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Review

Developmental effects of *in utero* metformin exposure

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ABSTRACT

According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, the intrauterine environment influences fetal programming and development, affecting offspring disease susceptibility in adulthood. In recent years, therapeutic use of the Type 2 diabetes drug metformin has expanded to the treatment of pre-diabetes, polycystic ovarian syndrome, and gestational diabetes. Because metformin both undergoes renal excretion and binds to receptors on the placenta, the fetus receives equivalent maternal dosing. Although no teratogenic nor short-term harmful fetal impact of metformin is known to occur, the effects of metformin exposure on longer-range offspring development have not yet been fully elucidated. This review encapsulates the (albeit limited) existing knowledge regarding the potential longer-term impact of intrauterine metformin exposure on the development of key organs including the liver, central nervous system, heart, gut, and endocrine pancreas in animal models and humans. We discuss molecular and cellular mechanisms that would be altered in response to treatment and describe the potential consequences of these developmental changes on postnatal health. Further studies regarding the influence of metformin exposure on fetal programming and adult metabolic health will provide necessary insight to its long-term risks, benefits, and limitations in order to guide decisions for use of metformin during pregnancy.

KEYWORDS: metformin, pregnancy, gestational diabetes, preeclampsia, polycystic ovarian syndrome, fetal growth restriction.

INTRODUCTION

Inspired by epidemiological findings in England and Wales that demonstrated a correlation between living in poorer areas and increased prevalence of ischemic heart disease [1], Dr. David Barker and colleagues analyzed health outcomes in the offspring of pregnant women who experienced the Dutch Hunger Winter of November 1944 through April 1945. The findings of these studies revealed that children exposed to undernutrition *in utero* were prone to the development of cardiometabolic disease and impaired glucose tolerance in adulthood, while offspring born to the same parents outside of the period of famine did not show these tendencies [2-4]. Thus, the Developmental Origins of Health and Disease (DOHaD) hypothesis was born, which postulates that the intrauterine environment alters fetal developmental programming in a way that influences susceptibility to metabolic disease in adulthood [5].

More recently, the increased incidence of obesity in women of childbearing age has spurred research that seeks to understand the influence of maternal

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overnutrition on the metabolic health of offspring. A meta-analysis of 23 published studies examining a combined total of over 1.3 million women across multiple ethnicities revealed that women in the United States and Europe have higher pre-pregnancy body mass index (BMI) and gain more weight during pregnancy than women of Asian descent, leading to an increased number of offspring that are large for gestational age in these populations [6]. Likewise, a study from Portugal revealed that offspring exposed to maternal overnutrition and obesity have increased BMI from infancy to two years of age [7]. Children with obesity are more likely to be obese as adults, which is associated with impaired glucose tolerance and increased mortality [8-10].

In non-human primate (NHP) offspring, a maternal calorically dense, high fat or Western style diet (WSD) results in reduced oxidative capacity in fetal skeletal muscle and impaired insulin-induced glucose uptake in the skeletal muscle of juvenile offspring, even in the absence of maternal obesity [11, 12]. In this same model, maternal WSD also alters the ratio of insulin-producing beta cells to glucagon-producing alpha cells in pancreatic islets, with a decrease in alpha cell number leading to an increased proportion of beta cells. Islets from NHP offspring exposed to overnutrition during development also show an over-secretion of insulin in response to a rise in glucose [13]. Of note, NHP offspring exposed to maternal WSD during development demonstrate increased reactive and ritualized anxiety, demonstrating an effect of maternal diet on offspring behavior in addition to physiology [14]. At a mechanistic level, we have shown that this largely occurs in humans and primates *via* two overarching means: 1) altered offspring gene expression driven by epigenetic changes to the early life "histone code" [15-21] and 2) alterations in the developing microbiome [22-24]. Taken together, these studies demonstrate that maternal WSD consumption, in both the absence and presence of obesity, impacts the development of various organ systems in offspring. The sum of the changes in fetal development predisposes offspring to metabolic disease and potentially to mental health issues later in life. Thus, lifestyle changes and treatments that reduce maternal obesity and overnutrition are predicted to have great impact on subsequent generations [25, 26].

In this review, we discuss what is known about metformin—a commonly used therapeutic for the treatment of Type 2 diabetes (T2D) whose use has recently expanded to include treatment of pre-diabetes, gestational diabetes mellitus (GDM), and polycystic ovarian syndrome (PCOS)—and its effects on fetal development and postnatal physiology. Additionally, we will explore how the mechanisms of action of metformin might influence the development of the fetal liver, brain, heart, gut (microbiome) and pancreas. Finally, we review clinical outcomes from studies of women who received metformin in pregnancy and their offspring, which suggest that the cellular processes by which maternal metformin acts may be disruptive to normal fetal growth and may ultimately result in childhood metabolic disease.

Metformin use in pregnancy

Women who are overweight or obese are prone to diseases associated with metabolic dysfunction, including PCOS, pre-diabetes, T2D, and GDM [27, 28]. These conditions are associated with impairments in insulin secretion and glucose homeostasis, introducing concerns regarding exposure of the fetus to hyperglycemia during pregnancy. Offspring of women with poorly managed GDM have increased adiposity, are large for gestational age, and have increased cardiometabolic risk [29, 30]. Long-term studies reveal that glycemic disorders in pregnant women predispose offspring to insulin resistance and T2D. These studies substantiate the notion that poor management of the maternal metabolic environment during pregnancy programs poor metabolic compensation in offspring later in life. Thus, it is imperative that maternal dysglycemia be appropriately managed and treated for the benefit of both the mother and her offspring.

Approved in 1994 by the Food and Drug Administration (FDA), metformin is the frontline therapeutic for T2D due to its low cost, oral delivery, and efficiency in reducing hyperglycemia [31]. Usual dosing of metformin starts at 500 mg nightly for one week, and increases to 500 mg twice daily up to a maximum dose of 2000 mg per day [32]. After

being absorbed in the jejunum *via* organic cation transporters, metformin targets the liver, where it reduces gluconeogenesis and glycogenolysis, thus helping to lower blood glucose. Unlike most other medications, metformin is excreted by the kidneys without first pass hepatic metabolism [33, 34]. Metformin also acts on adipose tissue and skeletal muscle to improve glucose uptake and insulin sensitivity by increasing translocation of the glucose transporter, GLUT4 to the plasma membrane [35, 36]. Well-tolerated by most people, metformin has been deemed a "miracle drug" for its anti-hyperglycemic and anti-obesity effects. However, 30-40% of patients experience clinically significant gastrointestinal (GI) side effects, including diarrhea, vomiting and bloating, and elevations of hepatic transaminases. These common GI side effects are due to two mechanisms: first, the high turnover of glucose and bile acids in the intestine as metformin accumulates; and second, the serious albeit rare side effect of lactic acidosis due to increased anaerobic metabolism of glucose secondary to increased glucose uptake [37]. Forslund *et al.* (2015) demonstrated that metformin-driven gut dysbiosis resulted from aberrant microbial-mediated fatty acid, butyrate, and tryptophan degradation [38].

Given its success in effectively treating hyperglycemia, therapeutic use of metformin has been expanded for the treatment of GDM, pre-gestational diabetes, PCOS, weight management, cancer, and infertility in patients undergoing *in vitro* fertilization (Figure 1) [39-41]. Metformin has also been trialed in the context of maternal obesity and pre-eclampsia to improve maternal and fetal outcomes [32, 42]. Use of metformin in the management of GDM occurs despite the lack of approval by the FDA for this clinical indication [43]. In light of this increased use among women who are pregnant, it is worthwhile recalling that metformin readily crosses the placental barrier through organic cation transporters, resulting in the developing fetus experiencing the full dose of metformin as administered to mothers during pregnancy [44]. There is also documented potential for increased bioavailability of metformin during pregnancy [45]. However, it is important to note that congenital anomalies associated with first trimester metformin treatment are rare [46].

Due to low rates of aerobic as compared to aerobic metabolism during early pregnancy, the embryo has few, immature mitochondria [47]. As such, in the first trimester, the embryo expresses very low levels of organic cation transporters which are responsible for transporting metformin into cells [47]. For this reason, metformin is likely safe in the first trimester. However, in the second and third trimesters, the placenta and fetus both express metformin transporters, exhibit high rates of aerobic metabolism, and are dependent on the activity of mature mitochondria [47]. Given metformin's inhibitory effects on the mitochondrial pathways, there is concern that metformin could adversely affect the function, growth, or differentiation of fetal or placental tissues into which metformin is transported [48]. These properties raise the possibility that metformin might induce fetal programming that may be maladaptive to metabolic health later in adulthood. Characterization of the cellular processes by which metformin acts on the developing fetus and placenta during pregnancy is crucial to understanding how this medication has the potential to induce developmental programming of metabolic disease in offspring and will be discussed in subsequent sections.

Mechanisms of metformin action

Intracellularly, metformin is thought to act mainly by altering the redox state of the cytoplasm to activate 5' adenosine monophosphate activated protein kinase (AMPK), a nutrient-sensing kinase that regulates cellular growth and metabolism. AMPK activation occurs *via* direct inhibition of Complex I of the mitochondrial electron transport chain, raising the intracellular AMP:ATP ratio while also increasing the presence of reactive oxygen species (ROS) [49]. Additionally, some studies suggest that AMPK is activated *via* direct phosphorylation by intracellular kinases that respond to the cellular redox status such as liver kinase B (LKB/STK11) and ATM *via* an indirect mechanism [50]. Interference with mitochondrial respiration increases glycolytic flux, resulting in the buildup of lactate, which accounts for the increased incidence of lactic acidosis observed in patients prescribed metformin [51, 52]. A study that analyzed effects of *in vitro*

Figure 1

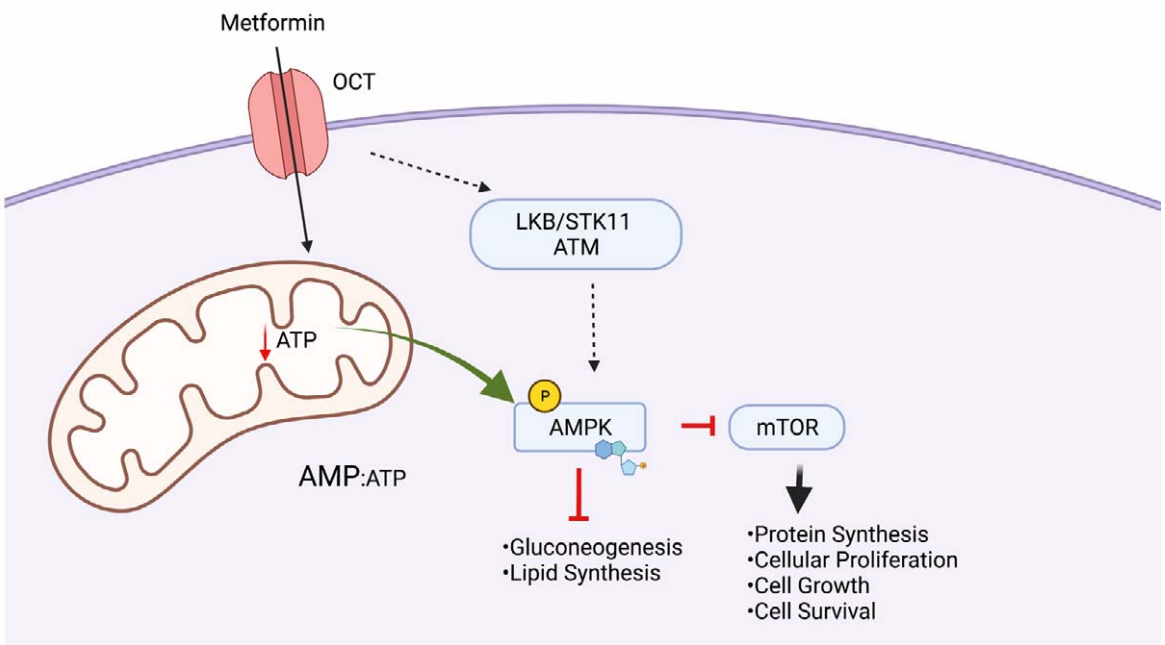

Figure 2

treatment of embryonic day (e) 8.5 mouse embryos with metformin for 6, 12, and 24 hours found increased glucose uptake and glycolysis in response as well as high lactate, indicating that metformin also induces high levels of lactate in embryos [53].

Another outcome of AMPK activation is the inhibition of protein synthesis regulated by the mammalian target of rapamycin (mTOR) complex, which is critical for fetal growth (Figure 2). The mTOR signaling pathway is a major nutrient-sensing mechanism in the placenta [47]. Consequently, by inhibiting the mTOR pathway, metformin could restrict nutrients like glucose and amino acids from reaching the feto-placental unit [54, 55]. In a study of late gestation mouse embryos (e18.5), offspring of dams that consumed a high fat diet (HFD) containing 60% kcal from fat and treated with a metformin *via* the drinking water unexpectedly weighed less than offspring of dams that consumed a control diet while treated with the same dose of metformin. Placental evaluation revealed a decrease in phosphorylated S6, a target of mTORC1 signaling [56], suggesting that metformin exposure, coupled with maternal HFD consumption, has inhibitory effects on the mTOR pathway. Nutrient restriction during fetal development has been previously established as a progenitor of adult diseases like T2D, obesity, and cardiovascular disease [57]. Thus, the effects of metformin on AMPK and the mTOR pathway and subsequent nutrient restriction could conceivably result in adult metabolic disease in offspring exposed to metformin *in utero*.

Overall impact of developmental metformin exposure

While initiation of pharmacological treatment of GDM is important to prevent neonatal complications like macrosomia, it is important to consider the underlying molecular mechanisms driving the fetal growth effects of hyperglycemia and the potential long-term cost of specific treatments. Long-term studies of children that were prenatally exposed to metformin reveal that as neonates they have lower birthweight and are small for gestational age but have increased BMI at 18 months of age and in early childhood due to catch-up growth [58-61]. Low birthweight is hypothesized to predispose offspring to glucose intolerance and insulin resistance in adulthood according to the thrifty phenotype hypothesis [62]. The MiTY study has demonstrated definitive concern for maternal metformin use resulting in low birthweight with disrupted post-natal growth velocity [63]. Thus, what could be interpreted as a metformin-driven "favorable" birthweight reduction in association with improved maternal glycemic control could instead be partly attributable to detrimental growth inhibition of the feto-placental unit by metformin with unknown long-term consequences [47].

Metformin has also been shown to lead to deficiencies in maternal vitamin B_{12} and folate, both of which are critical for regulating one-carbon metabolism for nucleotide, lipid, and amino acid synthesis [64, 65]. In development, folate and B_{12} are important for the prevention of neural tube defects *via* regulation of DNA methylation. Vitamin B_{12} deficiency during fetal development has been suggested to result in insulin resistance and increased adiposity in children [66]. Thus, *in utero* metformin exposure could alter growth *via* both metabolic and epigenetic mechanisms in both fetal and childhood development. Additionally, metformin inhibits the production of tricarboxylic acid (TCA)-derived metabolites needed for biosynthesis and growth while also impeding thiamine transport [67-69]. As such, the aforementioned anti-growth properties of metformin make this drug a good anti-cancer therapeutic; it

Legend to Figure 1. Reasons for metformin use in pregnancy. Metformin is increasingly prescribed beyond the treatment of Type 2 diabetes and gestational diabetes. Created with Biorender.com

Legend to Figure 2. Intracellular actions of metformin. Metformin enters the cell *via* organic cation transporters (OCT). Following entry, the cellular redox status results in phosphorylation of AMPK by intracellular kinases LKB/ATM/ STK11. Reduced ATP production by metformin-mediated Complex I inhibition in mitochondria also results in activation of AMPK *via* increased AMP:ATP ration. Activation of AMPK reduces anabolism in cells *via* downstream targets, ultimately inhibiting cell growth. Created with Biorender.com

has been evaluated in breast cancer, hepatocellular carcinoma, and gastric cancer [70-72]. However, these same anti-growth properties may not be beneficial in the context of the rapid growth that occurs during embryogenesis.

Given the antiproliferative and mitochondrial suppressive effects of metformin described above, some have postulated that metformin may be used to prevent or lessen the severity of pre-eclampsia, a hypertensive disease of pregnancy. While the pathophysiology of pre-eclampsia is still not well understood, metformin could prevent pre-eclampsia through its effects on cell metabolism, the anti-angiogenic state, and other processes associated with pre-eclampsia [73]. Cluver *et al*. (2021) performed a randomized control trial in 180 women who were pregnant (gravidae) with pre-term pre-eclampsia and found that the metformin-exposed cohort experienced a non-significant 7.6 day pregnancy prolongation compared to the placebo cohort [74]. Unfortunately, this study is likely underpowered for secondary analyses aimed at measuring the potential for longer-term harm in the offspring [63, 75]. This would have been a key outcome in this trial, given that any condition associated with placental insufficiency is a relative contraindication for its use due to concerns of lactic acidosis in the setting of the decreased placental perfusion which accompanies placental insufficiency [75]. As noted above, the MiTY trial noted a 2-fold increased risk of being born small for gestational age in the metformin arm of this T2D trial [63]. Before moving forward with prescribing metformin for any theoretical effects it may have on the treatment of pre-eclampsia, it is crucial to collect long-term safety data and fully explore any adverse childhood and/or adulthood metabolic effects associated with the drug.

In summary, metformin demonstrates some degree of effectiveness among gravidae in the management of glucose tolerance and possibly pre-eclampsia. However, these benefits may be accompanied by risk of longer-term harm to the metabolic health of the offspring as a result of fetal exposure to maternal metformin concentrations effectively restricting relative fetal growth and modifying fetal programming to predispose offspring to maladaptive effects later in life. Whether there is further additive or synergistic effects of a calorically-dense maternal or post-weaning diet remains unexplored, but is a key area of active investigation.

Effects of metformin on pancreas development and function

Detailed reviews of pancreas development can be found in [25, 76]. In brief, the pancreas forms as an evagination of the posterior foregut endoderm epithelium. Glucose homeostasis is primarily driven by the secretion of the endocrine hormones glucagon and insulin by the alpha and beta cells of the pancreatic islets of Langerhans, respectively, which make up approximately 2% of the total adult pancreas volume. The remaining 98% of the adult pancreas is comprised of exocrine tissue (acinar and ductal cells) that release and transport digestive enzymes. Both endocrine and exocrine cells are derived from the endodermal epithelium. Insulin, produced by pancreatic beta cells, encourages the uptake and utilization of glucose in peripheral tissues, while glucagon produced by alpha cells stimulates liver glycogenolysis and gluconeogenesis to increase blood glucose levels during times of fasting. New alpha and beta cells form from neogenesis from endocrine progenitors within the ductal epithelium during development. Just prior to birth in the mouse, neogenesis decreases and further increases in alpha and beta cell mass are due mainly to proliferation continuing into the postnatal period. Beta cell proliferation slows as individuals reach young adulthood and is very low in adult humans. However, beta cell proliferation can increase in response to proliferative stimuli such as weight gain and pregnancy. The number of beta cells at birth and the effectiveness of postnatal beta cell mass expansion contribute to the overall size of the beta cell pool in adulthood. Perturbations to the beta cell differentiation program by the intrauterine and postnatal environments could alter the size of the beta cell pool available to maintain glucose homeostasis in adulthood. In the setting of obesity, beta cell compensation leads to an increase in insulin production and secretion to counteract insulin resistance and hyperglycemia. Mechanisms of compensation include increased

beta cell size and proliferation, as well as increased insulin transcription. A smaller initial beta cell pool might contribute to beta cell failure and T2D.

Data evaluating the effect of developmental metformin exposure on beta cell development and function is conflicting. Studies by Gregg *et al.* using mouse models in which dams were administered metformin *via* the drinking water revealed an increase in multipotent pancreatic progenitor cells, endocrine progenitors, and an overall increase in pancreatic bud size in embryos analyzed at e14.0 [77]. This finding was consistent in pancreatic rudiments from e13.0 embryos cultured in metformin for 72 hours, though the number of endocrine pancreatic progenitors was decreased at the end of *in vitro* culture. The authors postulated that increased cell number could be due to increased signaling downstream of mTORC1 secondary to AMPK activation. This was supported by increased phosphorylated S6 and phosphorylated acetyl CoA carboxylase (p-ACC), a target of AMPK. At birth, offspring exposed to metformin had an increased beta cell fraction, indicating a positive effect of metformin on increasing the size of the beta cell pool at birth [77]. A later study by the same group observed that adult male offspring at six and nine weeks of age exposed to metformin *in utero* have improved glucose tolerance and insulin secretion due to increased L-type calcium channel transcription leading to a more robust calcium response in islets despite having similar beta cell mass to that of control offspring [78]. In the context of this study, intrauterine metformin exposure increases glucose-stimulated insulin secretion *via* proposed epigenetic modification to L-type calcium channels, though the increased beta cell fraction present in early postnatal life is no longer observed in offspring in adulthood.

Zebrafish embryos expressing cyan florescent protein (CFP) under control of the insulin promoter treated *in vitro* with metformin daily at 4-6 days post fertilization (dpf) have increased expression of the transcript for the hormone somatostatin, which is secreted by delta cells within the islets. Somatostatin is a counterregulatory hormone that reduces the secretion of insulin and glucagon. The increase in somatostatin production in response to metformin suggests that exposure may attenuate insulin secretion in zebrafish embryos, although functional studies evaluating insulin secretion would be required to support this idea. At six dpf, embryos exposed to metformin had increased beta cell number with no increase in insulin mRNA. The authors suggested that increased cell number in response to metformin is counterbalanced by attenuation of insulin production and secretion [79].

A recent study evaluating the consequence of *in vitro* treatment of cultured H9 human embryonic stem cells (hESCs) with metformin during a beta-like cell differentiation protocol demonstrates similar findings to those in zebrafish. Metformin treatment during pancreas/beta cell differentiation resulted in smaller clusters of beta-like cells and reduced expression of all hormone transcripts (*insulin*, *glucagon*, *somatostatin*, and *chromogranin A*). RNA sequencing of beta-like clusters from this study revealed that metformin treatment downregulated genes responsible for hormone production and secretion. Furthermore, metformin resulted in an increase in transcripts associated with mitochondrial respiration. Treatment of the immortalized human beta cell line, EndoC-BH1, with metformin had negative effects on mitochondrial function including decreased basal respiration, maximal respiration, spare respiratory capacity, and ATP production. Further, metformin treatment decreased glycolytic function in EndoC-BH1 cells. Therefore, in addition to reducing beta-cell number and expression of hormones, *in vitro* metformin treatment decreases mitochondrial respiration, an essential process for the ATP production needed to trigger insulin secretion [80].

Taken together, more studies evaluating the developmental effects of exposure to metformin on pancreas and islet development and function are necessary to elucidate whether metformin treatment is maladaptive or beneficial to the offspring pancreas. The differences observed in hormone regulation, beta-cell number, and glucose homeostasis in mice relative to human-derived cells demonstrate a need for studies in animal models more evolutionarily related to humans, such as the NHP. Islet architecture, proportion of

different islet cell types, and expression of some islet transcription factors are more similar between humans and NHP than they are between humans and rodents [81-84]. Furthermore, NHP demonstrate heterogeneity in their response to diets high in fat and carbohydrates resulting in varying susceptibility to metabolic disease, in a similar manner to humans [85]. Future studies in *ex vivo* islets and *in vivo* studies of glucose tolerance in offspring will provide more evidence as to the effect of *in utero* metformin exposure on the endocrine pancreas of the offspring.

Effect of metformin on hepatic cell growth and viability

The primary functional cells of the liver are derived from endodermal cells in the anterior foregut in response to inductive signals such as fibroblast growth factor (FGF) from the adjacent cardiac mesoderm. The endoderm undergoes extensive branching and growth into the mesoderm of the septum transversum, which contributes the mesenchymal component of the mature organ. Importantly, prior to formation of the bone marrow the liver is the site of fetal hematopoiesis, and thus, a smaller liver results in defects in blood development. A detailed review of the liver including developmental origins, cell types, and metabolism can be found in [86].

The consensus in the field is that in adults, metformin primarily acts on hepatocytes to reduce gluconeogenesis and glycogenolysis. Different mechanisms for this response have been proposed, including reduced cAMP response after interaction of glucagon with its receptor, inhibition of mitochondrial glycerol phosphate dehydrogenase (mGPD), and activation of AMPK [87-89]. Nevertheless, because many studies evaluate the effect of metformin on primary hepatocytes from adult animals, little data exists on the influence of metformin on the developmental program of the liver.

One study evaluating the effect of intrauterine metformin exposure in mouse embryos isolated at e18.5 revealed that metformin directly increases the expression of *Hnf4a*, a transcription factor important for hepatocyte differentiation and activation of the gluconeogenic program. This effect was correlated with decreased methylation in the *Hnf4a* promoter, mediated by increased expression of the long noncoding RNA, H19. Notably, metformin also decreased the expression of transcripts encoding the insulin receptor in these embryos as well [90]. Therefore, metformin has been shown to influence the developmental program of the liver *via* epigenetic modification of important transcription factor genes. The fact that these mice were born at lower birthweights than the non-exposed controls but then had a higher weight gain postnatally with consumption of a WSD is clinically concerning. In the offspring of gravidae treated with metformin, there is an increased risk in some cohorts for higher BMI during childhood when eating an ad lib diet [59, 91, 92]. These findings suggest that the effects of metformin on the hepatic epigenome (or gut microbiome) could result in long-term adverse hepatic function and metabolism.

While prenatal exposure of mice to metformin resulted in smaller birthweights compared to non-exposed mice, the metformin-exposed offspring fed a WSD post-weaning demonstrated an accelerated weight gain, greater measures of mesenteric fat, and more frequent occurrence of hepatomegaly [93]. The male mice exposed to metformin in this study later demonstrated impaired glucose tolerance and fasting hyperglycemia. In addition to these metabolic changes, the noted hepatomegaly is of clinical concern as there is a high risk of nonalcoholic fatty liver disease (NAFLD) in obese children [94]. NAFLD can progress into nonalcoholic steatohepatitis (NASH), one of the leading causes for liver transplant worldwide. Studies have shown that newborns from gravidae with both obesity and GDM have 68% increased hepatic fat measured by Magnetic Resonance Imaging/Magnetic Resonance Spectroscopy (MRI/MRS) when compared to infants born to gravidae with neither GDM nor obesity [95], which may serve as a “first hit” and increase their risk of developing NAFLD in the future [47].

Studies assessing the effects of metformin in *ex vivo* primary hepatocytes and *in vivo* liver function can be evaluated to speculate about potential effects

of metformin on the developmental program of the liver. Livers isolated from adult male mice treated with metformin after a fasting/refeeding paradigm showed inhibited activation of mTORC1 as measured by a reduction in phosphorylated S6K1, phosphorylated S6, and phosphorylated 4EBP1. This effect was reversed in mice lacking both alleles of AMPKa1 and AMPKa2, suggesting that the effect of mTORC inhibition is a result of AMPK activation in response to metformin. Further evaluation revealed reduced protein translation in response to metformin [96]. Reduction in mTORC signaling as the liver develops could result in growth restriction of the liver. Fetal hepatic metabolism is unique as the fetus develops in a low-oxygen environment with limited capacities for lipid and amino acid oxidation until birth [97]. The fetal liver has little or no gluconeogenesis, fewer mitochondria, and lower activity of carnitine palmitoyl-CoA transferase-1 (CPT1, the enzyme responsible for mitochondrial fatty acid transport) when compared to adult livers [97]. The developing and adult liver secrete insulin-like growth factor (IGF) which influences the development of other organ systems including the reproductive organs and the central nervous system. A reduction in the size of the liver and the attenuation of protein synthesis in hepatocytes would reduce the bioavailability of important growth factors for fetal organogenesis. This possibility needs to be directly evaluated in preclinical studies.

While the aforementioned studies suggest that metformin could interfere with normal cellular and organ growth during development, metformin is also protective against hepatic cell death in response to lipotoxicity. Immortalized human hepatocytes (HepG2 cells) and primary rat hepatocytes treated with metformin prior to exposure to the fatty acid palmitate were protected from palmitate-induced necrotic cell death. The authors proposed that metformin mediates these protective effects by reducing ROS accumulation *via* increasing transcription of the anti-oxidant enzyme superoxide dismutase 2 (SOD2), and mild inhibition of respiratory Complex I independent of AMPK activation [98]. During development, maternal HFD consumption is expected to increase the presence of fatty acids such as palmitate in the fetal circulation; therefore, metformin may protect developing hepatocytes from lipotoxicity induced by maternal overnutrition as well.

Taken together, these studies highlight the consideration that metformin can maintain cellular viability during stress; however, the anti-growth properties of metformin in primary hepatocytes from adult animals warrant the consideration of the role of metformin in fetal growth restriction. Moreover, the combined effects of metformin and WSD may have unintended long-lasting consequences on hepatic function.

Risk of neural tube defects in response to metformin exposure

The neural tube forms from folding of the ectodermal neural plate epithelium during development, eventually giving rise to the central nervous system (CNS; brain and spinal cord). Neural tube closure along the dorsal midline does not occur simultaneously along the entire anterior-posterior axis; the neural tube remains open for an extended time in both the anterior-most and posterior regions (called the anterior and posterior neuropores, respectively) [99]. Defects in completion of neural tube closure are some of the most common birth defects. Folate and vitamin B_{12} are critical nutritional cofactors for proper neural tube closure and women are recommended to consume folate supplements during pregnancy. Children born to mothers with reduced folate levels show a persistent reduction in total brain volume from the third trimester up to 10 years of age [100].

Individuals with T2D that take metformin exhibit deficiencies in vitamin B_{12} and folate due to reduced absorption from the gut [101-103]. Vitamin B_{12} is beneficial for hematopoiesis, nervous system maintenance, and methionine metabolism. In addition to reduction of serum B_{12} levels during pregnancy resulting from increased micronutrient demands and dilution, metformin intake may also reduce circulating B_{12} and its bioavailability to the fetus. A study of 120 Egyptian graviade, in which offspring were monitored for neural tube defects (NTD), and levels of serum homocysteine, methyl malonic acid, and vitamin B_{12} were measured, found

a correlation between low serum vitamin B_{12} and increased incidence of NTD such as anencephaly, encephalocele, and spina bifida [104]. Thus, folate levels in gravidae exposed to metformin should be monitored for the benefit of offspring CNS development.

Mouse models have also been used to investigate whether metformin could contribute to risk of occurrence of NTD. In an early study, delays in neural tube closure increased in a dose-dependent manner in e9.0 embryos cultured for 24-48 hours in metformin [105]. Importantly, metformin did not induce gross neural defects. The relevance of delayed neural tube closure in embryos in this study is unknown. Another study by the Loeken group revealed that neural tube defects are not observed in response to metformin culture [106]. In a mouse model of T2D in which dams were fed a HFD, administration of metformin reduced oxidative stress, ER stress, apoptosis, and the incidence of NTD in e8.5 embryos [107]. Hyperglycemia induced by T2D is known to increase the incidence of NTD, but metformin treatment reduces the incidence of NTD in this setting [108-110]. Therefore, the extent to which metformin delays neural tube closure or reduces risk of NTD should be considered in the context of the metabolic status of the mother.

In summary, metformin has not been associated with overt NTD; however, the finding that metformin causes delays in neural tube closure at a dose observed in the therapeutic range experienced in humans warrants more investigation into consequences of such delays. One should also consider the metabolic status of the mother, as the effects of fetal exposure to maternal diabetes can be attenuated by metformin, while different effects may be observed in the offspring of mothers consuming a healthy diet.

Effect of metformin on the developmental program of the heart

In adults, metformin protects against T2D-associated cardiovascular complications by reducing inflammation and by inducing autophagy to prevent cardiac hypertrophy [111, 112]. The extent to which this protection would be extended to the fetus is less known. Randomized, controlled studies in women that took metformin while pregnant suggest that intrauterine exposure to metformin does not result in gross effects on cardiac development and morphogenesis in offspring when assessed in early childhood. Four- and six-year-old offspring of obese pregnant women (in the absence of diabetes) exposed to metformin during gestation demonstrate improved cardiac function and hemodynamics in early childhood [113, 114]. Meanwhile, studies of offspring from women with GDM and PCOS that consumed metformin during pregnancy concluded that increased BMI in early childhood would predispose offspring to inferior cardiometabolic health as adults [60, 61]. The influence of metformin exposure during fetal development on cardiovascular disease susceptibility in adulthood is not known. Zebrafish embryos exposed to metformin displayed dysregulation of transcripts associated with cardiovascular development, dilation, enlargement, and function, in addition to cardiac edema when introduced from four hours post fertilization to five days post fertilization, a critical window of cardiac development [115].

As metformin is typically prescribed to women to manage gestational diabetes in pregnancy, one must consider the effect of hyperglycemia on cardiovascular development in the fetus. Exposure to maternal hyperglycemia and diabetes *in utero* has been associated with increased susceptibility to congenital heart defects and increased incidence of cardiovascular disease in offspring [116-120]. The mechanisms responsible for congenital heart defects in offspring of dams with Type 1 and Type 2 Diabetes include increased apoptosis, stress-induced oxidative stress, and endoplasmic reticulum stress [107]. Mouse models of gestational diabetes reveal that perturbed maternal glycemia results in cardiovascular defects due to the downregulation of genes responsible for cardiac neural crest development when insulin-producing beta cells are ablated with streptozotocin mid pregnancy [121]. Therefore, poor management of maternal diabetes makes the fetus susceptible to stress-induced changes to genes responsible for cardiovascular development. The glucose-lowering effect of metformin would be beneficial for evading

diabetes induced teratogenicity and embryopathy in offspring.

Long-term physiological/metabolic consequences of developmental metformin exposure

There is evidence that metformin places exposed fetuses at risk for developmental programming and lifetime alterations that may enable an obesogenic phenotype, particularly in a postnatal environment of nutritional and caloric excess [122, 123]. Several recent randomized clinical trials in women with GDM or PCOS suggest that exposure to metformin *in utero* results in a metabolic phenotype that increases offspring weight in childhood [59, 60, 91]. These studies, which are some of the first to examine the long-term consequences of fetal exposure to metformin, found that children whose mothers received metformin during pregnancy weigh more and manifest larger waist circumferences and higher fat mass at four and nine years of age [59, 60, 91]. In the PregMet study, mothers with PCOS were randomized to either metformin or placebo during pregnancy and the effects of metformin on offspring growth were determined for up to four years of age [91]. The 5-10 year follow-up of the PregMet study (the PedMed study) found that these same children (mean = 7.5 years) exposed to metformin *in utero* had significantly higher BMI, waist-to-height ratio, and waist circumference z-scores with borderline significantly higher body fat than their control peers [60]. A small follow-up study of offspring of women with PCOS who had been randomized to metformin or placebo demonstrated that although the children exposed to *in utero* metformin had significantly higher fasting blood glucose levels, they had lower low-density lipoprotein at eight years of age than the children exposed to the placebo [124]. These findings suggest that the actions of metformin *in utero* may ultimately worsen some long-term metabolic outcomes.

Similarly, the MiG trial randomized mothers with GDM to metformin (46% of whom also received insulin to achieve glycemic targets) versus insulin alone to examine the effects of *in utero* metformin exposure on fetal and childhood development [75]. In the follow-up to the MiG trial (MiG Tofu trial), children exposed to metformin had larger measures of subcutaneous fat without evidence of a decrease in visceral fat at age two [58]. These same children were assessed at nine years of age, and the metformin-exposed offspring were still noted to be statistically significantly larger on several measures including weight, mid-upper arm circumference, waist circumference, and waist-to-height ratio [59]. Thus, these randomized trials in women with PCOS and GDM provide evidence that is concurrent with animal studies and that supports the notion that metformin exposure *in utero* may ultimately result in or contribute to a metabolic phenotype that increases childhood weight or obesity. Further studies are needed to follow the offspring long-term for metabolic risk.

CONCLUSIONS

Recommendations for metformin use in pregnancy are growing in prevalence for the treatment of pregestational diabetes, GDM, PCOS, and preeclampsia. Metformin ameliorates the teratogenic effects of poor glycemic management in pregnancy as seen in the case of neural tube defects and cardiovascular defects in response to maternal diabetes. These recommendations are made based on findings from follow up studies in human trials that show that metformin does not cause gross anatomical abnormalities in offspring (for example, neural tube and cardiac defects).

Nevertheless, metformin alters the metabolic program of the fetus during development such that changes in the expression of genes responsible for the development of the pancreas, liver, central nervous system, and heart are observed. Maternal metformin use inhibits cellular processes that are crucial for normal fetal growth. When accompanied by maternal and/or postnatal nutrient excess, clinically apparent childhood metabolic disease ensues, as recent clinical studies suggest. The subtle changes to the epigenetic landscape of organs in development by metformin exposure combined with environmental exposures following birth could reasonably contribute to disease susceptibility in adulthood. Additionally, metformin inhibits cellular growth in disease, though this effect has not been

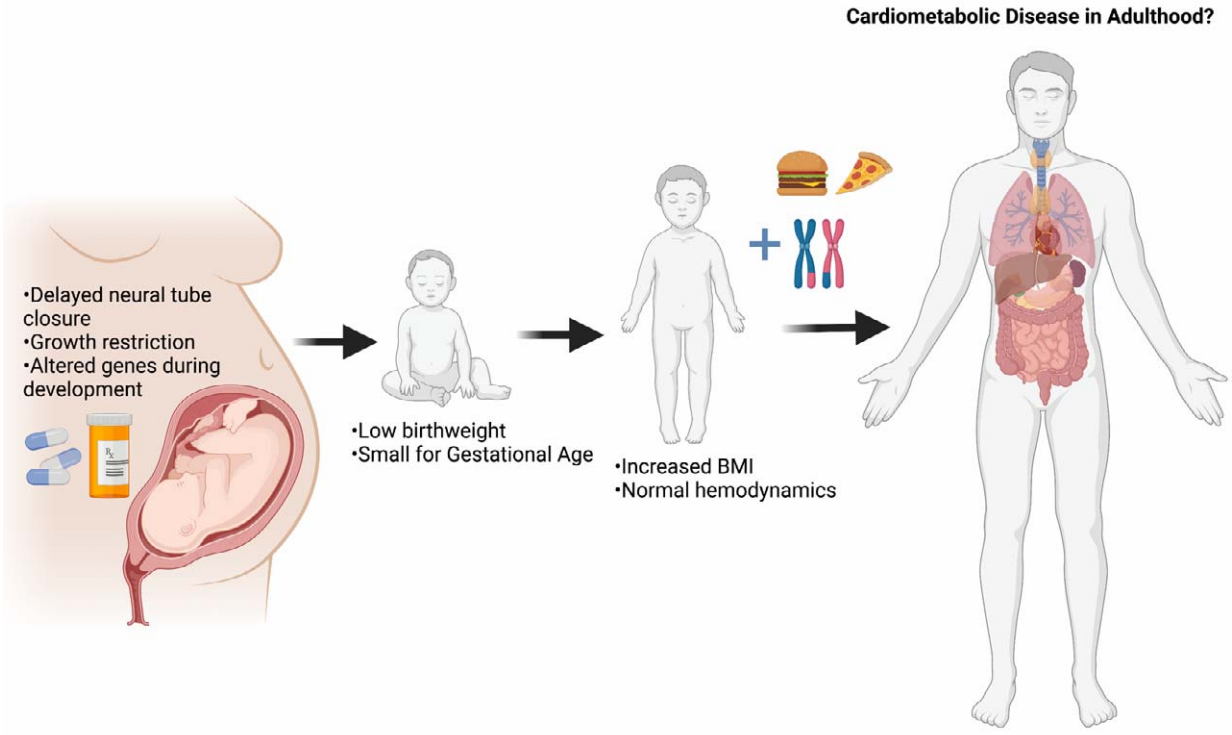

Figure 3. Effects of metformin on offspring health following intrauterine exposure. Exposure to metformin results in delayed neural tube closure, restriction of growth *via* inhibition of the mTOR pathway, and alteration of genes involved in development of the pancreas, liver, heart, and CNS. Human follow-up studies show that offspring are born small for gestational age with low birthweight in some women that take metformin while pregnant. Studies also show that offspring have increased BMI z-scores from 18 months of age into early childhood. The combination of intrauterine exposure to metformin, genetic predisposition, and offspring diet will influence the susceptibility of offspring to cardiometabolic disease in adulthood. Created with Biorender.com

thoroughly evaluated enough in fetal tissue after *in utero* exposure. Additional follow-up studies into adulthood of offspring whose mothers consumed metformin during pregnancy are imperative so that more data on long-term metabolic health is available. Animal studies should consider the timepoint at which metformin is introduced to dams, as critical periods of development differ for major organs. Future studies should follow up on the observation that metformin-exposed offspring have been shown to have low birthweight and increased BMI in early childhood (Figure 3). Fetal exposure to metformin may influence offspring metabolism to a similar degree as observed in response to maternal overnutrition and undernutrition. These studies highlight the necessity of not only considering the diet consumed by women while pregnant, but also the therapeutics that are employed to combat metabolic disease in pregnant women.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts to report.

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