Effect of Maternal Metformin Treatment in Pregnancy on Neonatal Metabolism: Evidence From Newborn Metabolic Screening

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OBJECTIVE

To investigate effects of maternal diabetes and metformin treatment on metabolic newborn screening (NBS) results of infants born to mothers with hyperglycemia during pregnancy.

RESEARCH DESIGN AND METHODS

Retrospective case-control study. NBS results of infants born to mothers treated with metformin for hyperglycemia during pregnancy were compared with diet-treated subjects with diabetes and matched normal control subjects. Exclusions: maternal type 1 diabetes, major fetal anomalies, and incomplete infant data. Inclusions: maternal hyperglycemia in pregnancy treated with diet alone or diet plus metformin. Results from the New South Wales Newborn Screening Program (dried infant blood spot sample, 24–72 h after birth) for 25 routinely studied analytes were measured using mass spectrometry. Data from metformin-exposed and control infants were compared using nonparametric methods and multiples of the median for each analyte.

RESULTS

A total of 574 case subjects were compared with 952 diet-treated case subjects with diabetes and 979 control subjects. Metformin-exposed infants had shorter gestational age (266 ± 7 vs. 272 ± 10 vs. 274 ± 9 days) ($P < 0.001$) and lower birth weights (3.28 ± 0.51 vs. 3.29 ± 0.49 vs. 3.33 ± 0.43 kg) ($P = 0.008$). Short-, medium-, and one long-chain acylcarntine (tetradecanoylcarnitine [C14]) concentrations were higher in the metformin-exposed group compared with normal control subjects. Comparison with diet-treated control subjects with diabetes (to eliminate confounding by hyperglycemia) continued to show raised butyrylcarnitine (C4), isovalerylcarnitine (C5), and glutarylcarnitine (C5D) in the metformin-exposed group. There was no evidence of vitamin B12 deficiency (low methionine and elevated propionylcarnitine [C3]) in metformin-exposed infants. All results were within normal population limits.

CONCLUSIONS

We have identified subtle (nonpathological) changes in neonatal metabolism that represent a signature effect of fetal metformin exposure.

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Metformin is an oral biguanide used in treatment of diabetes that has pleiotropic effects, including inhibition of hepatic gluconeogenesis, inhibition of intestinal glucose absorption, and multiple other effects beyond carbohydrate homeostasis (1). Weight-neutrality, tolerability, and oral dosing has made it an attractive agent for use in the treatment of gestational diabetes mellitus (GDM) and pregestational type 2 diabetes. Metformin is known to cross the placenta and is present in fetal plasma in similar concentrations to those in the maternal circulation (2,3). Metformin exerts antidiabetic effects through multiple mechanisms, which may include inhibition of complex I of the electron transport chain, inhibition of mitochondrial isoform-specific glycerophosphate dehydrogenase in the liver, cytosolic inhibition of AMP deaminase, and direct cytosolic activation of AMPK (1,4). These result in a redox state with increased hepatic cytosolic and mitochondrial AMP/ATP and NADH/NAD ratio, suppressing gluconeogenesis (1). Recently, metformin has also been shown to suppress expression of the hepatic plasma membrane citrate transporter (*SLC13A5*; Online Mendelian Inheritance in Man [OMIM] #608305), decreasing citrate uptake from the circulation, leading to decreased intracellular citrate promoting glycolysis and decreasing gluconeogenesis (4). Despite its multiple effects on metabolic networks, large-scale trials (5) have demonstrated safe maternal and fetal pregnancy outcomes with metformin use.

There are limited data on the effects of metformin on neonatal metabolism. Newborn screening (NBS) is a global public health initiative that provides screening for rare inborn errors of metabolism (IEM) within the first week after birth by analyzing metabolic intermediates that indicate major metabolic perturbations. In New South Wales (NSW), this screening process, performed 24–72 h after birth, includes assessment of concentrations of 25 acylcarnitines and amino acids that are intermediates of metabolic pathways affected in known IEM. We hypothesized that results of NBS may also provide insight on the effects of metformin exposure on neonatal metabolic pathways. Importantly, the timing of the test and sample (dried blood spot drawn from a heel-prick) minimizes maternal and placental influence on results and is taken while the newborn is in a catabolic state during the first few days of life due to transitioning from an in utero environment with a constant nutritional supply to intermittent enteral feeding. This is ideal for screening for disorders of fatty acid oxidation.

In Australia, metformin is categorized as a class C drug for safety in pregnancy ("suspected of causing harmful effects on the human fetus or neonate without causing malformations"). Metformin usage is highly variable between obstetric units, and safety concerns persist for long-term effects in metformin-exposed offspring. We sought to use available evidence from NBS to identify any effects of metformin exposure on metabolism in infants during the first few days of life. We identified case subjects from a busy obstetric diabetes service that routinely uses metformin as first-line treatment for hyperglycemia in pregnancy.

RESEARCH DESIGN AND METHODS

Study Population

This was a retrospective study undertaken at Blacktown Hospital, Sydney, NSW, Australia that included babies born 2007 through 2014. Metformin use was commenced in 2005. GDM is diagnosed through universal screening of women at 24–26 weeks' gestation with a 75-g oral glucose tolerance test and diagnostic cutoffs: fasting glucose $\geq$5.5 mmol/L and 2-h postprandial glucose $\geq$8.0 mmol/L. From 2010, women with a previous history of GDM or with high-risk ethnicity were offered an early 75-g oral glucose tolerance test between 16 and 18 weeks' gestation in addition to routine screening performed at 24–26 weeks' gestation (6). All women diagnosed with GDM were given dietary advice and diabetes education, taught glucose self-monitoring (treatment targets of fasting glucose $\leq$5.5 mmol/L and 2-h postprandial glucose $\leq$7.0 mmol/L), and reviewed fortnightly, increasing to weekly after 36 weeks' gestation. Tre-atment with either metformin or insulin (based on clinician discretion and patient preference) was added if blood glucose targets could not be achieved by diet and lifestyle modification. Treatment received, glycemic control, and maternal and fetal outcomes were recorded prospectively in an electronic database.

A database search was performed for women treated with metformin during pregnancy with available infant identifying data to allow a match with NBS results. Women taking metformin at the time of conception were included. Exclusions were: women with type 1 diabetes, pregnancies complicated by fetal malformations or chromosomal abnormalities (due to effects on NBS analytes), patients lost to follow-up, or those without data sufficient to link to NBS results. NBS data were obtained from the NSW Newborn Screening Program. Data linkage was performed by matching maternal identifiers with infant's date of birth. NBS workflow, including sample processing and analysis, has been previously published (7). Conditions screened and analyte panels are available in Supplementary Table 1. Underivatized sample preparation (rather than butanoic hydrochloric acid derivatization) was implemented in 2007. We allocated a pragmatic ratio of one metformin-exposed case subject to two control subjects. Normal control data were NBS results from two infants screened at the same/closest work date, batch, birth hospital (92% from Blacktown Hospital), matched as close as possible with the case for weight, sex, and gestational age to minimize analytical uncertainty. A post hoc analysis using the same methods was then undertaken comparing the NBS results of metformin-exposed infants with the results of infants born to mothers with GDM treated with diet only (all from Blacktown Hospital) to exclude confounding by hyperglycemia. Decenoylcarnitine (C10:1), hydroxyhexadecanoylcarnitine (C16OH), and hydroxyoctadecanoylcarnitine (C18OH) were excluded from analyses due to these analytes being present in very low levels and raising the possibility of type I errors.

We also hypothesized that metformin exposure at different stages of pregnancy might produce an analyte pattern reflective of the growing needs of the embryo and fetal cellular differentiation and maturation. We therefore analyzed separately the results of infants exposed from the first, second, or third trimester of pregnancy (with exposure continuing until delivery).

This project received ethics approval as part of a quality assurance project by the Western Sydney Local Health District

Human Research Ethics Committee (SAC-2018/3/6.12 [558:QA]).

Statistical Analysis

Analyses were undertaken using SPSS version 26.0 (IBM). Categorical data were compared using χ^2 testing. NBS analyte concentrations are not normally distributed and reported as medians with interquartile ranges with differences between groups assessed by the Mann-Whitney *U* test. Analytes were added to the NBS panel as the program progressed through the years and were included in the cohort analysis if performed and present in all three cohorts. Since results of absolute analyte concentrations can vary between laboratories, NBS laboratories worldwide have started to implement reporting of results as multiples of the median (MoM), in which individual analyte results are divided by the median concentration in the population screened with the ratio expressed as a whole number. Therefore, an MoM of 1.4 is interpreted as a result 1.4 times greater than the population median for that analyte. This allows standardization of values and decreases ambiguity during discussion among NBS laboratories worldwide. Using Microsoft Excel version 2002, we converted each individual analyte concentration into an MoM score (normalized against the median value for that analyte in the control group without diabetes). The median MoM score for each analyte is therefore 1.0 in the control group without diabetes, and the variance from 1.0 in the groups with GDM therefore indicates the magnitude of difference associated with either diet-treated GDM or metformin exposure.

The metformin-exposed case subjects were analyzed collectively as a group compared with normal control subjects and then divided into trimester-specific exposure groups and reanalyzed. Analysis was then repeated comparing the metformin-exposed case subjects with the diet-treated case subjects. All tests are two-tailed, with $P < 0.05$ taken as significant.

RESULTS

Characteristics

Table 1 shows the characteristics of the 574 metformin-exposed case subjects (483 monotherapy and 99 combined metformin/insulin), 978 normal matched control subjects, and 952 diet-treated case subjects with diabetes. Women treated with metformin had slightly shorter gestational periods and smaller infants. Mean (range) gestational age at commencement of metformin was 26 weeks (0–38) with a mean (range) dose of 1,326 (500–3,000) mg/day (Table 1), and metformin was usually continued until delivery.

Analyte Trends in the Metformin-Treated Cohort

None of the cohort were diagnosed with an IEM. All NBS results were within the normal reference ranges, and glutarylcarnitine (C5D) levels did not trigger diagnostic testing in any individuals. However, within these normal ranges, 9 of the 25 analytes showed significant differences between case and control subjects. Tables 2 and 3 show that metformin-exposed case subjects, compared with control subjects, had mildly but significantly higher concentrations of acylcarnitine species (short-, medium-, and one long-chain) and lower levels of leucine. Butyrylcarnitine (C4), isovalerylcarnitine (C5), and glutarylcarnitine (C5D) remained statistically significant in a comparison between metformin-exposed infants and diet-treated case subjects with diabetes, representing a putative metabolic signature of metformin exposure. Results of infants whose mothers required both insulin and metformin were analyzed separately, but the pattern of significant changes in acylcarnitines did not differ from the metformin group as a whole (data not shown) (Supplementary Table 5).

Trimester-Specific Changes

Comparison with normal control subjects again showed a similar pattern of higher concentrations of short-, medium-, and long-chain fatty acids, with some differences in amino acids. Isovalerylcarnitine (C5) was consistently elevated in all metformin-exposed case subjects versus control subjects without diabetes and diet-treated case subjects with GDM, regardless of the timing of commencement of treatment (Supplementary Tables 2–4).

CONCLUSIONS

We have shown, for the first time, subtle perturbations in concentrations of metabolic intermediates on 24–72-h-old

Table 1—Demographics of study groups

	Metformin-exposed case subjects with diabetes (*N* = 574)	Diet-treated case subjects with diabetes (*N* = 954)	Normal control subjects (*N* = 979)	Metformin-exposed case subjects with diabetes vs. normal control subjects, *P* value*	Metformin-exposed case subjects with diabetes vs. diet-treated case subjects with diabetes, *P* value*
Birth weight (kg), mean (± SD)	3.28 (± 0.51)	3.29 (± 0.49)	3.33 (± 0.43)	0.004	0.568
Gestational age at birth (days), mean (± SD)	266 (± 7)	272 (± 10)	274 (± 9)	<0.001	<0.001
Sex (female), %	47.4	47.5	47.4	0.941	0.791
Mean metformin dose, g/day	1,326				
Mean start of metformin treatment (weeks' gestation)	26				

*Continuous variables compared using *t* testing and discrete variables compared using χ^2 testing.

Table 2—NBS analytes (raw data) comparing metformin-exposed case subjects with diabetes with diet-treated case subjects with diabetes and metformin-exposed case subjects with diabetes with normal case subjects

Analyte (μmol/L)	Metformin-exposed case subjects with diabetes (*N* = 574)	Diet-treated maternal case subjects with diabetes (*N* = 954)	Normal control subjects (*N* = 979)	Metformin-exposed case subjects with diabetes vs. normal control subjects, *P* value*	Metformin-exposed case subjects with diabetes vs. diet-treated case subjects with diabetes, *P* value*
C4 (butyrylcarnitine)	0.31 (0.22–0.44)	0.29 (0.20–0.40)	0.27 (0.19–0.39)	**<0.001**	**0.003**
C5 (isovalerylcarnitine)	0.12 (0.09–0.17)	0.11 (0.08–0.15)	0.11 (0.08–0.15)	**<0.001**	**<0.001**
C5D (glutarylcarnitine)	0.08 (0.05–0.111)	0.07 (0.05–0.10)	0.07 (0.05–0.10)	0.080	**0.017**
C6 (hexanoylcarnitine)	0.06 (0.04–0.09)	0.06 (0.04–0.08)	0.06 (0.04–0.08)	<0.001	0.31
C8 (octanoylcarnitine)	0.09 (0.07–0.12)	0.09 (0.07–0.012)	0.08 (0.06–0.11)	0.003	0.803
C12 (dodecanoylcarnitine)	0.14 (0.09–0.20)	0.15 (0.11–0.24)	0.14 (0.09–0.22)	0.005	0.543
C14 (tetradecanoylcarnitine)	0.34 (0.26–0.44)	0.32 (0.25–0.41)	0.15 (0.11–0.22)	<0.001	0.440
Leucine (μmol/L)	131.11 (112.05–115.17)	140.68 (117.86–170.90)	144.72 (121.95–177.84)	0.003	0.554

Data presented as medians with interquartile ranges. Data presented as MoM unless otherwise indicated. Analytes in boldface represent significant analytes discriminatory for metformin. *Mann-Whitney *U* test.

newborns who had been exposed to metformin in utero. None of the cohort had analyte levels that triggered confirmatory testing or led to a diagnosis of an IEM. The effects of hyperglycemia, observed in both metformin-exposed and diet-treated case subjects with diabetes, produced a pattern similar to that seen in mild riboflavin (B2) deficiency (8) or multiple acyl-CoA dehydrogenase deficiency (OMIM #231680) with derangements in various short-, medium-, and long-chain acylcarnitines and lower concentrations of leucine. Comparison of the metformin-exposed group with inf-ants exposed to diet-treated GDM (no pharmacotherapy) showed statistically significant elevations in isovalerylcarnitine (C5) and was persistent regardless of when in the pregnancy metformin was started. The overall pattern of elevated butyrylcarnitine (C4), isovalerylcarnitine (C5), and glutarylcarnitine (C5D) in metformin-exposed case subjects not seen in diet-treated case subjects with diabetes does not mimic any IEM des-cribed to date and, if these findings are replicated in similar cohorts, can be considered an identifying pattern of in utero metformin exposure. An important cav-eat is these changes were very subtle and well short of changes that would be considered pathological or require remediation. However, taken together, these observations suggest an effect of metformin on fetal and neonatal metabolism that could have physiological significance.

This work has provided evidence on the effects of metformin on neonatal metabolism, particularly among the acylcarnitine species. These changes varied according to the gestational age at which metformin treatment was commenced and hence with duration of exposure.

The most consistent changes associated with metformin exposure were in isovalerylcarnitine (C5) concentrations, a short-chain fatty acid esterified to carnitine. These acylcarnitines are usually formed by catabolism of the branched-chain amino acids leucine and valine and can easily cross the mitochondrial membrane without the need for transporters, conserving cellular energy. Marked elevations of C5 are seen in isovaleric acidemia (OMIM #2435000), a disorder of leucine metabolism that has been misdiagnosed as diabetic ketoacidosis, as it presents with

Table 3—NBS analytes (normalized MoM data) comparing metformin-exposed case subjects with diabetes with diet-treated case subjects with diabetes and metformin-exposed case subjects with diabetes with normal control subjects

Analyte (μmol/L)	Metformin-exposed case subjects with diabetes (N = 574)	Diet-treated case subjects with diabetes (N = 954)	Normal control subjects (N = 979)	Metformin-exposed case subjects with diabetes vs. normal control subjects, P value*	Metformin-exposed case subjects with diabetes vs. diet-treated case subjects with diabetes, P value*
C4 (butyrylcarnitine)	1.15	1.07	1.00	**<0.001**	**0.003**
C5 (isovalerylcarnitine)	1.18	1.00	1.00	**<0.001**	**<0.001**
C5D (glutarylcarnitine)	1.06	1.00	1.00	0.080	**0.017**
C6 (hexanoylcarnitine)	1.00	1.00	1.00	<0.001	0.554
C8 (octanoylcarnitine)	1.13	1.13	1.00	0.003	0.803
C12 (dodecanoylcarnitine)	1.07	1.07	1.00	0.005	0.548
C14 (tetradecanoylcarnitine)	2.20	2.13	1.00	<0.001	0.440
Leucine	0.97	0.96	1.00	0.003	0.554

Data are MoM unless otherwise indicated. Analytes in boldface represent significant analytes discriminatory for metformin. *Mann-Whitney U test.

hyperglycemia and ketosis (9–11). Despite the qualitative similarity of our cohort's analyte pattern to this described IEM, it is of a significantly smaller magnitude that does not necessarily denote pathogenicity and, in fact, may indicate an adaptation to increase energy efficiency. Short- and medium-chain acylcarnitines pass easily through the mitochondrial membrane without needing the carnitine-acylcarnitine transporter system and contribute to the CoA pool needed to form metabolic intermediates allowing entry into the Krebs cycle. Therefore, the observed metabolite profile could represent metformin influence on metabolic networks to preferentially express these energy substrates, which may be advantageous in circumstances of insulin resistance or catabolic stress.

Metformin has been shown to reverse toxic effects of hyperglycemia on the placenta in vitro and in vivo and reverse placental epigenetic changes (through AMPK activation) that reduce mitochondrial biogenesis (12–14). A similar, timing-dependent response in fetal and neonatal metabolic networks may underlie the analytic differences seen in our cohort. Acylcarnitines are fatty acids esterified to an acyl group that allows transport of otherwise nonpolar substrates into the mitochondria for β-oxidation. The pattern of metabolite changes in hyperglycemia (higher concentrations of short- and medium-chain acylcarnitines and in tetradecanoylacylcarnitine [C14]) resembles a milder form of the disturbance described in association with riboflavin deficiency (8).

Two of metformin's targets, glycerophosphate dehydrogenase and complex I of the mitochondrial electron transport chain, use derivatives of riboflavin, such as flavin mononucleotide and flavin adenine dinucleotide. These derivatives form structural components of mitochondrial complex I and II and may in part explain the pattern of fatty acid oxidation that we observe in infants after metformin exposure. Concentrations of tetradecanoylcarnitine (C14) were two times higher than the median of the normal population observed in both metformin-exposed case subjects and diet-treated case subjects with diabetes. This is classified as a long-chain acylcarnitine and requires entry into the mitochondria via the carnitine transporter system. Higher concentrations

may be due to preferential metabolism of the simpler and shorter acylcarnitines, although another explanation could be that hyperglycemia activates different metabolite networks within the cellular system involving cross talk between mitochondria and peroxisomes, which are also involved in β-oxidation (15).

Cobalamin (vitamin B12) deficiency has been described in metformin use in adults (16). Therefore, it is noteworthy that no changes in markers indicative of cobalamin deficiency were observed in our group of metformin-exposed neonates (propionic acid, C3, and methionine). Thus, the potential effects of in utero metformin exposure on neonatal metabolism flavin derivatives appear to be restricted to minimal substrate accumulation of primarily short-chain acylcarnitines.

A limitation of this study is that all of the case and most control subjects (93%) came from one center and may not represent the entire patient population. However, this allowed verification that the control subjects used were from normal pregnancies, minimizing confounders. The center where the cases were drawn from is one of only two hospitals in NSW that routinely uses metformin in pregnancy, reducing chances that the few control subjects not from the same center had been unknowingly exposed to metformin. There were no data on parental ethnicity or method of delivery, although our method of selecting control subjects and using a 1:2 ratio reduced sampling bias and minimized measures of uncertainty. Furthermore, metabolic intermediates tested in NBS represent concentrations at one point in time, taken during a catabolic state following the physiologic stress of delivery and interruption of a constant nutritional supply. The results represent only one aspect of metabolism and its networks. Small sample volumes stored as a dried blood spot precluded an untargeted metabolomic approach, which would have more potential in identifying novel analytes and would have increased capability to quantify analytes with similar mass-to-charge ratios.

In this cohort, infants exposed to metformin had shorter gestational age as compared with diet-treated exposed infants and normal control subjects and weighed less, although the values were within normal limits. It is likely the slightly shorter gestational age is due to earlier obstetric intervention, rather than representing a phenomenon caused by metformin.

Our study has several strengths. It is the first to demonstrate the effects of metformin on neonatal metabolic networks and that changes may differ depending on timing of exposure during pregnancy. The lack of differences between groups in analytes involved in cobalamin deficiency provides some reassurance that this is not likely to be a detrimental effect on the developing fetus despite its association in adults treated with metformin. A key strength is that analytes have been measured in NBS samples 24–72 h after birth and not in cord blood. The latter often reflects a mixture of maternal and neonatal samples at the time of birth, a time of major physiological stress. NBS only reflects infant metabolism away from birth.

In summary, infants exposed to hyperglycemia have subtle but statistically significant changes in NBS analytes (short-, medium-, and long-chain acylcarnitines and leucine), while higher (but normal) butrylcarnitine (C4), isovalerlycarnitine (C5), and glutarylcarnitine (C5D) concentrations appear to be specific to maternal treatment with metformin during pregnancy. The changes seen are consistent with expected cellular actions of metformin, and it is possible that the subtle changes in pattern of acylcarnitines and amino acids with metformin exposure reflect a shift toward efficient cellular metabolism in a catabolic state. The changes observed are mild and well short of any effect that is likely to be harmful to the developing fetus or newborn infant.

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