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

\*Xiaoling He1 and \*Jamie V de Seymour2; Karolina Sulek2; Hongbo Qi1, Hua Zhang1, Ting-Li Han1,2, Silas G Villas-Bôas3; Philip N Baker1,2

1Department of Obstetrics and Gynaecology, The First Affiliated Hospital of Chongqing Medical University, China

2Liggins Institute, The University of Auckland, New Zealand

3School of Biological Sciences, The University of Auckland, Auckland, New Zealand

\*Joint first authors

Correspondence: Philip N Baker

Email: philip.baker@auckland.ac.nz

Phone: +64 9 923 3439

Fax: +64 9 373 8763

Liggins Institute,

Building 505

85 Park Road, Grafton

Auckland, New Zealand

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 H2SO4. 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:H2O. 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.

**References**

1. Reece, A E. The fetal and maternal consequences of gestational diabetes mellitus. The Journal of Maternal-Fetal and Neonatal Medicine, 2010; 23(3): 199–203
2. Federation ID. Diabetes Atlas 2013: <http://www.idf.org/diabetesatlas>.
3. Zhu WW, Yang HX, Wei YM, Yan J, Wang ZL, Li XL, et al. Evaluation of the value of fasting plasma glucose in the first prenatal visit to diagnose gestational diabetes mellitus in china. Diabetes Care. 2013 Mar;36(3):586-90.
4. Kenny LC, Broadhurst DI, Dunn W, et al. Robust early pregnancy prediction of later preeclampsia using metabolomic biomarkers. *Hypertension* 2010; **56**(4): 741-9.
5. Dudzik D, Zorawski M, Skotnicki M, et al. Metabolic fingerprint of Gestational Diabetes Mellitus. Journal of proteomics, 2014, 103: 57-71.
6. [de Seymour](http://www.researchgate.net/researcher/2051886943_Jamie_V_de_Seymour), J V, [Conlon](http://www.researchgate.net/researcher/2051884717_Cathryn_A_Conlon), C A, Sulek, K, [et al.](http://www.researchgate.net/researcher/2051883570_Silas_G_Villas_Boas) [Early pregnancy metabolite profiling discovers a potential biomarker for the subsequent development of gestational diabetes mellitus.](http://www.researchgate.net/publication/264245820_Early_pregnancy_metabolite_profiling_discovers_a_potential_biomarker_for_the_subsequent_development_of_gestational_diabetes_mellitus) Acta Diabetologica, 2014 [ePub ahead of print].
7. Sulek, K, [Han](http://www.researchgate.net/researcher/57954254_Ting-Li_Han), T-L, [Villas-Boas](http://www.researchgate.net/researcher/38546254_Silas_Granato_Villas-Boas), S G, et al. [Hair metabolomics: identification of fetal compromise provides proof of concept for biomarker discovery.](http://www.researchgate.net/publication/264245614_Hair_metabolomics_identification_of_fetal_compromise_provides_proof_of_concept_for_biomarker_discovery) Theranostics, 2014; 4(9):953-9.
8. Smart K F, Aggio R B M, Van Houtte J R, Villas-bôas S G. Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography–mass spectrometry. Nature Protocols, 2010, 5: 1709
9. Xia, J, Mandal, R, Sinelnikov, I, Broadhurst, D, Wishart, D S. MetaboAnalyst 2.0 - a comprehensive server for metabolomic data analysis. Nucl. Acids Res., 2012.
10. Metabocard for Adipic Acid (HMDB 00448) (n.d.).  Retrieved August 10, 2014, from Human Metabolome Database (HMDB) version 3.6.
11. Inouye, M, Mio, T, Sumino, K. Dicarboxylic acids as markers of fatty acid peroxidation in diabetes. Atherosclerosis, 2000, 148: 197–202
12. [Lappas, M](http://www.ncbi.nlm.nih.gov/pubmed?term=Lappas%20M%5BAuthor%5D&cauthor=true&cauthor_uid=21675877), [Hiden, U](http://www.ncbi.nlm.nih.gov/pubmed?term=Hiden%20U%5BAuthor%5D&cauthor=true&cauthor_uid=21675877), [Desoye, G](http://www.ncbi.nlm.nih.gov/pubmed?term=Desoye%20G%5BAuthor%5D&cauthor=true&cauthor_uid=21675877), [Froehlich, J](http://www.ncbi.nlm.nih.gov/pubmed?term=Froehlich%20J%5BAuthor%5D&cauthor=true&cauthor_uid=21675877), [Hauguel-de Mouzon, S](http://www.ncbi.nlm.nih.gov/pubmed?term=Hauguel-de%20Mouzon%20S%5BAuthor%5D&cauthor=true&cauthor_uid=21675877), [Jawerbaum, A](http://www.ncbi.nlm.nih.gov/pubmed?term=Jawerbaum%20A%5BAuthor%5D&cauthor=true&cauthor_uid=21675877). The role of oxidative stress in the pathophysiology of gestational diabetes mellitus. [Antioxid Redox Signal](http://www.ncbi.nlm.nih.gov/pubmed/21675877), 2011, 12: 3061-100
13. Smith C A, Want E J, O'Maille G, Abagyan R, Siuzdak G. XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching and identification, Analytical Chemistry, 2006, 78:779-787

***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

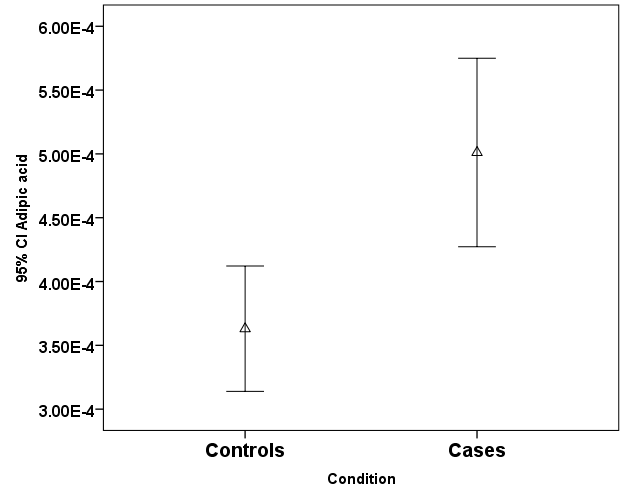
[2] median (25th percentile, 75th percentile)

[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 | | 2-Oxoglutaric acid | Lactic acid | | Adipic acid | Leucine | | Alanine | Malonic acid | | Aspartic acid | Methionine | | Citramalic acid | N-Acetylglutamic acid | | Citric acid | Octanoic acid | | Cysteine | Oxalic acid | | Fumaric acid | Palmitic acid | | Glutamic acid | Phenylalanine | | |  | | --- | | Proline | | Pyroglutamic acid | | Pyruvic acid | | Quinic acid | | Serine | | Stearic acid | | Succinic acid | | Threonine  Tyrosine | | 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*≤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** |