic kidney disease in the offspring.

2 | MATERIAL AND METHODS

2.1 | Animals

The study was conducted in Sprague-Dawley rats, including F0 generation (30 male rats, 45 female rats), F1 and F2 generation animals. The F0 generation rats were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China) and were delivered at the age of 4 weeks. All the animals were allowed free access to water and food. The experimental protocols were approved by the Experimental Animal Center of the Hunan Normal University (Changsha, Hunan, China).

2.2 | Study design

F0 male rats were randomly divided into two study groups: (1) control diet (CD, $n = 15$); (2) high-fat, high-sucrose and high-salt diet (HFSSD, $n = 15$). A detailed description of the diet compositions is indicated in our previous study[13] (Table S1).

15-week-old F0 founder male rats were fed either a CD or HFSSD for 9 weeks. Then they were mated with F0 normal-weight, naturally cycling CD-fed dams to produce F1 offspring. The presence of a vaginal plug was designated as gestational day 1. The rats were mated for 4 days. After mating and throughout the gestational period, all the F1 dams were fed CD. The number of F1 offspring per litter was 13.89 ± 0.3 (SE) and was not differ significantly between the groups. We sampled randomly 173 F1 offspring (86 males and 87 females) out of 540 total offspring. Thus, the sample size was around one-third of the entire offspring. The F1 male offspring was allocated into two study groups based on F0 paternal diet before mating: (1) paternal control diet group (PatCD group): male F1 offspring of CD-fed F0 founders received CD from birth until the 24th week of age and were mated with CD-fed dams and their F2 offspring represented the F0CD+F1CD group; (2) paternal high-fat, high-sucrose and high-salt diet group (PatHFSSD group): male F1 offspring of HFSSD-fed F0 founders received CD from birth until the 15th week of age followed by HFSSD until the 24th week of age and were mated with CD-fed dams and their offspring represented F0HD+F1HD group. The average litter size of F2 offspring was 13 and was not differ significantly between the groups. We sampled randomly 65 F2 offspring (33 males and 32 females) out of 150 total offspring. In summary, based on F0 paternal diet and F1 paternal diet before mating, the female F2 offspring can be divided into two study groups (Figure 1): (a) F0CD+F1CD group: female F2 offspring of F0 and F1 male founders fed a CD; (b) F0HD+F1HD group: female F2 offspring of F0 and F1 male founders fed a HFSSD. All F2 offspring (F0CD+F1CD and F0HD+F1HD) were fed CD from birth until the 24th week of age. Before sacrifice, F2 offspring were fasted for 8 h, then rats were placed in a metabolic cage and 24 h-urine was collected. At the 24th week, they were sacrificed under deep anesthesia induced by intraperitoneal injection of 3% (wt/vol.) sodium pentobarbital solution. Afterwards, blood samples were collected and organs were harvested.

Blood pressure measurements, metabolic analysis, analysis of kidney morphology, lncRNA-mRNA expression profiling, real-time quantitative PCR, correlation analysis of lncRNAs and mRNAs and functional prediction, gene-specific DNA methylation were described in detail in the electronic supplementary materials (ESM).

2.3 | Statistical analysis

Normality of data distribution was checked using Shaprio–Wilk test. Student's *t*-test (unpaired, two-tailed) and the Mann–Whitney test were used to compare parametric and non-parametric data, respectively, between groups. Pearson's correlation analysis was used to assess correlations between mRNAs and lncRNAs. Unless otherwise indicated, results are expressed as the mean ± SE. A p value of <.05 was considered statistically significant. The data were analyzed using SPSS version 20.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Heat maps were created using R version 3.5.3 (https://www.r-project.org/) and Cytoscape version 3.7.1 (https://cytoscape.org/) was used to construct the relational network.

3 | RESULTS

The primary outcome of the study in the F1 and F2 generations was a composite endpoint consisting of decreased GFR combined with increased urinary albumin to creatine ratio. Only if this endpoint was met, a detailed biochemical and morphological analysis was done.

3.1 | F0 founders

Detailed data on body weight gain, blood pressure and serum metabolite levels in the F0 male founders were presented in Table S2. The number of pups was similar in all groups; no effect of HFSSD on the number of pups, male/female ratio and birth body weight were detectable.

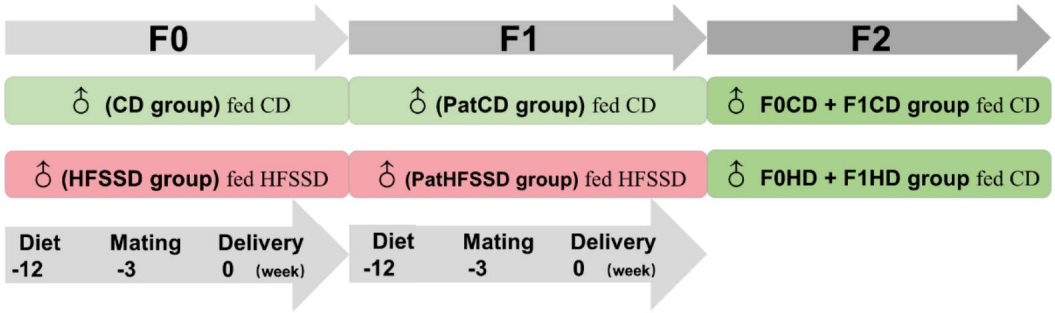

FIGURE 1 Study design for the F0, F1 and F2 generations. F0 founder male rats fed either a control diet (CD) or high-fat, high-sucrose and high-salt diet (HFSSD) for 9 weeks were mated with CD-fed dams to produce F1 offspring. The F1 male offspring was allocated into two study groups based on F0 paternal diet before mating: (1) Paternal control diet group (PatCD group): male F1 offspring of CD-fed F0 founders received CD from birth until the 24th week of age; (2) Paternal high-fat, high-sucrose and high-salt diet group (PatHFSSD group): male F1 offspring of HFSSD-fed F0 founders received CD from birth until the 15th week of age followed by HFSSD until the 24th week of age. Male PatCD group rats and male PatHFSSD group rats in 24th week of age were mated with female PatCD group rats to produce F2 offspring. Based on F0 paternal diet and F1 paternal diet before mating, the F2 offspring can be divided into two study groups: (1) F0CD+F1CD group: F2 offspring of F0 and F1 male founders fed a CD; (2) F0HD+F1HD group: F2 offspring of F0 and F1 male founders fed a HFSSD. All F2 offspring (F0CD+F1CD and F0HD+F1HD) were fed a normal diet (CD) for 24 weeks

TABLE 1 Effect of paternal HFSSD on kidney weights and kidney biomarkers in F1 offspring

	Female		Male	
Parameters	PatCD ($n = 7$)	PatHFSSD ($n = 11$)	PatCD ($n = 14$)	PatHFSSD ($n = 17$)
Body weight (100 days)	252.69 ± 4.47	266.96 ± 6.47	416.47 ± 8.50	425.20 ± 9.89
Relative left kidney weight (% to body weight)	0.32 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
Relative right kidney weight (% to body weight)	0.33 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
BUN (mmol L^{-1})	6.79 ± 0.33	6 ± 0.33	6.42 ± 1.03	5.27 ± 0.24
Plasma Cr (mmol L^{-1})	27.2 ± 1.03	24.05 ± 2.14	24.13 ± 3.72	24.3 ± 1.34
Plasma cystatin C (μg ml^{-1})	1.46 ± 0.11	1.53 ± 0.13	1.54 ± 0.18	1.75 ± 0.12
Urine volume (ml 24 h^{-1})	28.19 ± 3.69	6.23 ± 0.53**	45.46 ± 8.13	34.29 ± 6.26
Urinary microalbumin (μg 24 h^{-1})	82.56 ± 34.78	73.05 ± 14.01	65.99 ± 11.42	112.18 ± 33.61
Urine Cr (μmol 24 h^{-1})	65.49 ± 6.82	53.11 ± 4.51	95.66 ± 8.35	100.70 ± 9.47
Urinary albumin-to-creatinine ratio (μg μmol^{-1})	1.25 ± 0.43	1.37 ± 0.24	0.74 ± 0.14	1.13 ± 0.27
GFR/body weight (ml 24 h^{-1} g^{-1})	10.15 ± 1.07	7.77 ± 0.91	10.87 ± 1.52	9.29 ± 1.16

Note: Values are given as mean ± SE.

Abbreviations: BUN, blood urea nitrogen; Cr, creatinine; GFR, glomerular filtration rate.

**$p < .01$ vs. PatCD.

3.2 | Kidney function in F1 offspring

The urinary albumin-to-creatinine ratio was numerically higher in F1 offspring of the PatHFSSD group of both sexes. However, the differences did not reach statistical difference. The GFR corrected by body weight was numerically lower in F1 offspring of the PatHFSSD group of both sexes. However, the differences again did not reach statistical difference (Table 1). Therefore, we further observed the effect of HFSSD on kidney function in F2 offspring.

3.3 | Body weight and kidney function in F2 offspring

Compared with the F0CD+F1CD group, the body weight of the female F0HD+F1HD group increased significantly at the 9th, 15th, 18th, 22nd and 24th week (Figure S1A). However, no significant differences in body weight were observed between the F0CD+F1CD and F0HD+F1HD in F2 male offspring (Figure S1B). The urinary albumin to creatine ratio in female F2 offspring of F0HD+F1HD group was significantly increased compared with the F0CD+F1CD group ($p < .01$, Figure 2A). Furthermore, the ratio of GFR/body weight ratio at study end was significantly lower in female F0HD+F1HD group than that in F0CD+F1CD group ($p < .05$, Figure 2B). However, no significant differences related to renal function were observed between the F0CD+F1CD and F0HD+F1HD in male F2 offspring (Table 2). Since only female F2 offspring met the composite study endpoint, a detailed morphological and molecular assessment was next done in just female F2 offspring.

3.4 | Kidney morphology

The number and size of glomeruli was similar among the study groups of female F2 offspring (Figure 2C,D). In female F2 offspring born to grandfather and father exposed to the HFSSD diet prior to mating glomerulosclerosis score was increased by 31% and renal interstitial fibrosis area was increased by 52% ($p < .01$, $p < .05$ vs. control female counterparts, respectively, see Figure 2E–H).

3.5 | Expression of mRNA and lncRNA in the kidney

Microarray profiling in a subset of animals (see Section 2) was done to identify candidate genes being potentially differentially regulated. The results of microarray profiling showed that 285 mRNAs were differentially regulated. (156 up-regulated mRNA and 129 down-regulated mRNA) when comparing the F0CD+F1CD and the F0HD+F1HD group in female F2 offspring ($p < .05$ and |fold change| $\geqq 1.5$ was set as the threshold for significant differential expression) (Figures 3A and S2A).

Using the same criteria, we identified 129 lncRNAs (94 up-regulated lncRNA and 35 down-regulated graduated lncRNA) (Figure S2B). Protein-protein interaction analysis of these DEGs highlighted the key module of RT1 family (a family of MHC class Ib genes) in the relational network (Figure S2C). Functional enrichment and KEGG analyses revealed that antigen processing and presentation, immune response, and antiviral function in F0HD+F1HD group differed from that in the F0CD+F1CD group (Figure S3A,B), suggesting that renal dysfunction in F2 female offspring might be related to altered immunological properties of the kidneys.

From the 285 DEGs, we chose the most promising 18 candidate mRNAs based on a statistical criterion ($p < .01$, |fold change| $\geqq 2$) in the arrays for confirmation in the entire study population by qRT-PCR (Table S4). Expression of Enpp6, Tmem144 and Cd300lf was decreased whereas Actr3b gene expression was increased in female offspring born to grandfather and father exposed to the HFSSD (Figure 3B).

3.6 | Correlation analysis of lncRNA and mRNA

To analyze the relationship between lncRNA and the 4 confirmed differentially expressed mRNAs (Enpp6, Actr3b, Tmem144 and Cd300lf), we performed Pearson correlation analysis using the microarray data. Based on Pearson correlation coefficient >0.4 or <−0.4 and p value <.05, we found that 20 lncRNAs had significant correlations with Enpp6, Tmem144, Cd300lf and Actr3b. (Figure 4A). Subsequently, we conducted qRT-PCR to verify these correlations in the entire study. We found that lncRNA XR_146683.1 and uc.239+ were significantly down-regulated in female offspring born to grandfather and father exposed to the HFSSD (Figure 4B, Table S5). Further correlation analysis showed that expression of lncRNA XR_146683.1 had a significant positive correlation with that of Tmem144 ($r = 0.44$, $p = .027$) (Figure 4C).

3.7 | DNA methylation of specific target genes in the kidney

The methylation rate of CpG islands in the promoter region of Enpp6, Tmem144, Actr3b and Cd300lf genes was analyzed. Violin plot of differential methylation fragments showed that the methylation rate of CpG islands in the promoter region of Cd300lf was significantly higher (Figure 5A, $p = .001$) in the female F0HD+F1HD group as compared to the F0CD+F1CD group. Cd300lf gene promotor methylation was inversely correlated with Cd300lf mRNA expression ($r = -0.483$, $p = .012$) (Figure 5B). Cd300lf mRNA expression itself was inversely correlated with the urinary albumin-to-creatinine ratio in female F2 offspring (see Table S7 and Figure 5C, $r = -0.588$, $p = .005$).

We next analyzed the Cd300lf gene promotor methylation in more detail. We analyzed the CpG site specific

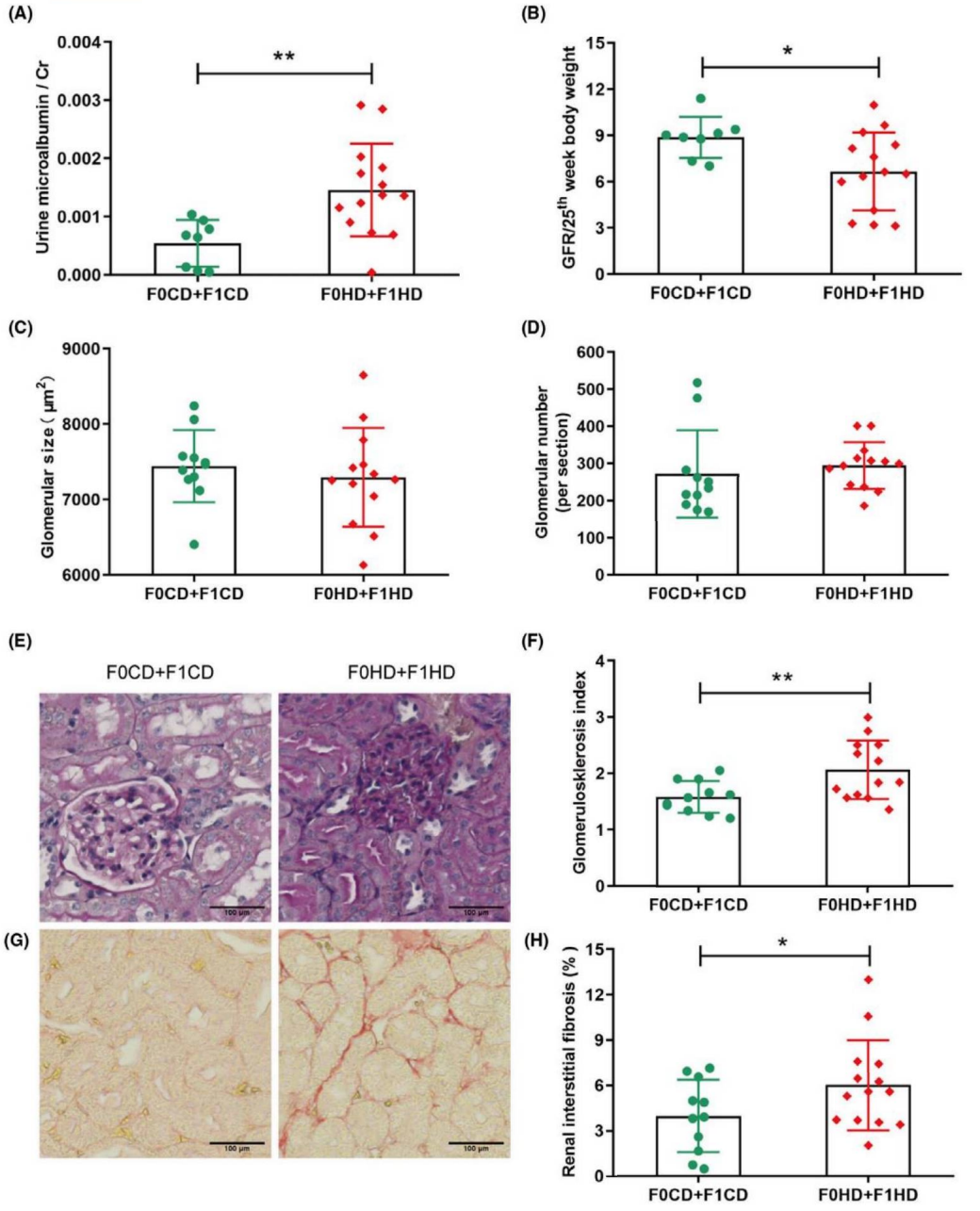

FIGURE 2 Effect of paternal HFSSD on urine indicators related to renal function and morphology of F2 female offspring. ratio of urine microalbumin and Cr (A); ratio of GFR and body weight of the 25th week (B); size of glomerulus (C); total number of glomerulus (D); representative photomicrographs of PAS-stained renal sections (magnification:×200) (E); glomerulosclerosis score (F); representative photomicrographs of picrosirius red stained renal sections (magnification:×200) (G); renal interstitial fibrosis area (H). F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *$p < .05$ vs. F0CD+F1CD, **$p < .01$ vs. F0CD+F1CD

TABLE 2 Effect of paternal HFSSD on blood pressure, kidney weights and kidney biomarkers in F2 offspring

	Female		Male	
Parameters	**F0CD+F1CD ($n = 11$)**	**F0HD+F1HD ($n = 15$)**	**F0CD+F1CD ($n = 18$)**	**F0HD+F1HD ($n = 19$)**
Body weight at the 24th week	308.1 ± 5.55	322.64 ± 3.71*	585.78 ± 7.98	604.77 ± 8.50
Heart rate (bpm)	442.2 ± 6.96	411.55 ± 9.5*	414.68 ± 8.34	406.95 ± 11.25
Systolic blood pressure (mm Hg)	135.58 ± 3.33	133.82 ± 2.28	137.61 ± 2.43	135.19 ± 2.96
Relative left kidney weight (% to body weight)	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.27 ± 0.01
Relative right kidney weight (% to body weight)	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.26 ± 0.01
BUN (mmol L^{-1})	8.43 ± 0.24	7.89 ± 0.21	7.22 ± 0.18	7.35 ± 0.17
Plasma Cr (mmol L^{-1})	27.82 ± 1.05	29.4 ± 0.88	26.44 ± 0.81	27.68 ± 0.87
Plasma cystatin C (μg ml^{-1})	1.14 ± 0.05	1.17 ± 0.05	1.32 ± 0.03	1.43 ± 0.08
Urine volume (ml 24 h^{-1})	44.48 ± 8.79	14.33 ± 3.38**	45.16 ± 6.09	26.07 ± 4.38*
Urinary microalbumin (μg 24 h^{-1})	43.9 ± 27.11	90.9 ± 43.73*	109.26 ± 85.82	85.01 ± 60.74
Urine Cr (μmol 24 h^{-1})	72.47 ± 4.37	62.59 ± 5.77	131.24 ± 4.39	105.19 ± 9.87*
Urinary albumin-to-creatinine ratio (μg μmol^{-1})	0.55 ± 0.14	1.69 ± 0.31**	0.80 ± 0.14	1.08 ± 0.21
GFR/body weight (ml 24 h^{-1} g^{-1})	8.86 ± 0.47	6.66 ± 0.67*	8.42 ± 0.40	6.41 ± 0.64*

Note: Values are given as mean ± SE.

Abbreviations: BUN, blood urea nitrogen; Cr, creatinine; F0CD+F1CD: F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD; GFR, glomerular filtration rate.

*$p < .05$ vs. F0CD+F1CD; **$p < .01$ vs. F0CD+F1CD.

methylation in the Cd300lf gene promotor region and could demonstrate that all CpG sites were differently methylated (different methylation rate) in all 7 promoter CpG sites (Figure 5D, Table S6). Pearson correlation analysis indicated that 5 CpG sites of Cd300lf showed negative correlation with the expression of Cd300lf (Figure 6).

We likewise analyzed whether the methylation rate of a given CpG site is correlated with other CpG sites methylation within the promoter region, Pearson correlation matrices were calculated and plotted as heat maps for each group. The resulting group-specific correlation patterns were clearly different. Regarding the correlation matrices of the methylation rate of CpG islands within the Actr3b and Cd300lf promoter, positive correlations, indicated by red, were more predominant in the offspring born to grandfathers and fathers on an unhealthy diet when compared with the offspring born to grandfathers and fathers on a normal diet (Figure 7A,B).

4 | DISCUSSION

The present study was designed to investigate the hypotheses that an unhealthy diet during spermatogenesis might have adverse effects on kidney function and morphology in the offspring. Several studies have already investigated and established the concept of maternal programming of cardio-metabolic and renal diseases,[1,2] while the evidence for paternal programming of kidney diseases is still limited.[11,14]

We fed male rats an unhealthy diet during spermatogenesis over two generations (F0 and F1) and studied the effects on kidney function in the offspring (F1 and F2). Impairment of both GFR and urinary albumin excretion was predefined as the endpoint of the initial phenotypic screening. If this composite endpoint was met, we performed a detailed morphological and biochemical analysis of the kidneys. F1 offspring demonstrated non-significant reduction of GFR and non-significant increase of urinary albumin excretion, while F2 offspring showed a significantly decreased GFR and a significantly increased urinary albumin excretion in females only. Thus, we investigated the female F2 offspring in more detail.

Studies on renal outcomes caused by paternal factors are limited and preliminary so far. In a rat model, paternal high fat diet resulted in increased triglyceride content in the kidneys of the offspring and tubular damage such as loss of brush border and cell sloughing, while there were no signs of glomerular damage.[11] Another study[14] showed that paternal exposure to synthetic glucocorticoids alter the expression and DNA methylation of the mineralocorticoid receptor-, estrogen alpha receptor-, and

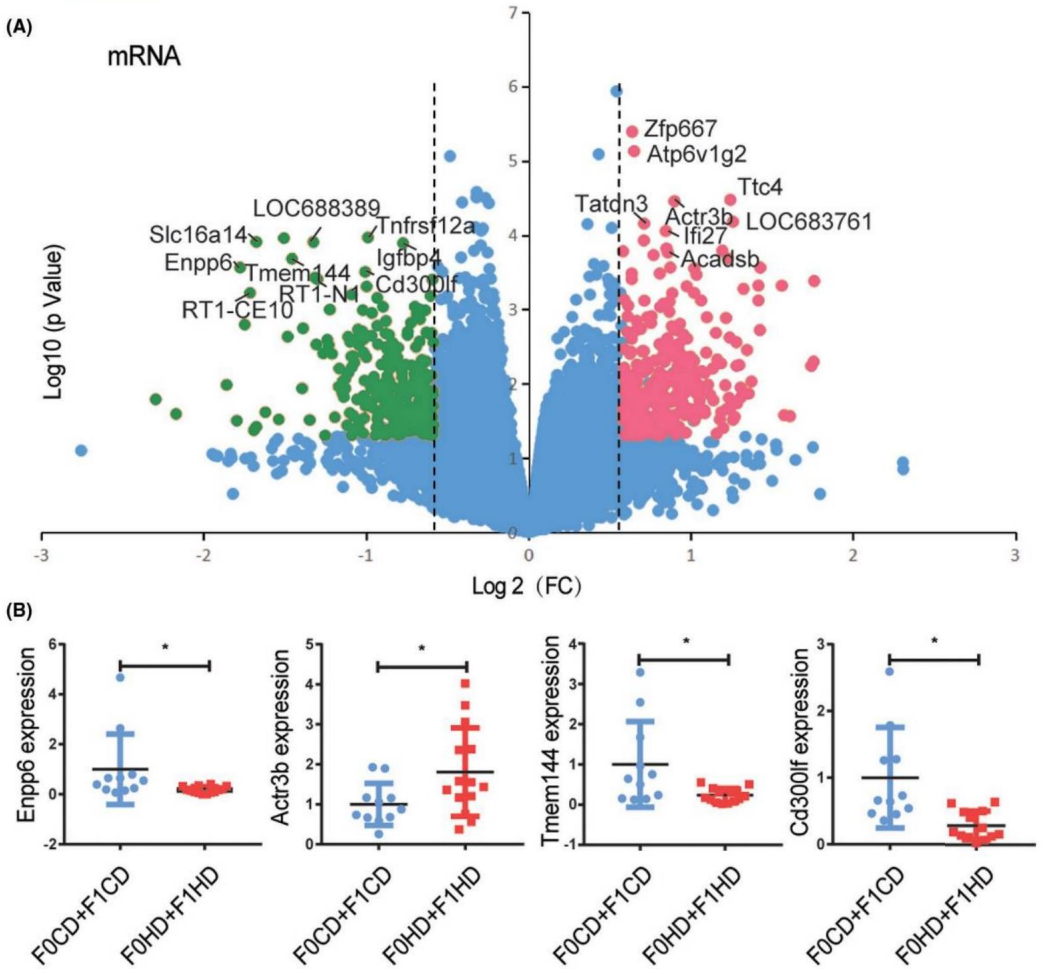

FIGURE 3 Analysis and verification of differentially expressed mRNAs. Volcano plot of differentially expressed mRNAs (A) based on |Fold change| ≥ 1.5 and $p < .05$ between the female offspring of F0CD+F1CD group and the F0HD+F1HD group. Blue dots indicate mRNA with no significant difference. Green dots indicate significantly down-regulated mRNAs, and red dots indicate significantly up-regulated mRNAs. The expression of *Enpp6*, *Cd300lf*, *Teme144* and *Actr3b* mRNA in kidney tissues of female F2 offspring was verified by qRT-PCR as only these four mRNAs showed significant differential regulation between the two groups (B). F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *$p < .05$ vs. F0CD+F1CD

glucocorticoid receptor gene in the hippocampus and kidney of offspring.

The molecular mechanisms of paternal programming of renal outcomes in the offspring are not well studied. However, several studies investigated paternal programming of other organs such as liver,[15–17] cardiovascular function,[18] pancreas,[19] adipose tissue,[20] and reproductive system.[21] There is now convincing evidence that the underlying molecular mechanisms are mediated via epigenetic alterations of the sperm such as sperm DNA methylation, histone modifications, noncoding RNAs in the tip of the sperm.[22–25] These epigenetic marks of the sperm cause after fertilization specific epigenetic alterations in target organs such as the endocrine pancreas and finally phenotypic alterations in the offspring. In other words, early epigenetic marks in the sperm induced by environmental factors as listed above cause specific epigenetic alterations in offspring target organs leading to a specific phenotype

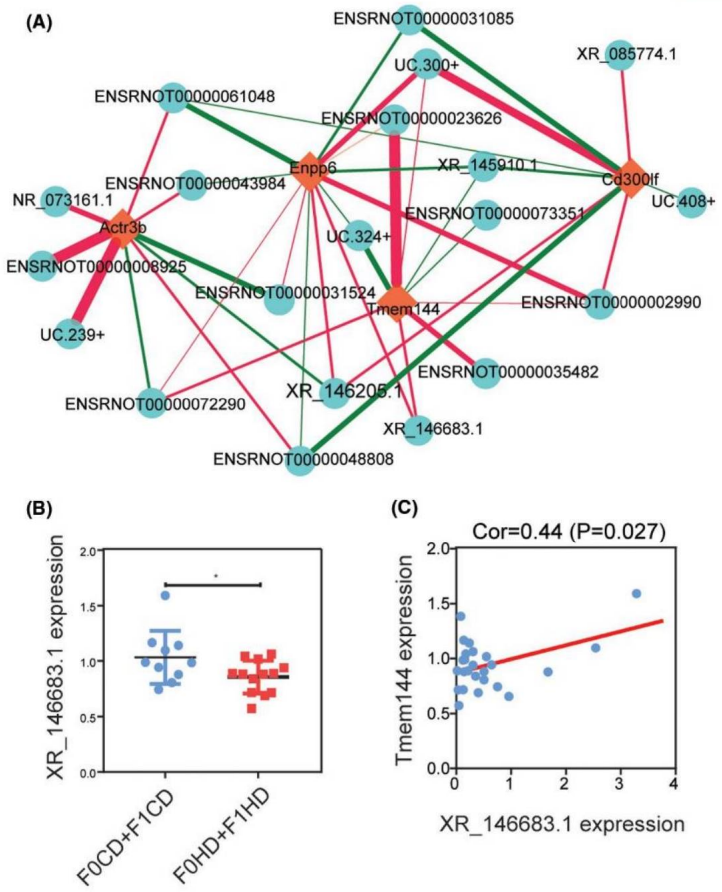

FIGURE 4 Correlation analysis of lncRNAs and mRNAs and verification of differentially expressed lncRNAs. (A) Correlation analysis of lncRNAs detected by the microarray approach and qPCR confirmed mRNAs. The circles represent lncRNAs and the diamonds represent mRNA. The red lines indicate positive correlation, and the green lines indicate negative correlation. The thicker the line, the higher the correlation coefficient. Pearson correlation coefficient >0.4 or <−0.4 and p value <.05 were set as screening criteria. (B) Renal lncRNA *XR_146683.1* expression in female F2 offspring. (C) Correlation analysis of renal lncRNAs *XR_146683.1 expression* and renal *Tmem144 mRNA expression*. F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *p < .05 vs. F0CD+F1CD

in the offspring later in life. This was best shown so far for metabolic conditions such as glucose metabolism/insulin resistance.

We did microarray profiling of mRNAs and lncRNAs in order to characterize the target organ specific epigenetic alterations in the offspring kidneys in those offspring who developed a renal phenotype (decreased GFR and increased urinary albumin excretion) in order to better understand the molecular mechanisms associated with the phenotype in the female F2 offspring of the fathers and grandfathers on an unhealthy diet during spermatogenesis. We identified 285 mRNAs and 129 lncRNAs differentially regulated by RNA sequencing. The statistically best candidates in terms of P-value and fold change were validated using qRT-PCR and hence four candidate genes (ENPP6: Ectonucleotide Pyrophosphatase/Phosphodiesterase 6 gene, TMEM144: Transmembrane Protein 144 gene, ACTR3B: Actin Related Protein 3B gene, and CD300LF: CD300 Molecule Like Family Member F gene) were finally proven to be differentially regulated. Moreover, the renal expression of lncRNA XR-146683.1 was significantly down-regulated in female F2 offspring of the F0HD+F1HD group compared with control female counterparts and its expression was correlated with the

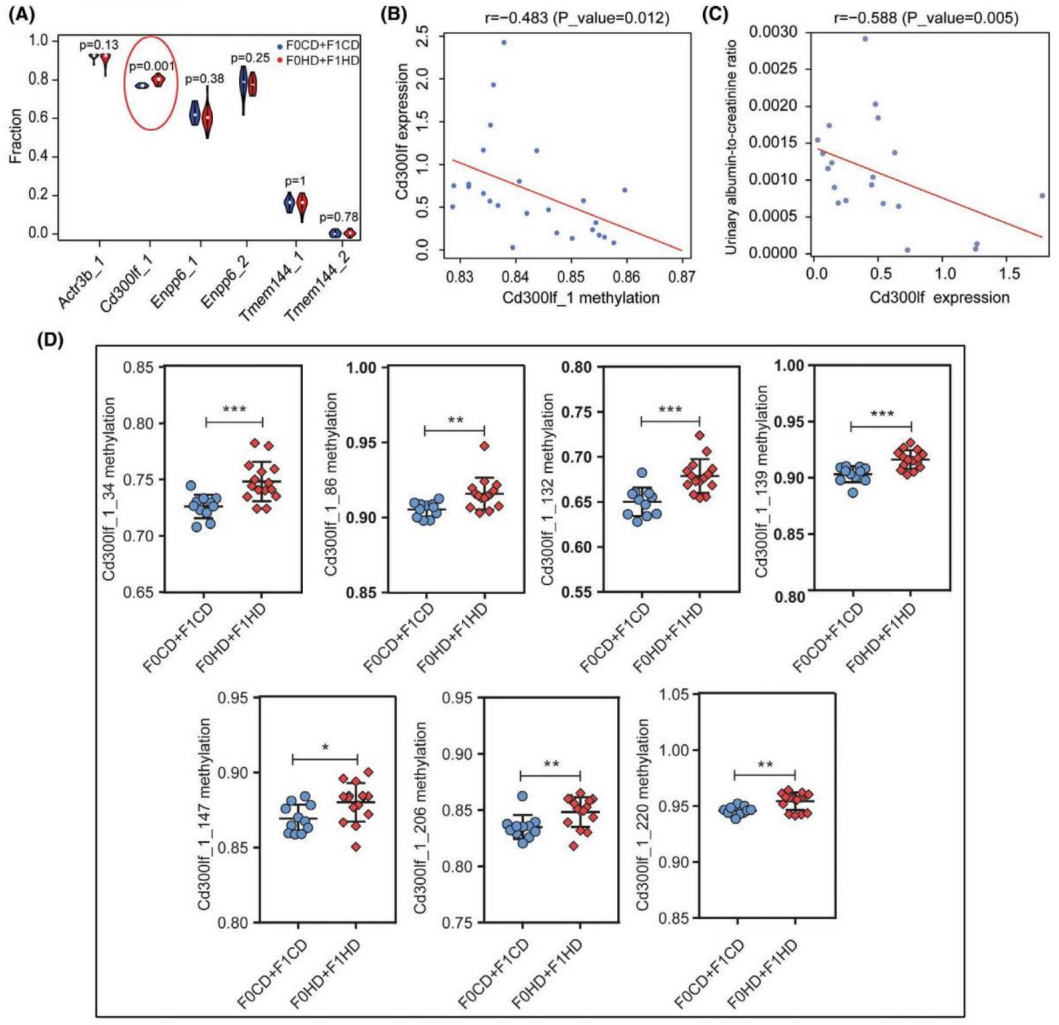

FIGURE 5 Relationship between *Cd300lf* DNA methylation—*Cd300lf* gene expression and urinary albumin excretion. (A) Comparison of overall *DNA methylation* in differently expressed genes between the F0CD+F1CD group and the F0HD+F1HD group in F2 female offspring. Fraction = methylated reads/Tatol reads detected in this fragment. (B) Correlation between methylation fragment of *Cd300lf* and the expression of *Cd300lf*. Differential CpG sites of *Cd300lf* between F0CD+F1CD group and F0HD+F1HD group in F2 female offspring kidney (C). (C) Correlation of *Cd300lf* gene expression and urinary albumin-to-creatinine ratio. (D) Comparison of methylation in kidneys of specific *Cd300lf* gene CpG sites between the F0CD+F1CD group and the F0HD+F1HD group of F2 female offspring. F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *$p < .05$ vs. F0CD+F1CD, **$p < .01$ vs. F0CD+F1CD

expression of Tmem144. The methylation rate of CpG islands in the promoter region was significantly affected in the Cd300lf gene, but not in other differently regulated genes in the F2 female offspring of the F0HD+F1HD group compared to control female counterparts. The methylation rate of CpG islands in the promoter region of Cd300lf was inversely correlated with Cd300lf mRNA expression in F2 female offspring. Moreover, Cd300lf mRNA expression was correlated to urinary albumin excretion suggesting that an unhealths died during spermatogenesis in the F0 and F1 generation affects methylation of the of CpG islands in the promoter region of Cd300lf in female

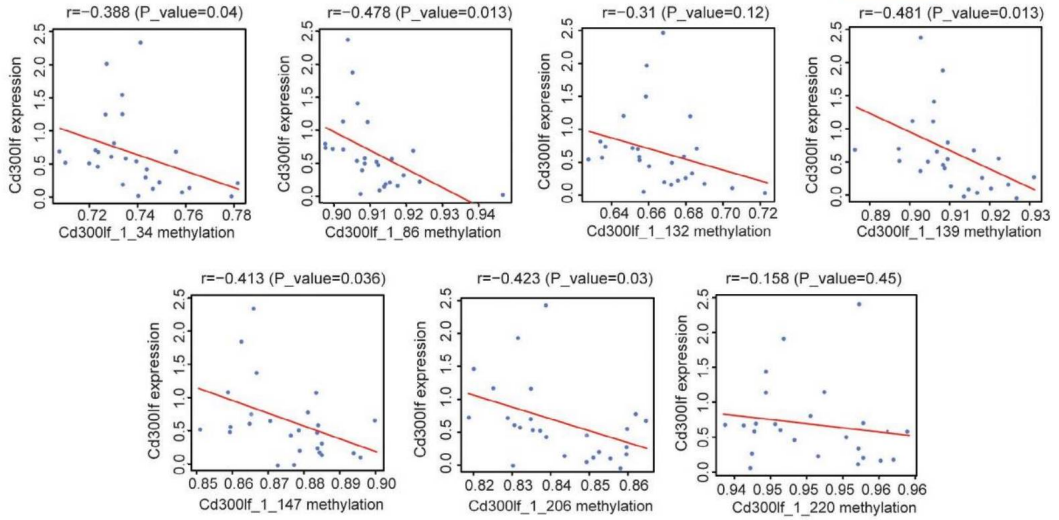

FIGURE 6 Correlation analysis between the degree of specific CpG site methylation and the expression of the corresponding *Cd300lf* mRNAs in F2 female offspring. A p value $< .05$ was considered statistically significant

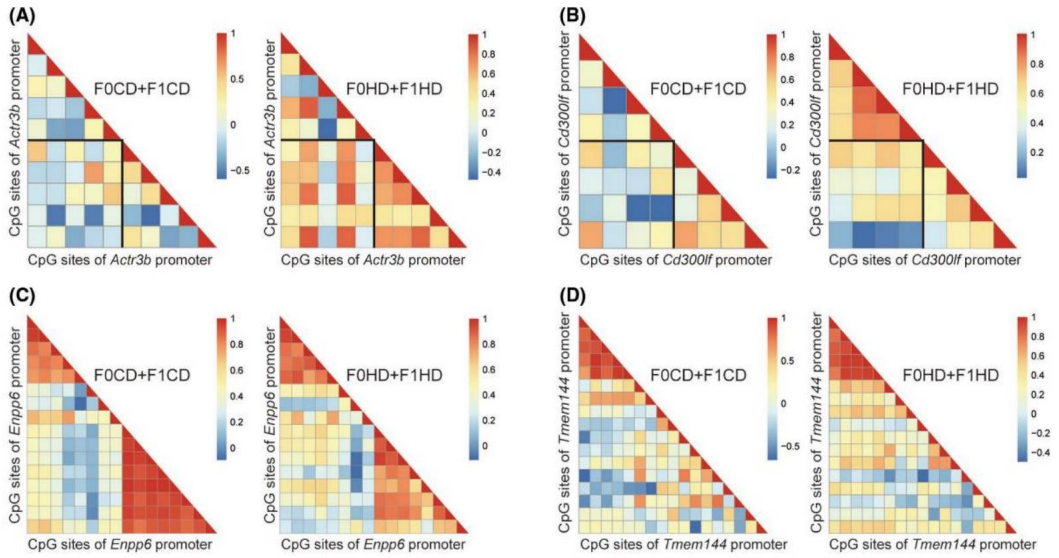

FIGURE 7 Heat maps of group-specific inter-CpG site correlation coefficients of DNA methylation of *Actr3b* (A), *Cd300lf* (B) *Enpp6* (C) and *Tmem144* (D) in female F2 offspring

offspring leading to alterations of Cd300lf gene expression and hence urinary albumin excretion. In addition, the methylation rate of two CpG sites (*Cd300lf*_1_132 and *Cd300lf*_1_147) in the promoter region of *Cd300lf* were positively correlated with the degree of glomerulosclerosis in F2 female offspring. Beside the degree of gene promoter methylation also the pattern of methylated CpG sites in a promoter of a gene might modulate its activity,[26] thus we analyzed treatment group-specific inter-CpG site correlation of DNA methylation and plotted the resulting correlation coefficients as heatmaps. Interestingly, different patterns were observed when comparing treatment

groups. Different correlation patterns between the degree of DNA methylation of one CpG site to another could result in a different net effect on gene expression by altering the attachment of transcription factors to gene promotors.

Cd300lf is expressed in monocytes and peripheral blood mononuclear cells.[27] *Cd300lf* acts as an inhibitory receptor for myeloid cells and mast cells.[28] It positively regulates the phagocytosis of apoptotic cells (efferocytosis) via phosphatidylserine (PS) recognition. It also plays an important role in the maintenance of immune homeostasis.[29] Phenotypes associated with *Cd300lf* include nervous system, homeostasis/metabolism and immune system phenotype,[30,31] but so far, few studies reported that this gene was involved in kidney diseases. Our findings are in agreement with studies suggesting that epigenetic modifications leading to glomerular and interstitial fibrosis through transcriptional regulation.[32,33] However, the specific molecular mechanism involved causing epigenetic changes of *Cd300lf* and subsequently alter kidney function and morphology in F2 female offspring need to be further analyzed.

The results of functional enrichment analysis of mRNAs and lncRNAs of our study showed that both differentially expressed mRNAs and lncRNAs were mainly enriched in antigen processing and presentation pathways. Similarly, a study by Jackson et al. found that maternal HFD can cause glomerulosclerosis and tubulointerstitial fibrosis in male offspring kidney by activating pro-inflammatory pathways.[34] A study conducted in a sheep model showed that maternal nutrient restriction during early fetal kidney development attenuates the effects of early onset obesity-related nephropathy, in part, through the downregulation of the innate inflammatory response.[35]

Our data suggest that paternal HFSSD over two generations resulted in functional alterations in the kidneys of female F2 offspring associated with increased glomerulosclerosis and tubulointerstitial fibrosis. The functional alterations observed in the F1 generation went into the same direction but did not reach statistical significance. For paternal programming induced by an unhealthy diet during spermatogenesis obviously two hits (high-fat, high-sucrose and high-salt diet during spermatogenesis in two subsequent paternal generations) are necessary. The effects were sex-dependent. Female F2 offspring are affected most. RNA sequencing followed by confirmatory qRT-PCR showed four differently regulated genes (Enpp6, Tmem144, Cd300lf, and Actr3b) in the kidneys of female F2 offspring. Methylation rate and pattern of CpG islands in the promoter region of Cd300lf was significantly invers correlated with Cd300lf mRNA expression in F2 female offspring ($r = -0.483$, $p = .0123$). Methylation pattern of the Cd300lf promotor was also altered, both effects might cause the observed effects on Cd300lf mRNA. Cd300lf mRNA expression itself was inversely correlated with the urinary albumin-to-creatinine ratio in female F2 offspring. Furthermore, the expression of lncRNA XR-146683.1 was significantly down-regulated in the F2 female offspring of fathers and grandfathers exposed to an unhealthy diet during spermatogenesis. Although we identified an epigenetic pathway linking Cd300lf methylation with urinary albumin excretion, it is more likely that complex alterations of gene expression are responsible for the observed renal phenotype in female F2 offspring of fathers and grandfathers on high-fat, high-sucrose and high-salt diet during spermatogenesis. In good agreement with this hypothesis, we saw that multiple renal genes (ENPP6, TMEM144, ACTR3B, and CD300LF) and also the non-coding RNA lncRNA XR-146683 are associated with the development of the renal phenotype of female F2 offspring (decreased GFR, increased urinary albumin excretion, glomerulosclerosis and renal interstitial fibrosis). Alteration of just a single pathway or even single genes as a result of fetal programming events during spermatogenesis and fetal development are rather uncommon, the environmental stimuli - high-fat, high-sucrose and high-salt diet during spermatogenesis in two subsequent paternal generations in our case—rather induce complex epigenetic marks[4,8,36–39] inducing the observed renal phenotype. How the interaction of these different epigenetic induced alterations in various gene expression patterns in the kidney finally causes the observed renal phenotype in female F2 offspring is yet unknown. Epigenetic alterations of paternal programming consist of two distinct epigenetic phenomena: the initial environmental factors—in our case the unhealthy pre-conceptional paternal diet—induced epigenetic alterations of the sperm (most important seem to be non-coding RNAs). This was first discovered in male rats exposed to a high fat diet. These initial alterations in the sperm causes later in life alterations in target organs such as the endocrine pancreas and fat tissues. DNA methylation of gene promotors of affected genes seems to be the main epigenetic alteration in this later stage of epigenetic changes.[19,40,41] We focused in our study on the late epigenetic effects in the kidney, see above. It is clearly a study limitation that we did not analyses epigenetic alterations in the sperm of the F0 and F1 generation and that we did not analyses epigenetic alterations in the less affected kidneys of the F1 generation.

Sex-dependency of fetal programming[37] and also paternal programming[19,40,41] is well known. A high paternal fat diet prior mating causes an impairment of glucose metabolism in particular in female offspring.[19] Potential underlying molecular pathways were reviewed recently.[37]

Taken together, our data suggest that a paternal pre-conceptional unhealthy diet might predispose in

particular female F2 offspring to chronic kidney disease. The mechanisms underlying this renal phenotype could involve differential regulation of the expression of mRNAs (Enpp6, Tmem144, Cd300lf, and Actr3b) and the lncRNA XR-146683.1 as well as alterations in the methylation pattern of CpG islands in the promoter region of the Cd300lf gene. This gene was reported to be involved in biological processes such as immune homeostasis and phagocytosis of apoptotic cells.

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DISCLOSURES

All authors declared no competing interests.

AUTHOR CONTRIBUTIONS

Berthold Hocher designed the study. Xiaoli Zhang, Ahmed A. Hasan, Suimin Zeng, Liping Liu and Li Xie performed the animal experiments and statistical analysis. Hongwei Wu performed bioinformatics analysis. Xiaoli Zhang, Ahmed A. Hasan, Jian Li and Berthold Hocher checked quality of the data. Xiaoli Zhang and Ahmed A. Hasan drafted the manuscript. Mohamed M. S. Gaballa, Jung Tobias, Grune Tilman, Bernhard K Krämer, Burkhard Kleuser, Jian Li and Berthold Hocher contributed to the revisions of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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2.4 Supplementary Material

Blood pressure measurement

Blood pressure measurements were performed on F2 female offspring at 9, 15 and 24 weeks of age using the CODA tail-cuff blood pressure system (Kent Scientific Corp., Torrington, CT, USA). Before the actual measurements, rats were trained to acclimate to the tail-cuff procedure. The tail blood pressure system was used in order to perform a longitudinal blood pressure analysis in rats throughout the study. Multiple measurements were performed with a minimum of 10 valid measurements for each rat.

Metabolic analysis

Blood samples were taken via aortic puncture, and serum was isolated. Blood urea nitrogen (BUN), creatinine (Cr) and Uric acid were measured using Hitachi 7020 automatic biochemistry analyzer (Hitachi High-Technologies, Tokyo, Japan). Serum cystatin C was measured by immunoassay (R & D systems, Minneapolis, USA). The 24-h urine of F2 offspring was collected using metabolic cage. The volume of urine was measured. After centrifugation, the supernatant was taken and urine microalbumin and urine creatinine were measured using Hitachi 7020 automatic biochemistry analyzer (Hitachi High-Technologies, Tokyo, Japan). The glomerular filtration rate (GFR) was evaluated by the creatinine clearance method[1,2] and calculated according to the following formula. GFR = (24-h urine volume * urine creatinine concentration) / serum creatinine concentration.

Kidney morphology

For histological analyses, kidneys were carefully harvested, total organ weight was measured. The samples of kidney were fixed in 10% (vol./vol.) neutral-buffered formalin, embedded in paraffin, and the paraffin blocks were then cut using a Jung RM 2025 microtome (Leica Biosystems, Wetzlar, Germany) to produce 4 µm thick sections on glass slides (Carl Roth, Karlsruhe, Germany) which were stained later to perform histological analysis. The slices of kidney were stained with periodic acid Schiff (PAS) staining for evaluation of the number and size of glomeruli and Sirius Red (SR) staining for fibrosis assessment. The samples were examined with light microscopy using BZ 9000 microscope by Keyence (Keyence, Neu-Isenburg, Germany). The number of glomeruli was determined by counting 20 non-overlapping consecutive fields (25 × 25µm^2 of cortex) in each of the three PAS-stained serial sections at 100× magnification by two different investigators who

were unaware of the specimen groups. Glomerular counting of each group was performed in 5 kidneys × 3 serial sections × 20 fields. [3]. Glomerular size was assessed by measuring the area of ≥50 glomeruli in each longitudinal PAS-stained kidney section using ImageJ software (National Institutes of Health, Bethesda, USA). Glomerulosclerosis was evaluated using PAS-stained kidney sections by rating the percentage of the PAS-positive areas within the glomerulus using a subjective, semi-quantitative scoring system (grade I–IV) performed by 2 investigators who were blinded to the study groups. Then, the glomerulosclerosis index was calculated according to the following formula: glomerulosclerosis index = [(the number of glomeruli rated with grade I × 1) + (the number of glomeruli rated with grade II × 2) + (the number of glomeruli rated with grade III × 3) + (the number of glomeruli rated with grade IV × 4)] / the total number of glomeruli. To analyze renal interstitial fibrosis, at least 10 microscopic fields, without glomeruli, blood vessels and big cavities, at 200× magnification per sirius-red-stained kidney section were captured using BZ-9000 compact fluorescence microscope (Keyence, Osaka, Japan). The thresholds for detecting the fibrotic area (sirius red positive area) per microscopic field were determined using a random subset of images with the aid of ImageJ software. Then, the percentages of the fibrotic areas per sections were calculated.

lncRNA-mRNA expression profile analysis

Microarray profiling of 14 kidney tissue samples (7 samples in each group) in F2 female offspring was carried out in collaboration with Oebiotech (China) using Agilent Rat lncRNA Microarray V2 (8*60K, Design ID: 062716).

Total RNA was quantified by the NanoDrop ND-2000 (Thermo Scientific) and the RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA were transcribed to double strand cDNA, then synthesized into cRNA and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned by the Agilent Scanner G2505C (Agilent Technologies). Feature Extraction software (version10.7.1.1, Agilent Technologies) was used to analyze array images to get raw data. Genespring (version 13.1, Agilent Technologies) were employed to finish the basic analysis with the raw data. To begin with, the raw data was normalized with the quantile algorithm. The probes that at least 50% out of every conditions have flags in "P" were chosen for further data

analysis. Differential analysis was performed using limma package[4] in R language. Genes with P values <0.05 and |fold change| ≧1.5 were considered significantly changed mRNAs and lncRNAs.

Real-time quantitative PCR

Total RNA was isolated from the kidney of the F2 offspring using Trizol reagent (TaKaRa, Dalian, Liaoning, China). The cDNA was synthesised using oligo-dT and random primers (TaKaRa, Dalian, Liaoning, China) for real-time quantitative PCR (qRT-PCR). qRT-PCR was performed using a Biorad CFX96 cycler (Bio-Rad laboratories, Hercules, CA, USA) and the SYBR Green mix (TaKaRa, Dalian, Liaoning, China). Primers for qRT-PCR were designed using the Primer 5 software and are presented in ESM Table 3. *β-Actin* was used to normalise selected mRNA expression and *Gapdh* was used to normalise selected lncRNA expression. The $2^{-[delta][delta]Ct}$ method was used to calculate the expression mRNA levels, where the Ct value represents the difference between cycle threshold values.

Correlation analysis of lncRNAs and mRNAs and functional prediction

The function prediction of lncRNAs is on the basis of the functional annotations of their expression-related mRNAs[5]. For each lncRNA, we calculated the Pearson correlation of its expression value with that of each mRNA and identified expression-related mRNAs of each lncRNA based on a Pearson correlation coefficient > 0.4 or < −0.4 and p <0.05. DAVID functional annotation database (https://david.ncifcrf.gov/), including gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, was applied to computed these correlative genes and then the enriched functional terms were used to predict functional terms of the corresponding lncRNAs. We analyzed the enrichment of functional term in annotation of coexpressed mRNAs using hypergeometric cumulative distribution function. Furthermore, lncRNA-transcription factor relationship pairs were predicted in ENCODE (Encyclopedia of DNA Elements) database[6].

Gene-specific DNA methylation in the kidney

DNA was extracted from the kidney and the concentration and purity were assessed spectrophotmetrically. Gene specific DNA methylation of CpG islands of the promoter region of Enpp6, Tmem144, Actr3b and Cd300lf was analysed using MethylTarget, based on Illumina next-

generation sequencing in combination with bisulfite treatment and DNA methylation mapping as previously described[7-9]. Illumina next generation sequencing was carried out in collaboration with Genesky Biotechnologies (Shanghai, China).

Supplementary Table 1. Composition of CD, and HFSSD

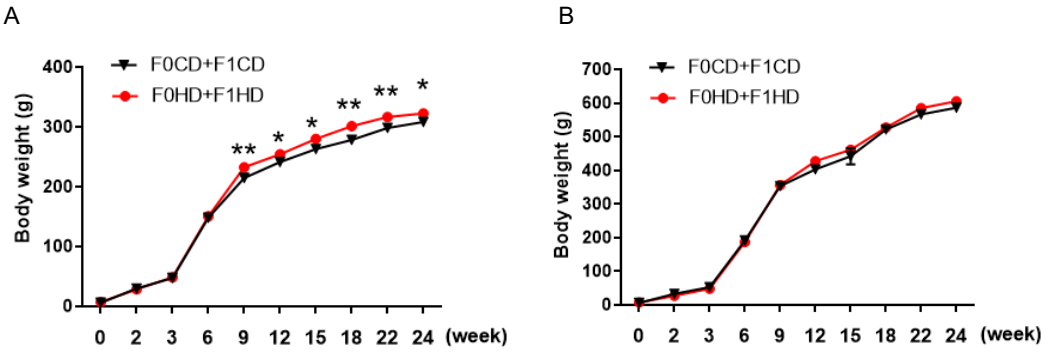
Components	CD	HFSSD
Casein(%, wt/wt)	20.0	23.3
Corn starch (%, wt/wt)	39.8	8.7
Maltodextrin (%, wt/wt)	13.2	10.5
Sucrose (%, wt/wt)	10.0	20.1
Fiber (%, wt/wt)	5.0	5.0
Soybean oil (%, wt/wt)	7.0	2.9
Lard (%, wt/wt)	0.0	20.7
Salt (%, wt/wt)	0.3	3.7
Minerals (%, wt/wt)	3.5	3.5
Vitamins (%, wt/wt)	1.0 (0.01 folate)	1.0 (0.01 folate)
Energy		
Energy value (kJ/kg)	16736	19539
Lipids (%)	15.8	45.5
Sucrose (%)	10.00	17.25
Proteins (%)	20.30	20.23

Abbreviations: control diet (CD), high-fat, high-sucrose and high-salt diet (HFSSD).

Supplementary Table 2. Body weight gain，blood pressure and serum metabolite levels in F0 male founder

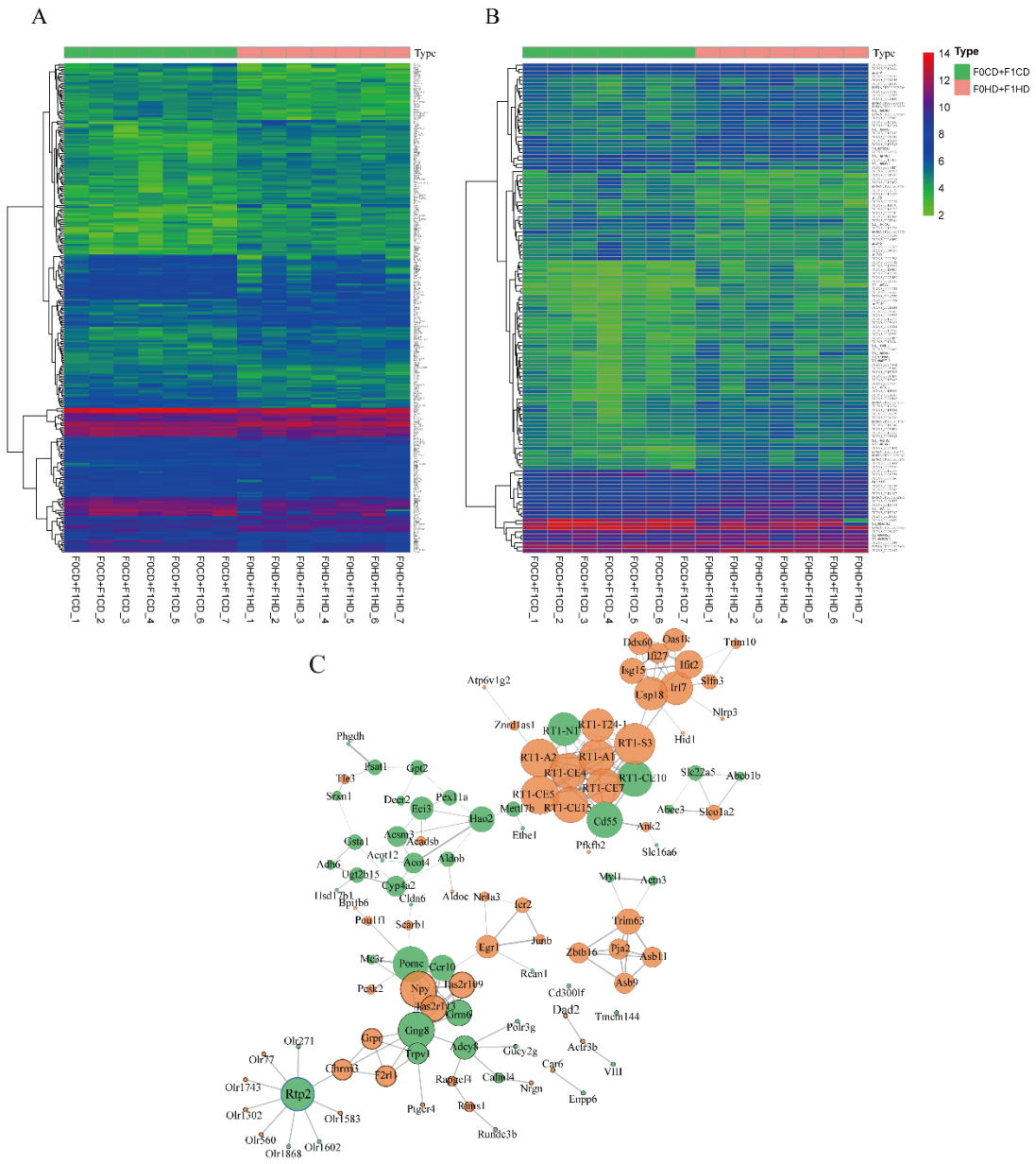
Parameter	CD (n=15)	HFSSD (n=15)
Body weight on arrival in the lab (g)	177.2 ± 5.2	175.5 ± 2.9
Body weight at the age of 9 weeks(g)	396.1 ±4.9	415.5 ±7.3
Body weight at the age of 12 weeks(g)	447.1±4.1	472.4±7.5**
Body weight at the age of 15 weeks(g)	475.8 ± 4.8	504.4 ± 8.1**
Body weight at the age of 18 weeks(g)	539.6 ± 4.1	562.6 ± 8.4*
Heart rate (bpm)	400.1 ± 9.4	416.0 ± 10.1
Systolic blood pressure (mmHg)	125.6 ± 3.0	132.4 ± 2.0
BUN (mmol L-1)	7.0 ± 0.3	8.3 ± 0.3**
Plasma Cr (mmol L-1)	35.3 ± 2.2	35.4 ± 2.1

Abbreviation: BUN, blood urea nitrogen; Cr, creatinine; control diet (CD), high fat-sucrose-salt diet (HFSSD). Values are given as mean ± SE. *: $P < 0.05$ *versus* CD, **: $P < 0.01$*versus* CD.



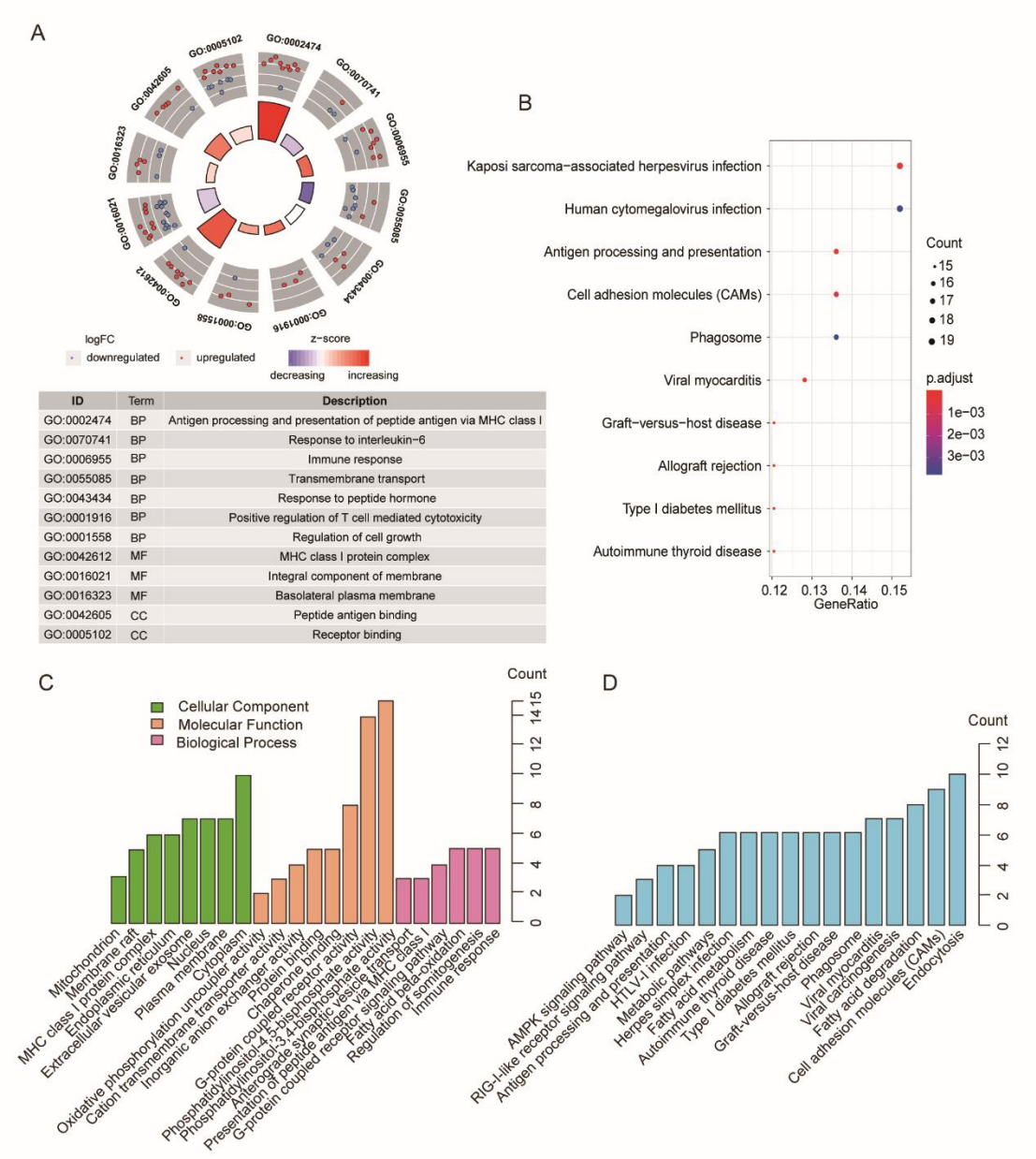
Supplementary Figure 1. The effect of paternal HFSSD on body weight of F2 female offspring (A) and male offspring (B).

*: P<0.05 *versus* F0CD+F1CD, **: P<0.01*versus* F0CD+F1CD.



Supplementary Figure 2 : Identification of DEGs. Heatmaps of Differentially expressed mRNAs (A) and lncRNAs (B) between F0CD+F1CD group and F0HD+F1HD group in F2 female offspring. |fold Change|≥1.5 and P-value < 0.05 were set as screening criteria. Protein-protein interaction network (C) of differentially expressed mRNA based on interaction score≥0.4.

Abbreviation: F0CD+F1CD: F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD: F2 offspring of F0 and F1 male founders fed a HFSSD.



ID	Term	Description
GO:0002474	BP	Antigen processing and presentation of peptide antigen via MHC class I
GO:0070741	BP	Response to interleukin−6
GO:0006955	BP	Immune response
GO:0055085	BP	Transmembrane transport
GO:0043434	BP	Response to peptide hormone
GO:0001916	BP	Positive regulation of T cell mediated cytotoxicity
GO:0001558	BP	Regulation of cell growth
GO:0042612	MF	MHC class I protein complex
GO:0016021	MF	Integral component of membrane
GO:0016323	MF	Basolateral plasma membrane
GO:0042605	CC	Peptide antigen binding
GO:0005102	CC	Receptor binding

Supplementary Figure 3：Functional enrichment analysis of DEGs and lncRNAs. Significant GO terms (A) and KEGG pathways (B) in the enrichment analyses of the 285 mRNAs. GO terms (C) and KEGG pathways (D) of the differential lncRNAs. The ordinate represents the frequency at which the lncRNA was enriched to each GO or KEGG entry.

Abbreviation: GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes.

Supplementary Table 3: Real-time polymerase chain reaction primers

Gene	Forward Primer	Reverse Primer
mRNA		
Enpp6	GTGGATTCAGGAACGAGGCT	TGTGATATATAACGGGCCCTTGG
Slc16a14	AGAGCCATGCTATTTTGGGACA	GGCAGGTGGATGAAAGGGAT
Iafbp7	AGCGTCGGCCAAAATTACAG	GTCAGGCAGGAGTGGGGTTA
LOC688389	TCCCAAGGCTGTGATTTCTTGT	AGGCAGTGGTGGTCAATGAT
LOC683761	TGCTGTGGCCATCATTAAAGC	AGCAGGGGTGTAGTCTCCTT
Tnfrsf12a	TCGGGTTGGTGTTGATACGC	TCCCAGAATGGGCCATAGCA
RT1-CE10	ACCCAGAGATGGGAGCCTTC	AACTACAAAGACAGCCAAAGGT
RT1-N1	GCCTGAAGGACAACTGCTCA	ATTCAACTGCCAGGTCAGGG
Apom	TCCTCTACAACCGATCGCCA	AGGACACAAGTGGAGTCACG
Atp6vla2	TGGAGGATCGCATCAAGGGA	AAGATGCTGTTCCGAAGGCG
Zfp667	GGAGGACGCTGTAGGGAGATAG	TCTGATTGGGACTCAGCCATTC
Ttc4	CTGAGGCTGTGAACTGGTGT	TTGAGAACTCACCTTGATGGC
Tatdn3	GTTCTGCTGCATGCGTTTGA	TCCGTGTCAGTTTTTCGGGT
Ifi27	GCTGGCACCGTTTTATCCAG	GCTAGAGAGGAGGCTGCAAT
Tmem144	GAGCTGCTCCCTGGTTGATA	CATGGTTGCAGGGTGGTAGA
Actr3b	GTACCAGGGCTCTACATTGCAGTA	CCTGCAATTGGGATGTGTTTGAT
Acadsb	CGATGCGAAGATCGGTACAAT	GACGTCAGTACTCTGCATCG
Cd300lf	TTCTCTTGCTGGTGGTCTCAC	GTGAGTCACAGAATCAGCCTGC
ß-Actin	CTGGCTCCTAGCACCATGAA	AAAACGCAGCTCAGTAACAGTC
lncRNA		
NR_073161.1	CCAAGTCGGGCTAGTTCGTG	CACTTGGGAAGACCACGCC
XR_145910.1	CGCCGAGCACCTTAAGTCTCAAG	AGAATAGACCAACACTGGCATCCG
XR_146205.1	GACTGGAAGATTGGTCCGTGTGC	GCAGTCCTCAGCCAGTCAACAC
XR_146683.1	CCTTCCTCCAGTCAGCCTCCTTG	AGAAGTCAGAGCCTGGTCTCACG
uc.324+	TCGGTGAAATCGCCTGTGTG	TGACACTCCAAAACCAGCGG
ENSRNOT00000048808	TGTAACTTCACGGAGAAGGCATCG	CCAGCTCAACCTTGGCGTAGTG

ENSRNOT00000008925	GACAAGGAAGGGAACGGCAC	CGTTGCTGTCCTCATAGCCC
uc.239+	CATGGCAGAATCCCTCACACA	CAGCGGGGTTCATTAAGGTCC
uc.300+	TTCTGGAAAGAATGGAAGGGAGG	GGCTGTGCCTGGCCTAAAT
ENSRNOT00000072290	CAGGCACAGCTCTCCAACTTCAAG	GTGTTCTGCTTCACCACGGTAAGG
ENSRNOT00000061048	TTCACCAGCGCCTTCAGCTA	TCACACCAAGGGCTTTCGGT
ENSRNOT00000031085	TGGCTACTCTTACAACGCACTCAC	GTTGGAGAGCAAGATGGTCCTGAC
ENSRNOT00000031524	CGGAGGTGTGAGGAGGAAGCC	CGCAGTGAGCCTGGAGATTAAGTC
ENSRNOT00000073351	CTTTCCTGGCCTGACGCAC	AAGAAGTAGAGGAGTTTGGGGGA
ENSRNOT00000043984	CCTCAAGGGCAAAGCCAAGT	AGCTCTTCTGCCTTCTCCACAT
ENSRNOT00000002990	CCTCAACCTTGCTCACCAACAGAG	TGTGTCAGCTTCATGGCTTCAGAG
uc.408+	CCGTGTCAGACTCCTCCGAA	GCGAAGCCCCCTTCTTTTCC
XR_085774.1	ATACACAGCGTGTGACAGCA	CCCGGCATTGCAAGAGAAAG
ENSRNOT00000023626	CACGATTCTCCGTCTGTGAGGAAG	AGGCGGAGGAGCAGAGAACG
ENSRNOT00000035482	TCGCTGGTGCTCAGAAGAATGTTG	CGTAGATGCTGAAGCATGGAGGTG
Gapdh	AGTGCCAGCCTCGTCTCATA	GGTAACCAGGCGTCCGATAC

Supplementary Table 4. Validation of the 18 candidate mRNAs detected by RNA sequencing of all samples from the F0CD+F1CD group and the F0HD+F1HD group in F2 female offspring by qRT-PCR.

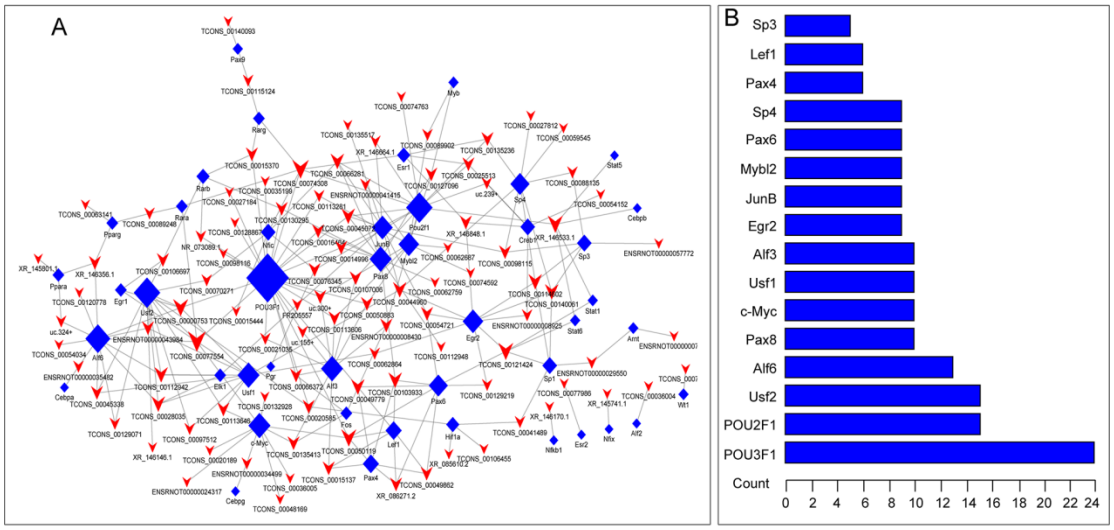
Gene	F0CD+ F1CD (n=11)	F0HD+F1HD (n=14)
Enpp6	1.00±0.42	0.17±0.04*
Slc16a14	1.00±0.38	0.75±0.27
Igfbp7	1.00±0.22	1.23±0.35
LOC688389	1.00±0.16	0.90±0.20
LOC683761	1.00±0.41	3.04±1.52
Tnfrsf12a	1.00±0.18	0.79±0.27
RT1-CE10	1.00±0.51	0.89±0.72
RT1-N1	1.00±0.20	0.74±0.10
Apom	1.00±0.12	0.96±0.16
Atp6vlg2	1.00±0.18	1.26±0.20
Zfp667	1.00±0.27	1.03±0.11
Ttc4	1.00±0.16	1.38±0.20
Tatdn3	1.00±0.13	1.37±0.17
Ifi27	1.00±0.15	1.11±0.28
Tmem144	1.00±0.32	0.23±0.05*
Actr3b	1.00±0.16	1.81±0.29*
Acadsb	1.00±0.24	1.46±0.37
Cd300lf	1.00±0.23	0.58±0.30*

Abbreviation: F0CD+F1CD: F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD: F2 offspring of F0 and F1 male founders fed a HFSSD. Values are given as mean ± SE. *P < 0.05 *versus* F0CD+F1CD.

Supplementary Table 5. Validation of the 20 candidate lncRNAs detected by RNA sequencing in all samples from the F0CD+F1CD group and the F0HD+F1HD group in F2 female offspring by qRT-PCR

Gene	F0CD+ F1CD (n=11)	F0HD+ F1HD (n=14)	Target mRNA	Correlation coefficient	P_value
NR_073161.1	1.00±0.15	0.98±0.08	*Actr3b*	0.154	0.46
XR_145910.1	1.00±0.14	0.98±0.08	*Enpp6*	-0.046	0.82
XR_146205.1	1.00±0.12	0.91±0.07	*Enpp6*	0.07	0.70
XR_146683.1	1.03±0.07	0.86±0.04*	*Tmem144*	0.44	0.02*
uc.324+	1.00±0.08	1.31±0.15	*Tmem144*	-0.25	0.22
ENSRNOT00000048808	1.00±0.15	0.97±0.08	*Cd300lf*	0.17	0.41
ENSRNOT00000008925	1.00±0.12	0.84±0.08	*Actr3b*	-0.05	0.79
uc.239+	1.00±0.06	0.84±0.04*	*Actr3b*	-0.06	0.77
uc.300+	1.00±0.16	1.09±0.14	*Enpp6*	-0.07	0.725
ENSRNOT00000072290	1.00±0.12	1.33±0.22	*Enpp6*	-0.27	0.23
ENSRNOT00000061048	1.00±0.12	1.17±0.20	*Enpp6*	-0.113	0.61
ENSRNOT00000031085	1.00±0.13	1.18±0.22	*Cd300lf*	0.15	0.50
ENSRNOT00000031524	1.00±0.15	1.21±0.18	*Enpp6*	-0.10	0.635
ENSRNOT00000073351	1.00±0.13	1.03±0.11	*Tmem144*	-0.13	0.57
ENSRNOT00000043984	1.00±0.12	0.88±0.09	*Actr3b*	-0.07	0.75
ENSRNOT00000002990	1.00±0.06	1.04±0.11	*Enpp6*	-0.19	0.42
uc.408+	1.00±0.14	1.12±0.17	*Cd300lf*	-0.01	0.96
XR_085774.1	1.00±0.12	1.14±0.15	*Cd300lf*	-0.22	0.31
ENSRNOT00000023626	1.00±0.11	0.98±0.10	*Tmem144*	-0.28	0.20
ENSRNOT00000035482	1.00±0.11	0.93±0.08	*Tmem144*	-0.06	0.78

Abbreviation: F0CD+F1CD: F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD: F2 offspring of F0 and F1 male founders fed a HFSSD. Values are given as mean ± SE. *P < 0.05 *versus* F0CD+F1CD.



Supplementary Figure 4. Correlation analysis between lncRNAs and TFs. Co-expression networks of lncRNAs and TFs (A). Blue nodes represent transcription factors and red nodes represent lncRNAs. The hub TFs associated with the expression of lncRNAs (B). The abscissa represents the frequency at which lncRNA enriched into each TF.

Abbreviation: TF, transcription factors.

Supplementary Table 6. Methylation level of differential CpG sites between F0CD+F1CD group and F0HD+F1HD group in F2 female offspring.

Target	Position	Type	Chr	Distance 2 TSS	Genome Position	Methylation level	P-value
*Cd300lf*_1	34	CG	10	759	103685120	Up	0.001
*Cd300lf*_1	86	CG	10	811	103685068	Up	0.006
*Cd300lf*_1	132	CG	10	857	103685022	Up	⩽0.001
*Cd300lf*_1	139	CG	10	864	103685015	Up	⩽0.001
*Cd300lf*_1	147	CG	10	872	103685007	Up	0.025
*Cd300lf*_1	206	CG	10	931	103684948	Up	0.009
*Cd300lf*_1	220	CG	10	945	103684934	Up	0.001

Abbreviation: Chr, chromosome. TSS, anscriptional start site. F0CD+F1CD: F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD: F2 offspring of F0 and F1 male founders fed a HFSSD.

Supplementary Table 7. Correlation analysis between differentially expressed mRNA / lncRNA, differential methylation site and the parameters related to kidney function / histology in F2 female offspring

	Urinary albumin-to-creatinine ratio	GFR / body weight (ml/24h/g)	Glomerulosclerosis score	Renal interstitial fibrosis area (%)
mRNA expression				
Enpp6	-0.401	0.256	-0.094	-0.357
Tmem144	-0.336	0.406	0.08	0.109
Actr3b	0.185	-0.144	0.06	0.357
Cd300lf	-.588**	0.01	-0.333	-0.243
lncRNA expression				
XR_146683.1	-0.09	0.127	-0.171	-0.111
Methylation				
*Cd300lf*_1_34	0.19	-0.328	0.185	0.181
*Cd300lf*_1_86	0.399	-0.104	0.195	0.187
*Cd300lf*_1_132	0.267	-0.286	.579**	0.188
*Cd300lf_1*_139	0.397	-0.323	0.258	0.306
*Cd300lf*_1_147	0.168	-0.194	.539**	0.305
*Cd300lf*_1_206	0.07	-0.189	0.387	0.213
*Cd300lf_1*_220	0.352	-0.366	0.294	0.251

* $P < 0.05$, ** $P < 0.01$.

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3 Publication 2：Paternal eNOS Deficiency affects Glucose Homeostasis and Liver Glycogen in male offspring without inheritance of eNOS Deficiency itself

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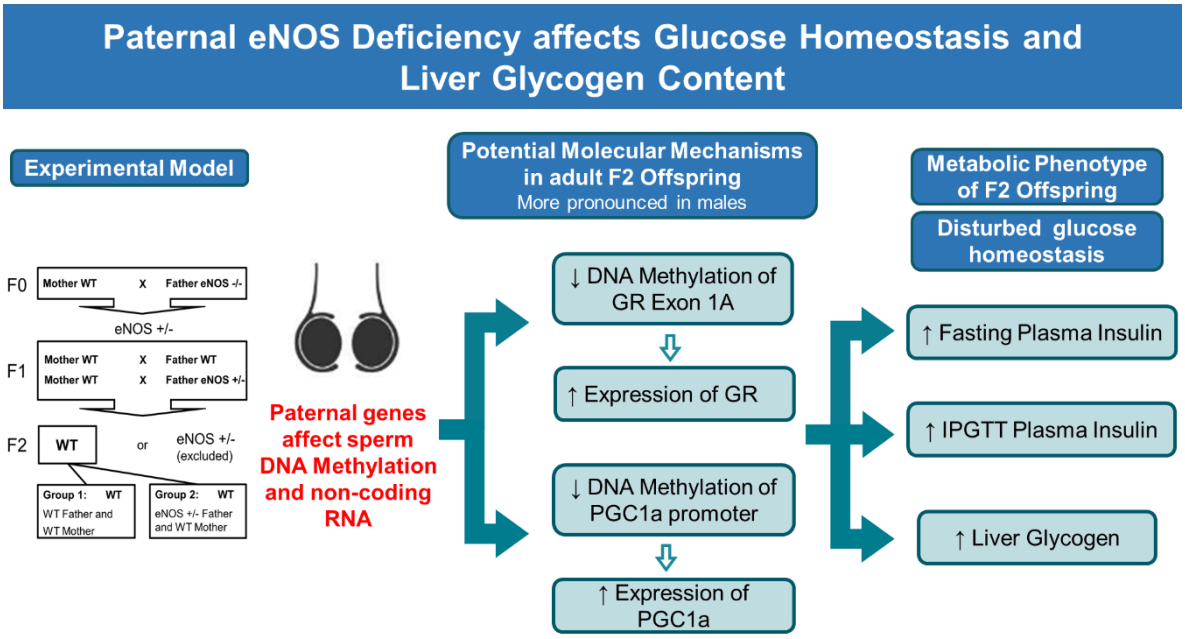
DOI: https://doi.org/10.1007/s00125-022-05700-x

3.1 Graphic abstract



3.2 Author's contribution

In this publication, Xiaoli Zhang made the following substantial contributions, including: 1) conducting portions of the animal experiments; 2) performing morphological analyses of mouse sperm, as well as DNA methylation and miRNA detection and analysis in sperm samples; 3) executing statistical analyses and creating figures and tables; 4) assisting with the manuscript submission process; 5) revising the manuscript.

3.3 Main content of the publication

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ARTICLE

Paternal eNOS deficiency in mice affects glucose homeostasis and liver glycogen in male offspring without inheritance of eNOS deficiency itself

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Abstract

Aims/hypothesis It was shown that maternal endothelial nitric oxide synthase (eNOS) deficiency causes fatty liver disease and numerically lower fasting glucose in female wild-type offspring, suggesting that parental genetic variants may influence the offspring's phenotype via epigenetic modifications in the offspring despite the absence of a primary genetic defect. The aim of the current study was to analyse whether paternal eNOS deficiency may cause the same phenotype as seen with maternal eNOS deficiency.

Methods Heterozygous (+/−) male eNOS (*Nos3*) knockout mice or wild-type male mice were bred with female wild-type mice. The phenotype of wild-type offspring of heterozygous male eNOS knockout mice was compared with offspring from wild-type parents.

Results Global sperm DNA methylation decreased and sperm microRNA pattern altered substantially. Fasting glucose and liver glycogen storage were increased when analysing wild-type male and female offspring of +/− eNOS fathers. Wild-type male but not female offspring of +/− eNOS fathers had increased fasting insulin and increased insulin after glucose load. Analysing candidate genes for liver fat and carbohydrate metabolism revealed that the expression of genes encoding glucocorticoid receptor (*Gr*; also known as *Nr3c1*) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1a*; also known as *Ppargc1a*) was increased while DNA methylation of *Gr* exon 1A and *Pgc1a* promoter was decreased in the liver of male wild-type offspring of +/− eNOS fathers. The endocrine pancreas in wild-type offspring was not affected.

Conclusions/interpretation Our study suggests that paternal genetic defects such as eNOS deficiency may alter the epigenome of the sperm without transmission of the paternal genetic defect itself. In later life wild-type male offspring of +/− eNOS fathers developed increased fasting insulin and increased insulin after glucose load. These effects are associated with increased *Gr* and *Pgc1a* gene expression due to altered methylation of these genes.

Keywords eNOS · Glucocorticoid receptor · Insulin resistance · Paternal programming · PGC1a

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Research in context

What is already known about this subject?

- Fetal programming can be caused by maternal genes affecting the offspring's phenotype in later life, independently of the offspring's genome
- Wild-type female offspring of heterozygous female eNOS knockout mice and wild-type male mice develop fatty liver disease and numerically lower fasting glucose. This is accompanied by sex-specific differences in expression and methylation of distinct genes

What is the key question?

- Are paternal genes, without transmission to the offspring, likewise able to affect the phenotype of the offspring?

What are the new findings?

- Mild paternal nitric oxide deficiency alters sperm DNA methylation and non-coding RNA profile
- Fasting glucose, fasting insulin, insulin response after glucose load, and liver glycogen storage were increased when analysing wild-type male and female offspring of +/− eNOS fathers
- Liver glucocorticoid receptor (*Gr*; also known as *Nr3c1*) and *Pgc1a* (also known as *Ppargc1a*) gene expression was increased while DNA methylation of *Gr* exon 1A and *Pgc1a* promoter was decreased in male wild-type offspring of +/− eNOS fathers

How might this impact on clinical practice in the foreseeable future?

- Using a gene knockout mouse model of reduced nitric oxide synthesis, this study shows that paternal genes that are not inherited by the offspring can still influence the phenotype of the offspring by altering the epigenome in the sperm and, later in life, in organs of the offspring

Abbreviations

eNOS	Endothelial nitric oxide synthase
FDR	False discovery rate
GR	Glucocorticoid receptor
iNOS	Inducible nitric oxide synthetase
L-NAME	$N(\gamma)$-nitro-L-arginine methyl ester
MeDIP	Methylated genomic DNA immunoprecipitation
miRNA	microRNA
NO	Nitric oxide
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
WT	Wild-type

Introduction

The 'fetal origin of diseases' hypothesis proposes that adulthood diseases originate through adaptation of the fetus to environmental conditions in early life [1]. Another mechanism responsible for programming events might be related to maternal genes affecting the fetal phenotype independently of the offspring's genome [2–14]. These clinical association studies stimulated the initiation of animal studies to identify the underlying molecular mechanisms. Heterozygous (+/−) female mice in which the *Nos3* gene, encoding endothelial nitric oxide synthase (eNOS), was knocked out and wild-type (WT) female mice were bred with male WT mice. Female offspring with normal *Nos3* gene status but born to heterozygous female eNOS knockout mice develop hepatic steatosis [15], causally demonstrating that maternal genes can epigenetically alter the offspring's phenotype without inheritance of the gene itself [15]. Paternal environmental factors prior to mating likewise affect the offspring's phenotype [1, 16]. It was shown that a pre-conceptional paternal high-fat diet results in impaired glucose tolerance in female offspring [17–21]. There are already studies suggesting that paternal genes without transmission to the offspring might likewise affect the offspring's phenotype [22–24] (Fig. 1).

We have chosen male heterozygous eNOS knockout mice to test this hypothesis, because eNOS plays an important role in the control of testicular vascular function, and hence heterozygous eNOS deficiency in male mice might create an unfavourable testicular microenvironment. We hypothesised that this could influence the offspring's phenotype. We analysed the impact of paternal nitric oxide (NO) deficiency on epigenetic alterations in sperm. Next, we analysed the phenotype of WT offspring of male heterozygous eNOS knockout mice, followed by analysis of candidate genes (both gene expression and related epigenetic alterations of

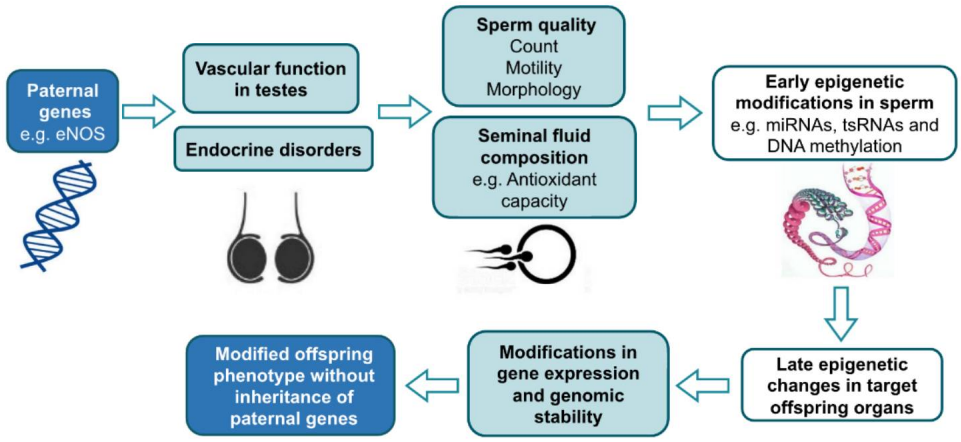

Fig. 1 Paternal programming hypothesis. The paternal genetic defects might impact on the offspring phenotype via genomic–epigenomic interactions without inheritance of the defective paternal genes. The paternal genetic changes might affect the endocrine system and vascular function in testes leading to alterations related to sperm quality and seminal fluid composition, which might in turn trigger early epigenetic modifications in sperm, e.g. miRNAs, tRNA-derived small RNAs (tsRNAs) and DNA methylation [18, 20, 36, 37, 64, 65]. These early epigenetic alterations might impact the offspring leading to late epigenetic changes in target offspring organs with modified gene expression and phenotype without transmittance of the paternal genetic change

differentially expressed genes) potentially responsible for the observed phenotype.

Methods

For detailed methods, please refer to the electronic supplementary material (ESM).

Breeding protocol and study protocol of eNOS-deficient mice Male heterozygous mice (C57BL/6 J background) in which the *Nos3* gene encoding eNOS was knocked out [25] were bred with C57BL/6 J female mice and their WT offspring were compared with offspring from healthy male and female C57BL/6 J mice. The breeding procedure is described in more detail in ESM Fig. 1. Study design and experimental protocols were conducted according to the local institutional guidelines for the care and use of laboratory animals and were approved by the animal welfare ethical committee of the state of Berlin.

Male and female offspring were kept for 24 weeks and analysed separately. Body weight, length, abdominal diameter, blood pressure and plasma creatinine were measured and IPGTT was performed. Experimenters were blind to group assignment and outcome assessment for the entire study.

Effects of NO deficiency on sperm development and epigenetic alterations in the sperm A total of 30 C57BL/6 J male mice were randomised into three groups and treated with different doses of *N*(γ)-nitro-L-arginine methyl ester (L-NAME) for 12 consecutive weeks.

Sperm total DNA methylation Mature sperm were isolated from cauda epididymis. Sperm total DNA methylation was performed as described before [26].

Sperm count and small RNA library construction Mature sperm were isolated from cauda epididymis of male C57BL/6 J mice and processed for RNA extraction as previously described [27, 28]. Small RNA libraries were constructed. After validation of library quality, sequencing was performed by Illumina HiSeq (Illumina, UK).

Testicular morphology Testes were fixed, processed and stained with haematoxylin and eosin, followed by computer-aided image analysis.

Liver morphology Livers were fixed, embedded in paraffin and cut into slices. Haematoxylin and eosin staining, Oil Red O staining and immunohistochemistry were performed, followed by computer-aided image analysis.

Pancreas morphology Pancreases were fixed, embedded in paraffin and cut into slices. Haematoxylin and eosin staining

and immunohistochemistry were performed, followed by computer-aided image analysis.

Liver glycogen content Glycogen content was determined using the amyloglucosidase method [29].

Quantitative real-time PCR Quantitative real-time RT-PCR was used to determine the relative expression levels of mRNAs as described recently [15]. Sequences of primers used are listed in ESM Table 1.

Quantification of gene-specific DNA methylation Quantification of gene-specific DNA methylation was achieved with methylated genomic DNA immunoprecipitation (MeDIP), with minor modifications as described by Weber et al [30].

Statistics For the statistical analysis of IPGTT glucose and insulin, two-way ANOVA test followed by Bonferroni post hoc test was conducted. The unpaired Student's *t* test and Pearson correlation analysis were applied for normally

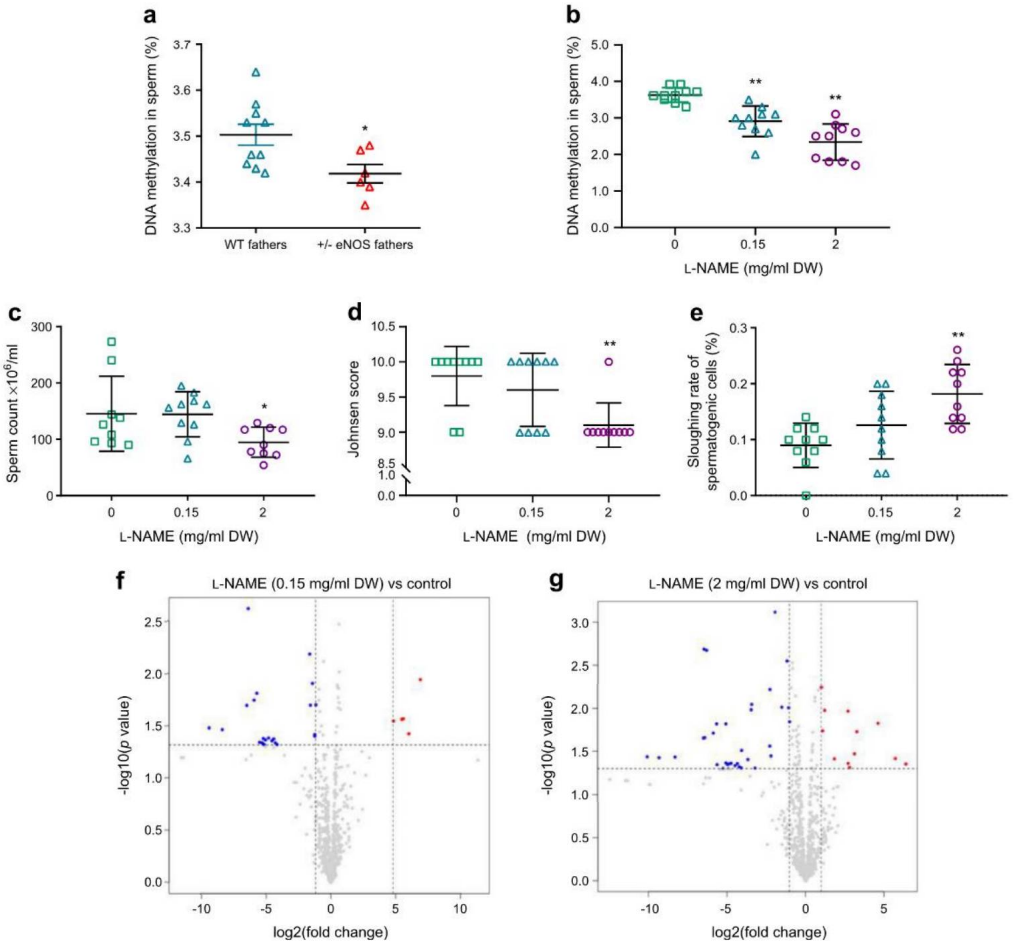

Fig. 2 Sperm DNA methylation in WT ($n = 10$) and +/− eNOS fathers ($n = 6$) (**a**), sperm DNA methylation in mice treated with different doses of L-NAME (**b**), sperm count (**c**), determination of testicular morphology: Johnsen score (**d**), sloughing rate of spermatogenic cells (**e**) and volcano plots of differentially expressed miRNAs: low-dose L-NAME (0.15 mg/ml drinking water [DW], $n = 10$) group vs control group ($n = 10$) (**f**) and high-dose L-NAME (2 mg/ml DW, $n = 10$) group vs control group (**g**). *$p<0.05$ vs WT fathers in (**a**); *$p<0.05$ and **$p<0.01$ vs control group in (**b**), (**c**), (**d**) and (**e**)

distributed data, while the Mann–Whitney *U* test and Spearman correlation analysis were used for non-normally distributed data. To correct for multiple testing in the gene expression analysis, a false discovery rate (FDR) cut off was set at 0.05 [31, 32]. Statistically significant differences were considered as $p \leq 0.05$.

Results

Effects on sperm under conditions of NO deficiency Sperm total DNA methylation in +/− eNOS fathers was lower than that in WT fathers (Fig. 2a). Mature sperm has a haploid chromosome set. Half of the spermatozoa from heterozygous eNOS knockout mice therefore have an inactivated *Nos3* gene; the remaining half have a normal *Nos3* gene. We therefore treated male WT mice with the identical genetic background as the eNOS knockout mice with L-NAME and then analysed the sperm. This sperm is a well-suited model to analyse effects of reduced eNOS activity in the testes on the maturation of genetically healthy sperm. We have chosen two dosages of L-NAME. The lower dose does not increase blood pressure, whereas the higher dose does. The mice treated with the lower dose can thus be regarded as a model of heterozygous eNOS knockout mice with sperm having only WT *Nos3* genes, because blood pressure is not increased in heterozygous eNOS knockout mice. Total DNA methylation in sperm of mice treated with L-NAME decreased in a dose-dependent manner (Fig. 2b). Sperm count, Johnsen scores and sloughing rate of maturing sperm cells were not altered in mice on low-dose L-NAME (Fig. 2c–e). Twenty-three microRNAs (miRNAs) were downregulated and five miRNAs were

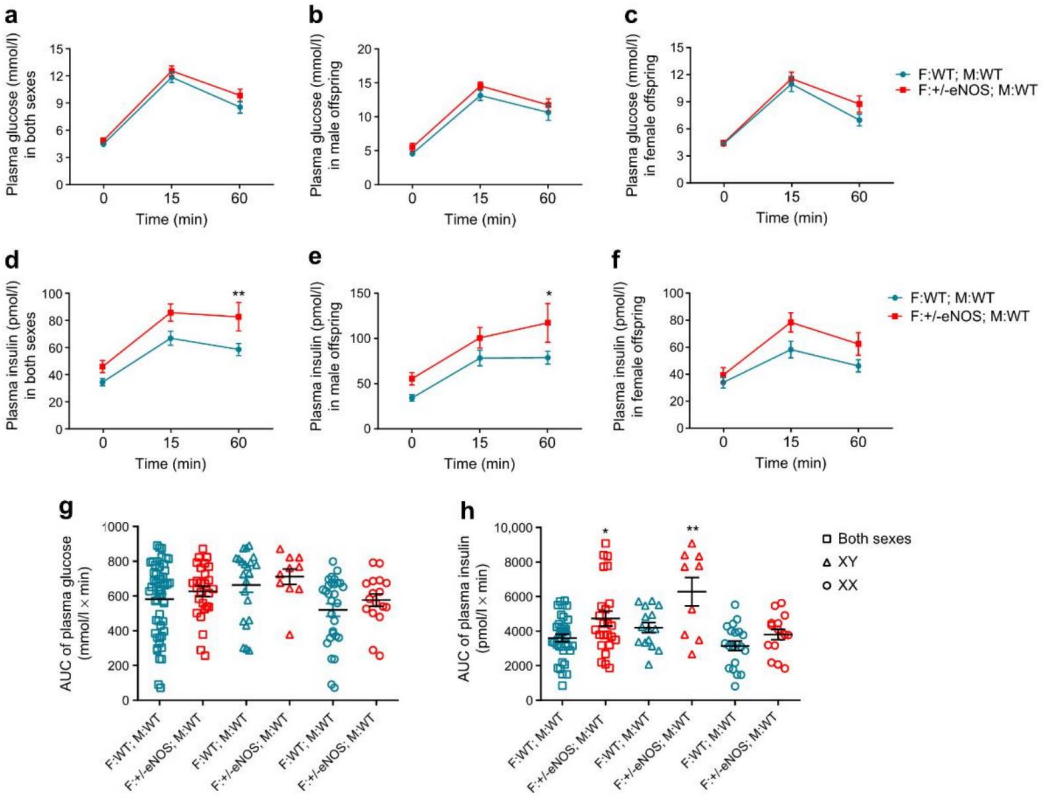

Fig. 3 Plasma glucose (**a**–**c**) and insulin concentrations (**d**–**f**) during IPGTT in all (**a**, **d**), male (**b**, **e**) or female (**c**, **f**) offspring; blue circles: father WT/mother WT; red squares: father +/− eNOS/mother WT; AUC for IPGTT plasma glucose (**g**) and IPGTT plasma insulin (**h**) in all (squares) (35 F:WT; M:WT and 24 F:+/−eNOS; M:WT), male (triangles) (15 F:WT; M:WT and 9 F:+/−eNOS; M:WT) or female (circles) (20 F:WT; M:WT and 15 F:+/−eNOS; M:WT) offspring; *$p<0.05$ and **$p<0.01$ vs father WT/mother WT. F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers; XX, female offspring; XY, male offspring

upregulated in the low-dose L-NAME group (Fig. 2f). Only six downregulated miRNAs were described previously (miR-615-3p, miR-193a-5p, miR-199b-5p, miR-144-3p, miR-132-3p, miR-8114) (ESM Tables 2–4).

Birth variables and adult body weight WT offspring born to +/− eNOS fathers and WT mothers showed no differences in birthweight, length and abdominal diameter when compared with controls (ESM Fig. 2). There was no difference among the groups regarding body weight (ESM Fig. 2, ESM Table 5).

Blood pressure and kidney function Neither blood pressure nor kidney function was different in WT offspring born to +/− eNOS fathers and WT mothers as compared with controls (ESM Table 5).

IPGTT During IPGTT, no differences in glucose concentrations could be observed (Fig. 3). Higher insulin concentrations after an i.p. glucose load, however, were found in animals born to +/− eNOS fathers and WT mothers. Sex-specific analyses showed higher insulin levels in male animals after 60 min of the IPGTT. The analyses of the insulin AUC for offspring from +/− eNOS fathers and WT mothers showed a higher AUC compared with controls. Considering offspring sex revealed that this effect was significant only in male offspring (Fig. 3).

Fasting plasma glucose and insulin WT offspring of +/− eNOS fathers showed higher fasting glucose concentrations. Sex-specific analyses revealed a numerically non-significant elevation of fasting glucose in female and male WT offspring of +/− eNOS fathers (Fig. 4a). Moreover, fasting plasma insulin was significantly higher in male WT offspring of +/− eNOS fathers (Fig. 4b).

Liver phenotype Liver weights, liver lobule dimensions, lobular inflammation connective tissue content and hepatic lipid content were similar in all groups (ESM Table 6). Liver glycogen content in both sexes, however, was higher in animals born to +/− eNOS fathers and WT mothers ($p<0.001$) (Fig. 4c). In male offspring, no significant correlation was found between liver glycogen and AUC of plasma glucose, or between AUC of plasma glucose and AUC of plasma insulin (Fig. 4d, e). However, AUC of plasma insulin was positively correlated with liver glycogen ($r = 0.452$, $p=0.03$) (Fig. 4f). For more details see ESM Table 7.

Liver eNOS/iNOS expression Liver eNOS (*Nos3*) and inducible nitric oxide synthetase (iNOS; encoded by *Nos2*) expression

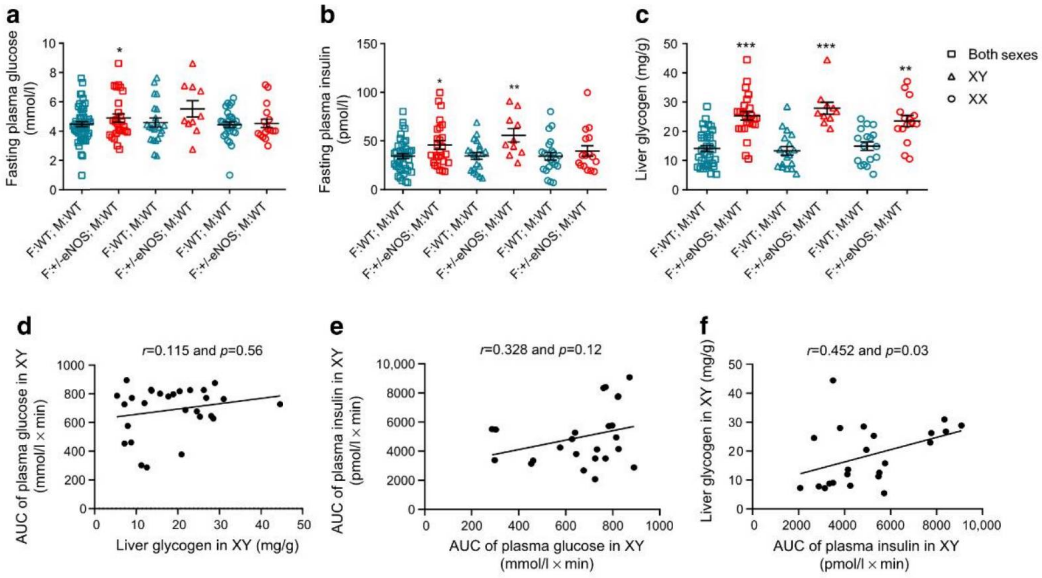

Fig. 4 Main phenotypic changes in the offspring: fasting plasma glucose (**a**), fasting plasma insulin (**b**) and liver glycogen (**c**) in all (squares) (35 F:WT; M:WT and 24 F:+/−eNOS; M:WT), male (triangles) (15 F:WT; M:WT and 9 F:+/−eNOS; M:WT) or female (circles) (20 F:WT; M:WT and 15 F:+/−eNOS; M:WT) offspring. Correlation analysis between liver glycogen and AUC of plasma glucose (**d**), AUC of plasma glucose and AUC of plasma insulin (**e**), and AUC of plasma insulin and liver glycogen (**f**) in male offspring. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs WT (F:WT; M:WT). F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers; XX, female offspring; XY, male offspring

were comparable in animals born to +/− eNOS fathers (ESM Table 8).

Pancreas morphology Size and density of pancreatic islets of Langerhans and beta cell content of islets were similar in all groups (Fig. 5).

Quantitative real-time PCR in the liver WT offspring of both sexes born to +/− eNOS fathers and WT mothers showed an altered expression of genes involved in lipid and carbohydrate metabolism. Seventeen genes were differentially expressed (Table 1). When analysing male offspring born to +/− eNOS fathers and WT mothers (p<0.05 and FDR <0.05), 19 genes were differentially expressed (Table 2), with *Gr* (which encodes glucocorticoid receptor [GR]; also known as *Nr3c1*) and *Pgc1a* (which encodes peroxisome proliferator-activated receptor gamma coactivator 1-alpha [PGC-1α]; also known as *Ppargc1a*) showing the lowest p values and FDR. Analysing female offspring born to +/− eNOS fathers and WT mothers revealed no differences (Table 3).

MeDIP methylation analysis in the liver MeDIP analysis revealed lower *Gr* exon 1A and *Pgc1a* promoter DNA methylation in WT male offspring of eNOS +/− fathers compared with controls (Figs 6, 7). Correlation analysis between the gene expression of liver *Gr* and *Pgc1a* and DNA methylation of *Gr* exon 1A and *Pgc1a* promoter in WT male offspring of eNOS +/− fathers revealed an inverse correlation.

Discussion

To test the advanced fetal programming hypothesis [2–13] for paternal genes, we used a comparable approach as in our previous study [15] by breeding male heterozygous eNOS knockout mice with female WT mice and comparing the phenotype of their WT offspring with the phenotype of offspring with WT parents. NO deficiency in male mice reduces sperm global DNA methylation and leads to complex changes in non-coding miRNAs in sperm. WT male offspring of +/− eNOS fathers had increased fasting insulin, increased insulin after glucose load and increased liver glycogen

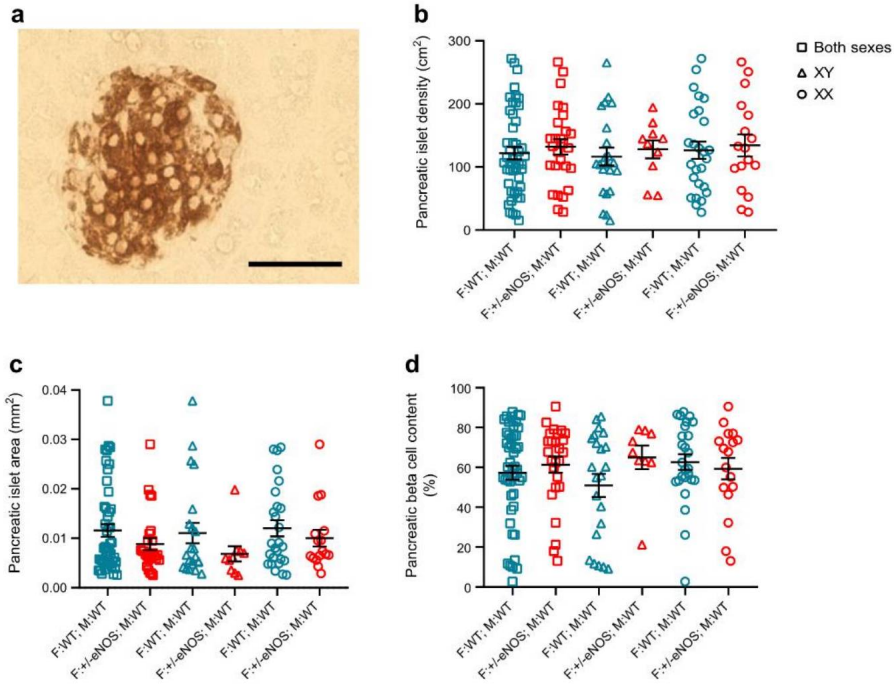

Fig. 5 Determination of pancreas morphology: example of insulin stain in beta cells of pancreatic islets of Langerhans (scale bar, 50 μm) (**a**), comparison of islet density (**b**), mean islet area (**c**) and beta cell content (**d**) of islets in all (squares) (35 F:WT; M:WT and 24 F:+/−eNOS; M:WT), male (triangles) (15 F:WT; M:WT and 9 F:+/−eNOS; M:WT) or female (circles) (20 F:WT; M:WT and 15 F:+/−eNOS; M:WT) offspring. F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers; XX, female offspring; XY, male offspring

Table 1 Liver gene expression in both sexes

Gene	F:WT; M:WT (n=20–50)	F:+/−eNOS; M:WT (n=20–26)	p value	FDR	Correlation with IPGTT insulin (AUC)		
					Spearman r	p value	FDR
Gr (*Nr3c1*)	1.00 ± 0.09	1.56 ± 0.14	4.9×10^{-6}	1.3×10^{-4}†	0.24	0.16	0.54
Igfbp2	1.00 ± 0.09	1.28 ± 0.12	1.9×10^{-4}	2.4×10^{-3}†	−0.11	0.54	0.81
Igfbp1	1.00 ± 0.25	2.15 ± 0.45	2.7×10^{-4}	2.4×10^{-3}†	0.26	0.13	0.50
Fbpase	1.00 ± 0.07	1.19 ± 0.11	4.7×10^{-4}	3.2×10^{-3}†	0.06	0.71	0.80
Ampk	1.00 ± 0.09	1.21 ± 0.13	6.6×10^{-4}	3.6×10^{-3}†	0.03	0.88	0.88
Pgc1a	1.00 ± 0.07	1.56 ± 0.19	8.3×10^{-4}	3.7×10^{-3}†	0.18	0.19	0.57
Cpt1	1.00 ± 0.08	1.11 ± 0.10	1.7×10^{-3}	6.4×10^{-3}†	−0.09	0.60	0.77
Tfam	1.00 ± 0.06	1.30 ± 0.08	2.7×10^{-3}	9.2×10^{-3}†	0.17	0.20	0.54
Ppar-A	1.00 ± 0.05	1.35 ± 0.12	3.2×10^{-3}	9.2×10^{-3}†	0.14	0.29	0.56
Acc1	1.00 ± 0.07	1.43 ± 0.13	3.4×10^{-3}	9.2×10^{-3}†	0.21	0.12	0.65
Acsl3	1.00 ± 0.11	1.64 ± 0.21	4.3×10^{-3}	1.1×10^{-2}†	0.23	0.09	0.61
Acsl4	1.00 ± 0.07	1.38 ± 0.12	5.3×10^{-3}	1.2×10^{-2}†	0.08	0.57	0.77
Ppar- Γ	1.00 ± 0.07	1.37 ± 0.13	8.4×10^{-3}	1.7×10^{-2}†	0.30	0.02	0.27
Hsl	1.00 ± 0.07	0.99 ± 0.10	9.8×10^{-3}	1.9×10^{-2}†	−0.27	0.12	0.54
Gys	1.00 ± 0.07	1.35 ± 0.13	1.0×10^{-2}	1.9×10^{-2}†	0.10	0.44	0.74
Cdkn1a	1.00 ± 0.11	2.20 ± 0.59	1.3×10^{-2}	2.1×10^{-2}†	0.09	0.53	0.84
Pdk4	1.00 ± 0.13	1.79 ± 0.42	2.5×10^{-2}	4.0×10^{-2}†	0.33	0.01	0.27
Nampt	1.00 ± 0.08	1.32 ± 0.14	3.8×10^{-2}	5.7×10^{-2}	0.12	0.37	0.67
Igfbp3	1.00 ± 0.12	0.88 ± 0.06	4.6×10^{-2}	6.6×10^{-2}	0.07	0.68	0.83
Srebf1c	1.00 ± 0.11	0.88 ± 0.09	5.7×10^{-2}	7.6×10^{-2}	−0.07	0.70	0.82
Nrf1	1.00 ± 0.03	1.11 ± 0.05	5.9×10^{-2}	7.6×10^{-2}	0.08	0.55	0.78
Gck	1.00 ± 0.06	0.81 ± 0.07	6.5×10^{-2}	7.8×10^{-2}	0.17	0.21	0.52
G6pase	1.00 ± 0.12	1.34 ± 0.12	6.6×10^{-2}	7.8×10^{-2}	0.16	0.24	0.50
Chrebp	1.00 ± 0.05	1.17 ± 0.10	9.9×10^{-2}	1.1×10^{-1}	0.24	0.06	0.54
Pck1	1.00 ± 0.07	1.13 ± 0.11	3.0×10^{-1}	3.2×10^{-1}	0.03	0.80	0.86
Pk-l	1.00 ± 0.05	1.06 ± 0.12	6.2×10^{-1}	6.4×10^{-1}	0.02	0.86	0.89
Fas	1.00 ± 0.08	0.94 ± 0.09	6.5×10^{-1}	6.5×10^{-1}	0.16	0.22	0.50

Data are given as mean ± SEM and normalised to the reference group (F:WT; M:WT)

† Significantly regulated gene with FDR <0.05. They are presented in ascending order according to FDR

F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers

content. Since there were no alterations in the endocrine pancreas and liver is the main site of insulin clearance [33, 34], we focused on the liver in our analysis. We found 19 genes differentially expressed in adult male offspring born to +/− eNOS fathers and WT mothers, with *Gr* and *Pgc1a* showing the lowest p value and FDR, whereas no differences in gene expression were seen in female offspring. DNA methylation of *Gr* exon 1A and *Pgc1a* promoter in male WT offspring of eNOS +/− fathers was decreased.

Offspring sex dependency of paternal eNOS deficiency The phenotype of female WT offspring of +/− eNOS fathers was less pronounced. Only liver glycogen storage was increased. Sex dependency of the offspring's phenotype is a common phenomenon in fetal programming [1]. One mechanism might be due to offspring sex-dependent transcriptional differences [1, 16]. During preimplantation development, male and female embryos display phenotypic differences that can only be attributed to the transcriptional differences resulting from their different sex chromosomes [35].

Opposite effects of maternal and paternal eNOS deficiency on glucose homeostasis The same parental stimulus (heterozygous eNOS deficiency that was not transmitted to the next generation) causes different phenotypes in the offspring. Male WT offspring of +/− eNOS fathers developed a phenotype consisting of increased fasting insulin, increased liver glycogen storage and increased insulin secretion after glucose load. It is remarkable that the effect on fasting glucose seems to go in opposite directions in WT offspring of heterozygous

Table 2 Liver gene expression in male offspring

Gene	F:WT; M:WT (n=10–22)	F:+/−eNOS; M:WT (n=10)	p value	FDR	Correlation with IPGTT insulin (AUC)		
					Spearman r	p value	FDR
Gr (*Nr3c1*)	1.00 ± 0.15	1.79 ± 0.11	1.80×10^{-7}	4.85×10^{-6}†	0.21	0.41	0.74
Pgc1a (*Ppargc1a*)	1.00 ± 0.10	1.96 ± 0.15	6.52×10^{-6}	8.80×10^{-5}†	0.16	0.45	0.76
Acsl4	1.00 ± 0.09	1.84 ± 0.17	4.17×10^{-5}	3.74×10^{-4}†	0.15	0.47	0.71
Acsl3	1.00 ± 0.15	2.37 ± 0.29	5.54×10^{-5}	3.74×10^{-4}†	0.24	0.25	0.68
Tfam	1.00 ± 0.08	1.58 ± 0.04	8.99×10^{-5}	4.85×10^{-4}†	0.18	0.39	0.75
Pdk4	1.00 ± 0.15	2.47 ± 0.37	1.25×10^{-4}	5.08×10^{-4}†	0.39	0.06	0.54
Igfbp1	1.00 ± 0.35	2.23 ± 0.43	1.32×10^{-4}	5.08×10^{-4}†	0.33	0.19	0.73
Igfbp2	1.00 ± 0.16	1.47 ± 0.20	1.93×10^{-4}	5.79×10^{-4}†	−0.10	0.69	0.81
Gys	1.00 ± 0.07	1.68 ± 0.19	1.93×10^{-4}	5.79×10^{-4}†	0.01	0.97	0.97
Fbpase	1.00 ± 0.10	1.22 ± 0.16	7.60×10^{-4}	2.05×10^{-3}†	0.02	0.94	0.98
Acc1	1.00 ± 0.12	1.71 ± 0.13	1.75×10^{-3}	4.22×10^{-3}†	0.23	0.28	0.69
Nampt	1.00 ± 0.12	1.83 ± 0.25	1.87×10^{-3}	4.22×10^{-3}†	0.30	0.16	1.08
Chrebp	1.00 ± 0.07	1.36 ± 0.06	3.09×10^{-3}	6.42×10^{-3}†	0.21	0.33	0.74
Ampk	1.00 ± 0.15	1.12 ± 0.14	4.61×10^{-3}	8.89×10^{-3}†	−0.13	0.63	0.77
Pck1	1.00 ± 0.07	1.44 ± 0.17	8.08×10^{-3}	1.45×10^{-2}†	−0.11	0.60	0.77
Nrf1	1.00 ± 0.05	1.21 ± 0.05	1.22×10^{-2}	2.06×10^{-2}†	0.15	0.49	0.70
G6pase	1.00 ± 0.15	1.64 ± 0.14	1.31×10^{-2}	2.08×10^{-2}†	0.28	0.18	0.97
Cdkn1a	1.00 ± 0.12	3.22 ± 1.37	2.27×10^{-2}	3.40×10^{-2}†	0.28	0.18	0.97
Cpt1	1.00 ± 0.12	0.92 ± 0.09	2.72×10^{-2}	3.87×10^{-2}†	−0.32	0.21	0.71
Hsl	1.00 ± 0.10	0.85 ± 0.10	5.59×10^{-2}	7.55×10^{-2}	−0.53	0.03	0.41
Srebf1c	1.00 ± 0.21	0.90 ± 0.11	6.07×10^{-2}	7.81×10^{-2}	0.02	0.93	1.00
Igfbp3	1.00 ± 0.19	0.83 ± 0.05	1.01×10^{-1}	1.24×10^{-1}	0.19	0.46	0.73
Ppar-α	1.00 ± 0.07	1.14 ± 0.09	2.57×10^{-1}	3.02×10^{-1}	−0.08	0.72	0.81
Ppar-γ	1.00 ± 0.10	1.10 ± 0.10	5.54×10^{-1}	6.23×10^{-1}	0.57	0.003	0.08
Pk-l	1.00 ± 0.10	0.96 ± 0.08	8.00×10^{-1}	8.64×10^{-1}	0.12	0.58	0.78
Fas	1.00 ± 0.16	1.04 ± 0.18	8.82×10^{-1}	9.04×10^{-1}	0.20	0.35	0.73
Gck	1.00 ± 0.10	1.02 ± 0.12	9.04×10^{-1}	9.04×10^{-1}	0.25	0.24	0.72

Data are given as mean ± SEM and normalised to the reference group (F:WT; M:WT)

† Significantly regulated gene with FDR <0.05, and genes are presented in ascending order according to FDR

F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers

eNOS-deficient mothers (see supplementary Table 2 of our previous publication [15] and Fig. 4). Fasting glucose was numerically lower in WT offspring of +/− eNOS mothers. In contrast, fasting glucose was significantly higher in WT offspring of +/− eNOS fathers compared with controls (Figs 3, 4). Epigenetic alterations were likewise different depending on the parental status of eNOS deficiency. In female WT offspring of +/− eNOS mothers liver fat content correlated significantly with fat storage-inducing transmembrane protein 1 (*Fitm1*) gene expression and *Fitm1* methylation was significantly decreased, whereas DNA methylation of *Gr* exon 1A and *Pgc1a* promoter in male WT offspring of eNOS +/− fathers in comparison with controls was lower while corresponding gene expression was increased.

Taken together, in parental eNOS deficiency the offspring phenotype strongly depends on whether the genetic defect was present in the mother or in the father. The different consequences of the same parental genetic defect (eNOS deficiency) can probably be explained by different impacts of eNOS deficiency for the maturation of the egg or the intrauterine development of the embryo (Figs 1, 2) [1, 16]. Paternal eNOS deficiency affects maturation and development of the sperm and finally alters the epigenome of the sperm, potentially causing long-lasting secondary epigenetic alterations resulting in an adult phenotype characterised by increased fasting insulin, increased insulin after glucose load and increased liver glycogen content. Studies showing that a preconceptional paternal high-fat diet results in an impaired glucose tolerance in female offspring due to epigenetic sperm

Table 3 Liver gene expression in female offspring

Gene	F:WT; M:WT (n=10–28)	F:+/−eNOS; M:WT (n=10–16)	p value	FDR	Correlation with IPGTT insulin (AUC)		
					Spearman r	p value	FDR
Ppar-α	1.00 ± 0.08	1.48 ± 0.18	8.45×10^{-3}	9.94×10^{-2}	0.41	0.02	0.27
Ppar-γ	1.00 ± 0.10	1.54 ± 0.19	9.70×10^{-3}	9.94×10^{-2}	0.44	0.01	0.27
Gck	1.00 ± 0.08	0.68 ± 0.08	1.10×10^{-2}	9.94×10^{-2}	0.02	0.89	1.00
Cpt1	1.00 ± 0.12	1.30 ± 0.16	2.36×10^{-2}	1.52×10^{-1}	0.29	0.26	1.00
Ampk	1.00 ± 0.10	1.30 ± 0.21	3.07×10^{-2}	1.52×10^{-1}	0.27	0.30	0.90
Gr (Nr3c1)	1.00 ± 0.12	1.32 ± 0.24	3.37×10^{-2}	1.52×10^{-1}	0.04	0.89	1.00
Fbpase	1.00 ± 0.11	1.16 ± 0.16	5.60×10^{-2}	1.89×10^{-1}	0.22	0.39	0.88
Igfbp1	1.00 ± 0.37	2.06 ± 0.82	5.60×10^{-2}	1.89×10^{-1}	−0.06	0.82	1.00
Igfbp2	1.00 ± 0.09	1.09 ± 0.09	6.47×10^{-2}	1.93×10^{-1}	−0.31	0.22	1.00
Hsl	1.00 ± 0.12	1.12 ± 0.16	7.15×10^{-2}	1.93×10^{-1}	0.002	1.00	1.00
Acc1	1.00 ± 0.09	1.25 ± 0.19	1.91×10^{-1}	3.97×10^{-1}	0.20	0.27	0.91
Pgc1a	1.00 ± 0.09	1.32 ± 0.27	1.91×10^{-1}	3.97×10^{-1}	0.13	0.46	0.83
Cdkn1a	1.00 ± 0.18	1.52 ± 0.32	2.01×10^{-1}	3.97×10^{-1}	−0.10	0.57	0.91
Igfbp3	1.00 ± 0.14	0.94 ± 0.11	2.06×10^{-1}	3.97×10^{-1}	−0.21	0.41	0.79
Tfam	1.00 ± 0.08	1.13 ± 0.10	3.28×10^{-1}	5.67×10^{-1}	0.22	0.23	1.00
Srebf1c	1.00 ± 0.11	0.85 ± 0.15	3.36×10^{-1}	5.67×10^{-1}	−0.22	0.39	0.88
Fas	1.00 ± 0.08	0.88 ± 0.11	3.64×10^{-1}	5.77×10^{-1}	0.16	0.38	0.93
Gys	1.00 ± 0.11	1.14 ± 0.15	4.64×10^{-1}	6.51×10^{-1}	0.13	0.47	0.79
Pk-L	1.00 ± 0.06	1.12 ± 0.19	4.78×10^{-1}	6.51×10^{-1}	−0.04	0.85	1.00
Pdk4	1.00 ± 0.20	1.37 ± 0.62	4.90×10^{-1}	6.51×10^{-1}	0.26	0.15	1.00
Acsl3	1.00 ± 0.16	1.18 ± 0.24	5.14×10^{-1}	6.51×10^{-1}	0.18	0.32	0.86
Nrf1	1.00 ± 0.04	1.05 ± 0.08	5.51×10^{-1}	6.51×10^{-1}	0.07	0.71	1.00
G6pase	1.00 ± 0.17	1.15 ± 0.15	5.54×10^{-1}	6.51×10^{-1}	0.05	0.79	1.00
Acsl4	1.00 ± 0.11	1.09 ± 0.12	5.93×10^{-1}	6.67×10^{-1}	−0.07	0.69	1.00
Pck1	1.00 ± 0.11	0.93 ± 0.13	7.00×10^{-1}	7.48×10^{-1}	0.04	0.84	1.00
Chrebp	1.00 ± 0.08	1.06 ± 0.15	7.21×10^{-1}	7.48×10^{-1}	0.28	0.11	0.99
Nampt	1.00 ± 0.11	1.00 ± 0.11	9.83×10^{-1}	9.83×10^{-1}	0.001	1.00	1.00

Data are given as mean ± SEM and normalised to the reference group (F:WT; M:WT)

No gene showed significant regulation with FDR <0.05, and genes are presented in ascending order according to FDR

F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers

and target organ alterations [17–19, 21] fit well with our findings.

Polymorphisms in the human *NOS3* gene (encoding eNOS) are associated with alterations in the composition of seminal plasma. eNOS deficiency-mediated changes in seminal plasma might thus also be a contributing factor [36, 37]. Maternal eNOS deficiency may affect egg maturation and intrauterine development [1, 15]. In this context, it is of note that parental diabetes has opposite effects on offspring birthweight [38], most likely due to the different effects of paternal and maternal diabetes on spermatogenesis and oocyte/intrauterine development, respectively.

Can the phenotype in male offspring be explained by an upregulation of GRs? The hepatic phenotype in WT male offspring of heterozygous eNOS-deficient fathers is in agreement with studies showing that exposure to hepatic GR inhibition lowers glucose in *ob*/*ob* mice [39] and that hepatic GR blockade decreases glucose production and improves insulin resistance [40, 41]. Excess glucocorticoid exposure causes hyperglycaemia and insulin resistance. Our finding of no differences in the glucose response to glucose load in WT male offspring of heterozygous eNOS-deficient fathers vs WT offspring of WT parents but marked differences with respect to insulin levels between WT male offspring of heterozygous eNOS-deficient fathers and controls (Figs 3, 4) suggests that paternal eNOS deficiency causes insulin resistance in the WT male offspring of male heterozygous eNOS knockout mice. In the liver, glucocorticoids increase glycogen storage [42]. We assume that an increased expression of the

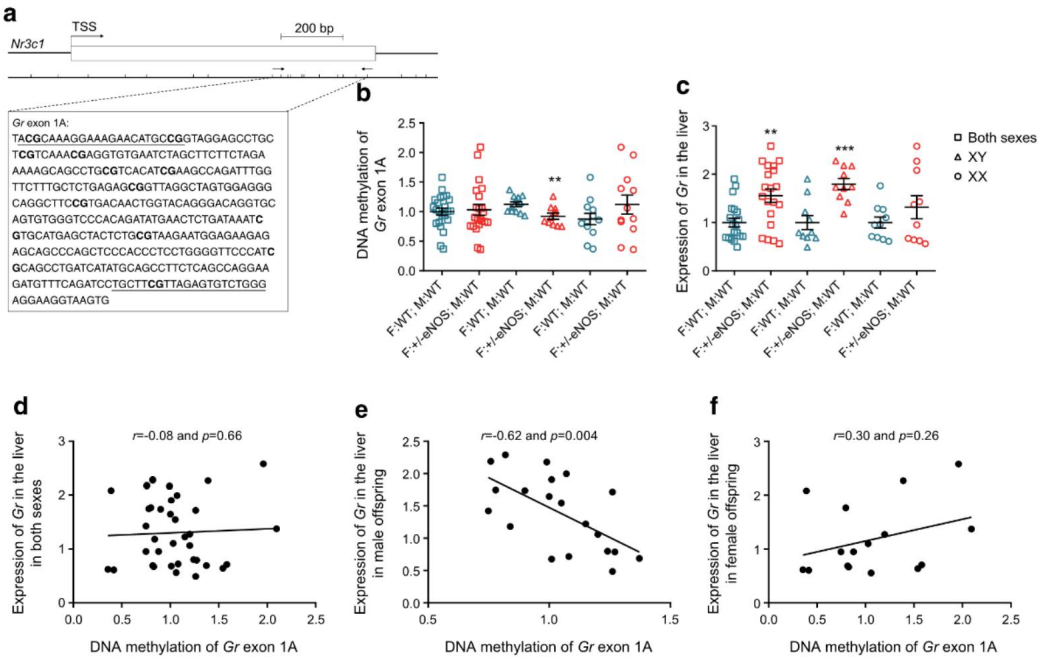

Fig. 6 MeDIP methylation analysis of *Gr* (*Nr3c1*) gene exon 1A and *Gr* expression in the liver: (**a**) genomic organisation of *Gr* exon 1A region with putative transcription start site (TSS) and beyond the position of CpG dinucleotides; amplified sequence is shown in the box (primer binding sites are underlined and analysed CpG dinucleotides are in bold letters); (**b**) degree of DNA methylation in amplified region; (**c**) hepatic expression of *Gr* in all (squares) (35 F:WT; M:WT and 24 F:+/−eNOS; M:WT), male (triangles) (15 F:WT; M:WT and 9 F:+/−eNOS; M:WT) or female (circles) (20 F:WT; M:WT and 15 F:+/−eNOS; M:WT) offspring (**$p<0.01$ and ***$p<0.001$ vs F:WT; M:WT); and correlation of DNA methylation and gene expression in all (**d**), male (**e**) or female (**f**) offspring. F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers; XX, female offspring; XY, male offspring

hepatic GR may have similar consequences. It was reported that GR interacts with insulin degrading enzyme [43]. Since the liver is the primary site for insulin clearance [33, 34], upregulated hepatic GR expression might likewise lead to increased insulin levels. This pathway is androgen-dependent [43]. Hepatic androgen-dependent GR effects on insulin might explain the observed phenotype of increased plasma insulin levels in male WT offspring of male heterozygous eNOS knockout mice.

The human *GR* gene (also known as *NR3C1*) comprises nine exons in which exons 2 to 9 are the protein-encoding region. This gene has a long complex promoter region (the 5′ untranslated region) which is similar to the mouse and rat *Gr* gene [44]. The mouse *Gr* gene has five distinct promoter regions which are 1A, 1B, 1C, 1D and 1E. Exon 1A is found 32 kb upstream from exon 2, and its expression was only detected in tissues with high GR content [45, 46]. Thus, *Gr* exon 1A was selected for DNA methylation analysis and indeed we saw decreased methylation of *Gr* exon 1A and increased *Gr* gene expression in the liver of male WT offspring of eNOS-deficient fathers. The *GR* gene in particular has been shown to be sensitive to early-life environmental conditions, and this effect has been attributed to epigenetic mechanisms [47].

***PGC1a* methylation and gene expression** DNA methylation of the *PGC1a* gene promoter modulates insulin resistance and is strongly associated with plasma fasting insulin [48, 49]. A study in patients with non-alcoholic fatty liver disease showed that *PGC1a* promoter methylation was inversely correlated with liver *PGC1a* mRNA expression. In addition, *PGC1a* promoter methylation was inversely correlated with HOMA-IR, fasting glucose and insulin. *PGC1a* promoter methylation was also inversely correlated with *PGC1a* promoter methylation [50]. A study done in a rat fetal programming model likewise found an alteration in DNA methylation and transcription of *Pgc1a*. The genetic and epigenetic modifications of *PGC1a* provide a potential mechanism linking early-life nutrition insult to long-term metabolic disease susceptibility [51].

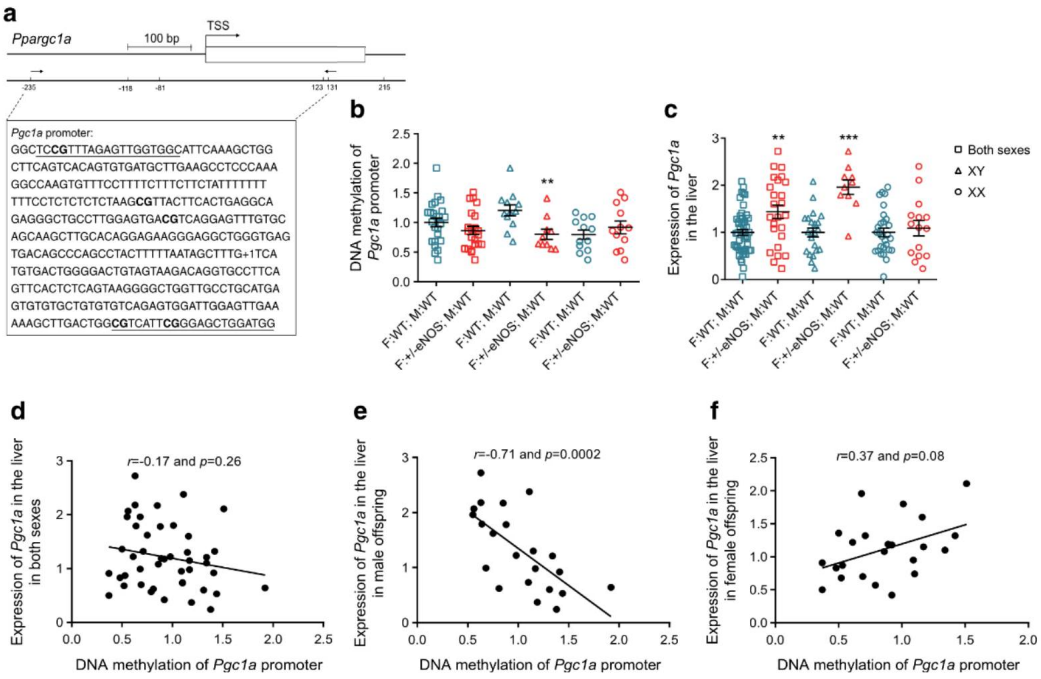

Fig. 7 MeDIP methylation analysis of *Pgc1a* (*Ppargc1a*) promoter and *Pgc1a* expression in the liver: (**a**) genomic organisation of *Pgc1a* proximal promoter region with putative transcription start site (TSS/+1) and beyond the position of CpG dinucleotides; amplified sequence is shown in the box (primer binding sites are underlined and analysed CpG dinucleotides are in bold letters); (**b**) degree of DNA methylation in amplified region; (**c**) hepatic expression of *Pgc1a* in all (squares) (35 F:WT; M:WT and 24 F:+/−eNOS; M:WT), male (triangles) (15 F:WT; M:WT and 9 F:+/−eNOS; M:WT) or female (circles) (20 F:WT; M:WT and 15 F:+/−eNOS; M:WT) (**$p<0.01$ and ***$p<0.001$ vs F:WT; M:WT); and correlation of DNA methylation and gene expression in all (**d**), male (**e**) or female (**f**) offspring. F:+/−eNOS; M:WT, WT offspring of eNOS heterozygous fathers and WT mothers; F:WT; M:WT, WT offspring of WT fathers and WT mothers; XX, female offspring; XY, male offspring

Pathophysiological and clinical implications Many studies have indicated that insulin resistance can be caused by fetal programming. Also, paternal factors prior to mating may influence the epigenome of the sperm and hence the adult offspring's phenotype [1, 16, 52, 53], as was observed in our study. Male heterozygous eNOS knockout mice might be a model of the human endothelial dysfunction sometimes observed in elderly fathers or fathers with hypertension [54–57]. If our findings can be translated to humans, paternal endothelial dysfunction in men might be a risk factor for developing insulin resistance in offspring. Our current study also supports the advanced fetal programming hypothesis as set out in our previous study [15], where we could demonstrate a maternal *Nos3* gene-driven epigenetic alteration of the offspring's phenotype. Our current study now proposes a non-environmental mechanism of fetal programming driven by altered paternal *Nos3/NOS3* gene function [58, 59] primarily affecting the sperm epigenome and later in life the methylation of offspring target organ genes, resulting in our case in a liver phenotype.

Our current study and the previous study [15] have some general implications:

(1) They break with the classical laws of inheritance. The phenotype of WT offspring born to either male or female heterozygous eNOS knockout mice should be identical to offspring from WT parents. However, this was not the case for offspring of either heterozygous eNOS knockout fathers as shown in this study or heterozygous eNOS knockout mothers as shown previously [15].

(2) They challenge a key research tool developed to understand gene function: murine transgenic or knockout animal models. Genetically altered animal models may not only reflect causality between a certain genetic alteration and a resulting phenotype. Altered parental genes may additionally induce epigenetic changes affecting the

offspring's phenotype. This notion is supported by human genome-wide association studies [60]. The clinical implications of our study should be further investigated in monogenic inherited diseases such as thalassemia.

Study limitations We used inbred mice for our experiments (see also ESM Fig. 1). However, founder fathers for the control group (WT offspring of WT fathers and WT mothers) and fathers for the investigated group (WT offspring of heterozygous eNOS fathers and WT mothers) are different. Thus, additional unknown genetic differences in the fathers used to generate the control group and the investigated group cannot be fully excluded. It is a study limitation that epigenetic changes caused by paternal NO deficiency were analysed at only two time points, in sperm and in the adult animal at the time of characterisation of the adult phenotype. Dynamic epigenetic changes, particularly during fetal development, and their impact on the adult phenotype are important topics of follow-up projects.

We performed IPGTT by measuring glucose and insulin at 0, 15 and 60 min and not for a longer duration on account of animal welfare. However, IPGTT for only 60 min showed differences among the groups and this duration for IPGTT was reported as a suitable approach previously [61–63]. Although group means for insulin AUC comparing WT male offspring born to heterozygous fathers and WT mothers with controls were clearly different (Fig. 3h), it needs to be mentioned that the variation of individual data in the groups was quite high. This might be due to the fact that this variable is calculated based on several measurements. There might be variations in the amount of glucose injected, the time of blood taking after glucose injection, the body weight of the individual animals as well as variation of the insulin ELISA used. All these variables potentiated the variability of insulin AUC. This is for example completely different from reports of organ weight. Here just the variability of the weight measurement method accounts for the variability of the variable. Moreover, we did not analyse fat tissue and muscles. Given the phenotype, this would have been of interest. Furthermore, we screened for differentially expressed genes by using a large but limited list of candidate genes known to be involved in liver fat and carbohydrate metabolism. We did this because the approach was successfully used in our initial study [15].

Conclusions This study shows that paternal genes without passing on to the offspring can influence the offspring's phenotype by altering the epigenome in the sperm and subsequently later in certain organs in adulthood. The same genetic defect in either the father or the mother without transmission to the next generation results in different offspring phenotypes. Our data specifically suggest that heterozygous eNOS deficiency in male mice might cause an unfavourable testicular environment influencing the sperm epigenome. These primary sperm epigenetic alterations may trigger long-lasting epigenetic and subsequent phenotypic alterations in offspring target organs (Fig. 1).

Supplementary Information The online version contains peer-reviewed but unedited supplementary material available at https://doi.org/10.1007/s00125-022-05700-x.

Data availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

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Contribution statement BH designed the study, wrote grant applications and got funding for this study. AAH and BH wrote the manuscript. BH, Y-PL, CR, XZ, OT, JR, LX, JL, LH, BKK and AAH provided feedback to the initial draft. Y-PL, CR, XZ, OT and JR did animal breeding and all animal procedures. Y-PL, CR, XZ, OT, JR, LX, JL and LH performed experiments. Data analysis was done by JR, LH, BKK, AAH and BH. All authors gave final approval of the version to be published. BH is the guarantor of the entire study.

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3.4 Electronic supplementary material (ESM)

ESM methods

1. Breeding protocol and study protocol of eNOS deficient mice

eNOS knockout mice of the C57BL/6J strain and their wild-type (wt) littermate were obtained from Gödecke et al. who described the whole procedure of their generation [1]. These animals were bred in-house according to the procedure described in the ESM Fig.1. Study design and experimental protocols were conducted according to the local institutional guidelines for the care and use of laboratory animals and approved by the animal welfare ethical committee of the state of Berlin.

Male and female offspring were kept for 24 weeks and analysed separately. Body weight, length and abdominal diameter of the F2 generation were measured daily until day 13, thereafter body weight daily until day 40 and weekly hereafter until week 20 of the experiment. Blood pressure was measured using the tail cuff method as previously described in week 24 [2]. Plasma creatinine was measured in week 23. Fasting glucose testing was performed in study week 21 and an intraperitoneal glucose tolerance test (IPGTT) in study week 24. Regarding IPGTT, the animals were fasted overnight, injected intraperitoneally with 2mg Glucose/g body weight, then blood samples were collected through the tail vein at 0, 15 and 60 minutes to measure plasma glucose and insulin as described previously [3-5]. Collecting blood samples after longer duration i.e 90 or 120 minutes was not done accounting for animal welfare and to avoid potential loss of animals.

2. Effects of nitric oxide deficiency on sperm development and epigenetic alterations in the sperm

A total of 30 C57BL/6J male mice were randomized into three groups and treated for a consecutive 12 weeks. The control group was given normal drinking water (n =10). The second group received drinking water containing L-NAME (0.15 mg/ml, approximately 15 mg/kg/day; n = 10; Sigma, St Louis, MO, USA). The third group received drinking water containing L-NAME (2 mg/ml, approximately 200 mg/kg/day; n =10). Daily water consumption was estimated individually for every animal 1 week before the experiment. A regular chow diet was used to feed all experimental subjects.

3. Liver morphology

Hematoxylin and Eosin Staining was done after washing the livers in PBS buffer, fixation in 4% (w/v) paraformaldehyde in PBS, embedding in paraffin and cutting into 3 μm slices using a Microm HM230 Microtom. For liver slices, the hepatic venules and their adjacent portal fields were identified by sinusoidal connection [5]. 10 lobules of every liver were thus identified using a Zeiss (Oberkochen, Germany) Axiovert 100 microscope (200x) and photographed with a Leica EC3 digital camera using LAS EZ software (Leica, Wetzlar, Germany). Linear lobular dimensions were measured from the centre of the hepatic vein to the centre of three related portal vein branches using ImageJ (version 1.410, NIH shareware). The mean radius of lobules was calculated for each animal. The extent of lobular inflammation was graded as described previously [6]: score 0, no inflammatory foci; score 1, fewer than two foci per × 200 field; score 2, two to four foci per × 200 field; and score 3, more than four foci per × 200 field.

Red Oil Staining was done as described elsewhere [7]. 30 pictures were taken per organ using an Olympus (Shinjuku, JP) BH-2 microscope (400x) and a digital camera CFW-1310C (Scion Corporation, Frederick, MD). The lipid content and the number and size of lipid droplets were quantified with the ImageJ program.

Liver immunohistohemistry: Liver sections were deparaffinized, boiled with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) with microwave for 10 min for antigen retrieval, and incubated overnight at 4°C with rabbit polyclonal anti-mouse CD68 antibody (ab125047, Abcam, Cambridge, UK) in 4% BSA/PBS. For immunostaining, an anti-rabbit staining kit (CTS005, R&D Systems, Minneapolis, MN) and peroxidase anti-peroxidase with the streptavidin-biotin system were used. Immunostaining was followed by hematoxylin for nuclear counterstaining. The number of CD68-positive macrophages in the liver was quantified as described previously [8].

4. Pancreas morphology

Hematoxylin and Eosin-stained pictures of whole tissue slide and of every islet of Langerhans were taken using Zeiss Axiovert 100 microscope (25x/200x) and Leica EC3 digital camera. The islets were counted, and the islet area was measured using ImageJ software to calculate the islet density and the mean islet area per slide.

Pancreas Immunohistochemistry: Beta cell content of islets of Langerhans was measured using immunohistological staining of insulin. We used an antibody against insulin (ab7842, abcam, Cambridge, UK) and a secondary antibody (ab6907, abcam) diluted in antibody diluent (Dako, Glostrup, DK) and for visualisation the ABC staining system (sc2023, Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. All islets per slide were photographed using an Olympus BH-2 microscope (200x) and CFW-1310C digital camera. Using ImageJ software, the total islet area and the beta cell area was measured (see also Figure 5). Content of β-cells in islets was expressed as the percentage of positively stained area in the total islet area.

5.Liver glycogen content

Glycogen content was determined using the amyloglucosidase method as described before [9]. Briefly, liver was incubated with 1N KOH (95°C, 30 minutes). Glycogen was precipitated using saturated sodium sulfate solution (Na_2SO_4) and 95% (v/v) Ethanol and washed twice in 60% (v/v) Ethanol. Resuspended glycogen was degraded with 0.1% (w/v) Amyloglucosidase (Sigma-Aldrich, St. Louis, MO) in acetate buffer (0.2 M sodiumacetate, 0.46% (v/v) acetic acid, pH 4.8) for 2 hours at 40°C. Glucose concentration was measured colorimetrically using the Glucose (HK) Assay Kit (Sigma-Aldrich). Double measurements were performed, and glycogen content was expressed in relation to liver weight.

6.Quantitative real time PCR

We analysed a list of candidate genes involved in liver fat and carbohydrate metabolism as described recently [10]. We have choosen this list, because we wanted to investigate whether a heterozygous paternal eNOS knockout that is not transmitted to the next generation has the same effect on the offspring's phenotype – a fatty liver phenotype – as we recently described in the offspring of female heterozygous eNOS mice [6]. RNA extraction from liver tissue, reverse transcription PCR and design of specific primer were done like previously described [11]with the exception that primer were obtained from Sigma-Aldrich, Eurofins (Ebersberg, GER) and Biolegio (Nijmegen, NE).

The PCR was performed on a Mx3000P thermal cycler (Stratagene, La Jolla, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), Sensi Mix or SensiFast low ROX kit (Bioline, London, UK) in accordance with instructions for use. All samples were analysed in triplicate. The PCR reaction efficiency has been proofed by linear regression method and the relative quantity of analysed genes was calculated with the ΔΔCt method as described elsewhere [12]. In short, the Ct values of gene of interest were normalized to the geometric mean of the reference genes HPRT, β-Actin and 18S. These values were normalized against the mean value of the reference group. Sequences of used primers are listed in ESM Table 1.

7. Quantification of gene specific DNA methylation

Quantification of gene specific DNA methylation was achieved with immunoprecipitation of methylated genomic DNA (MeDIP), with minor modifications as described by Weber et al. [13]. Briefly, genomic DNA was extracted from liver tissue by proteinase K treatment, RNAse digestion, phenol-chloroform extraction and precipitation with isopropanol. The DNA was sonicated to obtain random fragments between 300 and 1000 bp. 2 µg of the fragmented DNA was denatured for 10 min at 95°C and precipitated over night at 4°C with 10 µg of monoclonal antibody against 5-methylcytosine (Zymo Research) in IP buffer (10 mM sodium phosphate (pH 7.0), 140 mM NaCl, 0.05% Triton X-100). 20 µl of MagnaChip protein G magnetic beads (Millipore) were added and incubated for 2 h at 4°C to capture the antibodies. Magnetic beads were washed two times with IP buffer and treated with proteinase K for 3 h at 50°C. The methylated DNA was recovered by phenol-chloroform extraction and ethanol precipitation.

For the analysis of GR exon 1A and PGC1a promoter, specific primers were created (ESM Table 1). To calculate the proportion of methylated DNA in specific target sequences the content in enriched methylated DNA and input DNA were compared and normalized against the mean value of the reference group.

8. Sperm total DNA methylation

Mature sperm was isolated from cauda epididymis. Sperm total DNA methylation was performed as described before [14]. Briefly, DNA was extracted using a QIAamp DNA Mini Kit from Qiagen (Hilden, Germany). The concentration and quality of the RNA-free DNA solution were determined by a NanoDrop ND-1000 spectrophotometer. DNA hydrolysis was carried out using DNA

Degradase Plus from Zymo Research (Freiburg, Germany). DNA methylation was assessed by liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI/MS/MS) technique as described previously [15].

9. Sperm count and small RNA libraries construction

Mature sperm were isolated from cauda epididymis of male C57BL/6J mice and processed for RNA extraction as previously described [16, 17]. In brief, sperm were released from cauda epididymis into 5ml phosphate‐buffered saline (PBS) maintained at 37℃ for 15 min incubation, then 10 ul semen was taken and placed on a MAKLER sperm counting plate and observed by an optical microscope (Olympus BX 53) at ×400 magnification to assess sperm concentration. After incubation, nylon mesh (pore size: 70 mm) was used to filter the suspension. The sperm were then treated with somatic cell lysis buffer (0.1% SDS, 0.5% Triton X in DEPC H2O) for 40 min on ice to eliminate somatic cell contamination, after which the sperm be pelleted by centrifugation at 600g for 5 minutes. after removal of suspension, the sperm pellet was resuspended and washed twice. The sperm pellet was added with TRIzol reagent, homogenated, followed by RNA extraction. Small RNA libraries were constructed according to Small RNA Sample PreKit (Illumina), the small RNA libraries were prepared followed by library quality validation for sequencing.

Deep sequencing, quality control and Small RNA‐seq data analysis

For each RNA library, 10 million reads (raw data) were generated by Illumina Hi‐Seq. After quality control, small RNA tags were mapped to mouse genome to analyze their distribution and expression on the genome and annotated with miRNA, tRNA, rRNA and other small noncoding RNA from miRBase19, Genbank and Rfam databases using blastn. To analyze differential expression of small RNAs between L-NAME treated and normal mice sperm, miRNA reads were normalized by TPM（transcripts per million reads. Those miRNAs that had P value smaller than 0.05 and had the fold change of at least 2 were considered as significantly changed miRNAs.

10. testicular morphology

Small tissue samples of testicle were obtained, fixed and processed by routine histological techniques. Tissue sections of 5 μm thickness were stained with hematoxylin–eosin (H&E) and

observed under a microscope (Olympus BX53). Sections were evaluated according to the modified Johnsen scoring system as previously described [18, 19]. The sloughing rate of maturing sperm cells is also calculated (at least 50 seminiferous tubules per sample were analyzed) as previously described [20].

ESM Table 1: Primers used for real time PCR and MeDIP

Target Gene	Primer Pair	Length of amplification product (bp)
18S	fw 5‘CGGCTACCACATCCAAGGAA’ 3 rev 5‘GCTGGAATTACCGCGGCT ’ 3	187
Acc1 (Acaca, ENSMUSG00000020532)	fw 5’ TTTCACATGAGATCCAGCATG ‘3 rev 5’ GCCACAGTGAAATCTCGTTG ‘3	92
β-Actin (Actb, ENSMUSG00000029580)	fw 5' GATATCGCTGCGCTGGTC '3 rev 5' CATCACACCCTGGTGCCTA '3	123
Acsl3 (ENSMUSG00000032883)	fw 5’ CTGTTCCGGAAATCATGGA ‘3 rev 5’ GAAAACAAAGCGGTCACACA ‘3	158
Acsl4 (ENSMUSG00000031278)	fw 5’ CCAGAAAACTTGAGCGTTCC ‘3 rev 5’ TGTCCTTCGGTCCTAGTCCA ‘3	172
AMPK-α2 (Prkaa2, ENSMUSG00000028518)	fw 5‘AAAGACATACGAGAACATGAATGG’3 rev 5‘CTTCACAGCCTCATCGTCAA’3	105
Cdkn1a (ENSMUSG00000023067)	fw 5’ CAGACCAGCCTGACAGATTTC ‘3 rev 5’ GCAGGCAGCGTATATACAGGA ‘3	204
CHREBP (Mlxipl, ENSMUSG00000005373)	fw 5‘ GAAGATGCTTATGTTGGCAATG ’3 rev 5‘ GGCGGTAATTGGTGAAGAAA ’3	109
CPT1a (ENSMUSG00000024900)	fw 5‘CGCACATTACAAGGACATGG’3 rev 5‘TCTGCTCTGCCGTTGTTGT’3	158
eNOS (Nos3, ENSMUSG00000028978)	fw 5‘ GGGAAAGCTGCAGGTATTTG ’3 rev 5‘ GCTGAACGAAGATTGCCTCT '3	111
FAS (Fasn, ENSMUSG00000025153)	fw 5‘ GGATTCGGTGTATCCTGCTG ’3 rev 5‘ TGGGCTTGTCCTGCTCTAAC ’3	171
Fbp1 (FBPase)(ENSMUSG00000069805)	fw 5' ATCAAAGCCATCTCGTCTGC '3 rev 5' ATTTGCCCCTCTTCTCAGGT '3	217
G6Pase (G6pc, ENSMUSG00000078650)	fw 5' GACTGTGGGCATCAATCTCC '3 rev 5' TCACAGGTGACAGGGAACTG '3	165

Gck (ENSMUSG00000041798)	fw 5' AAGTCCCACGATGTTGTTCC '3 rev 5' CTTCCCTGTAAGGCACGAAG '3	100
GR (Nr3c1, ENSMUSG00000024431)	fw 5' ACACGTCAGCACCCCATAAT '3 rev 5' AGGCCGCTCAGTGTTTTCTA '3	160
Gys2 (ENSMUSG00000030244)	fw 5' GAGGAAGCCTGATGTAGTGACTC '3 rev 5' TCCAGATGACCATAGAAATGACC '3	138
Hprt (ENSMUSG00000025630)	fw 5'CAGGCCAGACTTTGTTGGAT '3 rev 5' TTGCGCTCATCTTAGGCTTT '3	147
HSL (Lipe, ENSMUSG00000003123)	fw 5'ACCTGCTTGGTTCAACTGGA'3 rev 5'CTGGCACCCTCACTCCATAG'3	111
IGF-BP 1 (Igfbp1, ENSMUSG00000020429)	fw 5' CAGCATGAAGAGGCAAAGG '3 rev 5' CTATAGGTGCTGATGGCGTTC '3	152
IGF-BP 2 (Igfbp2, ENSMUSG00000039323)	fw 5' AGGTCCTGGAGCGGATCT '3 rev 5' CATCTTGCACTGCTTAAGGTTG '3	125
IGF-BP 3 (Igfbp3, ENSMUSG00000020427)	fw 5' TGCTCCAGGAAACATCAGTG '3 rev 5' GGAGTGGATGGAACTTGGAA '3	110
iNOS (Nos2, ENSMUSG00000020826)	fw 5' TGACACACAGCGCTACAACA '3 rev 5' CCATGATGGTCACATTCTGC ' 3	152
Nampt (ENSMUSG00000020572)	fw 5' CACCGACTCGTACAAGGTTACTC '3 rev 5' TTTCACGGCATTCAAAGTAGG '3	83
Nrf1 (ENSMUSG00000058440)	fw 5' TCATCTCGTACCATCACAGACC '3 rev 5' TTTGTTCCACCTCTCCATCAG '3	182
Pdk4 (ENSMUSG00000019577)	fw 5' CTCTTCAAGAATGCCATGAGG '3 rev 5' TCGGTCAGAAATCTTGATTGTAAG '3	120
PEPCK (Pck1, ENSMUSG00000027513)	fw 5' ATACATGGTGCGGCCTTTC '3 rev 5' GACAACTGTTGGCTGGCTCT '3	204
PK-L (Pklr, ENSMUSG00000041237)	fw 5'AGTATGGAAGGGCCAGCA '3 rev 5'AGAGGTGTTCCAGGAAGGTG '3	130

PGC1a (Ppargc1a, ENSMUSG00000029167)	fw 5‘AGTCACCAAATGACCCCAAG’ 3 rev 5‘GGAGTTGTGGGAGGAGTTAGG’ 3	106
PPARγ (Pparg, ENSMUSG00000000440)	fw 5‘ CAGGCCTCATGAAGAACCTT’ 3 rev 5‘ GGATCCGGCAGTTAAGATCA’ 3	176
PPARα (Ppara, ENSMUSG00000022383)	fw 5’ TCTGGAAGCTTTGGTTTTGC '3 rev 5‘ TTCGACACTCGATGTTCAGG '3	176
SREBF1c (Srebf1, ENSMUSG00000020538)	fw 5‘CTGTCGGGGTAGCGTCTG’3 rev 5‘CGGGAAGTCACTGTCTTGGT’3	112
Tfam (ENSMUSG00000003923)	fw 5’ ACACCCAGATGCAAAACTTTC ‘3 rev 5’ CTTTGTATGCTTTCCACTCAGC ‘3	122
PGC1a promoter (5 CpG)	fw 5’ TCCGGTTTAGAGTTGGTGGC ‘3 rev 5’ CCATCCAGCTCCCGAATGAC ‘3	380
GR exon 1A (11 CpG)	fw 5’ ACGCAAAGGAAAGAACATGCC ‘3 rev 5’ CCCAGACACTCTAACGAAGCA ‘3	349

ESM Table 2. Differentially expressed miRNAs between low-dose L-NAME (0.15mg/ml DW) group and control group

miRNA	logFC	P Value
Down-regulated miRNA		
mmu-miR-615-3p	-1.188126408	0.019959695
mmu-miR-193a-5p	-1.27414962	0.038590132
mmu-miR-199b-5p	-1.275405992	0.039922001
mmu-miR-144-3p	-1.45308533	0.01248127
mmu-miR-132-3p	-1.610251375	0.020140258
mmu-miR-8114	-1.661256834	0.006551138
novel_184	-4.18149069	0.048205786
novel_187	-4.301847207	0.046457366
novel_158	-4.427062846	0.04252077
novel_189	-4.572081065	0.044273836
novel_145	-4.831490776	0.041621203
novel_141	-5.059573644	0.043502905
novel_228	-5.152814224	0.048448563
novel_137	-5.223232961	0.047526908
novel_170	-5.235373352	0.042099932
novel_258	-5.341004355	0.046318068
novel_238	-5.52641261	0.04578849
novel_147	-5.737488916	0.015507201
novel_248	-5.947660942	0.018018269
novel_156	-6.393873119	0.002402113
novel_152	-6.502969632	0.020218211
novel_139	-8.381870524	0.034513278
novel_133	-9.400424176	0.033183276
Up-regulated miRNA		
novel_66	4.793368	0.028573

novel_58	5.434276	0.027511
novel_29	5.556467	0.027116
novel_77	5.975698	0.037912
novel_31	6.866177	0.011476

ESM Table 3. Differentially expressed miRNAs between high-dose L-NAME (2mg/ml DW) group and control group

miRNA	logFC	P Value
Down-regulated miRNAs		
mmu-miR-1843a-5p	-1.022555086	0.014345528
mmu-miR-503-5p	-1.085633053	0.009853503
novel_33	-1.192791837	0.002824878
mmu-miR-7230-3p	-1.515984779	0.009723282
novel_28	-1.955217119	0.000764771
mmu-miR-5099	-2.205040054	0.035678986
mmu-miR-497a-3p	-2.269705466	0.006030488
novel_84	-2.280232855	0.027527084
mmu-miR-181d-3p	-3.226742279	0.049292565
mmu-miR-7015-3p	-3.4442631	0.009004629
mmu-miR-802-5p	-3.477390754	0.010386511
mmu-miR-1934-5p	-3.687785393	0.039205574
mmu-miR-128-1-5p	-4.083115839	0.030951033
novel_184	-4.109186325	0.049069552
novel_187	-4.230645211	0.047614594
novel_158	-4.352196753	0.043753659
novel_189	-4.498326021	0.046217523
novel_145	-4.757665544	0.043398889
novel_88	-4.772178861	0.043502864
novel_79	-4.923206711	0.04426357
novel_120	-4.947664744	0.049891703
novel_141	-4.991829808	0.044516861
novel_114	-5.085712365	0.042926355
novel_21	-5.086738329	0.015148642
novel_258	-5.271151919	0.049804242

novel_42	-5.63758224	0.044847908
novel_147	-5.670669564	0.015130277
novel_248	-5.886025363	0.019359849
novel_156	-6.321290758	0.002119395
novel_152	-6.43052072	0.022028445
novel_78	-6.483633127	0.002054752
novel_107	-6.510814481	0.022250835
novel_139	-8.312733009	0.036698075
novel_133	-9.333723567	0.037405496
novel_121	-10.0821985	0.036392588
Up-regulated miRNAs		
mmu-miR-320-3p	1.000850931	0.005684666
mmu-miR-365-3p	1.072714256	0.018210289
mmu-miR-146a-5p	1.207131152	0.010556529
mmu-miR-292a-5p	1.818042926	0.03852769
mmu-miR-219c-5p	2.686836576	0.010766331
mmu-miR-6970-5p	2.695362472	0.043526378
mmu-miR-218-1-3p	2.787253559	0.048315625
mmu-miR-7219-3p	3.09932527	0.033766784
mmu-miR-3060-3p	3.247223663	0.018640336
novel_66	4.592328379	0.014903218
novel_215	5.690671331	0.038178473
novel_77	6.36285523	0.044196678

ESM Table 4: Described functions of already decribed miRNAs identifyied in mouse sperm after low dose L-NAME treatment mimiking paternal heterozygous deficiency.

miRNA	Main findings	Reference
mmu-miR-615-3p	microRNA mmumiR-615-3p is reduced under conditions of endoplasmic reticulum (ER) stress, wherein it regulates the expression of C/EBP homologous protein (CHOP) and determines cellular sensitivity to cell death.	[21]
	CircZNF609 is involved in the pathogenesis of focal segmental glomerulosclerosis by sponging miR-615-5p	[22]
mmu-miR-193a-5p	miR-193a alleviates diabetic neuropathic pain in a mouse model through the inhibition of HMGB1 expression	[23]
	miR-193a/b-3p overexpression attenuates liver fibrosis through suppressing the proliferation and activation of HSCs.	[24]
	mmu-miR-193 influenced embryo implantation by regulating growth factor receptor-bound protein 7 expression.	[25]
mmu-miR-199b-5p	miR-199b-5p is an important regulator in medullary TEC proliferation through targeting Fzd6 to activate Wnt signaling and cell cycle signaling.	[26]
	miR-199b as a regulator of the phenotypic switch during vascular cell differentiation derived from iPS cells by regulating critical signaling angiogenic responses	[27]
	miR-199b is a direct calcineurin/NFAT target gene that increases in expression in mouse and human heart failure	[28]
mmu-miR-144-3p	miR-144 is involved in extracellular matrix remodeling post MI and its loss leads to increased myocardial fibrosis and impaired functional recovery.	[29]
	Downregulation of microRNA-144 inhibits proliferation and promotes the apoptosis of myelodysplastic syndrome cells through the activation of the AKAP12-dependent ERK1/2 signaling pathway	[30]
	miR-144 maybe a potential regulator of the development of atherosclerosis via changes in vimentin signaling.	[31]

	Circulating exosomal miR-144-3p inhibits the mobilization of endothelial progenitor cells post myocardial infarction via regulating the MMP9 pathway.	[32]
mmu-miR-132-3p	miR-132-3p priming enhances the effects of mesenchymal stromal cell-derived exosomes on ameliorating brain ischemic injury	[33]
	Targeted silencing of miRNA-132-3p expression rescues disuse osteopenia by promoting mesenchymal stem cell osteogenic differentiation and osteogenesis in mice	[34]
	Brown adipocyte-derived exosomal miR-132-3p suppress hepatic Srebf1 expression and thereby attenuate expression of lipogenic genes	[35]
	MiR-132 controls pancreatic beta cell proliferation and survival through Pten/Akt/Foxo3 signaling	[36]
mmu-miR-8114		No functional related literature found

ESM Table 5: Body weight, organ weights, systolic blood pressure and plasma creatinine

Variable	Both sexes		XY		XX	
	F:WT; M:WT (n=45-50)	F:+/-eNOS; M:WT (n=26-27)	F:WT; M:WT (n=20-22)	F:+/-eNOS; M:WT (n=10)	F:WT; M:WT (n=25-28)	F:+/-eNOS; M:WT (n=16-17)
Final body weight (g)	27.25±0.99	27.07±1.11	34.18±0.89	32.80±1.28	21.80±0.42	23.70±0.83 **
Relative Liver Weight (% of body weight)	4.31±0.06	4.26±0.08	4.56±0.07	4.44±0.10	4.11±0.08	4.16±0.11
Relative Kidney weight (% of body weight)	0.59±0.01	0.61±0.01	0.61±0.02	0.64±0.02	0.57±0.01	0.59±0.02
Relative Heart weight (% of body weight)	0.51±0.01	0.49±0.01	0.50±0.01	0.49±0.02	0.51±0.01	0.49±0.02
Systolic blood pressure (mmHg)	102.70±2.43	106.10± 2.16	95.65± 3.22	99.04± 2.14	108.30± 3.20	110.30± 2.76
Plasma creatinine (µmol/l)	52.16±0.02	49.50±0.03	48.62±0.02	46.85±0.03	55.69±0.03	50.39±0.04

XX: female offspring; XY: male offspring. Data are given as mean±SEM and **: $p<0.01$ vs. father wt/mother wt

ESM Table 6: Histological findings in the liver

Variable	Both sexes		XY		XX	
	F:WT; M:WT (n=48)	F:+/-eNOS; M:WT (n=26-27)	F:WT; M:WT (n=22)	F:+/-eNOS; M:WT (n=9-10)	F:WT; M:WT (n=26)	F:+/-eNOS; M:WT (n=17)
Liver Lobular Dimension (mm)	0.07±0.001	0.07±0.001	0.07±0.001	0.07±0.002	0.07±0.003	0.07±0.002
Liver Connective Tissue Content (% area)	0.16±0.02	0.17±0.03	0.12±0.03	0.11±0.04	0.18±0.03	0.20±0.04
Liver Fat Content (% area)	0.73±0.16	1.09±0.34	0.87±0.32	0.20±0.06	0.61±0.10	1.67±0.51
Liver Lipid Droplet Density (droplets/mm^2)	2701.0± 411.2	3066.1±689.0	2537.7±779.2	4980.7±255.5	2850.8±352.1	4406.6±969.9
Liver Lobular Inflammation (score)	0.31±0.13	0.33±0.14	0.29±0.18	0.33±0.21	0.33±0.21	0.33±0.21
Number of CD68-Positive Immune Cells (score) in the liver	0.85±0.10	0.92±0.08	0.86±0.14	1.00±0.00	0.83±0.17	0.83±0.17

XX: female offspring; XY: male offspring. Data are given as mean±SEM.

ESM Table 7: Correlation among plasma glucose, plasma glucose and liver glycogen.

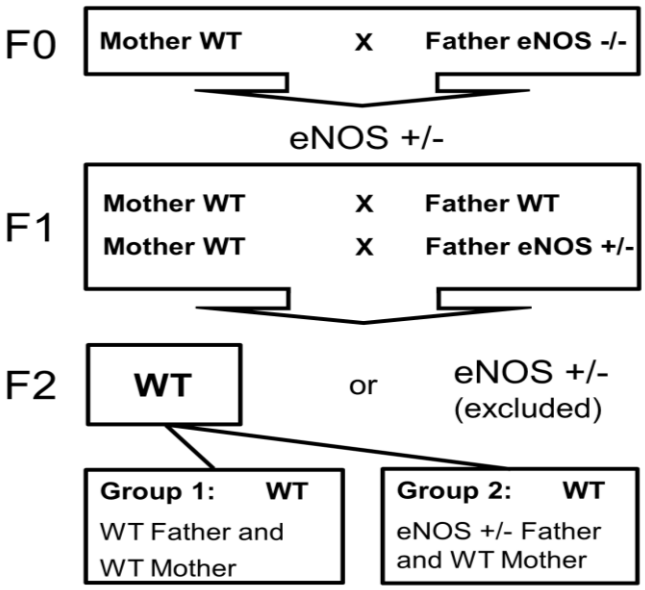
Parameter	Liver glycogen	IPGTT plasma glucose (0 min)	IPGTT plasma glucose (15 min)	IPGTT plasma glucose (60 min)	IPGTT plasma glucose (AUC)
Both sexes					
Liver glycogen	1.0	0.128	-0.017	0.161	0.087
IPGTT plasma insulin (0 min)	0.305*	-0.054	0.031	0.054	0.001
IPGTT plasma insulin (15 min)	0.2	-0.008	0.425**	0.399**	0.414**
IPGTT plasma insulin (60 min)	0.107	0.038	0.449**	0.536**	0.545**
IPGTT plasma insulin (AUC)	0.276	0.016	0.435**	0.449**	0.426**
XX					
Liver glycogen	1.0	-0.004	-0.183	0.143	-0.007
IPGTT plasma insulin (0 min)	0.201	-0.148	-0.033	0.330*	0.074
IPGTT plasma insulin (15 min)	0.114	-0.129	0.508**	0.428**	0.464**
IPGTT plasma insulin (60 min)	-0.059	0.105	0.354*	0.619**	0.504**
IPGTT plasma insulin (AUC)	0.183	0.07	0.396*	0.594**	0.430*
XY					
Liver glycogen	1.0	0.24	0.22	0.076	0.115
IPGTT plasma insulin (0 min)	0.34	0.04	0.135	-0.293	-0.114
IPGTT plasma insulin (15 min)	.419*	0.124	0.266	0.196	0.214
IPGTT plasma insulin (60 min)	0.337	-0.069	0.440*	0.254	0.391*
IPGTT plasma insulin (AUC)	0.452*	-0.035	0.473*	0.208	0.328

XX: female offspring; XY: male offspring. *: $p<0.05$, **: $p<0.01$.

ESM Table 8: Liver NOS expression, real time PCR quantification

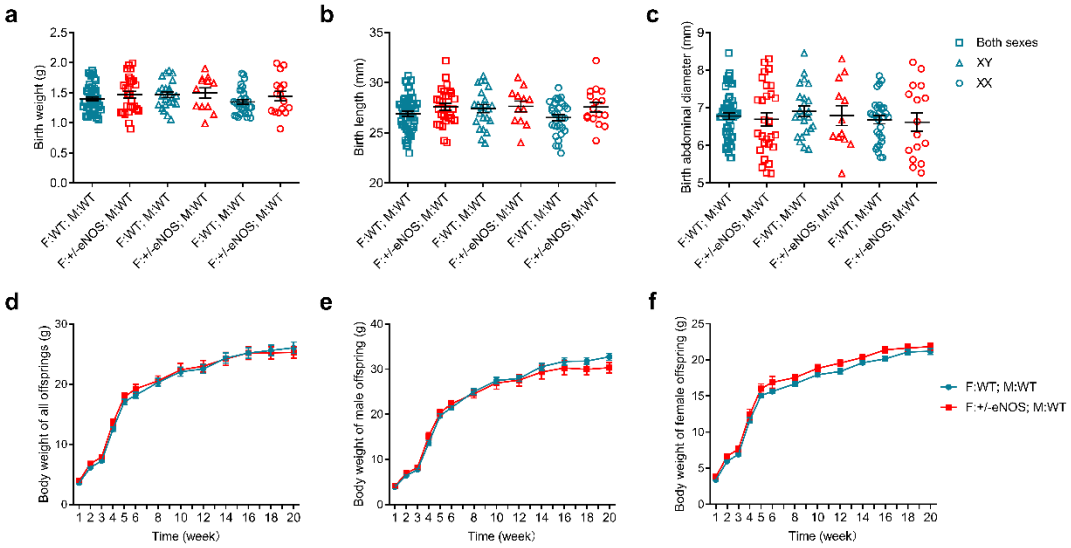
Variable	Both sexes		XY		XX	
	F:WT; M:WT (n=20)	F: +/- eNOS; M:WT (n=20)	F:WT; M:WT (n=10)	F: +/- eNOS; M:WT (n=10)	F:WT; M:WT (n=10)	F: +/- eNOS; M:WT (n=10)
eNOS (Nos3)	1.00 ± 0.06	1.03 ± 0.08	1.00 ± 0.07	1.04 ± 0.07	1.00 ± 0.11	1.02 ± 0.15
iNOS (Nos2)	1.00 ± 0.13	0.92 ± 0.12	1.00 ± 0.19	0.84 ± 0.10	1.00 ± 0.19	1.00 ± 0.22

XX: female offspring; XY: male offspring. Data are given as mean±SEM.



ESM Fig.1:

eNOS knockout mice of the C57BL/6J strain and their wild-type (wt) littermate were used. Female wt mice were cross-bred with homozygous male eNOS knockout mice. The resulting male heterozygous eNOS knockout ($eNOS^{+/-}$) mice were then again crossed with female wt mice. Only wt offspring of this breeding procedure (F2 generation) entered the study. These mice were compared to wt mice resulting from crossing male wt and female wt mice. Heterozygous animals used for breeding of the F2 generation were all derived from different dams i.e siblings were not used.



ESM Fig.2:

Birth weight, length and abdominal diameter as well as the change in body weight over the time XX: female offspring; XY: male offspring. F:WT; M:WT : wildtype offspring of wildtype fathers and wildtype mothers and F:+/-eNOS;M:WT : wildtype offspring of eNOS heterozygous fathers and wildtype mothers

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4 Publication 3：Parental sex-dependent effects of either maternal or paternal eNOS deficiency on the offspring's phenotype without transmission of the parental eNOS deficiency to the offspring

Xiaoli Zhang, Christoph Reichetzeder, Yvonne Liu, Johann-Georg Hocher, Ahmed A. Hasan, Ge Lin, Burkhard Kleuser, Liang Hu and Berthold Hocher

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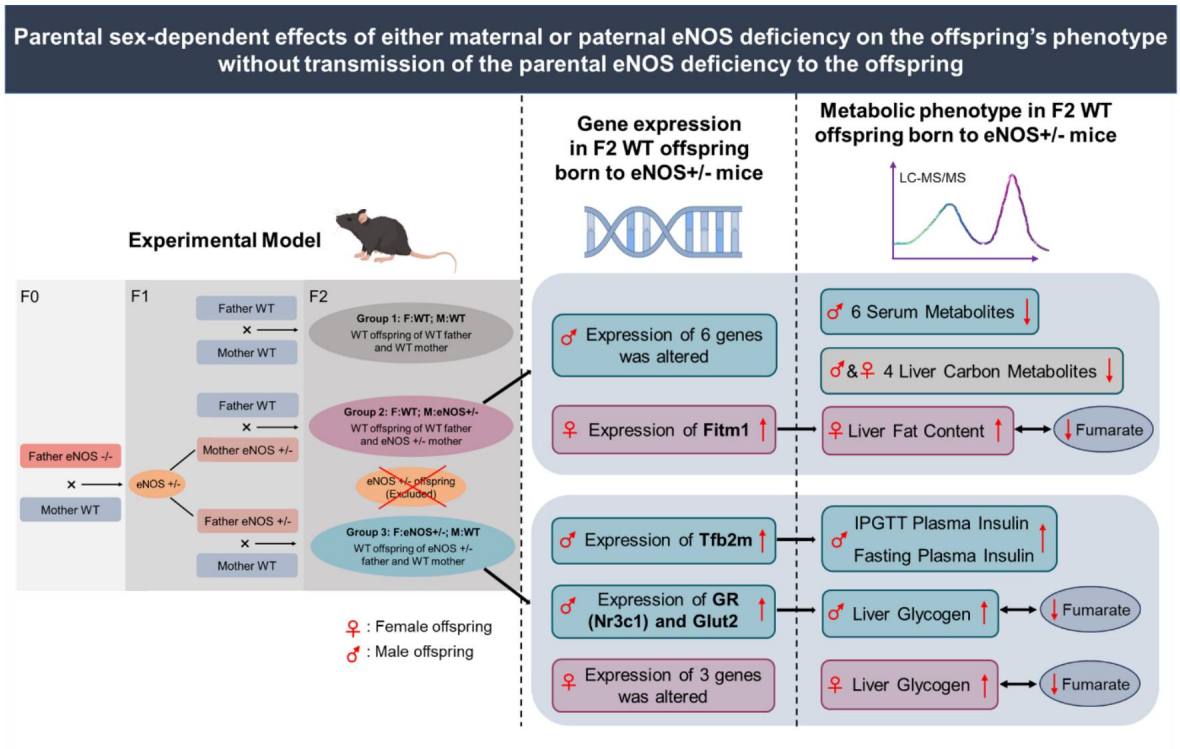
4.1 Graphic abstract



4.2 Author's contribution

In this publication, Xiaoli Zhang undertook the following crucial tasks: 1) analyzing and organizing the experimental data; 2) performing statistical analyses and creating figures and tables; 3) drafting the manuscript; 4) completing the submission process; 5) revising the manuscript and addressing reviewers' feedback.

4.3 Main content of the publication

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Parental sex-dependent effects of either maternal or paternal eNOS deficiency on the offspring's phenotype without transmission of the parental eNOS deficiency to the offspring

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Background: Preclinical animal studies and clinical studies indicate that both maternal as well as paternal genetic alterations/gene defects might affect the phenotype of the next-generation without transmissions of the affected gene. Currently, the question of whether the same genetic defect present in the mother or father leads to a similar phenotype in the offspring remains insufficiently elucidated.

Methods: In this head-to-head study, we crossbred female and male mice with heterozygous endothelial eNOS knockout (eNOS+/–) with male and female wild-type (wt) mice, respectively. Subsequently, we compared the phenotype of the resulting wt offspring with that of wt offspring born to parents with no eNOS deficiency.

Results: Wt female offspring of mothers with heterozygous eNOS showed elevated liver fat accumulation, while wt male offspring of fathers with heterozygous eNOS exhibited increased fasting insulin, heightened insulin levels after a glucose load, and elevated liver glycogen content. By quantitative mass-spectrometry it was shown that concentrations of six serum metabolites (lysoPhosphatidylcholine acyl C20:3, phosphatidylcholine diacyl C36:2, phosphatidylcholine diacyl C38:1, phosphatidylcholine acyl-alkyl C34:1, phosphatidylcholine acyl-alkyl C36:3, and phosphatidylcholine acyl-alkyl C42:5 (PC ae C42:5) as well as four liver carbon metabolites (fructose 6-phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate and fumarate) were different between wt offspring with eNOS+/– mothers and wt offspring with eNOS+/– fathers. Importantly, fumarate was inversely correlated with the liver fat accumulation in female offspring with eNOS+/– mothers and increased liver glycogen in offspring of both sexes with eNOS+/– fathers. The qRT-PCR results revealed that the gene expression patterns were different between wt

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offspring with eNOS+/− mothers and those offspring with eNOS+/− fathers. Different gene expression patterns were correlated with different observed phenotypic changes in male/female offspring born to mothers or fathers with a heterozygous eNOS genotype.

Conclusion: The identical parental genetic alteration (heterozygous eNOS deficiency), without being passed on to the offspring, results in distinct metabolic, liver phenotype, and gene expression pattern variations depending on whether the genetic alteration originated from the father or the mother.

KEYWORDS

maternal and paternal programming, eNOS, metabolomics, sex-dependent effects, offspring

Introduction

The "fetal programming" hypothesis suggests that during a crucial early stage of life, specific environmental and nutritional factors can lead to permanent changes in organ structure and function in response to environmental influences. These alterations may, in turn, increase the risk of developing cardiovascular and metabolic diseases later in life (Reichetzeder et al., 2016). The fundamental factors contributing to fetal programming encompass maternal undernourishment during pregnancy, overnutrition, obesity, diabetes, and exposure to harmful toxins (Reichetzeder et al., 2016). A novel mechanism, initially proposed by Hocher et al. (2000) and subsequently validated by other researchers (Masuda et al., 2002; van Beynum et al., 2006; Tsai et al., 2008; Miodovnik et al., 2012; Warrington et al., 2019), suggests that maternal genes may influence the fetal phenotype independently of the fetal genome. Similarly, existing studies have already suggested that paternal genes, even without being transmitted to the offspring, may also influence the phenotype of the offspring (Nelson et al., 2010; Chen et al., 2012; Li et al., 2016; Zhang et al., 2019; Liu et al., 2021; Zhang et al., 2022).

In our prior research, we conducted crossbreeding experiments involving female and male heterozygous eNOS knockout mice and male and female wt mice. We then compared the phenotype of the resulting wt offspring from these crosses with that of wt offspring born to parents who were both wt mice (Hocher et al., 2016; Hocher et al., 2022). We specifically selected eNOS knockout mice for these experiments due to the critical role that eNOS plays in regulating placental and vascular functions (Kulandavelu et al., 2012; Kusinski et al., 2012). Heterozygous eNOS deficiency has been conclusively linked to the development of an adverse intrauterine environment. Remarkably, this environmental factor can exert a significant influence on the vascular phenotype of offspring (Costantine et al., 2008). In addition, the presence of heterozygous eNOS deficiency in male mice can potentially lead to an unfavorable testicular microenvironment, primarily attributed to impaired testicular vascular function (Hocher et al., 2022). The findings from our prior research indicated that female offspring with wt genetics, born to mothers with heterozygous eNOS deficiency, developed fatty liver disease. Wt male offspring born to fathers with heterozygous eNOS deficiency exhibited elevated fasting insulin levels, as well as increased insulin levels following glucose ingestion (Hocher et al., 2016; Hocher et al., 2022). However, until now, we have not conducted a comprehensive study comparing the effects of maternal and paternal eNOS deficiency on genetically healthy offspring head-to-head. Our overarching goal is to gain a deeper understanding of phenotypic variations and uncover potential molecular mechanisms, particularly in relation to differences in glycemic control.

Metabolomics is a rapid, robust, and efficient research tool that analyses many small molecules of biochemical pathways in tissue, blood, urine, and other biological fluids (Hocher and Adamski, 2017; Lu et al., 2018; Yong-Ping et al., 2020). Metabolites are influenced by both endogenous regulatory mechanisms and the environment (Bachlechner et al., 2016; Feldman et al., 2017; Boone et al., 2019). In this study, we thus used metabolomics to gain a more profound understanding of the distinctions between genetically healthy offspring originating from either mother or father with heterozygous eNOS deficiency.

Materials and methods

Breeding and study protocol

The whole study protocol received approval from the animal welfare committee in Berlin, Germany, and was conducted in accordance with the relevant local institutional guidelines. ENOS knockout mice (strain B6.129P2-Nos3tm1Unc/J) and C57BL/6J control mice were sourced from Jackson Laboratories (Bar Harbour, ME). Animals were housed at a controlled environment (21°C ± 2°C, 50% ± 10% relative humidity and a 12:12h light-dark cycle) and had access to food and water *ad libitum*. A comprehensive description of the breeding procedure was shown in Supplementary Figure S1. Female wild-type (wt) mice were bred with male eNOS knockout mice (eNOS−/−) to produce F1 offspring with heterozygous eNOS (eNOS+/−) genotypes. Subsequently, female F1 mice with heterozygous eNOS knockout and male F1 mice with heterozygous eNOS knockout were once again bred with male and female wt mice, respectively. Heterozygous animals used for breeding of the F2 generation were all derived from different dams, i.e., siblings were not used. In addition, the F1 mice chosen for breeding the subsequent generation were matched in terms of age. Following parturition by the female, we promptly standardized the litter size to ten pups (comprising five males and five females). These newborns were nurtured until weaning at 21 days of age. Only the F2 wt offspring resulting from this breeding procedure were included in the study, and

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they were compared to wt offspring born to parents who were both wild type.

The male and female F2 offspring were raised for 24 weeks and subsequently underwent separate analyses, including measurements of birth weight, final body weight, and liver weight. Blood pressure was measured in the 24th week by using the tail-cuff method, as previously described (Quaschning et al., 2007). In the 21st week, an intraperitoneal glucose tolerance test (IPGTT) was conducted. In brief, the animals underwent an overnight fasting period, followed by intraperitoneal injection of 2 mg glucose per gram of body weight. Blood samples were then collected from the tail vein at 0, 15, and 60-min intervals to measure plasma glucose and insulin levels, following previously established protocols (Kim et al., 2010; Wang et al., 2010; Du et al., 2012). The trapezoid rule was used to determine the area under curve (AUC) for glucose and insulin concentrations in each animal.

Liver morphology

Liver morphology was analyzed under the microscope using two different stainings: Hematoxylin and Eosin (H&E), and Red Oil Staining.

a) H&E Staining: for preparation, the livers were washed with phosphate-buffered saline (PBS) buffer, then fixed with 4% (w/v) paraformaldehyde and embedded in paraffin. 3 µm thick slices were obtained using a Microm HM230 Microtomy and then stained with H&E. We identified hepatic venules and their adjacent portal fields by the sinusoidal connection between them. Ten lobules of each slide were identified using a Zeiss Axiovert 100 microscope (200 x), photographed with a Leica EC3 digital camera, and saved using LAS EZ software (Leica Microsystems). We measured the linear lobular dimensions by determining the distance from the center of the hepatic vein to the center of three associated portal vein branches using ImageJ (version 1.410, NIH shareware). Subsequently, we calculated the mean radius of lobules for each animal.
b) Oil Red O Staining was done as described before (Koopman et al., 2001). 30 pictures were taken per sample using an Olympus (Shinjuku, JP) BH-2 microscope (200x) and a digital camera CFW-1310C (Scion Corporation, Frederick, MD). The lipid content and the density of lipid droplets were quantified with ImageJ (version 1.410, NIH shareware).

Liver glycogen content

Liver tissue was incubated with 1N KOH (95°C, 30 min). Glycogen was precipitated using saturated sodium sulfate solution (Na_2SO_4) and 95% (v/v) ethanol and washed twice in 60% (v/v) ethanol. Resuspended glycogen was degraded with 0.1% (w/v) amyloglucosidase (Sigma-Aldrich, St. Louis, MO) in acetate buffer (0.2 M sodium acetate, 0.46% (v/v) acetic acid, pH 4.8) for 2 h at 40°C. Glucose concentration was measured photometrically using the Glucose (HK) Assay Kit (Sigma-Aldrich). Glycogen content was expressed in relation to liver weight.

Pancreas morphology

a) H&E Staining: pictures of whole tissue slides and of every islet of Langerhans were taken using the Zeiss Axiovert 100 microscope (25x/200x) and the Leica EC3 digital camera. The islets were counted, and the islet area was measured using ImageJ software to calculate the islet density and the mean islet area per slide.
b) Pancreas immunohistology: The beta cell content of islets of Langerhans was measured using immunohistological staining of insulin. We used an antibody against insulin (1:200, ab181547, abcam, Cambridge, United Kingdom) and a secondary antibody (1:500, ab97051, abcam, Cambridge, United Kingdom) diluted in antibody diluent (Dako, Glostrup, DK), and for visualization the ABC staining system (sc 2023, Santa Cruz Biotechnology, Santa Cruz, CA) following the instructions provided by the manufacturer. All islets per slide were photographed using an Olympus BH-2 microscope (200x) and a CFW-1310C digital camera. Thirty images for each sample were taken. The islet area and beta cell content were measured using ImageJ. The average islet size was obtained by total islet area/islet amount in each sample. The beta cell content was determined by the insulin positive staining area.

Metabolomic profiles in serum

At the end of the experiment, blood was obtained via retro-orbital collection. The blood samples were then centrifuged at 3,000 × g for 10 min at 4°C to obtain serum. Afterwards, we analysed serum metabolomic profiles using the Absolute IDQTM p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) and flow injection analysis-tandem mass spectrometry (FIA-MS/MS), following the kit's instructions. Detailed information on the procedure of metabolite quantification has been previously provided (Lu et al., 2018; Yong-Ping et al., 2020). We quantified a total of 163 targeted metabolites simultaneously from 10 µL of serum. These included 92 glycerophospholipids [comprising 15 lysophosphatidylcholines (LPC) and 77 phosphatidylcholines (PC)], 40 acylcarnitines (acylC), free carnitine, 14 amino acids (13 of which were proteinogenic, plus ornithine), hexoses, and 15 sphingolipids (SM) (Yong-Ping et al., 2020). The quantification of metabolite concentrations [µM] was based on internal standards.

Determination of central carbon metabolites in liver tissue

Liver tissues (15 to 30 mg each) were homogenized using a Fast Prep FP 120 homogenizer (Thermo Savant, Holbrook, NY) with 1 mL of phosphate buffer (pH 7.4) at a speed setting of 6.0, employing lysing matrix D. After homogenization, aliquots of the resulting homogenate were promptly frozen at −80°C until further analysis.

The concentrations of metabolites in the liver tissue homogenate were assessed using both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS), following previously established protocols (Hofmann et al., 2008; Maier et al., 2010). We spiked defined volumes of liver homogenate (as outlined below) with an internal standard solution.

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These samples were then subjected to evaporation until dryness using a stream of nitrogen, followed by derivatization. Subsequently, we conducted the analysis using gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was carried out using a 5975C inert XL MSD, coupled with a 7890A GC from Agilent Technologies in the Electron Impact (EI) mode.

Concentrations of 3-hydroxybutyrate, malate, citrate, and fumarate were determined in 5 µL liver tissue homogenate using the respectively labeled analogs 3-hydroxy-[$^2H_4$] butyrate, [$^{13}C_4$] fumarate, [$^{13}C_4$] malate, and [$^2H_4$] citrate as internal standards. Following the evaporation step, the samples underwent derivatization to form methyloxime tert-butyldimethylsilyl derivatives (Hofmann et al., 2008).

To determine glucose 6-phosphate (G-6-P) and fructose 6-phosphate (F-6-P) concentrations, 25 µL liver homogenate was spiked with the internal standards $^{13}C_6$-G-6-P and $^{13}C_6$-F-6-P, evaporated to dryness, and derivatized to the trimethylsilyl derivatives.

Concentrations of ribose 5-phosphate (Rib-5-P), ribulose 5-phosphate/xylulose 5-phosphate (Ribu-5-P), sedoheptulose 7-phosphate (Sed-7-P), phosphoenolpyruvate (PEP), 6-phosphogluconate (6-PG), 2-/3-phosphoglycerate (3-PG), and fructose 1,6-bisphosphate (FBP) were determined in 10 µL of liver homogenate and analysed by LC-MS-MS as described (Maier et al., 2010).

Quantitative real time PCR

We analysed a list of candidate genes involved in NO-synthase, metabolic process, energy homeostasis and fat storage, insulin-like growth factors and their binding proteins. RNA extraction from liver tissue and reverse transcription PCR were done like previously described (Hocher et al., 2022). Primers were obtained from Sigma-Aldrich, Eurofins (Ebersberg, GER) and shown in the Supplementary Table S1. The PCR was carried out on a Mx3000P thermal cycler (Stratagene, La Jolla, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), Sensi Mix or SensiFast low ROX kit (Bioline, London, United Kingdom) in accordance with the instructions. All samples were analysed in triplicate. The relative quantity of analysed genes was determined with the ΔΔCt method as described before (Hocher et al., 2022).

Statistics

Statistical analysis was performed using SPSS version 22.0. All values are presented as mean ± SEM. For all datasets, we applied a two-way analysis of variance (two-way ANOVA), followed by *post hoc* Tukey test. Metabolomics data of serum were analysed using MetaboAnalyst 3.0. To mitigate the false discovery rate (FDR), we employed the Benjamini–Hochberg (BH) procedure to adjust *p*-values. The BH procedure is defined as follows: Pm ≤ (m/M) × q, where "m" represents the rank of a given *p*-value, "M" is the total number of tests (M = 163), and "q" is the desired FDR threshold (set up at 0.05 in this study) (Madar and FastLSU, 2016). For normally distributed data, we used the Pearson correlation analysis. Statistically significant differences were defined as *p*-value ≤ 0.05.

Results

Parental eNOS deficiency had no impact on birth weight, body weight, organ weight, and blood pressure of wt offspring

Male and female wt offspring with either eNOS+/− fathers or eNOS+/− mothers showed no significant differences in birth weight, final body weight, relative liver weight, or blood pressure when compared to the control group (Supplementary Table S2).

Paternal eNOS deficiency led to an increase in fasting plasma insulin in male wt offspring

No differences in fasting plasma glucose were observed in male or female offspring born to eNOS+/− mothers/fathers. A significant increase in fasting plasma insulin was observed only in male wt offspring with eNOS+/− fathers (Supplementary Table S2).

Paternal eNOS deficiency led to increased plasma insulin after glucose intake in male wt offspring

IPGTT result showed that no differences in glucose concentrations at different time points after glucose intake and area under curve (AUC) of glucose in male or female offspring with eNOS+/− fathers/mothers (Figures 1A–C). Regarding insulin concentration, elevated insulin levels were observed at the 0 and 60 min after glucose intake in male offspring with eNOS+/− fathers (Figure 1D). Higher insulin levels at the 15 min after glucose intake were observed in female offspring with eNOS+/− fathers (Figure 1E). In addition, male offspring with eNOS+/− fathers had a significantly higher AUC of insulin (Figure 1F). No differences in insulin concentrations at different time points after glucose intake and AUC of insulin could be observed in male or female offspring born to eNOS+/− mothers.

Parental eNOS deficiency affected liver morphology of wt offspring

Liver lobule dimensions were consistent and similar among all groups. A significantly higher lipid droplet density and liver fat content were observed in female offspring born to eNOS+/− mothers (Supplementary Table S2; Figures 2A, B). However, the liver glycogen content was significantly higher in animals of both sexes born to eNOS+/− fathers (Figure 2C).

Parental eNOS deficiency had no impact on pancreas morphology of wt offspring

The size and density of pancreatic islets of Langerhans, as well as the beta-cell content within the islets, exhibited no significant differences among all groups (Supplementary Figure S2; Supplementary Table S2).

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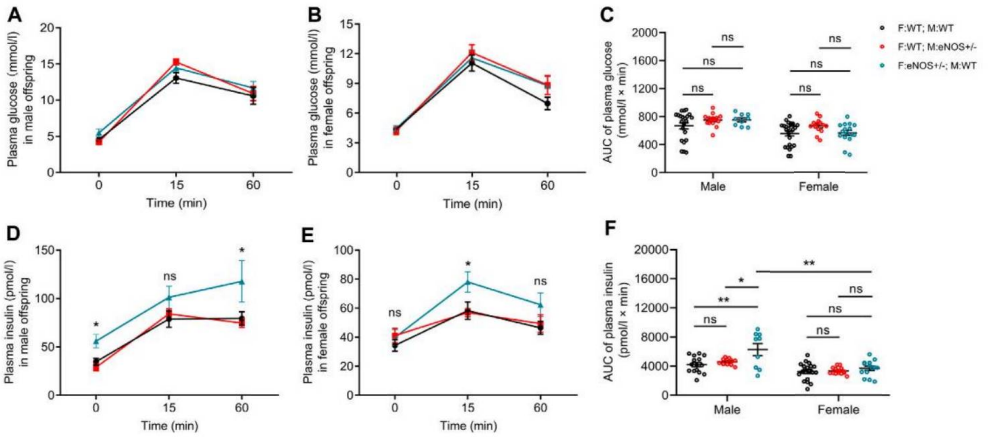

FIGURE 1
Comparison of plasma glucose **(A,B)** and insulin concentrations **(D,E)** during IPGTT in male **(A,D)** and female **(B,E)** offspring; AUC for IPGTT plasma glucose **(C)** and IPGTT plasma insulin **(F)** in male and female offspring. Black: F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers (male: n = 22 and female: n = 28); Red: F:WT; M: eNOS+/−: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: n = 15 and female: n = 14); Blue: F: eNOS+/−; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: n = 9 and female: n = 14). The data were presented as mean ± SEM and analysed by two-way ANOVA followed by *post hoc* Tukey test. *$p < 0.05$; **$p < 0.01$; ns: $p > 0.05$.

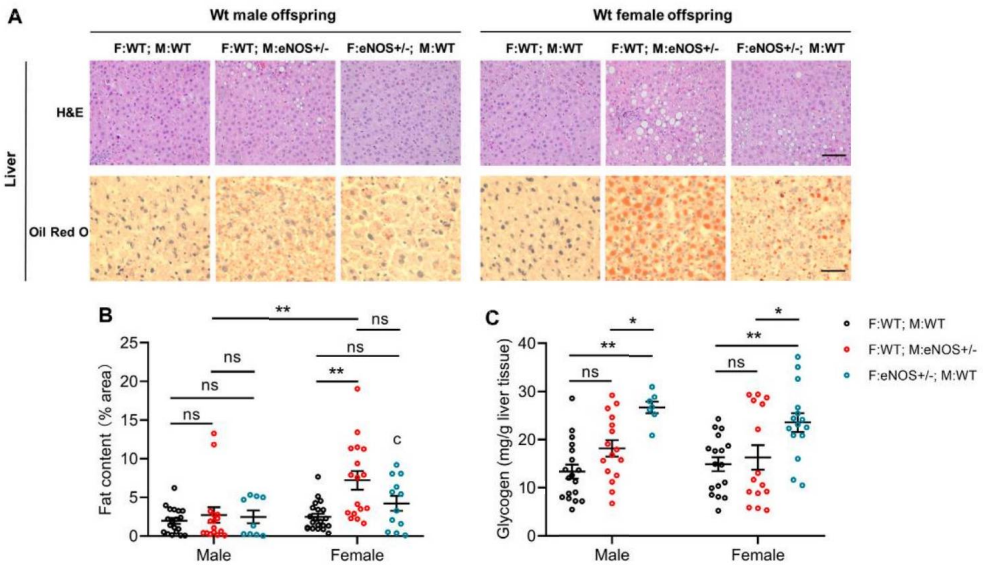

FIGURE 2
Morphological analysis of liver tissues. **(A)**: Representative images of haematoxylin and eosin (H&E) and Oil red O staining of liver sections (magnification: ×200 and scale bar: 50 μm); **(B)**: fat content and **(C)**: glycogen in the liver of wt offspring. Black: F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers (male: n = 19 and female: n = 21); Red: F:WT; M: eNOS+/−: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: n = 16 and female: n = 17); Blue: F: eNOS+/−; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: n = 9 and female: n = 12). The data in **(B)** and **(C)** were presented as mean ± SEM and analysed by two-way ANOVA followed by *post hoc* Tukey test. *$p < 0.05$; **$p < 0.01$; ns: $p > 0.05$.

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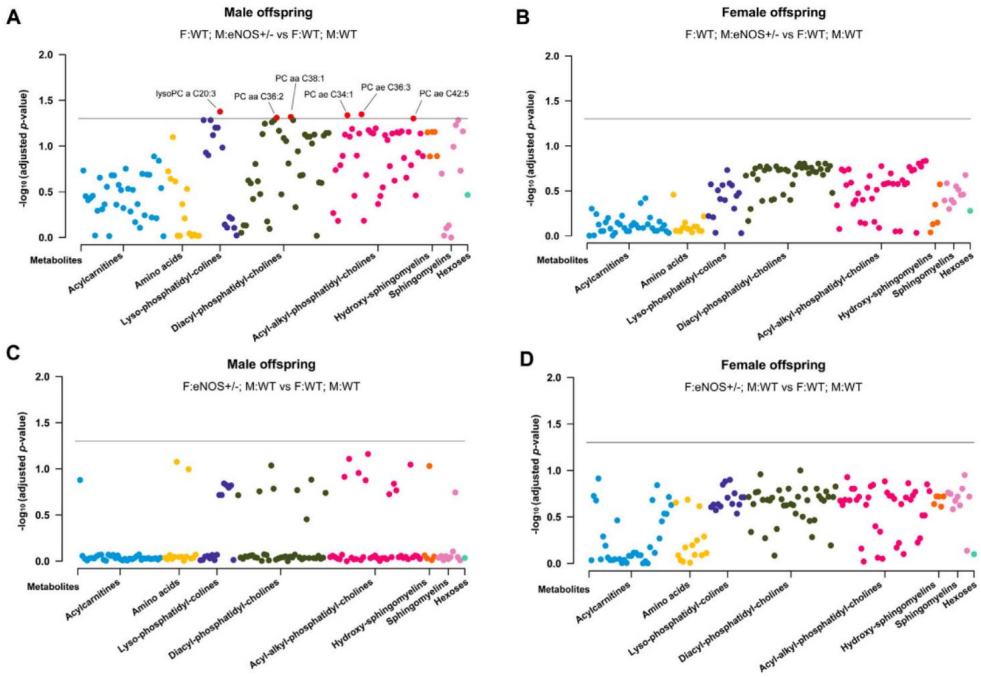

FIGURE 3
Manhattan Plot of offspring serum metabolites. **(A)** and **(B)**: serum metabolites in male **(A)** and female **(B)** wt offspring born to eNOS+/− mothers and wt fathers compared to those with wt parents; **(C)** and **(D)**: serum metabolites in male **(C)** and female **(D)** wt offspring born to eNOS+/− fathers and wt mothers compared to those with wt parents. The threshold line indicates the adjusted *p*-value of 0.05. F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers (male: $n = 25$ and female: $n = 28$); F:WT; M: eNOS+/−: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: $n = 13$ and female: $n = 18$); F: eNOS+/−; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: $n = 13$ and female: $n = 18$). The *p*-values were adjusted by Benjamini–Hochberg (BH) procedure.

Parental eNOS deficiency led to differing metabolomic profiles in the serum of wt offspring

After adjusting the *p*-values using the Benjamini–Hochberg procedure, six metabolites (lysoPC a C20:3, PC aa C36:2, PC aa C38:1, PC ae C34:1, PC ae C36:3, and PC ae C42:5) of the 163 targeted serum metabolites were significantly reduced in male offspring born to eNOS+/− mothers and wt fathers ($p < 0.05$ and FDR < 0.05) (Figure 3). No metabolite was significantly different in female offspring with eNOS+/− mothers or eNOS+/− fathers and male offspring with eNOS+/− fathers (for more details see Supplementary Table S3).

Parental eNOS deficiency led to differing liver carbon metabolites in liver tissue of wt offspring

We quantified selected substrates of glucose metabolism in liver tissue using both GC-MS and LC-MS-MS technology. In both male and female offspring with eNOS+/− mothers, we observed significantly lower concentrations of fructose 6-phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate and fumarate. Regarding the offspring with eNOS+/− fathers, both male and female offspring displayed significant reduction in fumarate concentration (Figure 4; Supplementary Table S4).

Correlation analysis showed the relationship between altered metabolites in liver/serum and phenotype in wt offspring

In wt offspring with eNOS+/− mothers, glucose 6-phosphate was negatively correlated with fat content in male offspring ($r = -0.401$, $p < 0.05$). Glucose 6-phosphate ($r = 0.393$, $p < 0.05$), fructose 6-phosphate ($r = 0.388$, $p < 0.05$), and fructose 1,6-bisphosphate ($r = 0.404$, $p < 0.05$) were positively correlated with glycogen content in female offspring. Fumarate was negatively correlated with the liver fat content in female offspring ($r = -0.338$, $p < 0.05$) (Figure 5A). LysoPC a C20:3 was

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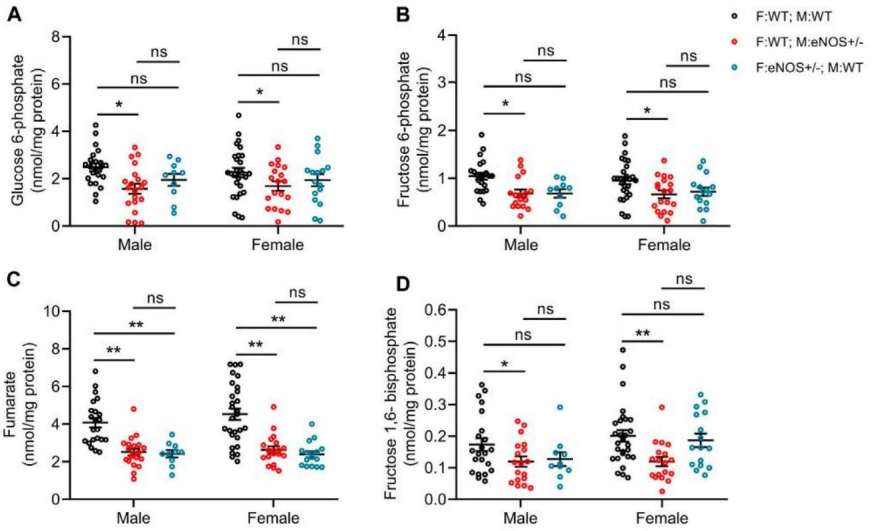

FIGURE 4
Comparison of carbon metabolite concentrations in liver tissue of offspring. **(A)**: glucose 6-phosphate; **(B)**: fructose 6-phosphate; **(C)**: fumarate; **(D)**: fructose 1,6-bisphosphate. Black: F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers (male: n = 21 and female: n = 27); Red: F:WT; M: eNOS+/−: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: n = 16 and female: n = 17); Blue: F: eNOS+/−; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: n = 9 and female: n = 15). The data were presented as mean ± SEM and analysed by two-way ANOVA followed by *post hoc* Tukey test. *$p < 0.05$; **$p < 0.01$; ns: $p > 0.05$.

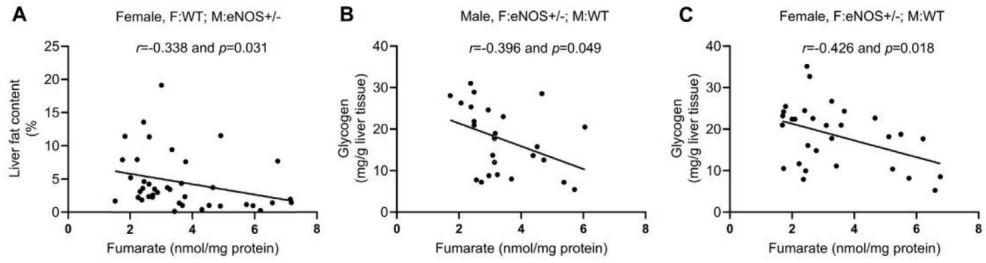

FIGURE 5
Correlation analysis between fumarate and phenotypic alterations in wild type offspring with eNOS+/− mothers/fathers. **(A)**: correlation between fumarate and liver fat content in female wt offspring with eNOS+/− mothers; **(B)** and **(C)**: correlation between fumarate and liver glycogen in male **(B)** and female **(C)** wt offspring with eNOS+/− fathers. F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers; F:WT; M: eNOS+/−: wildtype offspring of wildtype fathers and eNOS heterozygous mothers; F: eNOS+/−; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers.

positively correlated with the AUC of plasma insulin in female offspring (r = 0.448, $p < 0.05$). PC ae C42:5 was positively correlated with fat content in male offspring (r = 0.368, $p < 0.05$) (for more details see Supplementary Table S5).

In wt offspring with eNOS+/− father, fumarate was negatively correlated with glycogen content in both sexes ($r = -0.396$, $p < 0.05$; $r = -0.426$, $p < 0.05$) (Figures 5B, C). LysoPC a C20:3 had a positive correlation with fat content in male offspring (r = 0.394, $p < 0.05$) and glycogen content in female offspring (r = 0.375, $p < 0.05$). PC aa C38:1 was negatively correlated with glycogen content in male offspring ($r = -0.459$, $p < 0.05$). LysoPC a C20:3 (r = 0.460, $p < 0.01$), PC aa C38:1 (r = 0.404, $p < 0.05$), and PC ae C34:1 (r = 0.409, $p < 0.05$) were positively correlated with AUC of plasma insulin in female offspring (for more details see Supplementary Table S6).

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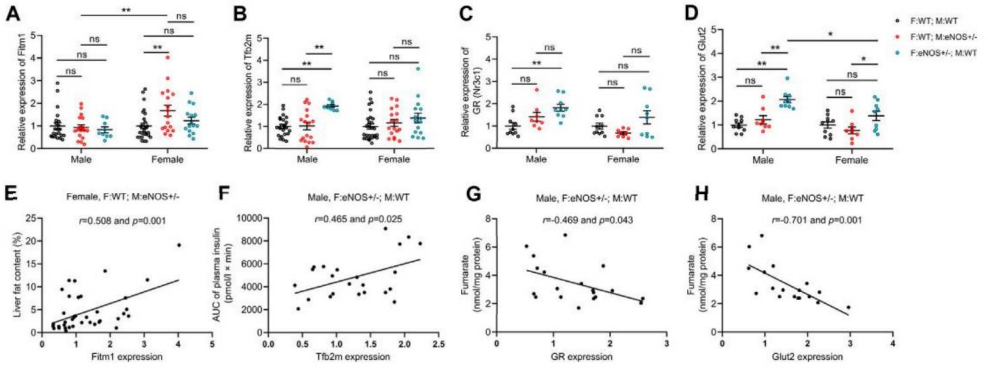

FIGURE 6
Gene expression (fold expression compared to control group) was analysed by real time PCR **(A–D)** and correlation analysis between altered genes and phenotypic changes **(E,F)** in wt offspring. **(G,H)**: correlation between altered genes and fumarate in male wt offspring with eNOS+/− fathers. Black: F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers (male: $n = 22$ and female: $n = 28$); Red: F:WT; M: eNOS+/−: wildtype offspring of wildtype fathers and eNOS heterozygous mothers (male: $n = 17$ and female: $n = 17$); Blue: F: eNOS+/−; M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers (male: $n = 9$ and female: $n = 15$). The data in **(A–D)** were presented as mean ± SEM and analysed by two-way ANOVA followed by *post hoc* Tukey test. $*p < 0.05$; $**p < 0.01$; ns: $p > 0.05$.

Parental eNOS deficiency resulted in different gene expression patterns in liver tissue of wt offspring

The qRT-PCR results revealed that the gene expression patterns were different between wt offspring with eNOS+/− mothers and those offspring with eNOS+/− fathers. In wt offspring with eNOS+/− mothers, PPARγ was significantly decreased while Pepck, Igf1, Igf2, Igfbp1 and Igfbp2 were significantly increased in male offspring. The expression of Srebf1c and Gck were significantly reduced while Fitm1 was significantly increased in female offspring. In wt offspring with eNOS+/− fathers, 12 genes were differentially expressed in male offspring and 3 genes were differentially expressed in female offspring. See more details in the Supplementary Table S7.

Correlation analysis showed the relationship between altered genes and phenotypic changes in wt offspring

As shown in Figure 6, the liver fat ratio showed a significant correlation with the expression of Fitm1 ($r = 0.508$, $p < 0.01$) in female offspring with eNOS+/− mothers. AUC of plasma insulin positively correlated with Tfb2m expression ($r = 0.465$, $p < 0.05$) in male offspring with eNOS+/− fathers. Seven genes (Chrebp, GR, Tfam, Tfb2m, G6Pase, Glut2 and Igf2) showed a significant correlation with increased liver glycogen in male offspring with eNOS+/− fathers (Supplementary Table S9). Fumarate negatively correlated with the expression of GR ($r = -0.469$, $p < 0.05$) and Glut2 ($r = -0.701$, $p < 0.01$) in male offspring with eNOS+/− fathers. See more details in the Supplementary Tables S8, S9.

Discussion

To investigate the advanced fetal programming hypothesis, which suggests that both maternal and paternal genes can influence the offspring's phenotype without the direct transmission of parental genes to the offspring, we employed a similar approach to our previous study. We crossed female and male heterozygous eNOS knockout mice with male and female wild type mice. Then, we conducted a head-to-head study to simultaneously analyse the paternal and maternal effects on the offspring's phenotype. Our study findings indicate that female wt offspring born to eNOS+/− mothers showed elevated liver fat accumulation. In contrast, male wt offspring born to eNOS+/− fathers displayed increased levels of fasting insulin, higher insulin levels following glucose intake, and elevated liver glycogen content (Figure 7). In addition, female wt offspring born to eNOS+/− fathers also showed an increased liver glycogen content. Pancreas morphology, including the endocrine pancreas, was not affected by parental eNOS deficiency. Our study identified six serum metabolites [lysoPhosphatidylcholine acyl C20:3 (lysoPC a C20:3), phosphatidylcholine diacyl C36:2 (PC aa C36:2), phosphatidylcholine diacyl C38:1 (PC aa C38:1), phosphatidylcholine acyl-alkyl C34:1 (PC ae C34:1), phosphatidylcholine acyl-alkyl C36:3 (PC ae C36:3), and phosphatidylcholine acyl-alkyl C42:5 (PC ae C42:5)] in male wt offspring and four liver carbon metabolites (fructose 6-phosphate, fructose 1,6-bisphosphate, glucose 6-phosphate and fumarate) in both sexes of wt offspring born to eNOS+/− mothers were significantly changed compared with those in wt offspring born to eNOS+/− fathers. These observations might be attributed to the adverse intrauterine environment in eNOS+/− mothers. Notably, further correlation analyses showed fumarate was inversely correlated with the fat accumulation in the liver in female offspring with eNOS+/− mothers and increased liver glycogen in offspring of both sexes with eNOS+/− fathers (Figure 7). Gene expression analysis

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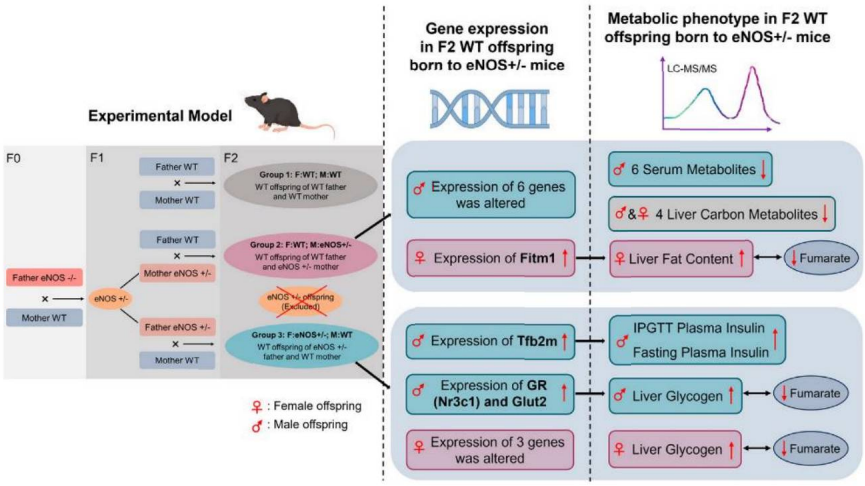

FIGURE 7
Graphical summary of the results obtained in this study. WT, Wild type; IPGTT, Intraperitoneal glucose tolerance test.

revealed an elevated expression of liver Fitm1 (fat storage inducing transmembrane protein 1) in female offspring with maternal eNOS deficiency, which was associated with increased liver fat accumulation in these offspring. Importantly, our study revealed that the expression of the Tfb2m (mitochondrial transcription factor B2) gene was increased in male offspring born to fathers with eNOS deficiency, which was significantly correlated with elevated insulin levels after glucose load in these male offspring. Seven genes were significantly correlated with increased liver glycogen in in male offspring born to fathers with eNOS deficiency. Furthermore, in these genes, the increased expression of GR (glucocorticoid receptor) and Glut2 (glucose transporter 2) genes was significantly correlated with the decreased levels of fumarate in male offspring with paternal eNOS deficiency (Figure 7).

Our data align with recent research (Nelson et al., 2010; Liu et al., 2021; Chen et al., 2022; Zhao et al., 2022), which suggests that maternal or paternal genes, even without direct transmission to the offspring, can influence the offspring's phenotype. This influence appears to depend on whether the gene defect was present in the mother or the father. One study investigated the transgenerational genetic effects of the fathers' Y chromosome on daughters' phenotypes and revealed that certain traits on the paternal Y chromosome (not inherited to daughters) significantly reduced anxiety levels among daughters (Nelson et al., 2010). Another study demonstrated that the mutation of paternal Usp26 (ubiquitin-specific peptidase 26) increased the risk of having children with Klinefelter syndrome (Liu et al., 2021). A recent study showed that the maternal environment affects offspring by influencing the level of oocyte TET3 (tet methylcytosine dioxygenase 3). This, in turn, has an impact on the reprogramming of the paternal genome within the zygote. The impairment of DNA demethylation and epigenetic inheritance specifically influences the expression of certain paternally hypermethylated genes involved in insulin secretion, including Gck (glucokinase), which plays a critical role in glucose metabolism. Consequently, this sensitizes the offspring to glucose intolerance (Chen et al., 2022). In addition, another study showed that deficiency of maternal Ezh1/2 (enhancer of zeste homolog 1/2) caused compromised H3K27me3 (tri-methylation of lysine 27 on histone H3 protein) and affected pluripotent epiblast cells within late blastocysts, which subsequently results in impaired embryonic development (Zhao et al., 2022). To sum up, genetic changes in the parental germline can affect the offspring's phenotype, even if those changes are not encoded in the DNA sequence of the offspring. These changes can be influenced by many factors such as diet, stress, and exposure to toxins. The molecular mechanisms underlying these epigenetic effects induced by parental genes may involve DNA methylation, histone modification, or mediation through small RNAs.

The key molecule in this study is eNOS, an enzyme that produces nitric oxide (NO) in endothelial cells. NO plays a crucial role in locally regulating vascular resistance, promoting angiogenesis, and is also considered a potential regulator of placental steroid biosynthesis and nutrient uptake (Vatish et al., 2006). eNOS deficiency is linked to the occurrence of intrauterine growth retardation (IUGR) due to impaired placental blood flow and nutrient delivery (George et al., 2022). Parental stimuli, characterized by heterozygous eNOS deficiency, yield diverse phenotypic outcomes in their offspring, contingent upon the sex of the parent carrying the deficiency, while not being passed on to the subsequent generation. Female offspring of mothers with the eNOS+/− genotype exhibited heightened liver fat accumulation, while male offspring of fathers with the same

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genotype showed increased fasting insulin levels and enhanced liver glycogen storage. These distinct outcomes arising from the identical parental genetic defect (eNOS deficiency) may be attributed to varying effects of eNOS deficiency on the reproductive organs of males and females. Maternal eNOS deficiency can impact various aspects of reproductive and maternal physiology, including egg maturation, intrauterine development, nursing behavior, and lactation (Pallares et al., 2008; Teichert et al., 2008). It is important to note that maternal eNOS deficiency has negative effects on liver fat ratio most likely due to the influence on oocyte/intrauterine development induced by maternal eNOS deficiency. Paternal eNOS deficiency can have far-reaching effects, potentially influencing both the maturation and development of sperm (Mostafa et al., 2015). Furthermore, it may trigger transmissible epigenetic alterations within these sperm, giving rise to enduring epigenetic changes. These modifications can ultimately result in an adult phenotype characterized by elevated fasting insulin levels, heightened insulin response following glucose intake, and increased liver glycogen content. This explanation aligns with previous research that demonstrates how a paternal high-fat diet before conception can lead to impaired glucose tolerance in offspring, primarily due to epigenetic modifications in sperm and consequential alterations in target organs (Ng et al., 2010; Terashima et al., 2015; Chen et al., 2016; Nembhard et al., 2018), which correspond to our findings. In our prior research endeavours, we were able to establish disparities in both overall DNA methylation levels and gene-specific DNA methylation patterns, along with variations in the expression of specific candidate genes among wild-type offspring with either maternal or paternal eNOS deficiency. Notably, we observed a conspicuous correlation between DNA methylation patterns and the observed liver phenotype in these offspring (Hocher et al., 2016; Hocher et al., 2022). In the current study, we showed that hepatic fat accumulation in female wt offspring of eNOS+/− mothers was associated with increased expression of liver Fitm1. This gene was reported in our previous study (Hocher et al., 2016). However, we added the corresponding expression data of this gene in wt offspring born to eNOS+/− fathers in this study. In addition, we found that increased expression of the Tfb2m gene in male offspring born to fathers with eNOS deficiency was significantly correlated with elevated insulin levels after glucose load in these male offspring. Tfb2m is a mitochondrial transcription factor involved in mitochondrial DNA transcription. Existing evidence have showed that Tfb2m played a critical role in insulin secretion (Adan et al., 2008; Fex et al., 2018). Our observations were consistent with these findings. What's more, we revealed that seven genes in male offspring born to fathers with eNOS deficiency were significantly correlated with increased liver glycogen in these male offspring. Particularly, we revealed the correlation among altered genes (GR and Glut2), metabolite (fumarate) and phenotypic changes (increased liver glycogen) in male offspring born to fathers with eNOS deficiency. These findings supplemented and added new evidence for a comprehensive understanding of how parental eNOS gene defects influence the phenotype of offspring, even when the offspring have not inherited the specific gene defect.

Sex differences on phenotypes were observed both in offspring of eNOS+/− mothers and of eNOS+/− fathers. Sexual dimorphism in fetal programming in response to identical stimuli has been extensively documented in previous studies (Reichetzeder et al., 2016). This divergence can be attributed to several factors. Firstly, both male and female sex steroid hormones, which are produced by both the fetus and the placenta, play a role in modulating the impact of mild NO deficiency on epigenetic and phenotypic changes in the offspring in a sex-specific manner. Additionally, sex-dependent transcriptional variations in the offspring may contribute to these observed differences (Bermejo-Alvarez et al., 2011; Li et al., 2016; Reichetzeder et al., 2016).

In the present study, we employed metabolomics to investigate further links between the observed offspring phenotypes and maternal/paternal eNOS deficiency. Characteristic metabolites associated with fatty liver disease and increased insulin and liver glycogen storage were identified. The precise pathophysiological significance of the serum metabolites we observed remains not fully elucidated. Phosphatidylcholines, including lyso-phosphatidylcholines, diacyl-phosphatidylcholines, and acyl-alkyl-phosphatidylcholines, are vital constituents of cell membranes and lipoproteins (Cole et al., 2012). Beyond their fundamental structural role, these molecules seem to play a role in various physiological processes. Notably, they are implicated in the liver's secretion of very low-density lipoproteins and are also associated with glucose regulation (Cole et al., 2012; Furse and de Kroon, 2015; Boone et al., 2019). Phosphatidylcholines have also been demonstrated to enhance the cell proliferation effects of insulin and insulin-like growth factor-1 (Kiss, 1999). Furthermore, alterations in the concentrations of phosphatidylcholines were linked to cardiometabolic changes triggered by an excess of liver and visceral fat (Floegel et al., 2013) and atherosclerosis (Matsumoto et al., 2007). In line with these studies, we identified that lysoPC a C20:3 was specifically associated with the AUC of plasma insulin in female offspring born to eNOS+/− mothers/fathers. PC aa C38:1 and PC ae C34:1 had a positive relation with the AUC of plasma insulin in female offspring with eNOS+/− fathers. lysoPC a C20:3 was positively correlated with glycogen content in female offspring, while PC aa C38:1 was negatively correlated with glycogen content in male offspring born to eNOS+/− fathers. In summary, while the specific functions of the majority of metabolites observed in our study remain uncertain, there appears to be an association between these metabolites and alterations in plasma insulin levels, liver fat, and glycogen, induced by maternal or paternal eNOS deficiency.

Particularly, we found that fumarate had a significant correlation with increased fat accumulation in liver in female offspring born to eNOS+/− mothers and increased liver glycogen in offspring of both sexes born to eNOS+/− fathers. Fumarate plays a pivotal role as a key intermediate in the tricarboxylic acid cycle (TCA), facilitating the interconnection of carbon and nitrogen metabolism (Araujo et al., 2011; Hengist et al., 2019). One way in which fumarate can indirectly affect blood glucose levels is by influencing insulin signaling. Studies have shown that fumarate can enhance insulin sensitivity and improve glucose uptake by cells, which may help to lower blood glucose levels (Franko et al., 2022). Additionally, fumarate has been shown to activate AMP-activated protein kinase (AMPK) is a critical enzyme that plays a central role

in the regulation of glucose metabolism (Li et al., 2011). With regards to fat accumulation, fumarate may indirectly impact this process by affecting mitochondrial function. Fumarate has been demonstrated to stimulate mitochondrial biogenesis and improve mitochondrial function, which may help to reduce fat accumulation (Wang et al., 2021). When it comes to glycogen, fumarate can impact glycogen storage in the liver indirectly through its effects on ATP production, AMPK activation, and insulin sensitivity (Noster et al., 2019). Consistent with these previous studies, our research also illustrated that fumarate was significantly related to the changes in liver fat accumulation and liver glycogen, induced by maternal/paternal eNOS+/− deficiency in offspring.

Our study has also several limitations. The primary goal of our study was to analyze potential sex-dependent effects of parental eNOS deficiency on the offspring's phenotype and we indeed could demonstrate that it matters whether the parental heterozygous eNOS deficiency was present in the father or mother in our head-to-head study. However, sex dependent effects of the origin of homozygous eNOS deficiency in the grandfather/grandmother's generation could also affect the phenotype in the F2 generation. There is some evidence that it would have affected the phenotype of the heterozygous eNOS mice coming from earlier studies by Longo M et al., showing that eNOS heterozygous offspring born to eNOS knockout mothers had higher blood pressure, effects on glucose tolerance and insulin levels compared to those offspring born to eNOS knockout fathers most likely because eNOS knockout mothers had an abnormal uterine environment (Longo et al., 2016). This uterine effect is absent when starting with homozygous eNOS deficient fathers. Therefore, we used male F0 eNOS−/− mice as origin of eNOS deficiency in our study. Given the study by Longo M et al., it is very likely that if the origin of eNOS deficiency would be the grandmother in the breeding protocol it would have affected the phenotype in the F2 generation, but this represents another research question that merits to be addressed in an independent study designed to address this topic. Epigenetic alterations of the adult phenotype are mainly caused by intrauterine epigenetic alterations of gene expression and subsequent alterations of the function and morphology of organs (Reichetzeder et al., 2016). This is clearly an important task for further studies to better understand the early life epigenetic mechanisms of parental eNOS deficiency. In addtion, we measured insulin and glucose levels at 0, 15, and 60 min during the IPGTT, with no further measurements to prioritize animal welfare. However, the duration of 60 min for IPGTT demonstrated differences among the groups and was also reported in previous studies (Kim et al., 2010; Wang et al., 2010; Hocher et al., 2022). What's more, the specific metabolomics platform used in this study restricted our choice of metabolites to investigate. On the other hand, an advantage of this platform is that it includes a collection of metabolites that are both biologically and analytically well-defined. Another limitation of our study is the absence of an analysis on the potential impact of eNOS heterozygosity on the metabolism of the parents (F1 generation) and an analysis on the hererozygous eNOS fetuses of F2 genetation. Some other studies already provided evidence on phenotype of eNOS heterozygous mice. Cook et al. have shown that $eNOS^{-/+}$ heterozygous mice were normotensive and had normal insulin sensitivity on a normal diet (Cook et al., 2004). Consequently, we did not investigate further on the metabolic phenotype of F1 eNOS heterozygous mice and mainly focused on the metabolic changes observed in the F2 generation with a healthy genotype. Additionally, we did not analyse the underlying epigenetic alterations. However, this was not done, because it was described recently by us using comparable experimental designs of the animal studies—increased liver glucocorticoid receptor and Ppargc1a gene expression attributed to altered methylation patterns of these genes when the father had eNOS deficiency and increased liver Fat Storage Inducing Transmembrane Protein 1 (*Fitm1*) and Cyclin-dependent kinase inhibitor 1A (Cdkn1a) gene expression resulted from altered methylation of these genes when the mother had eNOS deficiency (Kim et al., 2010; Wang et al., 2010; Hocher et al., 2022).

In conclusion, this head-to-head study demonstrated that the identical parental genetic modification (heterozygous eNOS deficiency) without transmission to the offspring causes an offspring metabolic and liver phenotype and liver gene expression pattern depending on whether the alteration was present in the father or the mother. Female offspring with wildtype genes from mothers with a heterozygous eNOS deficiency showed increased liver fat accumulation. In contrast, male offspring with wildtype genes from fathers with a heterozygous eNOS deficiency had higher fasting insulin levels, increased insulin response after a glucose load, and elevated liver glycogen content. We identified six serum metabolites and four liver carbon metabolites that differed between wt offspring with eNOS+/− mothers and wt offspring with eNOS+/− fathers. The most prominent effects were observed regarding fumarate (strong correlations between fumarate and changes on liver histology induced by maternal/paternal eNOS deficiency). Moreover, the gene expression patterns were different between wt offspring with eNOS+/− mothers and those offspring with eNOS+/− fathers. Importantly, the changes in specific gene expression were found to be correlated with the observed phenotypic alterations in wt offspring with eNOS+/− mothers/fathers. Our findings enhance the understanding of how parental genetic defects may impact the phenotype of genetically healthy offspring. This provides a foundation for more precise genetic counselling and screening. The clinical implications of our study should be further investigated in monogenic inherited diseases such as thalassemia.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Animal welfare committee of the state of Berlin, Germany. The study was

conducted in accordance with the local legislation and institutional requirements.

Author contributions

XZ: Data curation, Formal Analysis, Writing–original draft. CR: Data curation, Writing–review and editing. YL: Writing–review and editing. J-GH: Writing–review and editing. AH: Data curation, Writing–review and editing. GL: Writing–review and editing. BK: Supervision, Writing–review and editing. LH: Writing–review and editing. BH: Conceptualization, Project administration, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1306178/full#supplementary-material

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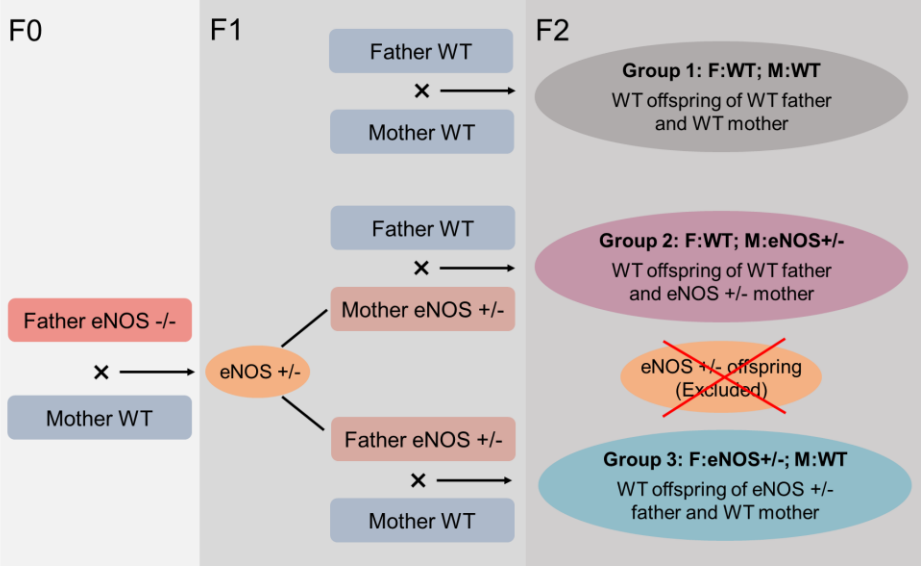
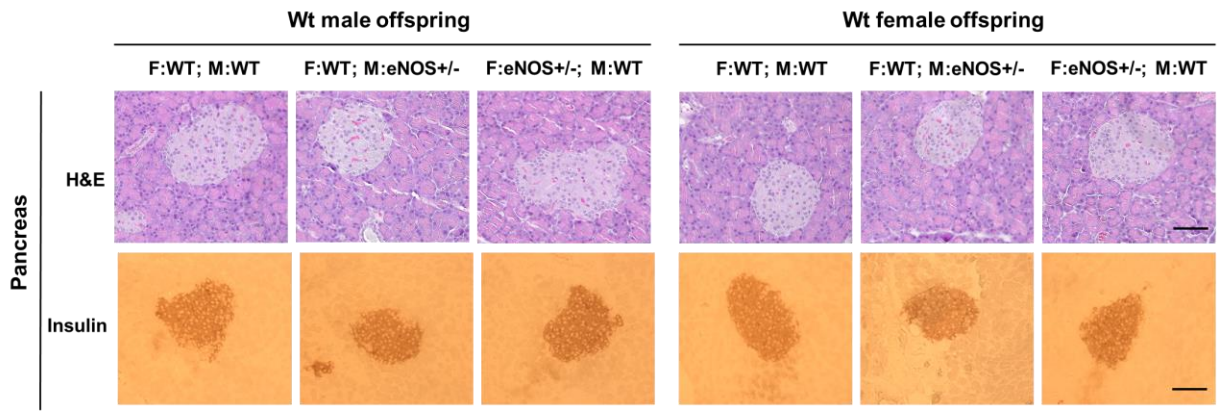
4.4 Supplementary materials



Supplementary Figure 1. Study design for the F0, F1 and F2 generations.

eNOS knockout mice of the C57BL/6J strain and their wild-type (wt) littermate were used. Female wt mice were cross-bred with homozygous male eNOS knockout mice. The resulting male and female heterozygous eNOS knockout ($eNOS^{+/-}$) mice were then crossed with female and male wt mice, respectively, to produce the F2 generation. Only wt offspring of this breeding procedure (F2 generation) entered the study. These mice were compared to wt mice resulting from crossing male wt and female wt mice. Heterozygous animals used for breeding of the F2 generation were all derived from different dams i.e. siblings were not used.



Supplementary Figure 2. Morphological analysis of pancreas tissues. Representative images of haematoxylin and eosin (H&E) and insulin staining of pancreas sections (magnification: 200× and scale bar: 50μm).

Supplementary Table 1. Primers used for real time PCR

Target Gene	Primer Pair
eNOS (Nos3, ENSMUSG00000028978)	fw 5‘ GGGAAAGCTGCAGGTATTTG ’3 rev 5‘ GCTGAACGAAGATTGCCTCT '3
iNOS (Nos2, ENSMUSG00000020826)	fw 5‘ TGACACACAGCGCTACAACA ’3 rev 5‘ CCATGATGGTCACATTCTGC ' 3
Chrebp (Mlxipl, ENSMUSG00000005373)	fw 5‘ GAAGATGCTTATGTTGGCAATG ’3 rev 5‘ GGCGGTAATTGGTGAAGAAA ’3
Srebf1c (ENSMUSG00000020538)	fw 5‘CTGTCGGGGTAGCGTCTG’3 rev 5‘CGGGAAGTCACTGTCTTGGT’3
PPARα (Ppara, ENSMUSG00000022383)	fw 5’ TCTGGAAGCTTTGGTTTTGC '3 rev 5‘ TTCGACACTCGATGTTCAGG '3
PPARγ (Pparg, ENSMUSG00000000440)	fw 5‘ CAGGCCTCATGAAGAACCTT’ 3 rev 5‘ GGATCCGGCAGTTAAGATCA’ 3
PPARγ Co1α (Ppargc1a, ENSMUSG00000029167)	fw 5‘AGTCACCAAATGACCCCAAG’ 3 rev 5‘GGAGTTGTGGGAGGAGTTAGG’ 3
GR (Nr3c1, ENSMUSG00000024431)	fw 5' ACACGTCAGCACCCCATAAT '3 rev 5' AGGCCGCTCAGTGTTTTCTA '3
Tfam (ENSMUSG00000003923)	fw 5’ ACACCCAGATGCAAAACTTTC ‘3 rev 5’ CTTTGTATGCTTTCCACTCAGC ‘3
Tfb1m (ENSMUSG00000036983)	fw 5’ AGGAAGTGGCTGAGAGACTTGT ‘3 rev 5’ GGTGCACCACTCCTACATCA ‘3
Tfb2m (ENSMUSG00000026492)	fw 5’ CGATCTGTACTCCTGCGAATC ‘3 rev 5’ ACCAAGGTTCCATGTGCAG ‘3
Nrf1 (ENSMUSG00000058440)	fw 5’ TCATCTCGTACCATCACAGACC ‘3 rev 5’ TTTGTTCCACCTCTCCATCAG ‘3
Nampt (ENSMUSG00000020572)	fw 5’ CACCGACTCGTACAAGGTTACTC ‘3 rev 5’ TTTCACGGCATTCAAAGTAGG ‘3
AMPK-α2 (Prkaa2, ENSMUSG00000028518)	fw 5‘AAAGACATACGAGAACATGAATGG’3 rev 5‘CTTCACAGCCTCATCGTCAA’3
Fbp1 (FBPase)(ENSMUSG00000069805)	fw 5' ATCAAAGCCATCTCGTCTGC '3 rev 5' ATTTGCCCCTCTTCTCAGGT '3
Gck (ENSMUSG00000041798)	fw 5' AAGTCCCACGATGTTGTTCC '3 rev 5' CTTCCCTGTAAGGCACGAAG '3
Pepck (Pck1, ENSMUSG00000027513)	fw 5' ATACATGGTGCGGCCTTTC '3 rev 5' GACAACTGTTGGCTGGCTCT '3
PK-L (Pklr, ENSMUSG00000041237)	fw 5’AGTATGGAAGGGCCAGCA ‘3 rev 5’AGAGGTGTTCCAGGAAGGTG ‘3
G6Pase (G6pc, ENSMUSG00000078650)	fw 5' GACTGTGGGCATCAATCTCC '3 rev 5' TCACAGGTGACAGGGAACTG '3
Glut 2 (Slc2a2, ENSMUSG00000027690)	fw 5’ GACGTCAATGGCACAGACAC ’3 rev 5’ GCCAACATTGCTTTGATCCT ’3
FAS (Fasn, ENSMUSG00000025153)	fw 5‘ GGATTCGGTGTATCCTGCTG ’3 rev 5‘ TGGGCTTGTCCTGCTCTAAC ’3
Acc1 (Acaca, ENSMUSG00000020532)	fw 5’ TTTCACATGAGATCCAGCATG ‘3 rev 5’ GCCACAGTGAAATCTCGTTG ‘3
Cpt1a (ENSMUSG00000024900)	fw 5‘CGCACATTACAAGGACATGG ’3 rev 5‘TCTGCTCTGCCGTTGTTGT ’3

HSL (Lipe, ENSMUSG00000003123)	fw 5‘ACCTGCTTGGTTCAACTGGA’3 rev 5‘CTGGCACCCTCACTCCATAG’3
Fitm1 (ENSMUSG00000022215)	fw 5’ TGCTTACGGCGCCTCTAC ‘3 rev 5’CACAAACTTTATGTTGAAGAAGTTGC ‘3
Fitm2 (ENSMUSG00000048486)	fw 5’ AAGCGCAACGTCCTCAAC ‘3 rev 5’ CAGATATACCAGATGGCTGTGC ‘3
Igf1 (ENSMUSG00000020053)	fw 5' CGTCTTCACACCTCTTCTACCTG '3 rev 5' CCTGTGGGCTTGTTGAAGTAA '3
Igf2 (ENSMUSG00000048583)	fw 5' TCTACTTCAGCAGGCCTTCAA '3 rev 5' GGGTATCTGGGGAAGTCGTC '3
Igfbp1 (ENSMUSG00000020429)	fw 5' CAGCATGAAGAGGCAAAGG '3 rev 5' CTATAGGTGCTGATGGCGTTC '3
Igfbp2 (ENSMUSG00000039323)	fw 5' AGGTCCTGGAGCGGATCT '3 rev 5' CATCTTGCACTGCTTAAGGTTG '3
Igfbp3 (ENSMUSG00000020427)	fw 5' TGCTCCAGGAAACATCAGTG '3 rev 5' GGAGTGGATGGAACTTGGAA '3
β-Actin (Actb, ENSMUSG00000029580)	fw 5' GATATCGCTGCGCTGGTC '3 rev 5' CATCACACCCTGGTGCCTA '3

Supplementary Table 2. Birth weight, body weight, liver weight, systolic blood pressure, fasting plasma glucose, insulin, and histological findings in the liver and pancreas.

Variable	Male offspring			Female offspring		
	F: WT; M: WT (n=22)	F: WT; M: eNOS+/- (n=15)	F: eNOS +/-; M: WT (n=10)	F: WT; M: WT (n=24)	F: WT; M: eNOS+/- (n=18)	F: eNOS +/-; M: WT (n=14)
Birth weight, body weight, liver weight and systolic blood pressure						
Birth weight (g)	1.46±0.04	1.28±0.04	1.53±0.08	1.35±0.04	1.30±0.04	1.43±0.07
Final body weight (g)	33.91±0.89	31.15±0.82	31.67±1.83	21.82±0.44	23.31±0.64	23.41±0.65
Liver weight (g)	1.54±0.05	1.41±0.04	1.42±0.08	0.89±0.02	1.00±0.04	1.00±0.05
Relative Liver Weight (% of body weight)	4.56±0.08	4.56±0.10	4.49±0.09	4.09±0.08	4.29±0.07	4.19±0.14
Systolic blood pressure (mmHg)	95.63±2.90	95.16±3.31	99.81±1.55	109.29±3.14	105.06±2.75	109.94±2.50
Fasting plasma glucose and insulin						
Fasting glucose (mmol/L)	4.52±0.35	4.60±0.32	5.43±0.59	3.98±0.20	4.60±0.30	4.86±0.38
Fasting insulin (pmol/L)	47.12±5.22	38.10±5.17	72.21±12.42[bc]	36.21±6.29	31.17±3.33	44.09±5.98
Histological findings in the liver						
Liver Lobular Dimension (mm)	0.07±0.001	0.07±0.001	0.07±0.003	0.07±0.002	0.07±0.002	0.06±0.003
Lipid droplet Density (droplets/mm2)	2239.75±747.54	2546.34 ±822.85	1585.9±569.46	3445.78±685.75	6624.64±1962.24[a]	3414.52±775.53[c]
Fat Content (% area)	1.96±0.38	2.71±1.00	2.47±0.83	2.49±0.38	7.24±1.20[a]	4.25±0.96
Glycogen	13.43±1.54	18.13±1.77	28.00±2.29[bc]	14.92±1.53	15.39±2.53	22.57±1.81[bc]
Histological findings in the pancreas						
Pancreatic island area (mm^2)	0.011±0.002	0.008±0.001	0.007±0.002	0.012±0.002	0.013±0.003	0.017±0.007
Pancreatic island density (islands/cm^2)	116.50±14.21	143.58±13.41	128.08±14.41	126.48±13.65	100.02±11.00	134.85±16.65
Pancreatic beta cell content (%)	50.95±5.79	52.77±5.26	65.07±5.93	62.66±3.96	55.11±5.43	58.74±5.09

F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers; F:WT;M:eNOS+/-: wildtype offspring of wildtype fathers and eNOS heterozygous mothers; F:eNOS+/-;M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers. Data are given as mean ± SEM. a: $p<0.05$, F:WT; M:eNOS+/- vs F:WT; M:WT; b: $p<0.05$, F:eNOS+/-; M:WT vs F:WT; M:WT; c: $p<0.05$, F:eNOS+/-; M:WT vs F:WT; M:eNOS+/-

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Supplementary Table 3. Metabolomic profiles in serum

Variable	Male offspring			Female offspring		
MetIQ Short Name	F: WT; M: WT (n=25) (μM)	F: WT; M: eNOS+/- (n=13) (μM)	F: eNOS+/-; M: WT (n=13) (μM)	F: WT; M: WT (n=28) (μM)	F: WT; M: eNOS+/- (n=18) (μM)	F: eNOS+/-; M: WT (n=18) (μM)
Acylcarnitines						
C0	31.18±11.2	24.3±11.69	25.72±8.54	29.45±11.38	29.37±12.04	28.83±11.89
C10	0.1±0.03	0.09±0.02	0.1±0.03	0.09±0.02	0.09±0.03	0.1±0.03
C10:1	0.08±0.02	0.09±0.02	0.09±0.02	0.08±0.02	0.08±0.02	0.09±0.02
C10:2	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01
C12	0.06±0.03	0.07±0.05	0.06±0.04	0.05±0.04	0.06±0.04	0.05±0.02
C12-DC	0.05±0.01	0.05±0.01	0.05±0.01	0.06±0.01	0.06±0.01	0.05±0
C12:1	0.1±0.03	0.11±0.04	0.12±0.04	0.11±0.04	0.11±0.03	0.12±0.02
C14	0.11±0.09	0.17±0.14	0.12±0.11	0.09±0.09	0.11±0.09	0.08±0.06
C14:1	0.11±0.06	0.13±0.08	0.12±0.07	0.09±0.07	0.11±0.06	0.1±0.05
C14:1-OH	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.01±0.01
C14:2	0.02±0.02	0.03±0.02	0.02±0.02	0.02±0.02	0.02±0.01	0.02±0.01
C14:2-OH	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
C16	0.24±0.19	0.36±0.28	0.26±0.22	0.21±0.21	0.25±0.2	0.19±0.15
C16-OH	0.01±0.01	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
C16:1	0.09±0.07	0.12±0.09	0.11±0.08	0.08±0.08	0.11±0.08	0.08±0.06
C16:1-OH	0.01±0.01	0.02±0.01	0.02±0.01	0.01±0.01	0.02±0.01	0.01±0.01
C16-2	0.02±0.02	0.03±0.02	0.02±0.02	0.02±0.02	0.02±0.01	0.01±0.01
C16:2-OH	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
C18	0.05±0.03	0.08±0.04	0.05±0.03	0.05±0.04	0.06±0.04	0.05±0.02
C18:1	0.19±0.18	0.28±0.23	0.19±0.16	0.18±0.22	0.22±0.19	0.15±0.14
C18:1-OH	0.02±0.02	0.03±0.02	0.02±0.02	0.02±0.02	0.02±0.02	0.02±0.01
C18:2	0.07±0.08	0.11±0.1	0.08±0.07	0.07±0.08	0.08±0.06	0.06±0.05
C2	19.3±5.16	20.36±6.17	20.69±6.1	17.93±5.51	20.06±6.22	17.93±3.64
C3	1.04±0.69	0.8±0.68	0.82±0.44	1.13±0.83	1.26±0.96	1.16±0.73
C3-DC (C4-OH)	0.26±0.11	0.27±0.1	0.28±0.15	0.21±0.11	0.27±0.18	0.21±0.08
C3-OH	0.03±0.01	0.02±0.01	0.02±0.01	0.03±0.02	0.02±0.01	0.03±0.01
C3:1	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
C4	1.36±1.4	0.7±0.38	1.02±0.83	1.54±2.56	1.26±0.95	1.22±0.94
C4:1	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
C4:1-DC (C6)	0.16±0.13	0.13±0.08	0.15±0.11	0.21±0.33	0.18±0.13	0.14±0.09
C6:1	0.02±0	0.01±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
C7-DC	0.02±0.01	0.02±0	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
C8	0.14±0.05	0.11±0.04	0.13±0.04	0.12±0.04	0.13±0.06	0.14±0.06
C8:1	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
C9	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
Amino acids						
Arg	115.44±46.25	87.07±49.75	117.95±47.63	117.23±40.61	139.08±61.98	138.39±43.99
Gln	628.28±168.55	524.54±234.83	585.77±178.89	620.21±186.03	603.41±198.32	643.06±187.89

Gly	304.6±76.5	244.69±70.25	293.46±68.74	300.32±78.67	294.41±79.48	296.83±63.39
His	79.67±23.66	67.5±23.41	73.21±23.3	75.81±22.77	77.81±21.96	75.24±14.06
Met	59.51±21.79	58.56±22.1	55.52±18.41	68.91±21.09	71.84±21.51	65.06±17.74
Orn	106.07±42.6	107.03±42.06	83.15±25.35	118±48.56	108.23±40.1	94.93±28.51
Phe	78.21±21.67	70.28±24.21	73.63±24.43	79.1±21.48	81.84±23.38	78.96±14.84
Pro	124.36±63.14	109.41±66.22	103.21±44.8	134.38±58.54	127.79±56.96	122.56±47.8
Ser	133.46±41.32	113.89±43.88	120.28±40.47	155.36±49.41	158.16±55.31	149.71±41.61
Thr	121.83±40.07	118.9±39.39	117.93±31.08	154.88±50.03	163.15±56.16	143.19±40.17
Trp	80.63±17.32	81.1±21.67	92.54±26.14	110.44±20.08	114.16±28.35	120.96±26.97
Tyr	104.42±49.78	101.38±46.7	102.26±42.61	112.52±46.24	117.16±44.68	118.11±50.33
Val	348.16±86.23	353.08±123.64	329.46±92.39	385.96±120.43	377.06±89.27	356.94±66.19
xLeu	356.44±91.4	352.85±129.19	341.46±94.57	365.39±102.54	393.35±103.54	351.56±68.04
Lyso-phosphatidyl-colines						
lysoPC a C14:0	3.25±1.14	2.21±0.68	3.64±1.54	3.29±1.28	3.67±1.55	3.99±1.89
lysoPC a C16:0	281.36±62.2	221.23±36.74	266±77.9	211.86±45.7	234.29±38.07	235.17±50.78
lysoPC a C16:1	8.26±2.18	5.61±1.34	8.48±2.06	4.75±2.05	5.26±1.72	5.65±2.13
lysoPC a C17:0	3.61±0.98	2.65±0.9	3.42±1.06	3.11±0.61	3.14±0.75	3.49±1
lyasoPC a C18:0	109.7±32.54	84.19±21.16	100.83±31.81	106.94±24.68	119.66±29.9	119.01±28.78
lysoPC a C18:1	59.17±17.86	39.18±9.35	62.31±23.75	41.93±12.2	47.11±13.58	53.74±20.78
lysoPC a C18:2	135.3±45.07	93.64±24.09	146.65±63.68	105.77±23.23	121.99±38.67	135.33±50.32
lysoPC a C20:3	11.46±5.87	5.48±2.7[a]	12.43±8.62	6.35±2.89	7.62±3.83	10±5.56
lysoPC a C20:4	41.26±16.48	30.71±7.4	48.88±17.28	39.45±10.87	49.94±16.3	62.17±23.32
lysoPC a C24:0	1.18±0.35	1.12±0.23	1.37±0.52	0.86±0.25	1.02±0.36	0.98±0.26
lysoPC a C26:0	1.52±0.71	1.43±0.44	1.96±1.08	0.92±0.31	1.14±0.56	1.14±0.37
lysoPC a C26:1	2.56±0.34	2.49±0.19	2.75±0.44	2.55±0.2	2.62±0.29	2.66±0.35
lysoPC a C28:0	0.85±0.34	0.78±0.23	1.06±0.53	0.59±0.24	0.72±0.38	0.71±0.25

lysoPC a C28:1	0.77±0.35	0.73±0.19	0.97±0.48	0.51±0.2	0.61±0.27	0.63±0.23
lysoPC a C6:0	0.03±0.01	0.03±0.01	0.03±0.03	0.02±0.01	0.02±0.01	0.03±0.01
Diacyl-phosphatidyl-cholines						
PC aa C24:0	0.33±0.17	0.34±0.11	0.42±0.21	0.23±0.1	0.31±0.16	0.29±0.1
PC aa C26:0	1.79±0.9	1.66±0.61	2±1.21	1.09±0.42	1.21±0.6	1.22±0.38
PC aa C28:1	0.47±0.25	0.44±0.17	0.6±0.38	0.32±0.13	0.37±0.2	0.38±0.12
PC aa C30:0	1.15±0.18	1.04±0.24	1.17±0.25	0.93±0.17	1.07±0.28	1.06±0.22
PC aa C30:2	1.24±0.22	1.14±0.16	1.22±0.35	0.8±0.17	0.92±0.19	0.92±0.2
PC aa C32:0	15.64±3.65	14.2±3.9	16.22±2.92	11.43±2.26	14.23±3.65	13.72±3.29
PC aa C32:1	9.39±3.67	7.25±2.38	8.81±3.35	4.87±1.64	6.21±2.26	5.78±1.63
PC aa C32:2	1.93±0.42	1.71±0.47	1.85±0.43	1.1±0.44	1.27±0.4	1.22±0.42
PC aa C32:3	0.23±0.06	0.2±0.04	0.22±0.07	0.15±0.05	0.18±0.05	0.18±0.06
PC aa C34:1	212.84±60.23	165.15±41.7	184.38±59.84	101.35±29.25	124.78±38.84	116.78±27.98
PC aa C34:2	375.72±64.19	310.08±61.11	345.54±72.47	229.93±41.68	263.24±53.04	257.94±56.53
PC aa C34:3	20.19±5.17	19.07±6.3	19.43±6.4	8.41±4.19	10.14±4.58	8.9±4.71
PC aa C34:4	0.63±0.18	0.61±0.2	0.65±0.2	0.3±0.15	0.36±0.15	0.35±0.15
PC aa C36:0	6.38±1.74	5.04±1.08	5.63±1.54	3.81±0.83	4.72±1.54	4.45±1.02
PC aa C36:1	55.34±16.36	40.28±9.52	45.86±15.35	31.18±7.32	37.21±12.38	35.39±8.34
PC aa C36:2	241.92±62.78	182.92±43.92[a]	211.54±61.84	153.13±29.38	178.65±49.72	169.62±43.56
PC aa C36:3	124.48±44.81	87.39±27.52	111.82±34.36	62.47±17.11	71.06±22.89	70.58±17.83
PC aa C36:4	174.28±42.83	157.38±31.71	164.85±29.14	105.17±29.3	132.71±34.86	127.95±31.2
PC aa C36:5	10.29±3.49	7.87±1.78	9.28±3.07	4.61±1.41	5.71±2.1	5.3±1.41
PC aa C36:6	0.55±0.13	0.46±0.1	0.5±0.13	0.26±0.09	0.3±0.11	0.3±0.09
PC aa C38:0	2.42±0.62	2.06±0.36	2.25±0.45	1.7±0.42	1.97±0.61	1.88±0.43
PC aa C38:1	6.99±1.66	5.39±1.16[a]	5.67±1.54	3.56±0.73	4.18±1.4	4.05±0.85
PC aa C38:3	38.32±14.93	25.74±6.97	33.05±12.48	22.14±5.39	27.3±9.54	26.87±5.86
PC aa C38:4	101.37±30.05	93.11±12.29	95.7±18.75	82.45±20.67	106.89±26.83	102.78±24.54
PC aa C38:5	56.2±16.88	45.48±8.84	53.82±11.1	29.65±8.57	36.25±10.84	36.07±8.03
PC aa C38:6	139.58±37.22	121.21±21.48	122.72±30.81	69.26±24.91	89.51±27.13	82.21±23.98
PC aa C40:1	0.65±0.17	0.56±0.09	0.64±0.15	0.43±0.08	0.5±0.13	0.46±0.08
PC aa C40:2	0.91±0.36	0.68±0.21	0.76±0.3	0.42±0.14	0.55±0.3	0.46±0.16
PC aa C40:3	1.66±0.76	1.06±0.46	1.38±0.73	0.49±0.12	0.6±0.21	0.53±0.11
PC aa C40:4	4.74±1.94	3.35±1.15	4.1±2.09	1.63±0.39	2.01±0.58	1.89±0.34
PC aa C40:5	12.78±3.97	9.96±1.47	11.18±4.4	6.88±2.06	8.71±2.42	8.03±1.66
PC aa C40:6	48.13±15.14	39.85±5.83	40.49±12.91	32.24±10.34	41.64±10.52	37.61±9.18
PC aa C42:0	0.25±0.06	0.24±0.04	0.25±0.07	0.22±0.06	0.28±0.11	0.25±0.07
PC aa C42:1	0.29±0.07	0.25±0.05	0.28±0.06	0.2±0.04	0.25±0.08	0.23±0.06
PC aa C42:2	0.44±0.13	0.38±0.09	0.42±0.12	0.21±0.06	0.29±0.11	0.25±0.07

PC aa C42:4	0.51±0.18	0.41±0.1	0.44±0.19	0.24±0.07	0.29±0.1	0.25±0.06
PC aa C42:5	0.88±0.36	0.6±0.25	0.73±0.4	0.28±0.06	0.33±0.09	0.31±0.05
PC aa C42:6	1.96±0.83	1.3±0.62	1.55±0.92	0.53±0.08	0.58±0.13	0.59±0.07
Acyl-alkyl-phosphatidyl-cholines						
PC ae C30:0	0.26±0.09	0.24±0.05	0.29±0.1	0.17±0.04	0.19±0.06	0.19±0.04
PC ae C30:1	0.65±0.22	0.53±0.15	0.68±0.25	0.54±0.13	0.56±0.17	0.6±0.13
PC ae C30:2	0.14±0.06	0.13±0.03	0.16±0.07	0.08±0.03	0.11±0.05	0.1±0.03
PC ae C32:1	0.94±0.19	0.84±0.13	0.94±0.21	0.69±0.14	0.8±0.2	0.82±0.15
PC ae C32:2	0.39±0.1	0.33±0.07	0.4±0.13	0.21±0.05	0.24±0.08	0.26±0.05
PC ae C34:0	0.9±0.16	0.75±0.15	0.87±0.15	0.63±0.12	0.74±0.21	0.74±0.15
PC ae C34:1	4.64±0.99	3.71±0.73[a]	4.11±0.97	2.75±0.59	3.14±0.85	3.21±0.64
PC ae C34:2	4.85±0.95	3.83±0.88	4±0.99	2.45±0.47	2.66±0.71	2.73±0.57
PC ae C34:3	1.27±0.32	0.97±0.35	1.07±0.36	0.72±0.14	0.81±0.3	0.81±0.19
PC ae C36:0	1.1±0.26	1±0.24	1.03±0.28	0.71±0.24	0.83±0.3	0.74±0.16
PC ae C36:1	6.01±1.77	4.9±1.03	5.28±1.42	3.31±1.14	3.55±1.2	3.34±0.76
PC ae C36:2	11.66±2.83	9.17±2.81	10.52±2.79	6.5±1.47	7.28±2.57	7.34±2.15
PC ae C36:3	2.62±0.49	2.1±0.48[a]	2.35±0.47	1.45±0.32	1.52±0.41	1.64±0.38
PC ae C36:4	4.73±0.95	4.55±0.74	4.71±0.79	3.43±0.86	3.84±0.81	4.11±0.98
PC ae C36:5	2.25±0.52	1.97±0.39	2.15±0.41	1.99±0.54	2.29±0.44	2.39±0.56
PC ae C38:0	4.32±1.31	3.34±0.63	3.71±0.78	1.9±0.55	2.15±0.67	2.08±0.48
PC ae C38:1	3.89±1.03	3.09±0.64	3.29±0.73	2.92±0.85	3.14±1.1	2.99±0.65
PC ae C38:2	8.38±2.5	6.37±2.23	7.39±2.43	3.9±0.97	4.51±1.7	4.24±1.26
PC ae C38:3	3.55±1.43	2.61±0.65	3.03±0.71	2.16±0.69	2.27±0.85	2.21±0.59
PC ae C38:4	4.94±1.26	4.55±0.84	4.75±0.8	3.73±0.87	4.34±0.94	4.55±1.02
PC ae C38:5	3.46±0.75	3.18±0.44	3.5±0.64	2.6±0.65	2.93±0.56	3.06±0.74
PC ae C38:6	2.92±0.57	2.68±0.36	2.74±0.6	1.93±0.5	2.18±0.43	2.24±0.52
PC ae C40:0	20.2±4.56	16.48±3.16	19.29±3.64	12.46±2.51	13.84±2.85	13.96±2.31
PC ae C40:1	3.34±0.95	2.66±0.69	2.99±0.83	1.71±0.3	2.07±0.56	1.89±0.35
PC ae C40:2	1.22±0.45	1.02±0.22	1.13±0.28	0.77±0.23	0.93±0.35	0.82±0.19
PC ae C40:3	1.58±0.74	1.18±0.28	1.29±0.37	1.18±0.69	1.13±0.52	1.04±0.34
PC ae C40:4	2.6±0.84	2.23±0.38	2.32±0.46	1.55±0.37	1.78±0.49	1.75±0.43
PC ae C40:5	2.55±1.24	1.87±0.42	2.01±0.46	1.55±0.58	1.5±0.45	1.48±0.34
PC ae C40:6	3.31±0.85	2.81±0.37	2.94±0.61	2.2±0.64	2.54±0.51	2.55±0.56
PC ae C42:0	1.39±0.45	1.03±0.34	1.26±0.41	0.57±0.11	0.64±0.17	0.65±0.1
PC ae C42:1	0.85±0.29	0.67±0.19	0.82±0.22	0.48±0.13	0.58±0.25	0.54±0.11
PC ae C42:2	0.8±0.28	0.66±0.18	0.78±0.23	0.43±0.13	0.56±0.25	0.5±0.11
PC ae C42:3	1.17±0.37	0.93±0.22	1.07±0.3	0.56±0.12	0.7±0.22	0.65±0.11
PC ae C42:4	0.59±0.21	0.5±0.1	0.54±0.15	0.34±0.1	0.42±0.15	0.36±0.1
PC ae C42:5	1.08±0.39	0.83±0.12[a]	0.88±0.16	0.82±0.32	0.81±0.22	0.75±0.16
PC ae C44:3	0.23±0.08	0.2±0.04	0.22±0.07	0.14±0.04	0.18±0.07	0.15±0.04
PC ae C44:4	0.24±0.07	0.2±0.04	0.23±0.07	0.14±0.03	0.18±0.07	0.16±0.04
PC ae C44:5	0.25±0.07	0.21±0.04	0.24±0.05	0.15±0.03	0.17±0.05	0.17±0.03
PC ae C44:6	0.25±0.05	0.22±0.03	0.24±0.05	0.17±0.03	0.21±0.06	0.19±0.04
Hydroxy-sphingomyelins						
SM(OH)C14:1	1.19±0.29	0.96±0.24	1.15±0.21	1.4±0.29	1.42±0.39	1.56±0.37
SM(OH)C16:1	0.39±0.07	0.35±0.05	0.41±0.07	0.46±0.1	0.47±0.09	0.52±0.1
SM(OH)C22:1	5.75±1.19	4.72±1.2	5.06±1.13	4.38±0.87	4.76±1.39	4.96±1.03

SM(OH)C22:2	2.87±0.68	2.33±0.4	2.6±0.42	2.21±0.44	2.32±0.67	2.42±0.52
SM(OH)C24:1	0.67±0.15	0.57±0.11	0.64±0.11	0.61±0.11	0.69±0.2	0.68±0.14
Sphingomyelins						
SM C16:0	20.31±4.41	18±2.82	18.84±2.49	22.91±4.57	24.69±4.84	25.88±4.29
SM C16:1	3.69±0.56	3.67±0.43	3.57±0.91	2.52±0.53	2.79±0.46	2.89±0.62
SM C18:0	2.19±0.52	2.25±0.47	2.31±0.54	4.5±1.62	4.99±1.36	5.15±1.23
SM C18:1	0.71±0.17	0.73±0.14	0.74±0.18	1.14±0.36	1.27±0.28	1.31±0.33
SM C20:2	0.29±0.09	0.29±0.09	0.31±0.1	0.2±0.08	0.23±0.06	0.24±0.07
SM C20:3	4.26±1.28	3.43±0.66	4.02±1.13	2.14±0.63	2.51±0.86	2.44±0.6
SM C24:0	12.3±2.46	9.88±1.63	9.91±2.19	7.06±1.47	7.93±2.29	8.22±1.54
SM C24:1	28.41±7.03	21.65±3.78	25.3±5.81	21.67±5.03	24.24±6.92	26.07±4.86
SM C26:0	0.4±0.14	0.34±0.08	0.37±0.11	0.25±0.08	0.3±0.17	0.27±0.09
SM C26:1	0.58±0.23	0.4±0.15	0.5±0.23	0.23±0.05	0.27±0.09	0.26±0.06
Hexoses						
H	10462.08±4387.31	8477.38±5533.04	10054.69±6835.55	8303.18±3210.57	9564±5260.55	8790.28±4042.01

F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers; F:WT;M:eNOS+/-: wildtype offspring of wildtype fathers and eNOS heterozygous mothers; F:eNOS+/-;M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers. PC = phosphatidylcholine; a = acyl; LPC = lysophosphatidylcholine; aa = diacyl; ae = acyl-alkyl; SM = Sphingomyelins; OH = hydroxy. Data are given as mean ± SEM. a: $p<0.05$, F:WT; M:eNOS+/- vs F:WT; M:WT.

Supplementary Table 4. Central carbon metabolites concentration (nmol/mg protein) in liver tissue

Variable	Male offspring			Female offspring		
	F: WT; M: WT (n=21)	F: WT; M: eNOS+/- (n=16)	F: eNOS+/-; M: WT (n=9)	F: WT; M: WT (n=27)	F: WT; M: eNOS+/- (n=17)	F: eNOS+/-; M: WT (n=15)
Ribose 5-phosphate	1.12 ± 0.06	1.25 ± 0.06	1.24±0.09	1.55 ± 0.10	1.56 ± 0.09	1.59±0.07
Glucose 6-phosphate	2.48 ± 0.17	1.58±0.22[a]	2.11±0.23	2.26 ± 0.20	1.61±0.20[a]	1.91±0.27
Fructose 6-phosphate	1.04 ± 0.08	0.67 ± 0.09[a]	0.72±0.08	0.94 ± 0.08	0.67 ± 0.08[a]	0.70±0.09
Phosphoenol-pyruvate	0.62 ± 0.04	0.55 ± 0.04	0.52±0.04	0.61 ± 0.04	0.51 ± 0.05	0.53±0.05
6-Phospho-gluconate	0.50 ± 0.05	0.52 ± 0.05	0.67±0.04	0.60 ± 0.04	0.44 ± 0.06	0.57±0.07
Fructose 1,6-bisphosphate	0.18 ± 0.02	0.12 ± 0.02[a]	0.14±0.02	0.20 ± 0.02	0.12 ± 0.01[a]	0.18±0.02
Seduheptulose 7-phoshate	1.96 ± 0.14	1.78 ± 0.17	2.09±0.33	1.99 ± 0.14	1.91 ± 0.22	2.20±0.28
Fumarate	4.07 ± 0.27	2.52±0.19[a]	2.53±0.16[b]	4.43 ± 0.30	2.66 ± 0.19 [a]	2.29±0.13[b]
Hydroxy-butyrate	1.54 ± 0.12	1.97+0.40	1.66±0.13	2.10 ± 0.16	2.35±0.35	1.68±0.06
Citrate	0.58 ± 0.08	0.56+0.05	0.54±0.04	0.72 ± 0.08	0.66±0.10	0.54±0.04
Malate	9.73 ± 0.55	8.59+0.43	9.92±0.47	9.21 ± 0.51	8.82±0.48	9.21±0.56
2- / 3-Phospho-glycerate	5.21 ± 0.33	4.99 ± 0.42	4.60±0.41	5.02 ± 0.26	5.04 ± 0.48	4.58±0.42
Ribulose 5-phosphate / Xylulose 5-phosphate	5.45 ± 0.72	5.76 ± 0.70	6.80±1.13	6.01 ± 0.68	6.34 ± 0.75	7.93±0.94

F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers; F:WT;M:eNOS+/-: wildtype offspring of wildtype fathers and eNOS heterozygous mothers; F:eNOS+/-;M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers. Data are given as mean ± SEM. a: $p<0.05$, F:WT; M:eNOS+/- vs F:WT; M:WT; b: $p<0.05$, F:eNOS+/-; M:WT vs F:WT; M:WT.

Supplementary Table 5. Correlation analysis between AUC of plasma glucose and insulin, fat content, glycogen, and metabolites in wild type offspring with eNOS+/- mothers.

Variable	Male offspring				Female offspring			
	AUC of plasma glucose	AUC of plasma insulin	Fat content	Glycogen	AUC of plasma glucose	AUC of plasma insulin	Fat Content	Glycogen
Glucose 6-phosphate	0.103	-0.028	-.401*	0.19	-0.098	0.129	0.047	0.393*
Fructose 6-phosphate	0.136	-0.008	0.119	0.111	-0.075	0.13	0.012	0.388*
Fumarate	-0.407*	0.285	-0.246	-0.193	-0.431**	-0.215	-0.338*	0.037
Fructose 1,6-bisphosphate	-0.013	0.073	-0.178	-0.059	-0.048	0.087	-0.069	0.404*
lysoPC a C20:3	0.016	0.036	0.158	-0.242	0.205	0.448*	0.22	0.03
PC aa C36:2	0.039	-0.007	0.318	-0.152	0.143	0.161	0.25	-0.049
PC aa C38:1	-0.177	-0.04	0.242	-0.177	0.218	0.357	0.183	-0.044
PC ae C34:1	0.086	0.01	0.316	-0.163	0.299	0.355	0.218	0.164
PC ae C36:3	0.142	0.004	0.064	-0.284	0.285	0.235	0.206	0.188
PC ae C42:5	-0.128	0.064	0.368*	-0.105	-0.24	-0.146	-0.111	0.001

AUC: area under curve. *: $p<0.05$, **: $p<0.01$.

Supplementary Table 6. Correlation analysis between AUC of plasma glucose and insulin, fat content, glycogen, and metabolites in wild type offspring with eNOS+/- fathers.

Variable	Male offspring				Female offspring			
	AUC of plasma glucose	AUC of plasma insulin	Fat content	Glycogen	AUC of plasma glucose	AUC of plasma insulin	Fat Content	Glycogen
Glucose 6-phosphate	0.239	-0.037	0.085	0.076	0.079	0.224	0.191	-0.12
Fructose 6-phosphate	0.23	-0.172	0.03	-0.098	0.14	0.26	0.068	-0.169
Fumarate	-.374*	-0.118	-0.318	-0.396*	-0.297	-0.332	-0.28	-.426*
Fructose 1,6-bisphosphate	0.033	-0.013	-0.176	-0.09	0.161	0.008	-0.032	0.206
lysoPC a C20:3	0.118	0.335	.394*	0.059	0.149	.460**	-0.052	.375*
PC aa C36:2	0.112	0.166	0.318	-0.165	0.066	0.322	0.089	0.206
PC aa C38:1	-0.218	-0.12	0.113	-.459*	0.072	.404*	0.138	0.135
PC ae C34:1	0.097	0.156	0.216	-0.238	0.22	.409*	0.053	0.252
PC ae C36:3	0.171	-0.028	-0.046	-0.324	0.26	0.291	-0.043	0.144
PC ae C42:5	-0.124	-0.013	0.155	-0.302	-.361	-0.241	-0.195	-0.191

AUC: area under curve. *: $p<0.05$, **: $p<0.01$.

Supplementary Table 7. Complete real time PCR results.

Variable	Male offspring			Female offspring		
	F: WT; M: WT (n=22)	F: WT; M: eNOS+/- (n=9)	F: eNOS+/-; M: WT (n=10)	F: WT; M: WT (n=25)	F: WT; M: eNOS+/- (n=13)	F: eNOS+/-; M: WT (n=10)
NO-synthase expression						
eNOS (Nos3)	1.00 ± 0.07	1.10 ± 0.14	1.06 ± 0.07	1.00 ± 0.11	0.83 ± 0.12	1.00 ± 0.16
iNOS (Nos2)	1.00 ± 0.19	0.84 ± 0.13	0.84 ± 0.11	1.00 ± 0.19	1.57 ± 0.75	0.95 ± 0.24
Genes involved in regulation of metabolic process, energy homeostasis and fat storage						
Chrebp (Mlxipl)	1.00 ± 0.07	0.95 ± 0.09	1.32±0.05[b]	1.00 ± 0.08	0.89 ± 0.08	1.03±0.16[c]
Srebf1c	1.00 ± 0.21	0.98 ± 0.15	0.91±0.12	1.00 ± 0.11	0.49 ± 0.05[a]	0.86±0.17[c]
PPARα	1.00 ± 0.07	0.79 ± 0.08	1.13±0.10[c]	1.00 ± 0.08	0.96 ± 0.13	1.47±0.19[bc]
PPARγ	1.00 ± 0.10	0.56 ± 0.05[a]	1.09±0.11	1.00 ± 0.10	1.04 ± 0.14	1.52±0.20[bc]
PPARγ Co1α	1+0.29	0.46+0.11	1.00+0.25	1+0.17	1.15+0.2	1.32+0.19
GR (Nr3c1)	1.00 ± 0.10	1.41± 0.19	1.82±0.16[b]	1.00 ± 0.13	0.68 ± 0.06	1.40±0.30[c]
Tfam	1.00 ± 0.08	0.99 ± 0.14	1.57±0.05[bc]	1.00 ± 0.08	1.20 ± 0.16	1.12±0.11
Tfb1m	1.00 ± 0.05	1.06 ± 0.14	1.00±0.05	1.00 ± 0.05	1.03 ± 0.10	0.87±0.04
Tfb2m	1.00 ± 0.09	1.02 ± 0.17	1.92±0.06[bc]	1.00 ± 0.12	1.16 ± 0.16	1.38±0.22
Nrf1	1.00 ± 0.05	1.15 ± 0.15	1.20±0.05	1.00 ± 0.04	1.00 ± 0.07	1.04±0.08
Nampt	1.00 ± 0.12	1.16 ± 0.21	1.68±0.22[bc]	1.00 ± 0.11	0.84 ± 0.12	1.00±0.12
AMPK (Prkaa2)	1.00 ± 0.15	1.32 ± 0.28	1.16±0.15	1.00 ± 0.10	0.73 ± 0.08	1.30±0.23[c]
Fbp1	1.00 ± 0.10	1.32 ± 0.15	1.24±0.17	1.00 ± 0.11	0.86 ± 0.08	1.14±0.17
Gck	1.00 ± 0.10	1.35 ± 0.39	0.99±0.18	1.00 ± 0.16	0.57 ± 0.08[a]	1.11±0.16[c]
Pepck (Pck1)	1.00 ± 0.07	1.41 ± 0.22[a]	1.41±0.29[b]	1.00 ± 0.11	0.72 ± 0.05	1.1±0.27
PK-L (Pklr)	1.00+0.1	0.98+0.08	0.94+0.08	1.00+0.06	1.18+0.09	1.13+0.20
G6Pase (G6pc)	1.00 ± 0.15	1.07± 0.15	1.54±0.11[b]	1.00 ± 0.17	1.21 ± 0.19	1.14±0.16
Glut2 (Slc2a2)	1.00 ± 0.09	1.22 ± 0.17	2.07±0.13[bc]	1.00 ± 0.13	0.77 ± 0.14	1.38±0.20[c]
FAS (Fasn)	1.00 ± 0.27	0.60± 0.09	0.77±0.11	1.00 ± 0.08	1.00 ± 0.17	1.62±0.33[bc]
Acc1 (Acaca)	1+0.12	0.83+0.11	1.68+0.15	1+0.09	1.03±0.13	1.27+0.20
Cpt1a	1.00 ± 0.12	1.10 ± 0.15	0.95±0.1	1.00 ± 0.12	0.86 ± 0.10	1.30±0.18[c]
HSL (Lipe)	1.00 ± 0.10	0.92 ± 0.11	0.90±0.10	1.00 ± 0.12	0.87 ± 0.16	1.15±0.17
Fitm1	1+0.07	1.02+0.12	0.93+0.06	1+0.12	1.68+0.24[a]	1.23+0.15
Fitm2	1+0.07	1.02+0.13	0.93+0.06	1+0.06	1.03+0.11	1.03+0.09
Insulin-like growth factors and binding proteins						
Igf1	1.00 ± 0.13	1.44 ± 0.17[a]	1.48±0.12[b]	1.00 ± 0.13	0.76 ± 0.09	1.08±0.17
Igf2	1.00 ± 0.08	1.75 ± 0.25[a]	1.51±0.25[b]	1.00 ± 0.14	0.83 ± 0.19	1.34±0.39
Igfbp1	1.00 ± 0.35	2.55 ± 0.58[a]	2.00±0.4[b]	1.00 ± 0.37	1.93 ± 0.66	1.61±0.76
Igfbp2	1.00 ± 0.16	2.16 ± 0.32[a]	1.47±0.23[b]	1.00 ± 0.09	1.02 ± 0.10	1.05±0.09
Igfbp3	1.00 ± 0.19	1.15 ± 0.15	0.81±0.06	1.00 ± 0.14	0.94 ± 0.07	0.90±0.11

F:WT; M:WT: wildtype offspring of wildtype fathers and wildtype mothers; F:WT; M:eNOS+/-: wildtype offspring of wildtype fathers and eNOS heterozygous mothers; F:eNOS+/-;M:WT: wildtype offspring of eNOS heterozygous fathers and wildtype mothers. Data are given as mean ± SEM. a: $p<0.05$, F:WT; M:eNOS+/- vs F:WT; M:WT; b: $p<0.05$, F:eNOS+/-; M:WT vs F:WT; M:WT; c: $p<0.05$, F:eNOS+/-; M:WT vs F:WT; M:eNOS+/-.

Supplementary Table 8. Correlation analysis between AUC of plasma glucose and insulin, fat content, glycogen, fumarate, and altered genes in wild type offspring with eNOS+/- mothers.

Variable	Male offspring					Female offspring				
	AUC of plasma glucose	AUC of plasma insulin	Fat content	Glycogen	Fumarate	AUC of plasma glucose	AUC of plasma insulin	Fat Content	Glycogen	Fumarate
Srebf1c	0.116	0.172	0.171	.684**	0.041	0.131	0.018	-0.308	0.375	0.407
PPARγ	0.029	0.026	.511**	-0.15	.371*	.403**	.447*	0.065	-0.171	-0.018
Gck	-0.264	0.064	-0.313	0.211	-0.203	-0.222	-0.319	-0.351	-0.161	0.032
Pepck (Pck1)	-0.036	0.288	-0.112	-0.079	-0.058	-0.078	-0.377	-0.099	0.079	-0.006
Fitm1	0.13	0.173	.443**	0.027	0.006	0.238	0.226	.508**	0.247	-0.264
Igf1	-0.057	0.396	-.555*	0.45	-0.383	-0.132	-0.269	-0.253	-0.186	0.027
Igf2	0.028	0.333	-0.319	0.442	0.089	-0.209	-0.425	-0.348	0.06	-0.014
Igfbp1	-0.257	0.114	-0.464	0.027	-0.022	0.19	-0.038	0.067	0.092	-0.103
Igfbp2	0.01	0.067	-.592*	0.123	-0.278	-0.269	-.681**	-0.202	-0.343	-0.153

AUC: area under curve. *: $p<0.05$, **: $p<0.01$.

Supplementary Table 9. Correlation analysis between AUC of plasma glucose and insulin, fat content, glycogen, fumarate, and altered genes in wild type offspring with eNOS+/- fathers.

Variable	Male offspring					Female offspring				
	AUC of plasma glucose	AUC of plasma insulin	Fat content	Glycogen	Fumarate	AUC of plasma glucose	AUC of plasma insulin	Fat Content	Glycogen	Fumarate
Chrebp (Mlxipl)	-0.259	0.201	0.225	.487*	-0.116	0.28	.357*	0.162	0.008	0.113
PPARα	-.382*	-0.097	0.123	-0.055	0.045	.351*	.411*	0.12	-0.045	-0.236
PPARγ	0.265	.723**	.589**	0.198	0.027	.358*	.423*	0.158	-0.011	-.306*
GR (Nr3c1)	-0.089	0.201	-0.187	0.481*	-0.469*	-0.096	0.25	0.084	-0.194	-0.236
Tfam	0.11	0.257	0.347	.469*	-0.281	0.189	0.207	0.279	0.132	0.073
Tfb2m	0.107	0.465*	0.364	.611**	-0.277	0.222	0.265	0.252	0.021	0.032
Nampt	0.014	0.251	0.058	0.371	-0.155	0.193	-0.003	0.032	-0.041	0.21
Pepck (Pck1)	-0.256	-0.157	-0.29	0.162	-0.258	0.163	0.192	-0.227	0.096	-0.139
G6Pase (G6pc)	-0.282	0.348	0.168	.388*	0.137	-0.256	-0.213	-0.127	0.34	0.059
Glut2 (Slc2a2)	0.124	0.126	0.129	.595*	-.701**	-0.248	0.085	0.332	-0.391	-0.204
FAS (Fasn)	0.307	0.179	0.146	0.316	0.1	-0.009	0.016	.556*	-0.123	-0.289
Igf1	0.014	0.345	-0.039	0.053	-0.319	-0.211	-0.214	-0.172	-0.312	-0.159
Igf2	0.055	0.21	-0.425	.600*	-0.147	0.001	-0.178	-0.336	-0.241	-0.208
Igfbp1	-0.05	0.293	-0.245	0.097	0.125	0.022	0.183	-0.443	0.195	0.025
Igfbp2	0.168	-0.1	-0.144	-0.184	-0.073	-0.224	-0.332	-0.428	-0.209	-0.106

AUC: area under curve. *: $p<0.05$, **: $p<0.01$.

5 Summary

The field of paternal programming offers intriguing prospects and implications across diverse areas of research and practical applications. It sheds light on the multigenerational health effects stemming from a father's experiences, exposures, and epigenetic changes, impacting not just immediate offspring (F1 generation) but also subsequent generations (F2, F3, etc.). This research deepens our understanding of epigenetic mechanisms underpinning the transmission of acquired traits, with potential implications in genetics, epigenetics, and developmental biology. Moreover, it highlights how environmental factors experienced by males can affect the health and development of their offspring, informing public health policies and lifestyle recommendations. While rodent models and human epidemiology have provided extensive evidence of paternal programming effects on offspring health, questions regarding the underlying mechanisms, the magnitude and persistence of these effects, and how to prevent adverse outcomes in offspring through intervention measures have remained areas of exploration. Our research team has been dedicated to investigating the mechanisms of paternal programming on offspring disease.

In developing as well as in developed countries, some men eat often a high-fat, high-sucrose and high-salt diet (fast food such as burgers etc.). Whether this is potentially harmful with regard to kidney function not just for themselves but also for their offspring is unknown. We thus analyzed in an animal model the hypothesis whether a paternal pre-conceptional unhealthy diet - high-fat, high-sucrose and high-salt diet - covering the period of spermatogenesis given to male rats for two generations might cause the incidence of chronic kidney disease in the F1 and/or F2 generation. We have chosen a high-fat, high-sucrose, and high-salt diet to mimic a fast food commonly consumed by men in reproductive age. We screened all offspring for the predefined primary outcome: decreased GFR in combination with increased urinary albumin excretion. Offspring fulfilling these criteria were then characterized in detail. The strongest effect was seen in female F2 offspring. They got a decrease in GFR combined with increased albumin excretion. These rats also develop glomerulosclerosis and interstitial fibrosis. The underlying epigenetic mechanisms were characterized by RNA sequencing followed by qPCR, DNA methylation analysis and system biology approaches to figure out the underlying mechanism. To the best of our knowledge, our study first established a paternal diet induced CKD rat model.

Subsequently, we proposed an advanced fetal programming hypothesis (shown in Figure 2 in the Introduction section). To investigate this hypothesis, we employed a methodology similar to our prior study. We crossbred male mice with a heterozygous eNOS genotype with female wild-type (wt) mice. We then compared the phenotypes of their wt offspring with those of offspring whose parents were wild type. The male mice lacking proper NO production exhibited reduced global DNA methylation in their sperm and experienced intricate alterations in non-coding miRNAs within their sperm. Male offspring born to fathers with an eNOS+/− genotype displayed elevated fasting insulin levels, increased insulin responses following a glucose challenge, and higher liver glycogen content. Since no significant alterations were observed in the endocrine pancreas, we directed our attention toward the liver for further analysis. We identified 19 genes with differential expression in male offspring born to fathers with eNOS+/− genotype. Among these genes, Pgc1a and Gr exhibited the most statistically significant differences, with the lowest p-values and false discovery rates (FDR). Interestingly, there were no significant differences in gene expression among female offspring. Furthermore, we observed a reduction in DNA methylation in the Pgc1a promoter and Gr exon 1A in male wt offspring born to fathers with an eNOS+/− genotype. Our data provide compelling evidence that a heterozygous eNOS deficiency in male mice can lead to an unfavorable testicular environment, which, in turn, impacts the epigenetic profile of sperm. These early epigenetic modifications in sperm have the potential to trigger enduring changes in epigenetic patterns, subsequently leading to alterations in the phenotypes of target organs in the offspring.

Based on our previous research, we delved into a detailed metabolomics study to assess the effects of maternal and paternal eNOS deficiencies on genetically healthy offspring. We found that hepatic fat accumulation in female wt offspring of eNOS+/- mothers was associated with increased expression of liver Fitm1. Moreover, we observed that in male offspring of eNOS-deficient fathers, elevated Tfb2m gene expression significantly correlated with increased insulin levels post-glucose load. Intriguingly, we identified seven genes in these male offspring that were significantly associated with enhanced liver glycogen levels. Notably, our study uncovered a connection between altered gene expression (GR and Glut2), a change in the metabolite fumarate, and the phenotypic shift of increased liver glycogen in male offspring of eNOS-deficient fathers. These discoveries not only enhance but also provide fresh evidence supporting our earlier findings. They offer a more detailed perspective on how parental eNOS gene anomalies can influence the

phenotypes of offspring, illustrating impacts that occur even in the absence of direct genetic inheritance of the gene defect.

In summary, our groundbreaking research presents a wealth of robust evidence supporting the concept of fetal programming, shedding new light on the profound influence of fathers' early-life conditions on the enduring health outcomes of their progeny. This multifaceted investigation delves into the intricate and intriguing interactions between genetics, epigenetics, and the environment, underpinning the complexity of how paternal factors can shape the health trajectories of future generations. Moreover, our findings not only contribute to the theoretical underpinnings of developmental and intergenerational health, but they also pave the way for the exploration of practical intervention measures. Understanding the mechanisms of paternal programming enables us to consider targeted strategies for ameliorating adverse health outcomes in offspring. By identifying specific points of intervention, we can develop evidence-based approaches to enhance the health and well-being of future generations. These insights hold promise for public health policies, medical interventions, and lifestyle modifications aimed at breaking the cycle of adverse health outcomes.

6 Outlook

The groundbreaking research highlighted in this study marks a pivotal shift in our understanding of paternal contributions to offspring health, underscoring a critical area of exploration in the realms of genetics and epigenetics. This work not only illuminates the often-underappreciated role of fathers in shaping the health trajectories of their children, but also reveals the intricate ways in which specific paternal gene defect can influence offspring phenotypes, despite the offspring's genotype is healthy.

This research lays a promising foundation for numerous future endeavors. It heralds an era of precision medicine, where personalized medical strategies can be developed based on the specific genetic and epigenetic influences of both parents, thereby enhancing treatment effectiveness, and minimizing side effects. It also paves the way for advanced genetic research, especially in understanding paternal influence on offspring health, delving into how paternal genes and lifestyle choices contribute to epigenetic changes. Public health initiatives stand to gain significantly from these insights, particularly in highlighting the importance of paternal health before conception and its lasting impact on children. There is a crucial opportunity for educational initiatives targeting potential and expecting fathers, raising awareness about the importance of good health practices, including nutrition, lifestyle, and environmental factors. Furthermore, our discovery forms a crucial base for refining genetic counseling practices and implementing more targeted genetic screening protocols. Specifically, these findings suggest that even when offspring are genetically healthy, they may still exhibit certain phenotypic characteristics or susceptibilities inherited from parents with specific genetic defects. This nuanced understanding has profound implications for families with a history of genetic disorders, as it underscores the importance of considering both maternal and paternal genetic backgrounds in assessing the risk and potential health outcomes for children. Genetic counseling can thus be tailored more accurately, considering the intricate ways parental genetics may affect offspring beyond direct inheritance of a specific gene mutation.

In the context of clinical applications, our study holds particular significance for monogenic inherited diseases such as thalassemia. Thalassemia, a condition often passed down through families, is characterized by the body making an abnormal form of hemoglobin, resulting in anemia. Understanding the role of parental genetic make-up in such diseases could lead to novel approaches in treatment and management. For instance, it may allow for the identification of at-

risk offspring earlier and more accurately, leading to timely interventions that could mitigate the disease's impact or even prevent the onset of more severe symptoms.

In essence, this study not only enriches our understanding of paternal influences on offspring health but also acts as a catalyst for future research and interventions aimed at enhancing the health and well-being of future generations.

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9 List of publications during doctoral study

1. **Zhang X,** Hasan A A, Wu H, Gaballa M M S, Zeng S, Liu L, et al. High-fat, sucrose and salt-rich diet during rat spermatogenesis leads to the development of chronic kidney disease in the female offspring of the F2 generation. FASEB J (2022) 36: e22259. doi: 10.1096/fj.202101789RR.

2. Hocher B, Lu Y P, Reichetzeder C, **Zhang X**, Tsuprykov O, Rahnenfuhrer J, et al. Paternal eNOS deficiency in mice affects glucose homeostasis and liver glycogen in male offspring without inheritance of eNOS deficiency itself. Diabetologia (2022) 65: 1222-1236. doi: 10.1007/s00125-022-05700-x.

3. **Xiaoli Zhang**, Christoph Reichetzeder, Yvonne Liu et.al. Parental sex-dependent effects of either maternal or paternal eNOS deficiency on the offspring's phenotype without transmission of the parental eNOS deficiency to the offspring. Frontiers in Physiology (2023) 14:1306178. doi: 10.3389/fphys.2023.1306178.

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9. Yvonne Liu, Johann-Georg Hocher, Huijun Chen, Liang Hu, **Xiaoli Zhang**, et al. The Degree of Prepregnancy Vitamin D Deficiency Is Not Associated with Gestational Diabetes in Women Undergoing ART. J Endocr Soc (2023) 7: bvad140. doi: 10.1210/jendso/bvad140.

10 CV of the author

For reasons of data protection, the curriculum vitae is not included in the online version.

11 Abbreviations

DOHaD	Developmental Origins of Health and Disease
ROS	reactive oxygen species
BMI	body mass index
T2DM	type 2 diabetes
IUGR	intrauterine growth restriction
CpG	cytosine-guanine
ncRNAs	non-coding RNAs
mRNA	messenger RNA
miRNA	microRNA
lncRNA	long non-coding RNAs
piRNA	PIWI-interacting RNA
circRNA	circular RNA
IGF-1	insulin-like growth factor 1
CD	control diet
CKD	chronic kidney disease
HFSSD	high-fat, high-sucrose and high-salt diet
UACR	urinary-albumin-to-creatinine ratio
GFR	glomerular filtration rate
ACTR3B	Actin Related Protein 3B gene
CD300LF	CD300 Molecule Like Family Member F gene
ENPP6	Ectonucleotide Pyrophosphatase/Phosphodiesterase 6 gene
TMEM144	Transmembrane Protein 144 gene
FDR	false discovery rate
NO	nitric oxide
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthetase

NOS3	Nitric Oxide Synthase 3
WT	wild type
eNOS+/-	heterozygous eNOS
IPGTT	intraperitoneal glucose tolerance test
L-NAME	N(γ)-nitro-L-arginine methyl ester
MeDIP	methylated genomic DNA immunoprecipitation
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
GR	glucocorticoid receptor
Fitm1	Fat Storage Inducing Transmembrane Protein 1
Tfb2m	mitochondrial transcription factor B2
Glut2	glucose transporter 2