Maternal hair metabolome analysis identifies a potential marker of lipid peroxidation in Gestational Diabetes Mellitus

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Keywords: Gestational Diabetes Mellitus, Metabolomics, Hair metabolome, Adipic Acid

Introduction

Gestational diabetes mellitus (GDM) is defined as an abnormal glucose tolerance that develops, or is first recognized during pregnancy; the development of GDM markedly increases risks of adverse obstetric and perinatal outcome (1). The immediate consequences include an increased likelihood of a Caesarean section, hypoglycaemia of the newborn, respiratory distress syndrome, and macrosomia (1). Longer-term implications of a pregnancy affected by GDM include a substantially increased risk of the mother developing type 2 diabetes postnatally, as well as the offspring having an increased susceptibility to obesity and related metabolic complications in adulthood (1). Within the Asia-Pacific region there are an estimated 76 million women at risk of having a pregnancy complicated by diabetes (2), with recent estimates suggesting up to 18% of pregnancies in China may be complicated by GDM (3).

Metabolomic profiling is a strategy for investigating the low weight molecules that represent the metabolome of a cell, tissue, or organism. The metabolome's position as a downstream product of gene expression enables the provision of a high-resolution multifactorial phenotypic signature of disease aetiology, manifestation, or pathophysiology, and has led to the search for metabolite biomarkers (e.g. 4). Previous studies have explored the GDM-specific metabolomic profile of blood samples, with promising results (5,6). However, the dynamic nature of biofluids can be influenced by many transient factors such as recent dietary intake, and hormonal changes. Analysis of blood requires invasive sampling, immediate processing and curation of samples under controlled circumstances if analysis is not immediate. Hair, in contrast, is a highly stable structure retaining endogenous compounds and reflecting environmental exposures for many months; moreover, hair sampling is non-invasive and the storage and processing of hair is much simpler, making it a particularly useful source of biomarkers in low resource settings. We have previously demonstrated the potential of the hair metabolome by identifying a metabolomic signature able to predict the subsequent development of fetal growth restriction (7).

The current pilot study aimed to investigate the maternal hair metabolome in relation to GDM outcome, to determine if maternal hair could be a source of metabolic information underlying the development of GDM.

Methods

Our study utilized hair samples from 94 pregnant women attending the first affiliated hospital of Chongqing Medical University, Chongqing, China. Samples were obtained at the time of Oral Glucose Tolerance Testing (OGTT) (24-28 weeks gestation) from the occipital lobe region, as previously described (7). The sample group contained 47 cases that were diagnosed with GDM (Fasting blood glucose ≥5.1mmol/l, OR blood glucose after 1 hour ≥10.0mmol/l, OR blood glucose after 2 hours ≥ 8.5mmol/l) and 47 controls with uncomplicated pregnancies. Case and control groups were matched for BMI and age (Table 1). Informed consent was obtained from the participants and ethical approval was granted by the Ethics committee of the first affiliated hospital of Chongqing Medical University.

The analytical component of the study was conducted at The University of Auckland, New Zealand. The samples were first washed in 10 ml of MQ water followed by a wash with 10 ml of methanol. Hair was weighed into 10mg (+/- 1mg unless the subject had <10mg of hair, in which case, all hair was used) portions and placed into glass vials. The samples were incubated for 23 hours at 54˚C in 1ml of 1M KOH. The dissolved hair specimen was then neutralized by the addition of 84 µl of 9M H_2SO_4. After centrifugation to remove the salts, the supernatant was concentrated in a SpeedVac Concentrator with a Refrigerated Vapor Trap (Thermo Scientific, New Zealand). Samples then underwent extraction with 1ml of 70% v/v MeOH:H_2O. After centrifugation at 2000 g for 5 min the supernatent was again concentrated on the SpeedVac. Dried samples were

derivatised using the alkylation procedure described in Smart et al. (8) and then analysed on the Thermo Scientific Trace Gas Chromtaogram Ultra coupled to an ISQ Mass Spectrometer GC-MS at the Centre for Genomics, Proteomics and Metabolomics at the University of Auckland, using settings previously described (8).

Chromatographic data was deconvoluted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) (online software distributed by the National Institute of Standards and Technology, USA, - http://www.amdis.net/) before metabolite identification and relative quantitation was performed using in-house R xcms based software and an in-house MS library of commercially available standards (13). The data was normalized by biomass and sum-normalization before statistical analysis was performed using SPSS version 21.0 and Metaboanalyst 2.0 (9). An Independent Samples T-test taking into account unequal variance was conducted to compare metabolite levels between groups (the threshold $P<0.01$ was applied, to account for multiple comparisons). Correlations between the hair metabolome and the potential confounders- age and BMI, were explored using a Pearson's Product-Moment correlation. An ANCOVA analysis was performed to assess the effect of age and BMI on the metabolites that differed between groups.

Results

Thirty metabolites were identified, using an in-house mass spectral library (Table 2).

One metabolite was found to differ significantly in GDM cases as compared to controls: adipic acid (P=0.002; FDR= 0.065) (Figure 1). The area under the ROC curve was 0.662.

Octanoic acid, leucine, and phenylalanine demonstrated a significant negative correlation with BMI, and adipic acid and citric acid a significant positive correlation with age (Table 3) in the control group only.

An ANCOVA analysis demonstrated that adipic acid remains significant when adjusted for BMI and age (P=0.009).

Discussion

Findings from the current pilot study provide evidence of a distinguishable difference in the hair metabolome between women that develop GDM and those with uncomplicated pregnancies. To the authors' knowledge, this study is the first to investigate the GDM-related hair metabolome using an untargeted approach.

Adipic acid was found at significantly higher levels in the hair of GDM cases in our study when compared to controls. Adipic acid is a dicarboxylic acid used in the food industry as an additive for its flavour and texture components. The identification of adipic acid in transient biofluids such as blood and urine is often considered a result of recent food consumption (10). However, previous studies have reported higher adipic acid levels in the urine of subjects with Type 2 diabetes: it was suggested that the elevated levels were the end product of omega oxidation resulting from lipid peroxidation that occurs in the oxidative stress environment observed in diabetes (11). This detrimental environment also presents itself in GDM (12).

A limitation of our study was the analysis of the full length of each hair sample; the hair metabolome identified was thus influenced by metabolism in early pregnancy and prior to pregnancy. However, this suggests that earlier sampling is likely to identify women who subsequently develop GDM. Future studies should investigate whether analysis of hair grown only during the time of pregnancy/initial pre-pregnancy period, similarly demonstrate increased concentrations of adipic acid.

Our finding that some metabolites found in the hair metabolome were significantly correlated with age and BMI provides justification for the matching of case and control groups by these two potential confounding factors in future studies. Despite these relationships only occurring as statistically significant in the control groups, an assessment of the 95% confidence intervals show an overlap between the groups, indicating that despite a varied

significance, there is little evidence of discordant correlation between the groups.

Currently, hair metabolome analysis is in its infancy. We are endeavoring to develop a method for LC-MS analysis so that a comprehensive profile of the metabolites in hair can be elucidated using a range of analytical platforms.

Validation of our preliminary findings, augmented by an investigation of the hair metabolome during specific time periods, could provide potential clinical applications for the prediction of GDM.

Acknowledgements:

The authors wish to thank Gravida: National Centre for Growth and Development for funding the project. All metabolome analyses were carried out at the Centre for Genomics, Proteomics and Metabolomics of the University of Auckland.

Funding:

The study was funded by Gravida: National Centre for Growth and Development (New Zealand). KS and PNB are supported by Gravida: National Centre for Growth and Development (New Zealand); JVDS by the Agnes Paykel Trust (New Zealand).

Contribution Statement:

The concept for this study originated with PNB. XH was responsible for participant recruitment and sample collection. KS, T-LH, and SGV-B developed the method for hair analysis and provided technical assistance. JVDS performed the GC-MS analysis and statistical analysis. JVDS drafted the manuscript and all authors critically reviewed the results and the manuscript prior to submission.

Conflict of Interest: None

Statement of Human and Animal Rights:

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

Statement of Informed Consent:

Informed consent was obtained from all patients included in the study.

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Table 1.

		P-value
BMI		
GDM	26.27 ± 3.23 [1]	
Controls	26.32 ± 3.28 [1]	0.938 [a]
Age		
GDM	30 (27, 32) [2]	
Controls	29 (27, 32) [2]	0.721 [b]

[1] mean ± standard deviation

[2] median (25th percentile, 75th percentile)

[a] Independent Samples t-test

[b] Mann-Whitney U test for non-parametric data

Table 2. List of metabolites identified in hair samples

Metabolites		
2-Oxobutyric acid	Glycine	Proline
2-Oxoglutaric acid	Lactic acid	Pyroglutamic acid
Adipic acid	Leucine	Pyruvic acid
Alanine	Malonic acid	Quinic acid
Aspartic acid	Methionine	Serine
Citramalic acid	N-Acetylglutamic acid	Stearic acid
Citric acid	Octanoic acid	Succinic acid
Cysteine	Oxalic acid	Threonine
Fumaric acid	Palmitic acid	Tyrosine
Glutamic acid	Phenylalanine	Valine

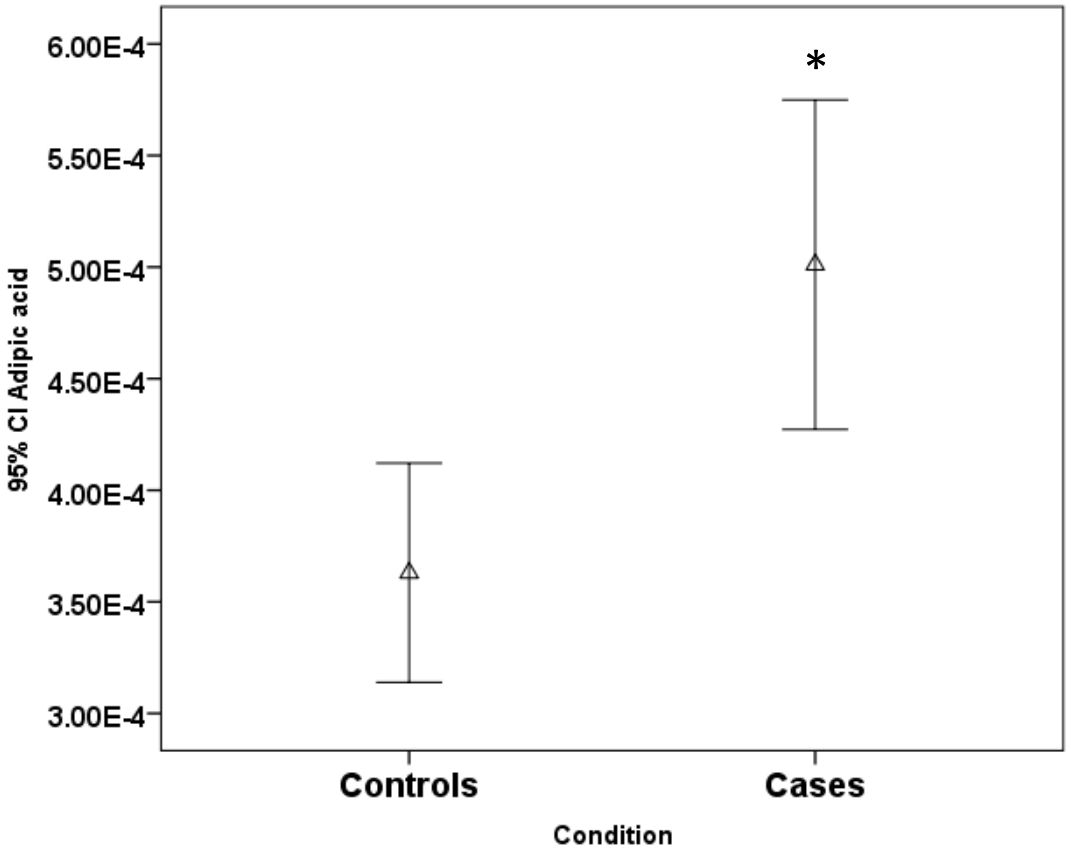

Figure 1. The distribution of Adipic acid levels among participants as displayed by the sum-normalized mean.
The triangles represent the mean relative abundance in each condition, and the bars represent the 95% confidence interval of the mean

* Statistically significant difference $P \leq 0.01$

Table 3. Correlation of hair metabolites with BMI and age

	Cases			Controls		
	95%CI	*R*	*P-value*	*95%CI*	*R*	*P-value*
BMI						
Octanoic Acid	-0.515 – 0.016	-0.23	0.14	-0.530 – -0.039	-0.30	**0.04**
Leucine	-0.461 – -0.043	-0.25	0.11	-0.550 – -0.074	-0.34	**0.02**
Phenylalanine	-0.425 – 0.008	-0.21	0.18	-0.543 – -0.046	-0.33	**0.03**
Age						
Adipic Acid	-0.233 – 0.396	0.07	0.64	0.093 – 0.565	0.33	**0.02**
Citric Acid	-0.368 – 0.204	-0.09	0.54	-0.062 – 0.588	0.29	**0.05**