Biochemical clinical factors associated with missed abortion independent of maternal age
A retrospective study of 795 cases with missed abortion and 694 cases with normal pregnancy

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Abstract
The incidence of fertile women with missed abortion dramatically increased in recent years, while very few serum indices have been identified for the diagnosis of missed abortion. The aim of this study was to identify related factors for missed abortion through a retrospective study of serum indices.

A total of 795 cases of women with missed abortion and 694 cases of women with normal pregnancy between March 2014 and March 2017 were included in the present study. The diagnosis of missed abortion was based on clinical history, clinical examination, and transvaginal ultrasound findings. The final diagnosis of missed abortion was based on assessment of pregnancy structures (i.e., a gestational sac without fetal heart rate) via transvaginal ultrasound. We evaluated the clinical values of 4 serum indices and their relationship to missed abortion: gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH), adenosine deaminase (ADA), and fibrinogen (FIB).

The serum levels of GGT, ADA, and FIB showed statistically significant differences comparing women who experienced missed abortion with women who had normal pregnancies (controls). Among women with missed abortion, the levels of GGT and ADA were dramatically increased (GGT: P < .0001; ADA: P = .0459), while FIB levels were slightly lower (P = .0084) compared to controls. The LDH levels exhibited a non-significant trend toward lower levels in the missed abortion group (P = .3951). Interestingly, the observed significant increase in serum GGT levels among women with missed abortion was not affected by maternal age.

This study found that GTT may be a useful marker which was associated with missed abortion, indicating its potential clinical roles in missed abortion.
1. Introduction

Miscarriage, also known as spontaneous abortion, is a common complication in pregnancy and there is still a lack of biomarkers with predictive value for asymptomatic patients before the event occurs. Missed abortion (MA) is a specific type of miscarriage, and refers to embryonic or fetal death with failure of the retained intrauterine products of conception to be discharged naturally. Women with MA may have no obvious symptoms, yet it occurs in approximately 8 to 20% of clinically diagnosed pregnancies. The MA may cause maternal morbidity, including endometrial injury, coagulative dysfunction, depression, and anxiety. Presently, multiple etiologic factors including maternal chromosomal abnormalities, immunological factors, endocrine disorders, uterine abnormalities, hereditary thrombophilia, infections, and environmental factors have been identified for MA, and these conditions may occur in up to 50% of all women with miscarriages.[3]

Recently, a few gene expression and functional studies revealed a correlation between genetic factors and MA. Evidences indicated that early gestation is associated with a hypoxic environment, which may encourage angiogenesis, but severe hypoxia may inhibit angiogenesis. Placental angiogenesis is dependent upon various growth factors (including VEGF and its receptors, and so on). For instance, aberrant Delta-like ligand 4/Notch (Dll4/Notch) and Hypoxia inducible factor 1a/Vascular endothelial growth factor (HIF-1α/VEGF) signaling may have a role in MA.[4] The expression of HIF-1α and VEGF was lower in the MA, and the levels of HIF-1α/VEGF mRNA and protein in HTR8/SVneo cells were significantly enhanced under hypoxia.[5] Moreover, microRNA 575 (miR-575) has been demonstrated to be upregulated in maternal placenta in patients who have experienced a miscarriage. Abnormal expression of miR-575 may lead to MA by influencing apoptosis and angiogenesis. Inhibition of miR-575 may inhibit apoptosis and promote angiogenesis in MA.[6] Tumor necrosis factor-α-induced protein-8 like-2 (TIE2), identified as a member of the TNFAIP8 family, is a negative regulator of inflammation and immunity.[7] A TIE2 could play important roles in maintaining maternal-fetal tolerance, and decreased TIE2 expression in the decidua may be related to the development of MA.[8] Another study aimed to explore the expression of suppressor of cytokine signaling (SOCS3), transforming growth factor β1 (TGFβ1), and indole-amine 2,3-dioxygenase (IDO), and to analyze the association between SOCS3 and TGFβ with IDO expression in chorionic villi and decidua at the maternal-fetal interface during early pregnancy. In the normal physiological state of pregnancy, SOCS3 and TGFβ may be involved in the regulation of immune tolerance by positive or negative regulation of IDO expression at the maternal-fetal interface.[9] Taken together, these studies indicated that many biological processes (i.e., angiogenesis, hypoxia, apoptosis, inflammation, and immunity) and their regulatory pathways are crucial for the pathogenicity of MA.

And these studies begin to offer some insights, but the exact mechanism of early pregnancy loss in MA remains to be elucidated. However, none of them have analyzed the role of plasma metabolites as promising biomarkers of MA.

Using UHPLC-MS based metabolomics screening, plasma metabolic profiles identify plasma metabolite biomarkers (i.e. glyceral acid, indole, and sphingosine) as having perfect accuracy for diagnosis of MA, which make it possible to explore promising biomarkers in plasma.[11] Here, in this study, 4 serum indices represent markers for metabolic homeostasis of the human body and are involved in oxidative stress reaction, apoptosis, and multiple metabolic processes. Gamma-glutamyltransferase (GGT) is a transferase that catalyzes the transfer of gamma-glutamyl functional groups from molecules such as glutathione to acceptor molecules, forming glutamate.[12] The GGT induction has been shown to be related to oxidative stress and apoptosis[13] also GGT plays a key role in the gamma-glutamyl cycle, a pathway for the synthesis and degradation of glutathione and drug and xenobiotic detoxification.[14] Adenosine deaminase (ADA) is an enzyme involved in purine metabolism. It plays a key function in the development and maintenance of the immune system in humans.[15] It has also been recognized that the activity of ADA protein is upregulated in mouse hearts overexpressing HIF1α, suggesting a role in oxygen-related homeostatic signaling.[16] Lactate dehydrogenase (LDH) is an enzyme found in nearly all living cells, and is broadly expressed in body tissues including blood cells and heart muscle. The LDH is a valuable biomarker for common injuries and disease based on its release during tissue damage. Fibrinogen (FIB) is a glycoprotein that in vertebrates circulates in the blood. A study with a small population showed that serum FIB levels showed a small but non-significant decrease in women with MA.[17] According to long-term clinical observations, these serum indices may be influenced by the pathogenesis of MA.

The aim of this study was to identify potential predictive factors for MA through a retrospective study of serum indices. In this study, we systemically analyzed the association between 4 serum indices (GGT, ADA, LDH, and FIB) and MA in order to assess whether they could be used as early prediction factor(s) for MA.

2. Materials and methods

2.1. Study design and participants

This study was a retrospective study. A total of 795 cases of women aged from 17 to 49 years with MA at 7 to 10 gestational weeks at The Fourth People’s Hospital of Zhenjiang (Zhenjiang, China) between March 2014 and March 2017 were included in the present study. An additional 694 cases of fertile women aged between 17 and 48 years with no history of MA were used as controls.

The diagnosis of MA was based on the clinical history, clinical examination, and transvaginal ultrasound (TVU) results. In cases...
where pregnancy structures (a gestational sac without fetal heart rate) were identified by TVU, the final diagnosis of MA was made. In control group, pregnancy structures are normal.

Inclusion criteria were a gestational age at 7 to 10 weeks (based on the 1st day of the last menstrual period) and no history of recurrent spontaneous abortions, chromosomal abnormalities, endocrine diseases, anatomical abnormalities of genital tract, infections, immunologic diseases, trauma, internal diseases, hereditary disorders, maternal diseases, psychological factors, or any chemical agent intake before their elective terminations. No obvious other internal medical and surgical disease was found in both groups.

### 2.2. Definition of age groups

For reducing possible biases affecting the relationship between serum levels and MA, the overall study population was divided into different age groups, including age group 1 (17–27 years), age group 2 (28–38 years) and age group 3 (39–49 years).

### 2.3. Detection of serum indices

The samples (5 ml) poured into the testing tube without anticoagulant, to divide serum. In order to decrease the time of keeping samples in the laboratory conditions, during 5 min of incubation in the environmental temperature, the sample immediately became centrifuged (4000 rpm for 5 min) and the serum solution was divided by blood clot. Then the resulted serum was used to test serum levels of GGT, LDH, ADA, and FIB. Serum indices were measured at the same time point with an automated biochemical analyzer (Beckman Coulter AU5800).

### 2.4. Statistical analysis

Values were measured using the mean with standard deviation (SD). Differences between 2 groups were calculated by student’s t test. Odds ratios (ORs) and 95% confidence interval (CI) were calculated by using logistic regression. Data were analyzed by using SPSS software, version 22 (IBM, IL) and PASS software, Version 11.0.7 (NCSS).

### 3. Results

#### 3.1. Study populations

Our retrospective study includes 694 cases of healthy fertile women with normal pregnancies and 795 cases of women with MA. Mean age between control (30.0 ± 4.6 years) and MA (30.6 ± 5.7 years) subjects showed no statistically significant difference (P = .08). Twenty-nine cases (4.2%) in the control group and 34 cases (4.3%) in the MA group had lower abdominal pain (P = .93). Five cases (0.7%) in the control group and 6 cases (0.8%) in the MA group had at least 1 occurrence of vaginal bleeding (P = .94). A comparison of baseline general and gynecologic characteristics among the control and MA groups is summarized in Table 1.

#### 3.2. Serum indices analysis identifies 3 potential biomarkers for missed abortion

Our results show that the levels of serum GGT and ADA significantly increased in women with MA (GGT: 17.68 ± 9.85 IU/L; ADA: 6.05 ± 3.94 IU/L) when compared to women with normal pregnancies (GGT: 14.57 ± 7.93 IU/L, P < .0001; ADA: 5.67 ± 3.36 IU/L, P = .0459). Moreover, serum FIB levels were modestly but significantly lower in the MA group compared to controls (MA: 4.31 ± 0.91 g/L; control: 4.44 ± 0.96 g/L; P = .0084). Serum LDH levels did not differ among the MA and control groups (MA: 148.05 ± 30.46 IU/L; control: 149.34 ± 27.88 IU/L, P = .3951). Taken together, these data suggest that 3 out of the 4 key metabolism-related biochemical indicators evaluated in this study (GGT, ADA, and FIB) may be potential predictive markers for MA (Table 2).

### Table 1

Characteristics of study population: women with normal pregnancy and women with MA.

| Characteristic                  | Control   | MA         | P   |
|--------------------------------|-----------|------------|-----|
| Participants, n                | 694       | 795        |     |
| Age, y, Mean±SD                | 30.0±5.7  | 30.6±5.7   | .08 |
| 17–27 (y), n (%)               | 282 (40.6%) | 275 (34.59%) | .94 |
| 28–38 (y), n (%)               | 333 (47.98%) | 431 (54.21%) | .91 |
| 39–49 (y), n (%)               | 79 (11.38%) | 89 (11.20%) | .64 |
| Lower abdominal pain, n (%)    | 29 (4.18%) | 34 (4.28%) | .9253 |
| Vaginal bleeding, n (%)        | 5 (0.72%) | 6 (0.75%) | .9386 |

The data were evaluated for statistical differences using student’s t test. MA = missed abortion, SD = standard deviation.

### Table 2

Comparison of 4 serum indices in women with normal pregnancy and women with MA.

| Serum Index | Control Mean±SD (IU/L) | MA Mean±SD (IU/L) | OR (95% CI) | P   |
|-------------|------------------------|-------------------|-------------|-----|
| GGT (IU/L)  | 694 14.57±7.93         | 793 17.68±9.85    | 1.046 (1.031–1.061) | .0001 |
| LDH (IU/L)  | 694 149.34±27.88       | 795 148.05±30.46  | 0.996 (0.995–1.002) | .3951 |
| ADA (IU/L)  | 694 5.67±3.36          | 793 6.05±3.94     | 1.040 (0.998–1.084) | .0459 |
| FIB (g/L)   | 694 4.44±0.96          | 795 4.31±0.91     | 0.863 (0.773–0.964) | .0084 |

The data were evaluated for statistical differences using student’s t test. ORs and 95% CI were calculated by using logistic regression. AD = adenosine deaminase, CI = confidence interval, FIB = fibrinogen, GGT = gamma-glutamyltransferase, LDH = lactate dehydrogenase, MA = missed abortion, ORs = odds ratios, SD = standard deviation.
3.3. GGT levels in MA are independent of maternal age

Genetic and metabolic risk factors increase significantly with age. To explore early predictive factors that are independent of age, we divided our study population into 3 age groups, comprising 17 to 27 years, 28 to 38 years, and 39 to 49 years. Among age-specific subgroups, 3 serum indices exhibited extremely similar serum level patterns among women ages from 17 to 49 years. The serum concentrations of GGT and ADA significantly increased, while FIB levels were decreased among women with MA. Surprisingly, a similar pattern was observed for women ages from 17 to 27 years. On the other hand, mean GGT serum level was significantly increased among all age groups of women with MA (Table 3). Our results demonstrate that GGT may be a potential predictive factor for MA independent of maternal age.

4. Discussion

The MA is one of the most common types of early pregnancy loss (EPL), and several reasons have been identified for the failure of these pregnancies. The incidence of fertile women with MA dramatically increased in the last few years, while very few serum indices have been identified for the diagnosis of MA. In this study, we initially started with 4 candidate serum indices (GGT, ADA, FIB, and LDH) and evaluated the relationship between clinical values of these serum indices and MA (Table S1, http://links.lww.com/MD/C697 and Table S2, http://links.lww.com/MD/C697). Comparing women who experienced MA with control women who had healthy pregnancies, the serum enzyme levels of GGT, ADA, and FIB showed statistically significant differences. Surprisingly, although GGT levels remained within the normal range (8–87 IU/L), these levels were significantly higher in women with MA compared to controls, independent of age. These observations demonstrate for the 1st time that GGT serum level may be a novel predictive biomarker for the diagnosis of MA.

The GGT enzyme is widely distributed in the human body, including the kidneys, bile duct, pancreas, gallbladder, spleen, heart, brain, and seminal vesicles, and is frequently localized to the plasma membrane with its active site directed toward the extracellular space.[21] Serum GGT activity has already been used as a biomarker for liver diseases or alcohol consumption in clinical practice. However, serum GGT is more than a marker of liver diseases or alcohol consumption. Epidemiological studies have found a strong association between serum GGT levels and many cardiovascular disease risk factors.[20,21] In addition, several prospective studies have shown that baseline serum GGT level is an independent risk factor for the development of heart disease, hypertension, stroke, and type 2 diabetes.[21,22]

An increase of serum GGT might be interpreted as a defense mechanism reflecting the induction of cellular GGT activity under oxidative stress.[23,24] There is evidence that cellular GGT plays an important role in antioxidant defense systems and mediates oxidative stress-induced cell death.[25,26] Other studies show that the increased resistance of GGT-expressing cells to H2O2-induced apoptosis results from activation of ASK-1/p38 signaling and increased expression of cellular catalase, resulting in decreased formation of reactive oxygen species (ROS) and thereby protection against ROS-induced DNA damage.[27,28]

The correlation between mRNA and protein levels is typically insufficient to predict protein expression levels.[22] A previous study using 2D gel-based proteomics identified only 13 proteins dysregulated in placental villous tissues of EPL: Fas inhibitory molecule (FAIM), S100 calcium-binding protein A11 (S100A11), and RNA-binding protein regulatory subunit were downregulated, and 5 proteins, including ubiquitin-conjugating enzyme E2N (UBE2N) and the proteasome beta-subunit were significantly upregulated.[19] Ni et al conducted a proteomics analysis to identify differentially expressed proteins in placental villous tissues from normal pregnant women and EPL patients, and found 51 differentially expressed proteins, of which 22 proteins were upregulated and 29 proteins were downregulated; these proteins participate in cell migration, angiogenesis, oxidative stress, apoptosis, and metabolic pathways.[19] Another study performed by Xin et al identified 5952 proteins in placental villi, 588 of which were differentially expressed in women who

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**Table 3**

Comparison of 4 serum indices by age in women with normal pregnancy (control) and women with missed abortion (MA).

| Age group 1 (17–27 y) | Control | MA |
|-----------------------|---------|----|
| n | Mean±SD | n | Mean±SD | OR (95% CI) | P |
| GGT (IU/L) | 282 | 13.97±7.46 | 274 | 16.47±8.89 | 1.049 (1.022–1.076) | .0001 |
| LDH (IU/L) | 282 | 148.46±28.90 | 275 | 146.67±25.40 | 0.998 (0.991–1.004) | .4382 |
| ADA (IU/L) | 281 | 5.67±2.12 | 275 | 6.15±3.22 | 1.082 (1.002–1.168) | .0378 |
| FB (g/L) | 282 | 4.50±0.92 | 275 | 4.26±0.70 | 0.720 (0.589–0.883) | .0011 |

| Age group 2 (28–38 y) | Control | MA |
|-----------------------|---------|----|
| n | Mean±SD | n | Mean±SD | OR (95% CI) | P |
| GGT (IU/L) | 333 | 14.58±7.65 | 430 | 17.92±10.43 | 1.049 (1.028–1.070) | <.0001 |
| LDH (IU/L) | 333 | 147.84±25.59 | 431 | 148.10±32.50 | 1.000 (0.995–1.005) | .904 |
| ADA (IU/L) | 333 | 5.65±4.29 | 429 | 5.98±4.58 | 1.021 (0.979–1.065) | .3005 |
| FB (g/L) | 333 | 4.39±1.03 | 431 | 4.36±1.00 | 0.963 (0.837–1.109) | .6051 |

| Age group 3 (39–49 y) | Control | MA |
|-----------------------|---------|----|
| n | Mean±SD | n | Mean±SD | OR (95% CI) | P |
| GGT (IU/L) | 78 | 16.71±10.18 | 89 | 20.27±11.76 | 1.033 (1.000–1.066) | .0391 |
| LDH (IU/L) | 79 | 158.90±31.71 | 89 | 152.02±34.36 | 0.994 (0.984–1.003) | .1879 |
| ADA (IU/L) | 79 | 5.75±2.30 | 89 | 6.08±2.38 | 1.085 (0.933–1.216) | .349 |
| FB (g/L) | 79 | 4.81±0.74 | 89 | 4.25±0.83 | 0.768 (0.520–1.136) | .186 |

The data were evaluated for statistical differences using student’s t test. ORs and 95% CI were calculated by using logistic regression. ADA = adenosine deaminase, CI = confidence interval, FB = fibrinogen, GGT = gamma-glutamyltransferase, LDH = lactate dehydrogenase, MA = missed abortion, ORs = odds ratios, SD = standard deviation.

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experienced EPL. Further bioinformatics analysis indicated that these differentially expressed proteins participated in a variety of signaling pathways, including the focal adhesion pathway and ribosome pathway. These identified proteins, especially those with known roles in apoptosis and oxidative stress, may be novel predictive markers for MA.

Several studies suggest that regulation of apoptosis is critically important for the successful development and outcome of a pregnancy. Low rates of apoptosis in placental villi tissues are a normal physiologic phenomenon, while high levels of apoptosis may result in miscarriage. Oxidative stress is a common pathological background for different etiologies of EPL, and it has been suggested that elevated ROS trigger endoplasmic reticulum (ER) stress by influencing ER function. Accumulation of ROS and regulation of protein folding are closely linked events, and alterations in redox status or generation of ROS can affect ER homeostasis and protein folding. The ER stress induces apoptosis in part via activation of caspase-4 and caspase-affecting ER homeostasis and protein folding. Oxidative stress and regulation of protein folding are closely linked to apoptosis, which may result in miscarriage. Oxidative stress is a common pathological background for different etiologies of EPL, and it has been suggested that elevated ROS trigger endoplasmic reticulum (ER) stress by influencing ER function. Accumulation of ROS and regulation of protein folding are closely linked events, and alterations in redox status or generation of ROS can affect ER homeostasis and protein folding. The ER stress induces apoptosis in part via activation of caspase-4 and caspase-12. Liu et al provided evidence that sustained ER stress occurs in EPL decidua cells (DCs), and the potentially deleterious relationship between ER stress and oxidative stress is likely to play an important role in the development of EPL.

While providing important insights into the etiology of EPL, none of the aforementioned studies systematically evaluated serum analytes in women with EPL. Additionally, our study assessed GTT levels during an earlier stage of gestation. Our study demonstrates that apoptosis and oxidative stress-related serum index GTT may improve the efficiency of diagnosis of MA, representing a new and possibly independent tool in prediction assessment. Additional investigation of this relationship with larger populations will help to refine the present findings. Gynecologists and obstetricians should be aware of the potential added value of GTT for counseling and predicting risk of MA during pregnancy. Our study demonstrates that GTT may be a useful marker associated with MA and may improve the diagnostic efficiency of MA.

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