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## Spontaneous miscarriage in first trimester pregnancy is associated with altered urinary metabolite profile



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### ABSTRACT

Threatened miscarriage is the most common gynecological emergency, occurring in about 20% of pregnant women. Approximately one in four of these patients go on to have spontaneous miscarriage and the etiology of miscarriage still remains elusive. In a bid to identify possible biomarkers and novel treatment targets, many studies have been undertaken to elucidate the pathways that lead to a miscarriage. Luteal phase deficiency has been shown to contribute to miscarriages, and the measurement of serum progesterone as a prognostic marker and the prescription of progesterone supplementation has been proposed as possible diagnostic and treatment methods. However, luteal phase deficiency only accounts for 35% of miscarriages. In order to understand the other causes of spontaneous miscarriage and possible novel urine biomarkers for miscarriage, we looked at the changes in urinary metabolites in women with threatened miscarriage. To this end, we performed a case-control study of eighty patients who presented with threatened miscarriage between 6 and 10 weeks gestation. Urine metabolomics analyses of forty patients with spontaneous miscarriages and forty patients with ongoing pregnancies at 16 weeks gestation point to an impaired placental mitochondrial  $\beta$ -oxidation of fatty acids as the possible cause of spontaneous miscarriage. This study also highlighted the potential of urine metabolites as a non-invasive screening tool for the risk stratification of women presenting with threatened miscarriage.

### 1. Introduction

Threatened miscarriage is one of the most common gynecological emergencies occurring in 15–20% of pregnancies [1]. It is defined as an ongoing pregnancy with vaginal bleeding and may be accompanied by abdominal pain [2]. Although most women who presented with threatened miscarriage go on to have healthy births, approximately 25% of them progress to spontaneous miscarriage [3,4]. The exact cause of these spontaneous miscarriages remains unknown, although usage of antidepressants and the presence of uterine fibroids have been shown to increase the risk of miscarriage, as early as the first trimester [5–7].

To date, studies have identified multiple maternal serum biological markers, medical and psychosocial factors as potential prognostic

markers for miscarriage [1,8,9]. In addition, recent investigations have demonstrated the importance of hormones and endocrine-immune interactions in maintaining early pregnancy [2,10]. One such hormone is progesterone, which promotes maternal immune tolerance to the fetal semi-allograft, sustains decidualization and controls uterine contractility [3,4,11]. Progesterone triggers the expression of Progesterone Induced Blocking Factor (PIBF) by lymphocytes and decidual cells. PIBF has been shown to exhibit anti-abortive effects *in vivo* and is a pivotal mediator in progesterone-dependent immunomodulation [12,13]. The activation of the immune response triggers inflammation which also plays a pivotal role in increasing the risks of pre-term birth [14]. Both progesterone and PIBF contribute to the success of early pregnancy, and several earlier studies have reported that the risk of miscarriage is significantly higher in women with lower levels of serum progesterone

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and PIBF [8,9,15–17]. Our group has also shown that women with a serum progesterone > 35 nmol/L have a relatively low risk of miscarriage, with a corresponding negative predictive value of 92% [18,19]. However, only about 35% of women with recurrent pregnancy losses are attributed to luteal phase deficiency resulting in inadequate levels of progesterone. Hence, there may be other pathways contributing to a spontaneous miscarriage, and elucidation of these pathways could lead to the development of novel biomarkers and targeted treatment of spontaneous miscarriage.

Other than serum biomarkers, metabolic profiling has also been used to assess the risk of ectopic pregnancies. Horgan et al. used ultra-performance liquid chromatography – mass spectrometry (UPLC-MS) to look at plasma metabolites in rats and identified discriminatory metabolites associated with small-for-gestational age syndrome [20]. Kenny et al. was also able to identify signatory metabolic differences between pregnant women with preeclampsia and those who went on to have healthy births [21]. Notably, Diaz et al. was able to identify several metabolites associated with prenatal disorders, such as gestational diabetes and pre-term delivery, using urine samples of patients [22].

The relative success of urine  $\beta$ -human Chorionic Gonadotropin ( $\beta$ hCG) over serum  $\beta$ hCG in diagnosing pregnancy is testament to the utility of urine metabolites as a non-invasive diagnostic or prognostic marker of pregnancy outcomes.  $\beta$ hCG has also been shown to be predictive of birth weights from as early as the first trimester [23]. However, unlike  $\beta$ hCG, progesterone is metabolized mainly in the liver and only its metabolites are excreted in urine. Although much work has been done in metabolites associated with pregnancy complications, there is no study on urine metabolites associated with spontaneous miscarriage to date. With existing pre-natal care unable to accurately identify women at high risk of spontaneous miscarriage with sufficient accuracy, there is a need to further understand the underlying causes so as to better predict and eventually prevent spontaneous miscarriage. Thus, we conducted a case-control study of eighty patients, half of whom went on to have healthy births, whilst the other half had spontaneous miscarriages. From the discriminatory metabolites profile, we proposed a panel of urine metabolites associated with spontaneous miscarriage and possible mechanisms responsible for women presenting with threatened miscarriage, progressing to spontaneous miscarriage.

## 2. Materials and methods

### 2.1. Patient recruitment

An approval from the Singhealth Centralised Institutional Review Board was obtained (CIRB REF: 2013/320/D) before patient recruitment began from 6 June 2013 to 17 September 2015. At the end of the recruitment period, a total of 40 patients who went on to have miscarriage were recruited. We then randomly selected 40 patients who went on to have healthy births from the same pool of recruited patients and performed a case-control study of 80 pregnant women, aged 21 years and above. Patients presenting at the KK Women's and Children's Hospital (KKH) Singapore, 24-h Women's Clinic from September 2013 to June 2015 were recruited. Inclusion criteria were (i) patients with a single intrauterine pregnancy between 6 and 10 weeks of gestation (confirmed and dated by ultrasonography) and (ii) patients presenting with pregnancy-related per vaginam bleeding. Women with previous episodes of per vaginam bleeding or women treated with progesterone for previous per vaginam bleeding in the current pregnancy, or women diagnosed with inevitable miscarriage, missed miscarriage, blighted ovum or women who are planning to terminate the pregnancy were excluded.

Maternal blood samples were taken to measure serum progesterone level at presentation. Blood was collected in plain tubes and centrifuged for 10 min at 3000g within 2 h of collection. Serum progesterone level was measured in the KKH clinical laboratory using a commercial ARCHITECT progesterone kit (Abbott, Ireland). Urine samples were

collected at presentation for metabolite analysis. Covariates for the analysis were maternal demographics, health, obstetric and lifestyle factors collected by an investigator administered questionnaire in either English or Mandarin.

### 2.2. Outcome measures and follow-up

The primary outcome measured was spontaneous miscarriage, defined as self-reported uterine evacuation after inevitable or incomplete miscarriage, or complete miscarriage with an empty uterus, by the 16th week of gestation. All participants were contacted at the 16th week of pregnancy to verify their pregnancy status. 40 patients experienced spontaneous miscarriage whilst pregnancy was ongoing in 40 patients.

### 2.3. Urine metabolite profiling using UPLC-MS

Methods of urine metabolite profiling were adapted from a previously published protocol [24], and performed on ACQUITY UPLC/Xevo G2-XS QToF (Waters, Manchester, UK) equipped with an electrospray source operating at either positive (ESI+) or negative ionization mode (ESI-). The source temperature was set at 120 °C with a cone gas flow of 50 L/h and a desolvation gas temperature of 450 °C with a desolvation gas flow of 1000 L/h. The capillary voltage was set to 2 kV in the positive ionization mode, and 1.8 kV in the negative ionization mode. The cone voltage was set at 30 V. 3  $\mu$ L of sample was injected into 100 mm  $\times$  2.1 mm, 1.7  $\mu$ m HSS T3 column (Waters) held at 40 °C using the ACQUITY UPLC system from Waters. Elution was performed with a linear gradient of 1–15% B over 1–3 min, 15–50% B over 3–6 min, 50–95% B over 6–9 min, and finally the gradient was held at 95% for 1.1 min. In both the positive and negative ionization modes, mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The column flow rate was 0.5 mL/min. Profile data were collected from 50 to 1200  $m/z$  for both the positive and negative ionization mode with a scan time of 0.15 s over a 12 min analysis. Leucine enkephalin, at a concentration of 200 ng/mL, was used as the lock mass with a flow rate of 5  $\mu$ L/min. It had a  $m/z$  of 556.2771 and 554.2615 in the positive and negative ionization mode respectively [25]. MassLynx software from Waters was used to control the system and data acquisition. The UPLC-MS analysis in this study employed a QC strategy that was previously described [26]. Firstly, to condition the column, QC sample was run 10 times before initiating the runs for the actual samples. Next, the QC sample was injected every time after the injection of 5 samples, and at the start and end of the analysis run. During the sample analysis, a total of 17 QC samples were injected, for the purpose of monitoring instrument stability and analyte reproducibility. After sample analysis, a series of diluted QC samples (1:9, 1:4, 1:2, 1:1) in the reconstitution solvent mixture was injected. Finally, a blank sample was injected at the start and end of the analysis.

### 2.4. Data preprocessing

Preprocessing of MS data (in RAW format), which includes automatic alignment using retention time, peak picking, and deconvolution, was performed using Progenesis QI v2.0 (Nonlinear Dynamics, Newcastle, UK). Samples were median normalized and log transformed [27]. Features near the solvent front, with a retention time < 0.55 min, and chromatographic peak width < 0.03 min were not included for further analysis. Features with an intensity of < 3000 were also discarded. A data matrix containing the samples analyzed versus detected features and their corresponding raw and normalized abundance values was produced for downstream analysis and processing in Python and MATLAB (Mathworks, Natick, MA). Using the QC samples, the unreliable features were removed following the procedures outlined in a previous publication [24]. Features were only accepted if they were present in all of the QC samples, and revealed a coefficient of variation

(COV) < 10%. Finally, raw abundance of features that did not display good linearity in the dilution QC samples, as defined by  $R^2 < 0.9$  and  $p$  value  $\geq 0.05$ , were also excluded from downstream analysis.

### 2.5. Multivariate data analysis

Principal component analysis (PCA) was performed in MATLAB and Python to visualize clustering and identify outliers. Orthogonal projection to latent structure (OPLS) analysis was performed to maximize separation between case and control samples whilst minimizing variability unrelated to the separation using the “ropIs” package implemented in R [28]. The measurement values were standardized prior to OPLS analysis and adjusted for age and BMI. The optimal number of orthogonal components was determined using 5-fold cross validation. The R2Y was calculated to provide an indication of the variability explained by the model and the cross validated Q2Y was calculated to indicate the model performance in cross validation datasets. After statistical modeling, top ranking features with FDR adjusted  $p$ -value  $\leq 0.05$ , were selected for further downstream identification.

### 2.6. Statistical analysis

*t*-Tests were used for statistical comparison of differences in maternal characteristics and metabolite levels between the miscarriage and full-term birth groups. Analysis of co-variances was used to determine if metabolite levels were linked to gestation age.

## 3. Results

### 3.1. Patients' characteristics

Eighty patients who presented with threatened miscarriage between 6 and 10 weeks gestation were recruited. 40 patients had spontaneous miscarriages before 16 weeks gestation (case) and 40 patients had ongoing pregnancies beyond 16 weeks (control). The mean serum progesterone levels were significantly higher in women with ongoing pregnancy ( $66.5 \pm 24.2$  nmol/L) than in women with spontaneous miscarriage ( $32.0 \pm 21.0$  nmol/L) ( $p < 0.0001$ ) (Table 1). Notably, other than gestation age, there were no significant differences in maternal characteristics between the two groups.

### 3.2. Urine metabolites analysis

We then used UPLC-MS to analyze the metabolites present in the urine of both control and case cohorts. Briefly, each sample was first passed through the UPLC column for separation of the various metabolites, before analysis via mass spectrometry (Fig. 1). The UPLC-MS analysis in this study employed a QC strategy that was previously

described by Gika et al. [29] for the purpose of monitoring instrument stability and analyte reproducibility (Fig. 2).

Next, peak alignment, peak picking, peak deconvolution, median normalization, and log transformation were applied on the raw UPLC-MS data. A total of 1291 and 1153 retention time-exact mass pairs (i.e. features) were found, in positive electrospray ionization mode (ESI+) and negative electrospray ionization mode (ESI-) respectively. The stability and the reproducibility of the sample analysis were visualized using Principal Component Analysis (PCA) score plots as shown in Fig. 3. The QC samples were found to be tightly clustered in the score plots, and there was no drift in their PCA scores. After removing features that were not present in all the QC samples, the coefficient of variation (CV) or relative standard deviation (RSD) was calculated for all features. In both positive and negative ionization mode, 89% of the features' CVs were < 10. Finally, the linearity of the features in the dilution QC samples was tested. 77% and 87% of features in positive and negative ionization were retained for downstream analysis when the threshold for acceptance was set at  $R^2 > 0.9$ . After these steps, a total of 791 features were obtained from positive ionization, and 1004 features from negative ionization. To obtain a list of features capable of defining the variation of the metabolic profiles of urine in case and control subjects, data acquired in both ionization modes were analyzed using OPLS-DA model [30] (Fig. 4). Prior to the analysis, the data were standardized. The OPLS-DA modeling was performed with 5-fold cross-validation adjusted for age and BMI. An explained variance (R2Y) of 0.67 and predictability (Q2Y) of 0.22 was obtained for ESI+. For ESI-, R2Y = 0.70 and Q2Y = 0.19. Finally, the metabolites were selected based on the absolute value of the model coefficients and the  $p$  value of the one-way ANOVA after 5% false discovery rate (FDR) correction.

These short-listed features were further subjected to LC MS/MS and the spectra obtained used for their identification with comparison to open source databases. We picked up a total of six metabolites, belonging to three different families, with significant differences in levels between women who went on to have healthy births and those who had spontaneous miscarriages (Table 2). Two metabolites were found to be higher levels in women who had spontaneous miscarriage as compared to women who had healthy births. These two metabolites are tetrahydrocortisone, a stress-induced hormone, and hexanoylcarnitine, a metabolite belonging to the carnitine family and involved in fatty acid metabolism. On the other hand, four metabolites were found to be at lower levels in women who had spontaneous miscarriages. One of them is  $3\alpha,20\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide, the glucuronide conjugate of pregnenediol and an inactive metabolic product of progesterone. As such, lower urinary levels of  $3\alpha,20\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide would thus be reflective of lower progesterone levels in the mum's body, as indeed seen in the maternal characteristics (Table 1). We have also identified three other carnitine products, viz. propionylcarnitine, isovaleryl carnitine and 3-methylglutaryl carnitine,

**Table 1**

Maternal characteristics and serum progesterone levels of women with ongoing pregnancy or with spontaneous miscarriage at 16 weeks gestation.

Maternal characteristics	Ongoing pregnancy at 16 weeks gestation	Spontaneous miscarriage at 16 weeks gestation	p Value
	Cohort (N = 40)	Cohort (N = 40)	
Serum biological markers			
Progesterone, mean $\pm$ SD (nmol/L)	66.5 $\pm$ 24.2	32.0 $\pm$ 21.0	< 0.0001
Demographics			
Age, mean $\pm$ SD (years)	30.8 $\pm$ 3.9	31.8 $\pm$ 5.6	n.s.
Health, obstetric and lifestyle factors			
Gestation age (GA) at recruitment, mean (wks)	7.6 $\pm$ 1.7	6.7 $\pm$ 1.2	< 0.05
Previous miscarriage (%)	35	25	
BMI, mean $\pm$ SD (kg/m <sup>2</sup> )	22.5 $\pm$ 4.4	24.0 $\pm$ 4.5	n.s.
Medical comorbidities			
Hypertension (%)	0	0	
Smoking during pregnancy (%)	10	5	n.s.
Alcohol during pregnancy (%)	0	0	

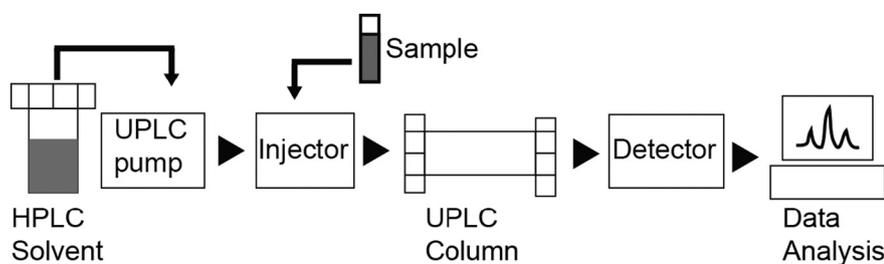


Fig. 1. Workflow of urine metabolite analysis. Samples from both cohorts were randomly injected into the system for separation by ultra-performance liquid chromatography (UPLC) before analysis by mass spectrometry (MS).

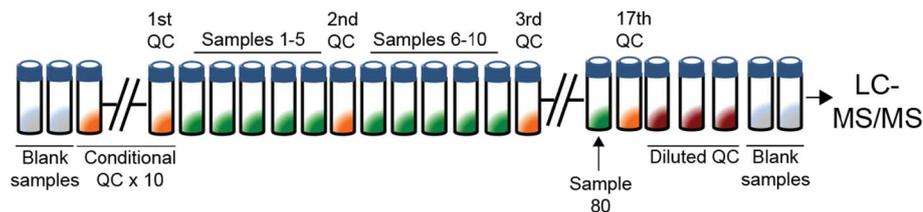


Fig. 2. Schematic of set-up for the identification of metabolites between case and control groups. Quality Control (QC) samples were interspersed between the 80 samples to ensure that the resolution of the column is not compromised.

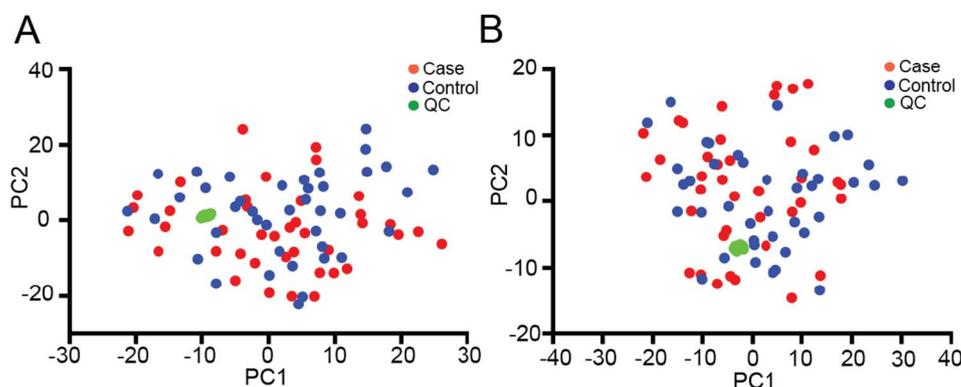


Fig. 3. PCA was applied to the metabolomics data after preprocessing. Visual inspection of the clustering of the quality control (QC) samples and drift of the run order QCs in the PCA scored plots were performed to assess the data integrity by tight clustering of the QC samples on the PCA score plots. The QC samples were found to be well clustered near the center of the scores plot for both (A) positive and (B) negative ionization mode. Case and control samples, however, do not exhibit any significant separations. Quality of the dataset is first assessed using principal component analysis (PCA).

whose levels were lower in mums who miscarried. Together, these results seem to hint that lower progesterone levels, higher stress and deficiencies in fatty acid metabolism are the reasons behind spontaneous miscarriages in our cohort.

Earlier, we found that other than progesterone levels, gestation age was also significantly different between the two cohorts. We then utilized co-variance analysis to determine if the levels of these six metabolites were correlated to gestation age differences (Table 2). We found that only 3 $\alpha$ ,20 $\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide was correlated to gestation age. This was due to the fact that progesterone levels change with regards to gestation age and thus, we can expect levels of 3 $\alpha$ ,20 $\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide, a progesterone metabolite, to correlate with gestation age. After adjusting for gestation age differences, the level of 3 $\alpha$ ,20 $\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide remained significantly different between the two cohorts (Table 2). The remaining five metabolites have been shown to be unaffected by

gestation age and therefore were not adjusted for their levels. Notably, all five metabolites were significantly different between the two cohorts (Table 2).

The identities of these metabolites were then confirmed by comparing their MS/MS spectra with that of commercial standards purchased (Supplementary Fig. S1). Key features in the MS/MS spectra for commercially purchased 3 $\alpha$ ,20 $\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide, tetrahydrocortisone, propionylcarnitine and isovalerylcarnitine matched those seen in the urine samples, thus confirming their identities. However, additional features were seen in the urine samples of 3-methylglutaryl carnitine and hexanoylcarnitine when compared to the purchased standards, suggesting that they might be structural isomers of the metabolites identified in the samples.

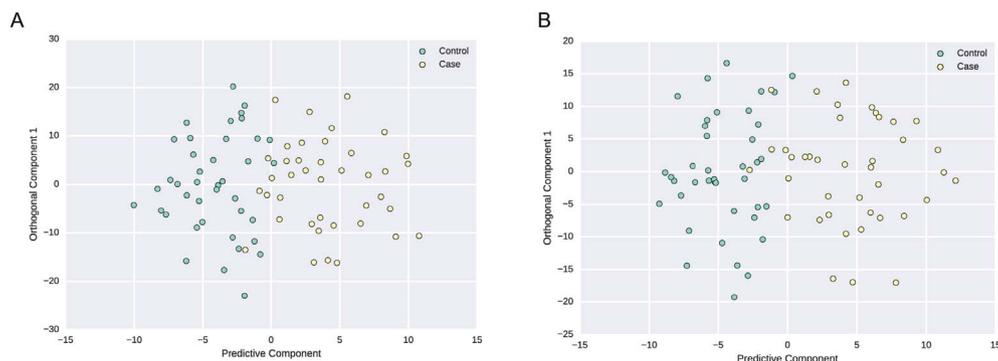


Fig. 4. Multivariate analysis of urine metabolomics data. (A) Scores plot of ESI+ measurements. The urine samples from case and control subjects were found to be well separated along the predictive component axis with an explained variance  $R^2Y = 0.67$  and predictability  $Q^2Y = 0.22$ . (B) Scores plot of the ESI- experiment, with a  $R^2Y = 0.70$  and  $Q^2Y = 0.19$ .

**Table 2**

Top feature hits with significant differences in levels between women who went on to have healthy births and those with spontaneous miscarriage. The fold change indicates the differences in the mean relative abundance with a negative value indicating lower levels in women who miscarry as compared to women with healthy births.

Name	Family	Role	Mean relative abundance (full term)	Mean relative abundance (miscarriage)	Fold change	p-Value	Co-relation to GA (p-value)
3 $\alpha$ ,20 $\alpha$ -Dihydroxy-5 $\beta$ -Pregnane-3-glucuronide	Progesterone	Immune tolerance	410,901	286,680	– 0.30	< 0.001	0.01
Tetrahydrocortisone	Cortisol	Stress axis	5376	7527	0.40	0.0027	n.s.
Propionylcarnitine	Carnitine	Fatty acid metabolism	8841	5187	– 0.41	< 0.0001	n.s.
Isovalerylcarnitine	Carnitine	Fatty acid metabolism	3989	2709	– 0.32	< 0.001	n.s.
3-Methylglutaryl carnitine	Carnitine	Fatty acid metabolism	2579	1069	– 0.59	< 0.001	n.s.
Hexanoylcarnitine	Carnitine	Fatty acid metabolism	1653	2286	0.38	0.0016	n.s.

#### 4. Discussion

This study compares urine metabolites between pregnant women with and without spontaneous miscarriage. Our findings contribute to the assembly of metabolic profiles of women with healthy pregnancies and women with spontaneous miscarriages. Understanding the interactions of the derivatives of urine metabolites can lead to a better understanding of the pathophysiology behind spontaneous miscarriage, and can eventually serve as a powerful predictive and diagnostic tool for the possibility of miscarriage. Through the screening and comparison of urine metabolite profiles, we found significant differences in six urine metabolites amongst pregnant women with and without spontaneous miscarriages.

##### 4.1. Progesterone metabolite

3 $\alpha$ ,20 $\alpha$ -Dihydroxy-5 $\beta$ -pregnane-3-glucuronide, a progesterone-derived steroid-glucuronide, was found to be significantly lower in women with spontaneous miscarriages. This finding was supported by Sauer et al. who also found that the same urinary metabolite was significantly lowered in ectopic pregnancies [31].

Glucuronidation is a metabolic pathway for the degradation of steroids, and is an important step for the conversion of steroids to hydrophilic molecules to facilitate excretion [32]. Since steroid-glucuronides are the metabolites of steroids, their levels in urine are reflective of steroidal levels in the bloodstream. A low 3 $\alpha$ ,20 $\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide level reflects low progesterone levels in women with spontaneous miscarriages. Progesterone promotes uterine quiescence and has been shown to be critical for the continuation of pregnancy and reduction in uterine contractions [33]. Clinical studies have also shown that women who miscarry have significantly lower serum progesterone [8,16,18,19]. Progesterone not only supports endometrial development, it enhances blood flow and oxygen delivery through greater nitric oxide production [34]. The administration of a progesterone receptor antagonist, mifepristone, has also been shown to cause miscarriage [35], further cementing the pivotal role of progesterone in maintaining pregnancy.

Progesterone triggers the expression of PIBF. The fetal presence in the maternal body triggers an immune response as it contains cells from the paternal side as well. A successful pregnancy would thus require suppression of the maternal immune response against the fetus. PIBF has been shown to modulate the cytokine production from a pro-inflammatory Th1 response to that of an anti-inflammatory Th2 response [36]. Moreover, PIBF directly modulates the cytotoxic effect of decidual lymphocytes, protecting the fetus from these immune cells [37]. Several studies have also shown that lower PIBF levels result in pathological pregnancies such as pre-term birth or pre-eclampsia [38]. Thus, the indispensability of progesterone in pregnancy aligns well with the finding of low progesterone metabolites in the urine of women who

miscarry.

With the large volume of work supporting the importance of progesterone and PIBF in maintaining a healthy pregnancy, several studies have also proposed the use of these molecules to predict miscarriage or pre-term birth [15,18,37,39]. Thus, urinary 3 $\alpha$ ,20 $\alpha$ -dihydroxy-5 $\beta$ -pregnane-3-glucuronide, a downstream metabolic product of progesterone excreted in urine, is a potentially novel urinary biomarker for predicting spontaneous miscarriage.

##### 4.2. Tetrahydrocortisone

From our analysis, levels of urinary tetrahydrocortisone were ~40% higher in women who had spontaneous miscarriage compared to those who went on to have healthy births. Tetrahydrocortisone is a urinary metabolite of cortisone derived from the reduction of cortisone by 5 $\beta$ -reductase. Cortisone itself is converted from cortisol via the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 2 [40]. Cortisol binds to mineralocorticoid and glucocorticoid receptors to regulate homeostasis in several important cellular process such as energy homeostasis, metabolism, triggering adequate responses to stress and limiting inflammation [41,42]. Cortisone, on the other hand, also binds to mineralocorticoid and glucocorticoid receptors, albeit with lower affinity. Thus, with high levels of cortisol present, cortisone is produced to modulate the activation of these receptors. Cortisol levels increase when stress levels are high. Studies in pregnant sheep showed that higher levels of cortisol led to alterations in uterine blood flow and maternal glucose concentrations [43,44]. This in turn led to changes in uteroplacental metabolism and consequently affected fetal nutrition, leading to impaired fetoplacental growth and poor fetal viability. An increase in cortisol level has also been shown to reduce the fetal umbilical uptake of glucose due to a larger uptake by the uteroplacental tissues in the maternal uterus in pregnant sheep [45]. In its place, the maternal ewe increases lactate production and switches the fetal metabolism to that of aerobic glycolysis, a metabolic strategy of the early embryo [46,47]. This metabolic strategy may be detrimental to fetal health as seen in studies with increased cortisol levels in pregnant ewes [43]. In humans, higher cortisol levels have also been associated with pregnancy loss [48], indicative of higher fetal stress in women who eventually miscarry.

In addition to a direct effect on fetal metabolism, cortisol affects progesterone levels as well. Cortisol stimulates placental enzymes responsible for the biosynthesis of estradiol from pregnenolone, causing an increase in estrogen secretion and a subsequent decrease in progesterone production [49]. This might explain the decrease in progesterone levels we observed in the women who miscarried. In addition, higher estrogen levels induce the release of prostaglandin F2 $\alpha$  from the placenta, causing enhanced myometrial responses to oxytocin and stimulating contractions [50]. This may cause unfavorable pregnancy conditions.

### 4.3. Carnitine family

Carnitine biosynthesis occurs mainly in the liver and kidneys from the amino acids lysine and methionine [51]. Carnitines play an important role in fatty acid metabolism [52]. They are obligatory cofactors in the transport of fatty acids with long chain acyl groups into the mitochondria. These fatty acids are then broken down via fatty acid oxidation to produce energy [53]. In our analysis, we saw a higher level of hexanoylcarnitine (C6) in women who miscarry when compared to women who went on to have healthy births. The increase in carnitine levels in urine in the form of acylcarnitine is characteristic of secondary carnitine deficiency and is most likely caused by an accumulation of organic acids [53]. Secondary carnitine deficiency can occur due to or in association with defects in fatty acid oxidation metabolism [54]. Hexanoylcarnitine is converted from the long chain acyl coenzyme A (acyl-CoA) via carnitine palmitoyltransferase-1 (CPT1), which swaps the CoA moiety for carnitine [52]. Acylcarnitine from the cytosol is then exchanged with carnitine from the mitochondria using carnitine-acylcarnitine translocase (CACT). After acylcarnitine is successfully transported into the mitochondria, acylcarnitine is converted back to acyl-CoA and carnitine, catalyzed by carnitine palmitoyltransferase-2 (CPT2) [52]. Subsequently, acyl-CoA can proceed to participate in  $\beta$ -oxidation and the citric acid cycle (Fig. 5). Accumulation of acylcarnitines has been linked to fatty acid oxidation deficiency due to a defect in carnitine-acylcarnitine translocase [55], and acylcarnitines directly reflect the oxidation rate of fatty acid [56,57]. Previously, a clinical report of a mother who has had previous miscarriages but gave birth to a baby with a lethal deficiency of CACT indicated that maternal heterozygosity for CACT deficiency with fetal homozygosity for the same deficiency may contribute to impaired metabolism and toxic metabolites formation in both fetus and placenta [58]. Thus, there is strong support for the major role that fatty acid oxidation plays in pregnancy maintenance.

We also found that propionylcarnitine (C3) levels were ~40% lower in women who miscarried as compared to those who went on to have healthy births. This implies a lower level of carnitine and thus, lower levels of fatty acid metabolism in women who miscarry. Shekhawat et al. showed that enzymes involved in  $\beta$ -oxidation exhibited increased activities in the placenta early in gestation and were less active nearing delivery, thus reflecting the importance of fatty acid oxidation to the placental provision of energy [59]. Since the fetus draws considerable energy from the placenta during development, lower levels of carnitine would imply lower energy for the fetal growth, survival and metabolic processes, leading to unfavorable pregnancy conditions. This view is supported by others who have shown that fatty acid oxidation plays a major role in energy generation by the placenta [60–63]. In addition, impairment in mitochondrial  $\beta$ -oxidation of fatty acids can cause fasting induced hypoglycemia and cardiovascular collapse [64]. In mice, deficiency of the mitochondrial trifunctional protein (MTP) responsible for catalysis in the last three steps of fatty acid  $\beta$ -oxidation causes stunting of fetal development, hypoglycemia and sudden death in neonates, further illustrating the importance of unimpeded  $\beta$ -

oxidation in ensuring fetal survival [65]. Studies in mice further reveal that  $\beta$ -oxidation enzymes are present in trophoblast cells, and defects in the OCTN2 transporter result in reduced carnitine accumulation in both fetus and placenta, adversely affecting fatty acid metabolism important to fetal and placental development [66]. Carnitines may also play important roles in preventing over-accumulation of acyl compounds occurring in organic acidemia including propionic acidemia and isovaleric acidemia, which would be toxic to the cell [67,68].

Lower levels of isovalerylcarnitine in women who miscarry may also indicate lower isovaleryl-CoA, a metabolite of leucine, and correspondingly lower levels of leucine [69]. Leucine, an essential amino acid, is important for protein synthesis and a significant decrease in the rate of transamination of leucine contributes to maternal protein and nitrogen accretion [70]. Thus, lower levels of leucine may adversely affect fetal development and contribute to eventual miscarriage.

Reduced levels of 3-methylglutaryl carnitine, a urine metabolite of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA), signals lower HMG-CoA levels in women who miscarry. HMG-CoA is converted to mevalonate, under catalysis by HMG-CoA reductase [71]. Mevalonate is a precursor molecule to many biologically important molecules, including cholesterol and steroid hormones [71]. Reduced HMG-CoA levels may be a direct cause of lower progesterone levels, since pregnenolone, synthesized from cholesterol, is an important precursor for progesterone [72]. In addition, possible shunting of pregnenolone to produce cortisol through the glucocorticoid pathway, at the expense of progesterone, may have contributed to the raised cortisol levels and reduced progesterone levels in women who miscarry as well.

Together, these changes in carnitine metabolite levels indicate defects in amino acid oxidation and/or fatty acid oxidation process and can be caused by any of the several players involved in the pathway [73]. This reduces the energy output by the maternal placenta and directly affects the fetus negatively. In addition, with unmet fetal metabolic demands, fetal stress will likely be induced, contributing to raised tetrahydrocortisone levels and the eventual miscarriage of the fetus.

## 5. Conclusion

Our analysis of patients presenting with threatened miscarriage in this study presents compelling evidence pointing to progesterone deficiency, increased stress hormones and loss of energy production via fatty acid oxidation in the placenta as primary pathways culminating in spontaneous miscarriage. Urine metabolites may provide a simple non-invasive test enabling better risk stratification of women with threatened miscarriage. In addition, dietary carnitine supplementation for pregnant women with threatened miscarriage may reduce the risk of spontaneous miscarriage. As this study is a pilot trial, further investigation with a larger patient cohort and experiments on the role of carnitines in spontaneous miscarriage will be necessary to verify these novel findings.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbaci.2017.07.003>.

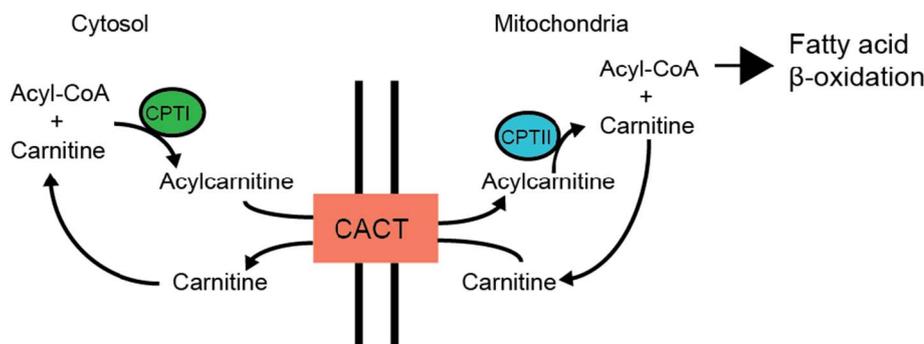


Fig. 5. Pathway showing the interconversion of carnitine and acylcarnitine and their transport from the cytosol into the mitochondria. CPTI: carnitine palmitoyltransferase-1, CPTII: carnitine palmitoyltransferase-2, CACT: carnitine-acylcarnitine translocase.

## Transparency document

The <http://dx.doi.org/10.1016/j.bbacli.2017.07.003> associated with this article can be found, in online version.

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