Short Communication

Metabolomic profiling of maternal hair suggests rapid development of Intrahepatic Cholestasis of Pregnancy

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Abstract:
Introduction: Intrahepatic Cholestasis of Pregnancy (ICP) is a common maternal liver disease; development can result in devastating consequences, including sudden fetal death and stillbirth. Currently, recognition of ICP only occurs following onset of clinical symptoms.
Objective: Investigate the maternal hair metabolome for predictive biomarkers of ICP.
Methods: The maternal hair metabolome (gestational age of sampling between 17+4 weeks and 41 weeks) of 38 Chinese women with ICP and 46 pregnant controls was analysed using gas chromatography–mass spectrometry.
Results: Of 105 metabolites detected in hair, none were significantly associated with ICP.
Conclusion: ICP development was not detectable in full-length hair strands, suggesting that ICP has rapid development - only distinguishable later in pregnancy.

Keywords: Intrahepatic Cholestasis of Pregnancy; Metabolomics; Hair; Gas Chromatography-Mass Spectrometry

1. Introduction

Intrahepatic Cholestasis of Pregnancy (ICP) is a pregnancy-related liver disease characterized by intense maternal itching, usually on the hand palms and feet soles, accompanied by elevated liver enzymes (alanine aminotransferase ALT and aspartate aminotransferase AST), and high serum bile acid levels[1]. The onset of symptoms typically occurs in the late second or early third trimesters[2]. Despite maternal symptoms residing after delivery, the development of ICP can have long term implications for both mother and offspring, including: fetal hypoxia, sudden fetal death, stillbirth, preterm delivery, meconium staining of the amniotic fluid, and an increased likelihood of additional maternal pregnancy complications such as gestational diabetes and preeclampsia[2,3]. The etiology of ICP remains unknown, but is hypothesized to be multifactorial, involving genetic, hormonal, and environmental components. This uncertainty of pathogenesis has limited the identification of women at risk of ICP development prior to the onset of symptoms. An investigation into biomarkers of ICP would allow for the development of an early pregnancy screening tool to identify risk of being diagnosed with ICP later in pregnancy and could also help elucidate mechanisms underlying ICP development and progression, highlighting potential therapeutic targets.

Hair samples have been utilized in forensic science for the detection of exogenous compounds including drugs, poisons, and toxic metal[4], but have also been analysed in the biomedical field for endogenous compounds such as cortisol, a stress hormone[5]. In comparison to transient biological specimens such as urine and plasma, hair offers unique chemical stability that captures long-term exposures, as hair grows at a rate of approximately one centimetre per month[6]. In turn, sustained environmental exposures, stress, and nutrition can affect the chemical composition of human hair. Additional benefits of analyzing hair samples compared to other biological specimens include its simple, non-invasive collection, low-maintenance storage, and relative sanitation.

Metabolomics is the study of low molecular weight compounds; the metabolome refers to the collection of these compounds (known as metabolites) that can be detected from a biological sample[7]. The metabolome reflects both endogenous processing as well as being influenced by environmental and lifestyle exposures, offering a snapshot of phenotype. The maternal hair metabolome has been able to accurately differentiate women that developed the pregnancy complication fetal growth restriction, from healthy controls[8]. A separate study has also identified a potential biomarker of gestational diabetes mellitus development in maternal hair[9].

The current study analysed the maternal hair metabolome in search for biomarkers of ICP development that could be useful for early detection of ICP risk. We hypothesise that the maternal environment plays a role in the development of ICP and that early detection of women at risk of ICP development could benefit from early intervention, provided a robust screening tool was available.

2. Materials and Methods

2.1 Study participants

Participants included in this case-control study were recruited from the First Affiliated Hospital of Chongqing Medical University, China (gestational age of sampling ranged between 17+4 weeks and 41weeks). Patients were diagnosed with ICP according to the criteria used in China (symptoms of itching accompanied by elevated total bile acids (>10 μmol/L), ALT (>40 U/L) and/or AST (>40 U/L))[10]. Ethics approval was granted by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (date: 2014-01-06) and informed consent was obtained from all participants.

2.2 Sample collection and preparation

Hair samples were collected from participants at recruitment, by cutting a small section of hair from the occipital region, 0.5cm from the scalp. The samples were stored at -20°C prior to chemical processing. All chemicals used in this study were of analytical grade.

To prepare the hair samples for metabolomic analysis, they were washed in 10 ml Milli-Q (MQ) water, followed by a 30 sec vortex, then in 10 ml methanol, followed by a 30 sec vortex. The two-step washing procedure was repeated. Hair samples from each participant were weighed (range 5.0-

5.3 mg) and placed into glass vials, followed by the addition of 400 µl of 1 M potassium hydroxide and 20 µl of 2,3,3,3-d4-alanine, which was used as an internal standard. Samples underwent an incubation period of 30 min at 54°C, centrifugation at 4000 rpm for 5 min, then a final incubation for 19 hr at 54°C. Following incubation, digested hair samples were neutralized with sulphuric acid. To extract metabolites, 1000 µl of methanol was added and the samples were centrifuged at 4000 rpm for 5 min. A 500 µl aliquot of the supernatant from each sample was transferred to a microcentrifuge tube and 200 µl of the remaining supernatant was transferred to a conical tube, to form a pooled quality control sample. Five negative controls were also prepared. The samples, negative controls, and QC samples were dried to completeness using a speedvac concentrator (Savant SPS121P Speedvac, Thermo) at a pressure of 0.8 kPa.

Methyl Chloroformate (MCF) derivatization was performed on all samples, according to Smart et al.[11]. In brief, samples were resuspended in 200 µl of sodium hydroxide (1 M) and vortexed for 30 sec; 167 µl of methanol was added and the samples were vortexed for 30 sec. The resulting solutions were transferred into silanized glass tubes, and 34 µl of pyridine was added. The derivatization started with the addition of 20 µl of MCF followed by a 30 sec vortex. A further 20 µl of MCF was added, followed by another 30 sec vortex. A 400 µl volume of chloroform was added and the derivatised sample was vortexed for 15 sec, followed by the addition of 400 µl of sodium bicarbonate (50 mM) and a 15 sec vortex. Samples were centrifuged for 2 minutes at 2000 rpm. The upper aqueous layer was removed and sodium sulfate added. The chloroform phase was transferred into amber glass GC vials, containing glass inserts.

2.3 Gas chromatography-mass spectrometry (GC-MS) analysis

The hair metabolome was characterized in this study using an Agilent 7890A gas chromatograph coupled to a 5975C inert mass spectrometer with a split/splitless inlet. GC-MS instrument parameters were based on the parameters described by Smart et al.[11] (listed in Online Resource 1).

2.4 GC-MS data pre-processing

The freely available Automated Mass Spectral Deconvolution and Identification System software[12] (AMDIS v2.66) was used to deconvolute the peaks from the GC-MS chromatograms, and an in-house library of MCF-derivatised metabolite standards was used to identify metabolites within the chromatogram, matching the peaks to the library standards according to the MS spectrum of the metabolite and its respective retention time. The relative abundance of the identified metabolites was determined using peak height. Metabolites were normalized using peak height of the internal standard (2,3,3,3-d4-alanine) and hair biomass.

2.5 Statistical analysis

Metabolite levels were converted to z-scores before statistical analysis was performed. Principal component analysis (PCA) of the complete hair metabolomic profiles was performed using Metaboanalyst v3.0[13], to check for outliers. Following removal of outliers, a non-parametric Mann Whitney test was conducted in SPSS v24 to test differences in metabolite levels between cases and controls. Metabolites with a P-value<0.05 were considered statistically significant. The Benjamini Hochberg procedure[14] was used to account for multiple comparison testing, generating false discovery rates for each metabolite (FDR). Receiver Operating Characteristic (ROC) curves were produced using Metaboanalyst v3.0 for each significant metabolite and their area under the ROC curve (AUC) was calculated, to determine their ability to correctly classify ICP cases from controls. Significant metabolites were entered into logistic regression models with confounding variables to check if their relationship with ICP remained significant.

3. Results and Discussion

84 participants were included in the study; 38 cases diagnosed with ICP and 46 healthy pregnant controls. Two participants were excluded from the study due to missing data and one was removed as they were deemed an outlier following the visual inspection of the PCA (Online Resource 2-3). Participant characteristics are displayed in Table 1. Gestational age at delivery was significantly earlier in women who developed ICP when compared to controls; maternal age and body mass index

(BMI) were significantly lower in cases; and the levels of TBA, ALT, and AST were significantly higher.

Table 1. Participant Characteristics

	ICP (n=38)	Healthy (n=46)	P-value
Maternal age (years)	27.9 ± 3.9	29.1 ± 3.8	0.03*
Maternal BMI (kg/m^2)	24.5 ± 2.9	26.0 ± 3.1	0.02*
Gestational age at delivery (weeks)	32.8 (29.9, 35.4)	36.3 (34.7, 37.3)	5.2E-05*
TBA (μmol/L)	21.9 (9.3, 36.0)	2.2 (1.4, 3.7)	7.9E-12*
ALT (U/L)	121.0 (19.0, 218.0)	15.0 (10.0, 22.0)	1.9E-07*
AST (U/L)	67.0 (28.0, 113.0)	17.0 (15.0, 22.0)	5.8E-09*

Variables with a normal distribution are reported as mean ± standard deviation; Variables with a non-normal distribution are reported as median (lower quartile, upper quartile)

The metabolites identified in the hair metabolome of participants in this study included a range of amino acids, fatty acids, and organic acids (Online Resource 4 contains a full list of metabolites identified). Of the 105 metabolites detected, 71 were identified. Adipic acid was associated with ICP, found in significantly lower levels in ICP cases when compared to controls (P-value = 0.047; Figure 1); it no longer remained significant after adjusting for the potential confounding variables of maternal age, BMI, and gestational age at sample collection. No significant associations were found between the hair metabolites and ICP development in this study. Online Resource 5 displays an Orthogonal-Partial Least Squares Discriminant Analysis (O-PLSDA) plot which also demonstrates that there was no discrimination between case and control groups after dimension reduction.

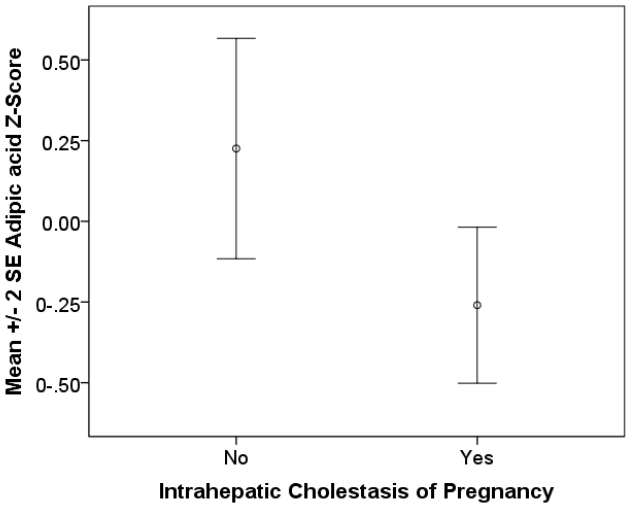

Figure 1. Comparison of the levels of adipic acid in the ICP case and control groups, displayed using the mean of the adipic acid Z-score in each group (P-value = 0.047; FDR = 0.93; AUC (95% Confidence Interval (CI)) = 0.63 (0.50, 0.75); Odds ratio adjusted for maternal age and maternal BMI (95% CI) = 0.56 (0.32, 0.97)). The circles represent the mean relative abundance in each group, and the bars represent two standard errors (SE) either side of the mean.

Subgroup analysis was performed on participants who met all ICP diagnostic criteria of elevated ALT, AST and itching (n=14) compared to the control group. When comparing these participants, succinic acid was significantly lower in cases (P-value = 0.03; FDR=0.89; AUC (95% CI) =0.70 (0.53, 0.84)). However, succinic acid did not remain significantly associated with ICP when it was entered into a logistic regression model adjusted for maternal age and BMI.

This study was the first to use the hair metabolome to search for biomarkers of ICP development. Despite identifying two metabolites with P-values <0.05, their FDRs indicated that their significant P-values were likely to be false positive findings and therefore that adipic acid and succinic acid were

not reliable biomarkers of ICP. The findings from this pilot study suggest that ICP has a rapid onset which deems it undetectable in hair, a stable biospecimen that reflects the accumulation of metabolic information over a series of months (depending on the hair length – in this study the full hair strands were analysed). We had hypothesised that differences in the metabolite profile might reflect dietary or environmental predispositions to ICP and we did not predict that a negative result would occur. The likelihood of rapid onset makes the search for biomarkers of ICP better suited to more transient biospecimens such as blood (serum/plasma) or urine.

The plasma metabolome has proven effective for classifying cases of nonalcoholic fatty liver disease, steatosis, and steatohepatitis in a study conducted using both GC/MS and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC/MS/MS) to characterize the metabolome[15]. Blood and/or urine could indicate changes in the early stages of ICP development resulting from the increased circulation and/or excretion of metabolites produced as the liver disease develops. It is biologically plausible that if the progression to ICP involved the production of unfavourable byproducts, that these would be excreted before having time to accumulate in the hair.

There were limitations of this study worth mentioning. Firstly, the sample size was small and this may have contributed to the inability to detect significant differences in the metabolomic profile of ICP cases compared to controls. However, it can be argued that if there were any sensitive, clinically useful biomarkers then they should be identifiable in a sample of this size. Secondly, using a single metabolomic analysis technique limits the classes of metabolites that can be detected. Future studies would benefit from exploring other techniques that could identify different classes of compounds eg. liquid chromatography coupled to mass spectrometry could be used for a comprehensive lipidomic analysis.

Future studies investigating biomarkers of ICP should consider exploring the metabolome of more transient biological specimens in a longitudinal prospective study. This study design could be used not only to identify biomarkers at one point in time but to also pinpoint how the metabolome changes over time in those women that go on to develop ICP, understanding the metabolic trajectory of ICP and identifying the earliest stage of gestation where at-risk women can be identified.

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Author Contributions: KS, PB, and HZ conceived and designed the study; XH recruited participants and collected the samples; ST performed the experiment with the assistance of T-LH and KS; JdS and ST analysed the data; JdS wrote the paper; all authors revised the manuscript prior to submission.

Compliance with Ethical Requirements:

Conflicts of Interest - The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Research involving Human participants - All procedures performed in this study were in accordance with the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent – Informed consent was obtained from all participants.