DOI:
10.1016/j.clinbiochem.2018.11.016

Document Version
Peer reviewed version

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PII: S0009-9120(18)30123-1
DOI: https://doi.org/10.1016/j.clinbiochem.2018.11.016
Reference: CLB 9884

To appear in: *Clinical Biochemistry*

Received date: 2 February 2018
Revised date: 2 November 2018
Accepted date: 29 November 2018

Please cite this article as: Negar Maghsoodi, Nicholas Shaw, Gemma F. Cross, Jamshid Alaghband-Zadeh, Anthony S. Wierzbicki, Jonathan Pinkney, Ann Millward, Royce P. Vincent , Bile acid metabolism is altered in those with insulin resistance after gestational diabetes mellitus. Clb (2018), https://doi.org/10.1016/j.clinbiochem.2018.11.016

Original Article

Bile Acid metabolism is altered in those with Insulin Resistance after Gestational Diabetes Mellitus

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Abstract

Background: Bile acids (BAs) are known mediators of glucose metabolism that are altered in type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus (GDM). We hypothesised that post-prandial BA fractions are changed in women with Insulin resistance (IR) after recovery from GDM using homeostatic model assessment (HOMA-IR).

Methods: 45 women median age 44(31-47) with previous GDM, including 20 with HOMA-IR >2.8 and 25 age-matched controls with HOMA-IR ≤ 2.8 were studied. After an overnight fast, all underwent an oral glucose tolerance test. Blood samples were collected at baseline and every 30min for 120min and analysed for glucose on automated platform and for total BAs, their conjugates and fractions using liquid-chromatography tandem mass-spectrometry. Baseline samples were analysed for insulin on automated platform. Delta (Δ) change (difference between baseline and maximal post-prandial response) were calculated. Data is presented as median (IQR).

Results: Fasting primary and unconjugated BAs were higher in women with HOMA-IR >2.8 vs. those with HOMA-IR ≤ 2.8 [0.24 (0.16-0.33) vs 0.06(0.04-0.22) µmol/L and 0.91(0.56-1.84) µmol/L vs. 0.69(0.32-0.89) µmol/L respectively. Δ taurine-conjugated BAs was higher in women with HOMA-IR ≤ 2.8 than those with HOMA-IR>2.8 [0.33(0.20-0.54) vs 0.23(0.13-0.34) µmol/L]. Fasting glucose and non-12α-hydroxylated BAs were negatively correlated in women with HOMA-IR >2.8 (all $p<0.05$).

Conclusions: Following GDM, individuals with HOMA-IR >2.8 have altered conjugated and non-12α-hydroxylated fractions of BAs. It remains to be elucidated if the altered BA metabolism is a contributing factor to the pathogenesis or a consequence of GDM.

Keywords: Bile Acids; Gestational Diabetes Mellitus; Insulin Resistance.

Introduction:

Bile acids (BAs) play an important role in the digestion and absorption of fat and fat soluble vitamins in the small intestine, but also act as receptor-mediated hormones in the metabolism of glucose and energy homeostasis (1). Around 50% of daily cholesterol is converted to water-soluble BAs in the hepatocytes as primarily cholic acid (CA) and chenodeoxycholic acid (CDCA). The major pathway of BA synthesis begins with the hydroxylation of cholesterol by cholesterol 7α-hydroxylase (*CYP7A1*). Subsequent metabolism via the intestinal bacterial flora on primary BAs, results in the formation of secondary BAs; deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA). The glycine or taurine conjugation steps give rise to a total of 10 species of conjugated BAs. CA and DCA are 12α-hydroxylated BAs which contain a hydroxyle group on C12 position whereas in the non-12α-hydroxylated BAs (CDCA, DCA and LCA) this is absent (2).

Bile acids signal through two receptors: the nuclear farnesoid X receptor (FXR) and the membrane receptor TGR5 (a G-protein coupled receptor) in the liver, intestine, gall bladder, adipose tissue, skeletal muscle and pancreas. FXR is involved in glucose homeostasis as well as lipid metabolism. In the intestine BAs stimulate L-cells via TGR5 receptors, activating the glycogen-like peptide 1 (GLP-1) pathway. The incretin GLP-1 promotes insulin secretion and enhances glucose metabolism (3).

Gestational diabetes mellitus (GDM) is characterised by impaired glucose tolerance, recognised for the first time during pregnancy. It occurs in 3-5% of all pregnancies. Most women with GDM recover normal glucose metabolism after delivery, but are still at increased risk (~50%) of developing type 2 diabetes (T2DM) (4). Many studies have shown alterations in BA metabolism in patients with T2DM. Furthermore, modifications of the BA pool may help improve glycaemic control in patients with T2DM (3). Patients with uncontrolled T2DM have altered BA pool size and composition. Despite the discrepancies between studies, current data suggest that alterations in the metabolism of BAs may either be a cause or an effect of metabolic changes associated with T2DM (3).

Obstetric cholestasis (OC) is a pregnancy–induced complication affecting 1 in 200 pregnant women. OC is characterised by maternal pruritus and raised bile acids and associated with impaired glucose tolerance test, dyslipidaemia and increased foetal growth leading to macrosomia. Aberrant homeostasis of BAs in OC may be responsible for impaired glucose metabolism, by affecting FXR and TGR5 signalling pathways (5). Few studies have examined the association between GDM and the composition and pool size of BAs during pregnancy in humans.

Glucose tolerance tests are commonly used to define the clinical spectrum of glucose-related disorders. Individuals with pre-diabetes manifesting either as impaired fasting glucose or impaired glucose tolerance show an increased predisposition to develop T2DM. The gold standard method to assess glucose disposal and insulin resistance is euglycaemic hyperinsulinaemic clamp but this is impractical for large studies. A number of simpler surrogate measures of insulin resistance exist of which the Homeostasis Index of Insulin Resistance (HOMA-IR) is well established where HOMA-IR is calculated using the following equation:

Fasting glucose (mmol/L) × Fasting insulin (mIU/L) / 22.5

In the US National Health and Nutrition Survey (NHANES) individuals with HOMA-IR > 2.8 (75^{th} centile cut-off) showed an increased predisposition to develop T2DM and higher cardiovascular mortality (6).

We hypothesised that BAs pool and composition as well as post-prandial BA metabolism are altered in women with previous history of GDM and with current insulin resistance (HOMA-IR > 2.8) compared with those with normal glucose metabolism. We also aimed to describe the relative kinetics of BAs in the fasting and post-prandial period in recipients of OGTT and examine the temporal correlation between fasting BAs with fasting insulin and glucose concentrations. We assessed BA profiles by measuring primary and secondary BAs, 12α-hydroxylated and non-12α-hydroxylated fractions, as well as their glycine and taurine conjugates.

Subjects and Methods:

- **Subjects:**

This case-control study was approved by the local ethics committee of Devon and Cornwall REC / Plymouth University Hospital, UK (Ref: 1978, UKCRN number 4444) and was conducted according to the principles of the Helsinki declaration. All patients gave written informed consent. The power calculation was based on a previous study by our group comparing post-prandial BA response between patients with T2DM and normal individuals (7).

Power calculation: 19 participants in each group are required if the mean post-prandial delta (*Δ*) change (difference between baseline and maximal response) of total BA in women with HOMA-IR>2.8 is 4.28 µmol/L and women with HOMA-IR≤ 2.8, 0.88 µmol/L with a standard deviation of 4.45 and 1.08 µmol/L respectively; at a significance level of 0.05 this would give a power of 90%.

This case-control study recruited 45 women aged between 31 and 57 years with a history of previous GDM. All recruitment was carried out at Plymouth University Hospital. All were diagnosed with GDM 10 years prior to the study but had recovered to be euglycaemic at the time of study. The women were divided based on their HOMA-IR value into two groups in order to investigate whether there is a difference in post-meal BA response and other parameters between those with HOMA-IR > 2.8 vs. ≤ 2.8 (control group). All women underwent a formal oral glucose tolerance test (OGTT). After an overnight fast an in-dwelling cannula was inserted into the antecubital fossa. 75 g glucose powder was dissolved in 200 ml warm water to be drunk over 5 minutes. Blood samples (serum and plasma) were collected at baseline and then at 30, 60, 90 and 120 minutes. Samples were stored for six months at -80 °C until analysis. Samples were analysed for glucose, primary and secondary BA as well as their glycine and taurine conjugates at all the time points. Insulin was measured on fasting samples.

- **Methods**

1. BA measurement:

BAs were measured in three separate batches by an established in-house method using liquid chromatography tandem mass spectrometry (LC-MS/MS) in the department of Clinical Biochemistry (Viapath Analytics) at King's College Hospital NHS Foundation Trust, London, UK. A Jasco high performance liquid chromatography (HPLC) system with three PU-pumps, MX-2080-32 solvent mixing module, AS-2059 autosampler and CO-2067 column oven was used (Tokyo, Japan). The HPLC system was connected to an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Cheshire, UK) operated with an electrospray ionisation (ESI) source. All solvents and chemicals used were of analytical grade.

1.a. LC-MS/MS method

LC-MS/MS assay was performed by modification of an existing protocol for plasma BA fractionation. Chromatography was performed using an Acentis fused-core C18 analytical column (150 x4.6mm, particle size 2.7µm, Sigma-Aldrich) incubated at 40°C. Mobile phase A was made from methanol + 0.012% formic acid (v/v) +5mM ammonium acetate (w/v) PH 4.50. Mobile phase B was made from deionised H2O + 0.012% formic acid (v/v) + 5mM ammonium acetate (w/v) PH 7.31. Mobile phase gradients were delivered at a flow-rate of 0.6 mL/min, with 70% solvent A and 30% Solvent B at time 0 min, 95% solvent A and 5% solvent B at time 10 min and 95% solvent A and 5% solvent B at time 14 min. Negative ion mass spectra of the fractionated BAs were recorded in multiple reaction-monitoring (MRM) mode. The transitions were as following:

m/z 391.2/391.2 for DC, CDC and UDC,
m/z 407.1/407.1 for CA,
m/z 375.2/375.2 for LC,
m/z 448.2/74.1 for GDC, GCDC and GUDC,
m/z 464.2/74.1 for GCA,
m/z 432.1/74.1 for GLC,
m/z 498.2/80.0 for TDC, TCDC and TUDC,
m/z 514.0/80.0 for TCA,
m/z 482.2/80.0 for TLC,
m/z 395.2/395.2 for D4-DC,

m/z 452.1/74.1 for D4-GDC

m/z 502.2/80.0 for D4-TDC.

AnalystTM Software version 1.4.2 (Applied Biosystems) was used to acquire data and quantitation was carried out using peak area analysis corrected by comparison to internal standards (unconjugated, glycine- or taurine-conjugated d4-DCA). The column was washed between injections with 100µL of autosampler wash (70% (v/v) methanol, 30% (v/v) H2O).

1. a. 1. Standard preparation

A stock solution containing all BAs was prepared at a concentration of 4mM in methanol and used to make 20, 10, 5, 1, 0.1 and 0.05µM calibration standards by serial dilution in phosphate buffered saline (PBS). 250µL aliquots were stored at -20°C. This method is linear for all the BAs between 0.17 and 10 µmol/L.

. BAs with shown retention times for each fraction (Table 1) were adequately separated (Figure 1).

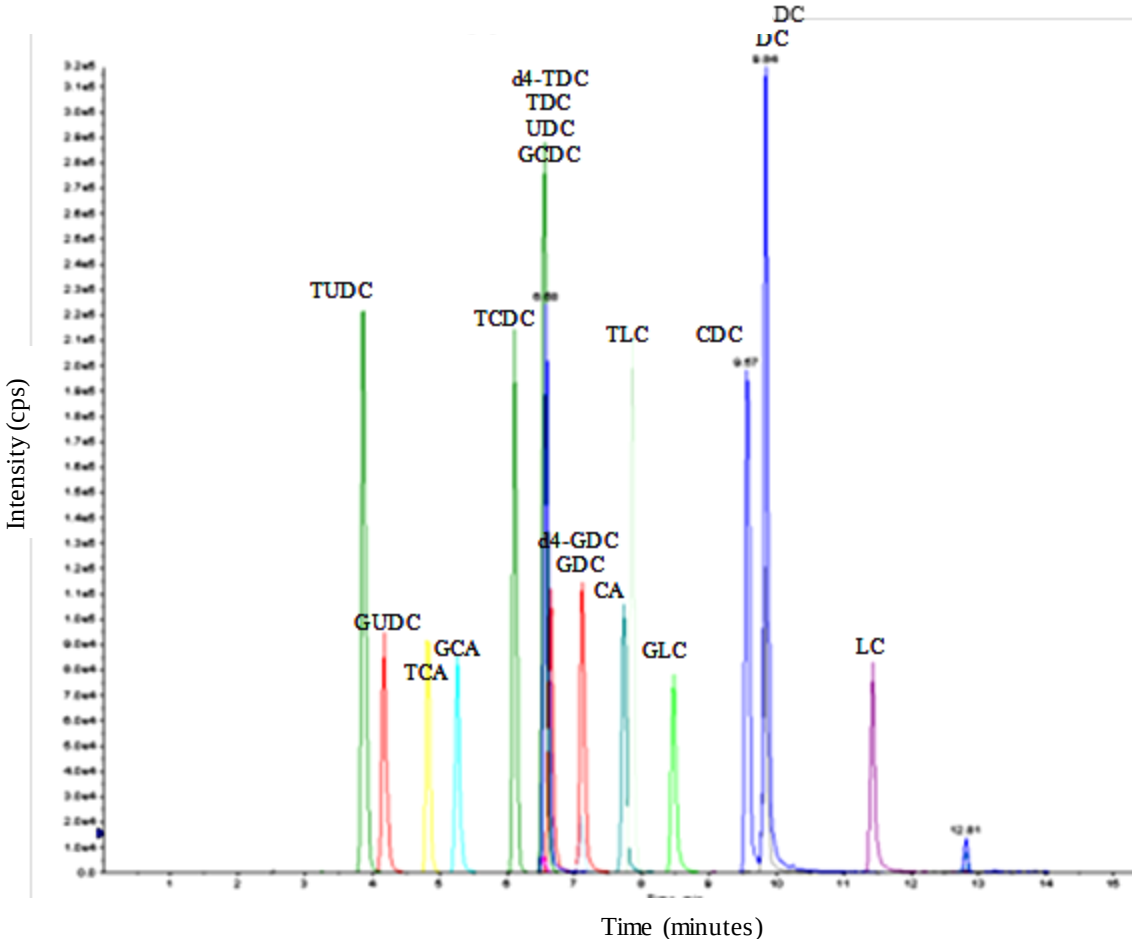

Figure 1. Bile acid chromatogram

A chromatogram of a 1µM calibration standard showing all the bile acid analytes measured. *CA* Cholic acid; *CDCA* Chenodeoxycholic acid; *d4-DCA* Deoxycholic-2,2,4,4-d4 acid; *d4-GDCA* Glycodeoxycholic-2,2,4,4-d4 acid; *d4-TDCA* Taurodeoxycholic-2,2,4,4-d4 acid; *DCA* Deoxycholic acid; *GCA* Glycocholic acid; *GCDCA* Glycochenodeoxycholic acid; *GDCA* Glycodeoxycholic acid; *GLCA* Glycolithocholic acid; *GUDCA* Glycoursodeoxycholic acid; *LCA* Lithocholic acid; *TCA* Taurocholic acid; *TCDCA* Taurochenodeoxycholic acid; *TDCA* Taurodeoxycholic acid ; *TLCA* Taurolithocholic acid; *TUDCA* Tauroursodeoxycholic acid; *UDCA* Ursodeoxycholic acid.

Table 1. Bile acid retention times

Column retention times for each of the bile acid fraction and internal standards. *BA* Bile acid.

BA	Retention time (min)
TUDCA	3.91
GUDCA	4.27
TCA	4.85
GCA	5.27
TCDCA	6.53
D4-TDCA	6.60
TDCA	6.60
UDCA	6.60
GCDCA	6.60
D4-GDCA	6.76
GDCA	7.10
CA	7.77
TLCA	7.69
GLCA	8.55
CDCA	9.60
D4-DCA	9.84
DCA	9.83
LCA	11.50

1. a. 2. Plasma sample preparation

After extraction of BAs from plasma, 800µL of protein precipitating solution (acetonitrile containing the three internal standards) was added to 250µL plasma and vortex-mixed. Samples were centrifuged at 13,000 rpm for 10 minutes and 900µL of the supernatant was removed. The supernatant was then dried down using compressed air at 60°C. The residue was re-suspended in 250µL of a 50:50 (v/v) mix of the mobile phases (A&B). 10µL of this solution was injected onto the column (equivalent to 8.57µL of the original plasma sample).

1. b. Linearity checks were carried out with aliquots of pooled serum spiked with a combined BA stock at different concentrations and then serially diluted with pooled plasma. Creation of a blank matrix proved difficult; endogenous BA could not be removed from plasma by charcoal stripping and BA was found to be present in bovine serum albumin (BSA). Therefore the lower concentrations of the linear range were established by serial dilution with PBS solution. The linear ranges were variable, but for the purpose of simplicity the range 0.1 - 10µM was used as this was valid for all bile acids with R^2 value ranging from 0.9886 – 0.9999.Lower and upper limits of linearity in umol/L for each bile acid were as follows; CA, Cholic acid (0.08-20.0); CDCA, Chenodeoxycholic acid (0.07-20.0); GCA, Glycocholic acid (0.1-20.0); GCDCA, Glycochenodeoxycholic acid (0.16-20.0);

GDCA, Glycodeoxycholic acid (0.14-20.0); GLCA, Glycolithocholic acid (0.17-10.0);

GUDCA, Glycoursodeoxycholic acid (0.06-20.0); LCA, Lithocholic acid (0.16-20.0);

TCA, Taurocholic acid (0.07-20.0); GCA, Glycocholic acid (0.1-20.0); TCDCA, Taurochenodeoxycholic acid (0.07-20.0); TDCA, Taurodeoxycholic acid (0.07-20.0); TLCA, Taurolithocholic acid (0.17-10.0); GLCA, Glycholithocholic acid (0.17-10.0);

TUDCA, Tauroursodeoxycholic acid (0.05-10.0); UDCA, Ursodeoxycholic acid (0.07-10.0); DCA, Deoxycholic acid (0.08-10.0). The coefficient of variations (CV) for all BAs was acceptable and ranged from 3.6% to 12% at the lower limit of quantitation. The inter-assay CV was 3.6-8.0%.

1. c. interferences

The most likely interferences are posed by ion suppression and isobaric contaminants. Ion suppression and isobaric contaminants exists between 2 to 2.5

minutes and after 14 minutes on each sample injection and thus is not a problem for this assay as the analytes elute between these times. No effects from the possible interferences such as haemolysis, lipaemia and icterus are seen in this method (recoveries ranged from 91 -109 %). We did not evaluate for any specific drug interferences but to our knowledge there are no known interactions. Use of high dose uersodeoxycholic acid can alter the BA pool and composition (8) however; none of the patients in our study were on this medication.

1. d. Carry-Over/ Carry-Under

As a result of the incorporation of a wash run into the protocol and alteration of the auto-sampler flush settings and flush solution, no carry-over or carry-under was seen.

1. e. Recovery

The method has good recovery for all concentrations ranging from 0.5, 1, 5 and 10 µM for all BAs (recoveries ranging from 91– 115%).

2. Glucose measurement:

Glucose was measured by enzymatic hexokinase method on the Siemens Advia 2400 (Frimley, UK). Inter-assay CVs% were 1.6% at 6.4 mmol/l and 1.0% at 16.0 mmol/l.

3. Insulin measurement:

Insulin was measured by a two-site sandwich immunoassay using direct chemiluminometric method on the Siemens Centaur (Frimley, UK). Intra-assay CVs% were 4.6, 3.2 and 3.3 % and inter-assay precisions 5.9, 2.6 and 4.8% at 14.68, 45.72 and 124.51 mU/L respectively.

Statistical analysis

Statistical analysis was performed using Analyse-It® version 2 (Leeds, UK). Data were tested for normality using the Shapiro-Wilk W test with a confidence interval of 95%. Given the nonparametric data, groups were compared using Mann Whitney U test and correlations by Spearman's Rank. The 12α-hydroxylated and non-12α-

hydroxylated BA fractions, HOMA-IR and delta (Δ) change (difference between baseline and maximal post-prandial response) were calculated. All data is reported as median and inter-quartile range (IQR). A p value ≤ 0.05 was taken as statistically significant.

Results:

Patient characteristics are shown in Table 1 and correlation studies between women with HOMA-IR ≤2.8 and >2.8 in Table 2. Body mass indices and fasting insulin concentrations were higher in individuals with insulin resistance (both p<0.001). Fasting glucose concentrations were similar between the two groups (HOMA-IR ≤2.8, p=0.08 and >2.8, p=0.13).

Table 2. Comparisons of the analytes between women with HOMA-IR ≤2.8 and >2.8.; HOMA-IR Homeostasis model assessment-estimated insulin resistance index; F Fasting; BAs Bile acids. Δ Delta change (The difference between basal and maximal post-prandial response); Values are median concentration with IQR.

Comparisons	HOMA-IR ≤ 2.8 (n=25)	HOMA-IR> 2.8 (n=20)	p-value
Age (year)	42 (38-44)	43 (39-47)	0.51
BMI (Kg/m^2)	25 (23-27)	30 (27-37)	**<0.001**
Insulin (mIU/L)	5.61 (3.8-8.2)	**161 (12.9-9)**	**<0.0001**
Glucose (mmol/L)	5.0 (4.4-4.8)	5.0 (4.6-5.6)	0.08
HOMA-IR	1.2 (0.8-1.6)	3.6 (3.1-4.3)	**<0.0001**
Fasting total BAs			
F total BAs (µmol/L)	1.53 (1.12-1.91)	1.43(1.04 -1.98)	0.63
F total primary BAs (µmol/L)	0.06 (0.04-0.22)	0.24 (0.16-0.33)	**0.004**
F total secondary BAs (µmol/L)	0.19 (0.14-0.23)	0.16 (0.14-0.32)	0.83
F total glycine-conjugated BAs (µmol/L)	0.89 (0.54 -1.38)	0.80 (0.49-1.22)	0.86
F total taurine-conjugated BAs (µmol/L)	0.27 (0.16-0.41)	0.21(0.17-0.29)	0.56
F total unconjugated BAs (µmol/L)	0.69 (0.32-0.89)	0.91 (0.56-1.84)	**0.02**
F total 12α-hydroxylated BAs (µmol/L)	0.21 (0.15-0.44)	0.22 (0.18-0.29)	0.97
F total non12α-hydroxylated BAs (µmol/L)	0.16 (0.08-0.21)	0.19 (0.14-0.22)	0.17
Δ Total BAs			
Δ total BAs (µmol/L)	2.61 (1.74-3.79)	2.62 (1.85-4.50)	0.96
Δ total primary BAs (µmol/L)	0.13 (0.03-0.50)	0.29 (0.07-0.60)	0.25
Δ total secondary BAs (µmol/L)	0.18 (0.07-0.31)	0.21 (0.05-0.55)	0.43
Δtotal glycine-conjugated BAs (µmol/L)	2.24 (1.34-2.89)	1.76 (1.42-2.74)	0.64

Δtotal taurine-conjugated BAs (µmol/L)	0.33 (0.20-0.54)	0.23 (0.13-0.34)	**0.03**
Δtotal 12α-hydroxylated BAs (µmol/L)	0.15 (0.05-0.53)	0.09 (0.04-0.35)	0.63
Δ total non12α-hydroxylated BAs (µmol/L)	0.09(0.025-0.24)	0.10 (0.06-0.29)	0.35

Fasting primary BAs and Δtaurine-conjugated BA were higher in women with HOMA-IR≤ 2.8 (p=0.03). No difference was seen between the two groups in post-prandial changes of primary and secondary BAs, glycine-conjugated, 12α-hydroxylated and non-hydroxylated fractions (all p>0.05).

Table 3. Correlation studies between women with HOMA-IR ≤2.8 and >2.8.; *HOMA-IR* Homeostasis model assessment-estimated insulin resistance index; Values are median concentration with IQR.

Correlations		**HOMA-IR ≤ 2.8 (n=25)**	**HOMA-IR > 2.8 (n=20)**
BMI	secondary BAs	R^2= 0.44, **p=0.05**	R^2= -0.41, p=0.10
BMI	primary BAs	R^2= 0.17, p=0.42	R^2= -0.30, p=0.23
Glucose	total primary BAs	R^2= 0.32, p=0.11	R^2= -0.30, p=0.23
Glucose	total secondary BAs	R^2= -0.01, p=0.95	R^2= -0.41, p=0.10
Glucose	12α-hydroxylated BAs	R^2= 0.35, p=0.07	R^2= -0.12, p=0.64
Glucose	12α-non-hydroxylated BAs	R^2= 0.22, p=0.26	R^2= - 0.54, **p=0.03**
Insulin	glycine-conjugated BAs	R^2= 0.51, **p=0.007**	R^2= 0.02, p=0.91
Insulin	taurine-conjugated BAs	R^2= 0.54, **p=0.003**	R^2= -0.11, p=0.64
HOMA-IR	glycine-conjugated BAs	R^2= 0.41, **p=0.03**	R^2= 0.36, p=0.15
HOMA-IR	taurine-conjugated BAs	R^2= 0.44, **p=0.03**	R^2= 0.15, p=0.6
HOMA-IR	12α-hydroxylated BAs	R^2= 0.04, p=0.84	R^2= 0.22, p=0.38
HOMA-IR	non-12α-hydroxylated BAs	R^2= 0.09, p=0.67	R^2= -0.06, p=0.81

There was a positive correlation between BMI and secondary BA concentration and between fasting insulin and HOMA-IR with glycine- and taurine-conjugated BAs in individual with insulin resistance (Table 2) (All p≤0.05). A negative correlation was observed between fasting glucose and non-12α-hydroxylated BAs in individuals with HOMA-IR>2.8.

Median concentration of total BAs and total taurine conjugated BAs as well as other BA components were measured following a test meal in women with HOMA-IR ≤ 2.8 and >2.8 at different time points. The post-prandial response of total and total taurine-conjugated BAs between the HOMA-IR≤ 2.8 and >2.8 are shown in Figures 1 and 2 respectively. Figure 1 shows that total BAs concentration was not different at given time points between the case and control group, however; this difference was observed in total taurine-conjugated BAs concentrations between the two groups as presented in Figure 2 and shown in Table 1.

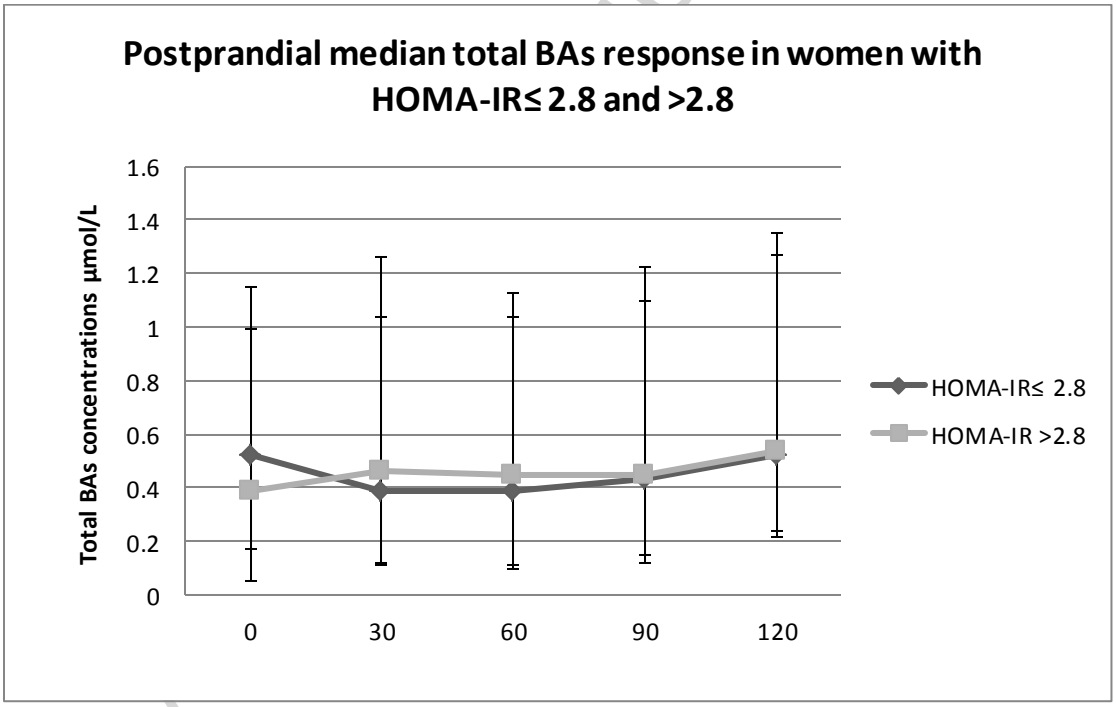

Figure 2. Post-prandial median total BAs in women with HOMA-IR ≤ 2.8 and >2.8. Median concentration of total BAs following a test meal in women with HOMA-IR ≤ 2.8 and >2.8 at different time points (minutes). *BAs* Bile acids; *HOMA-IR* Homeostasis model assessment-estimated insulin resistance index.

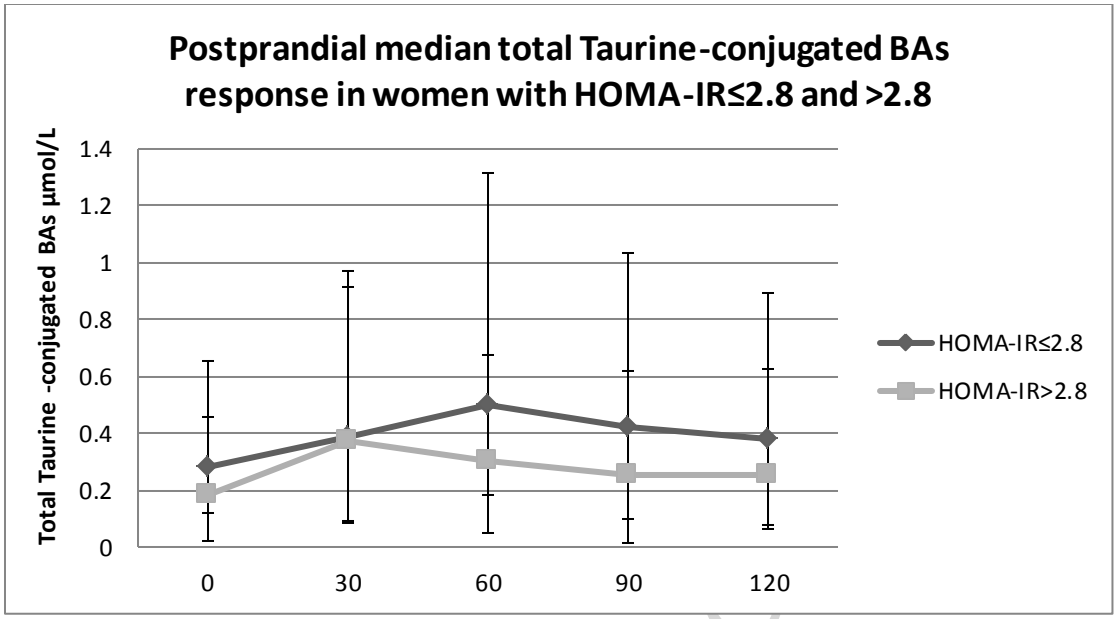

Figure 3. Post-prandial median total taurine-conjugated BAs in women with HOMA-IR ≤ 2.8 and >2.8. . Median concentration of taurine-conjugated BAs following a test meal in women with HOMA-IR ≤ 2.8 and >2.8 at different time points (minutes). *BAs* Bile acids; *HOMA-IR* Homeostasis model assessment-estimated insulin resistance index.

Discussion

Our study demonstrates that BA metabolism is altered in those with continued insulin resistance after GDM. Fasting total primary BAs and total unconjugated BAs were increased in women with HOMA-IR >2.8. The Post-prandial changes of taurine-conjugated BAs were lower in women with HOMA-IR >2.8. A recent study has shown that total taurine-conjugated BAs show an inverse association with glycaemic status with a strong discriminative power between control and GDM pregnant women (4) as was seen in this study. Another study reported that concentrations of total taurine-conjugated BAs were higher in patients with T2DM compared with normoglycaemic controls (10) but our study did not find any difference between fasting taurine-conjugated BAs concentration in women between the two groups. However, it is worth considering that our study samples were taken from non-diabetic individuals which may explain the discordance between the reported data. Nonetheless, post-prandial taurine-conjugated BAs response was increased in the individuals with HOMA-IR≤ 2.8 in keeping with the findings of Wewalka *et al* (9). Further analysis with a larger cohort will possibly better explain trends in BA species.

Pregnancies associated with maternal insulin resistance may progress towards GDM (4). BA metabolism is altered in T2DM (3) but, little is known about BAs metabolism and composition in normal pregnancies or GDM. Recent studies have focused in BAs subspecies. The enzymes *CYP7A1* (rate limiting) and *CYP8B1* (12α hydroxylase) drive the BA pool and composition respectively (10). Non-12α-hydroxylated primary BA, CDCA, and secondary BA LCA have greater affinity for the receptors FXR and TGR5 respectively (11). Our study investigated the changes of 12α-hydroxylated and non 12α-hydroxylated BAs in a group of women with a history of GDM with HOMA-IR> 2.8 compared to those with normalised insulin metabolism. Studies suggest that 12α-hydroxylated BAs are higher in insulin resistant individuals compared with healthy subjects (10, 11). In our study no difference was seen between the fasting concentrations of total 12α-hydroxylated and non 12α-hydroxylated BAs between the two groups. However, fasting total non-12α-hydroxylated BAs showed a negative correlation with glucose in women with HOMA-IR>2.8. Thus an increase in non 12α-hydroxylated BA may contribute to improved glucose metabolism after GDM.

Intestinal bacterial flora converts primary BAs to secondary BAs (2). Gut microbiota have been shown to be altered in a number of metabolic conditions such as obesity, diabetes and cardiovascular disease (1, 9). A study showed that T2DM and increased resistance to insulin associate with elevated secondary BA, DCA and decreased primary BA, CDCA likely due to altered intestinal flora activity (1, 11). This study did not investigate changes in intestinal flora in GDM. Our findings are compatible with those studies in showing an increase in fasting total primary BAs in the group of individuals with HOMA-IR> 2.8 compared with subjects with HOMA-IR≤ 2.8.

A positive correlation was seen between BMI and fasting total secondary BAs in subjects with HOMA-IR≤ 2.8. A study in obese subjects showed that GLCA (a conjugated secondary BA) levels decreased after bariatric surgery (12). The relative composition of the gut flora during early life can predict the subsequent development of obesity (13). Levels of lipopolysaccharide (LPS), an endotoxin derived from the cell walls of a gram-negative bacterium are increased in obese

individuals. Changes in LPS and intestinal bacterial flora after bariatric surgery have been postulated to alter BA metabolism (13). In our study total primary BAs in the group were increased in patients with continuing insulin resistance (HOMA-IR >2.8) compared with those with normal glucose metabolism.

Limitations and further work

The power calculation was adequate to compare the changes in primary and secondary BAs but maybe inadequate for multiple comparisons and to resolve changes in individual BAs. Hence, some of the potentially significant changes in the concentrations of BA fractions may have resulted only on relative changes in our study. Furthermore individuals with HOMA-IR >2.8 had higher BMI values compared to those with HOMA-IR ≤2.8 and on average were more overweight to obese. Therefore, the altered BA metabolism could be either cause or effect of higher BMI.

A meta-analysis suggested that elevated concentrations of inflammatory markers such as interleukin-6 (IL-6) and C-reactive protein (CRP) are associated with increased risk of T2DM (14). BAs are known to have regulatory impacts on the innate immune system. Innate immune system includes macrophages which secrete inflammatory markers such as IL-6 and tumour necrosis factor-α (TNF-α) (15). We did not measure inflammatory markers in this study. A history of concomitant medications and other co-morbidities were not well documented in our cohort.

Alterations in sex hormone metabolism are associated with elevated levels of BAs in OC (16). Measurements of oestrogen, progesterone and their metabolites may provide further insights into alterations of BAs and glucose metabolism during pregnancy. This study was performed in a post-partum cohort diagnosed with GDM in their previous pregnancies 10 years ago. More informative studies might be useful during pregnancy or immediately post-partum.

In patients with previous GDM this study shows that BAs metabolism is altered in those with insulin resistance (HOMA-IR >2.8) compared with those who have lower

HOMA-IR values. Changes in 12α-hydroxylated and non-12α-hydroxylated BAs in women with HOMA-IR ≤2.8 or >2.8 were investigated. In women with HOMA-IR>2.8 a negative correlation between glucose concentrations and non-12α-hydroxylated BAs were seen, which is compatible with the hypothesis of alteration of non 12α-hydroxylated fractions of BAs in insulin resistance. Post-prandial dynamic changes in BAs after GDM were investigated. Women with HOMA-IR≤2.8 had elevated post-prandial taurine-conjugated BAs response compared with those with higher HOMA-IR.

In conclusion, following GDM, individuals with HOMA-IR values of >2.8 have altered conjugated forms of BAs as well as non-12α-hydroxylated fractions compared to those in whom insulin metabolism had normalised. However, it remains to be elucidated if the altered BA metabolism is a contributing factor to the pathogenesis or a consequence of GDM.

Disclosure: The authors report no conflicts of interest in this work.

Acknowledgments

AM, NS and JP were involved in conceiving the clinical study design, patient recruitment and ethical approval of the study. RV, JA and NM conceived the bile acid study design. NM carried out literature review, sample analysis and statistical analysis. GC was involved in analytical work. RV was involved in statistical analysis of the data. NM wrote the first draft of the manuscript and JA, RV, ASW, JP and NS read and edited the manuscript. All authors approved the final version for submission.

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