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Mass spectrometry of water-soluble vitamins to establish a risk model for predicting recurrent spontaneous abortion

Bitao Wu^{1,2,4}, Zhenghao Li^{1,2,4}, Bi Peng^{3,4}, Qiang Yang^{1,2}, Wenqiang Jiang^{1,2}, Ying Ma^{1,2}✉, Jie Tang^{1,2}✉ & Yuwei Yang^{1,2}✉

The adverse pregnancy outcomes, including recurrent spontaneous abortion (RSA), are strongly correlated with water-soluble vitamins, but how to predict RSA occurrence using them remains unsatisfactory. This study aims to investigate the possibility of predicting RSA based on the baseline levels of water-soluble vitamins tested by ultra-liquid chromatography-tandem mass spectrometry. A total of 918 pregnant women was consecutively enrolled in this cross-sectional study. According to the miscarriage numbers, they were divided into normal first pregnancy (NFP, n = 608), once spontaneous abortion (OSA, n = 167), and continuous spontaneous abortion (CSA, n = 143) groups. The Cox proportional-hazards regression model was employed to establish a risk model for predicting RSA. The RSA occurrence was 6.54% in overall pregnant women, with a prevalence of 12.57% in the OSA group and 27.27% in the CSA group. Significant differences were observed in baseline deficiencies of vitamin B3, B5, B6, and B9 among NFP, OSA, and CSA groups ($\chi^2 = 12.191 - 37.561$, all $P < 0.001$). Among these vitamins, B9 ($HR = 0.89$ and 0.88 , all $P < 0.001$) and B6 ($HR = 0.83$ and 0.78 , all $P < 0.05$) were identified as independent factors in both the OSA and CSA groups; whereas B5 was identified as an additional independent factor only in the CSA group ($HR = 0.93$, $P = 0.005$). The Cox proportional-hazards model established using these three vitamins exhibited poor or satisfactory predictive performance in the OSA (Sen = 95.2%, Spe = 39.0%) and CSA (Sen = 92.3%, Spe = 60.6%) groups, respectively. However, B5, B6, and B9 compensatory levels were not associated with RSA occurrence (all $P > 0.05$). Our study presents a highly sensitive model based on mass spectrometry assay of baseline levels in B vitamins to predict the RSA occurrence as possible.

Keywords Recurrent spontaneous abortion, Water-soluble vitamins, Baseline, Mass spectrometry, Risk model

Spontaneous abortion (SA), an accidental miscarriage occurring without external intervention prior to 20~24 weeks gestational age, affects more than 20% of clinically recognized pregnancies¹. It can subdivide into threatened, inevitable, incomplete, missed, septic, and complete abortions^{1,2}, and manifest imperceptible embryo loss even life-threatening shock during a pregnancy course². When consecutive spontaneous pregnancy losses occur, it is referred to as recurrent spontaneous abortion (RSA), which affects approximately 1~5% of reproductive women^{3,4}. The definition of RSA in relation to the number of miscarriages varies among different countries. The European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine define RSA as two or more accidental miscarriages, while the Royal College of Obstetricians and Gynaecologists defines it as three or more^{4,5}. Additionally, recent epidemiological surveys provide evidence to suggest that the COVID-19 pandemic has potentially accelerated the occurrence of RSA, with a prevalence rate reaching up to 6.5~9.5%^{6,7}.

¹Mianyang Central Hospital, affiliated to School of Medicine, University of Electronic Science and Technology of China, Mianyang, China. ²NHC Key Laboratory of Nuclear Technology Medical Transformation (Mianyang Central Hospital), Mianyang, China. ³Sichuan Mental Health Center, The Third Hospital of Mianyang, Mianyang, China. ⁴These authors contributed equally: Bitao Wu, Zhenghao Li and Bi Peng. ✉email: lizza2222@126.com; tjandbetty@sc-mch.cn; yyw318@sc-mch.cn

Although the etiology of RSA can often be attributed to fetal, maternal, or external factors, in many cases, the specific etiology remains never to be identified. Numerous studies have demonstrated a significant association between water-soluble vitamins and adverse pregnancy outcomes, including anemia, nausea and vomiting, and malnutrition in pregnant women, and/or fetal neural tube defects, other central system disorders, and poor embryonic development^{8–10}. The water-soluble vitamins include nine compounds, that is, eight B vitamins (B1, B2, B3, B5, B6, B7, B9, and B12) and one vitamin C¹¹. Of them, B vitamins have been found to potentially induce chromosomal abnormalities^{12,13}, as well as a broad spectrum of endocrine, immune, and metabolic disorders^{14,15}, thereby impeding proper embryo development during pregnancy, potentially increasing abortion risk. The systematic review and Meta-analysis on vitamin and mineral Supplementation during pregnancy have demonstrated a significant impact of vitamin B9 supplementation on enhancing pregnancy outcomes^{16–18}, while the role of other vitamin supplementation remains a subject of controversy^{19–22}.

Up to now, the progress in predicting and preventing RSA is unencouraging, despite the relative simplicity of its diagnosis³. The lack of standardized definitions, the uncertainties surrounding pathogenesis, and the highly variable clinical presentation, as well as the compensatory vitamin levels, and the accuracy of the measurement technique, all contribute to challenges in predicting RSA. The isotope-dilution liquid chromatography tandem mass spectrometry offers a highly sensitive, selective, and specific testing method for water-soluble vitamins²³. Therefore, based on this technique for assessing the baseline levels of water-soluble vitamins in pregnant women, this study aims to investigate the potential for predicting RSA. According to the number of accidental miscarriages, we defined once SA (OSA) as only once occurrence, and continuous SA (CSA) as twice or more occurrences. Our OSA definition is consistent with the definition of RSA in most countries.

Methods

Participants

The study enrolled pregnant women with a SA history who underwent long-term follow-up at Mianyang Central Hospital, School of Medicine, University of Electronic Science and Technology of China from Jan. 2021 to Dec. 2023. Inclusion criteria: (1) pregnant women with clinically recognized pregnancies at 4 and 6 gestational weeks; (2) their baseline levels of water-soluble vitamins were tested at 4–6 gestational weeks; (3) with well-documented pregnancy outcomes. Exclusion criteria: (1) pregnant women who had previously undergone an induced abortion or were deciding an artificial abortion based on personal choice; (2) pregnant women who had taken complex or individual B vitamins, excluding vitamin 9, prior to conception and prior to the examination of water-soluble vitamins; (3) pregnant women requiring termination of their pregnancies due to severe illness; (4) pregnant women who were lost to follow-up. During the same period, pregnant women with a first pregnancy further a successful delivery were consecutively enrolled referring to the inclusion and exclusion criteria for pregnant women with SA history.

Finally, a total of 918 pregnant women was enrolled in this study (See Supplementary Fig. 1). According to the SA experience, all participants were divided into three groups: normal first pregnancy (NFP, pregnant women with a first pregnancy further a successful delivery, n = 608), once spontaneous abortion (OSA, pregnant women with once SA history, n = 167), and continuous spontaneous abortion (CSA, pregnant women with two or more SA history, n = 143). After conducting baseline testing, the OSA and CSA groups were administered with a low dosage of B complex vitamin supplementation until 24 gestational weeks. The daily dosage of B complex vitamin supplementation was as follow: 1.4 mg of B1, 1.4 mg of B2, 36 mg of B3, 4 mg of B5, 7 mg of B6, 30 µg of B7, 360 µg of B9, and 6 µg of B12.

Sample collecting and processing

Aliquots of 3.0 ml fasting blood were collected in an SST-II vacuum tubes (BD, USA) during 4 ~ 6 gestational weeks and after 4 weeks of vitamin supplementation. Serum was separated within 30 min via centrifuging at approximately 1500 × g for 10 min, and used for the quantification of water-soluble vitamin levels within a time-frame of 3 days. Prior to quantification, the serum was stored at – 20 °C.

Before mass spectrometry, the thawed serum was preprocessed using a water-soluble vitamin assay kit (CAT. NO. YS20020009, Shandong Yingsheng Biotechnology Co., Ltd., CHN). The sample releaser III from the kit was mixed with the freshly dissolved internal standard concentrate at a volume ratio of 200:1. 60 µl of this mixture was combined with 60 µl of serum and vortexed for 5 min, followed by centrifugation at 12000 × g for an additional 5 min. Subsequently, 70 µl of supernatant was transferred into a microplate, and sealed for chromatographic analysis.

Chromatography-tandem mass spectrometry assay of water-soluble vitamin

Nine water-soluble vitamins were measured on a EXT9050MD ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (Shandong Yingsheng Biotechnology Co., Ltd., CHN). The chromatography conditions were as follows: C18 column at 40 °C, 30 µl injection volume, and density gradient elution with 0.5 ml/min flow rate by mobile phase A of 0.1% formic acid in deionized water and mobile phase B of 0.1% formic acid in methanol. The percentage of mobile phase B was 1% from 0 to 1 min, 98% from 1 to 2 min, 98% from 2 to 3.5 min, 1% from 3.5 to 4.5 min, 0% from 4.5 to 5.0 min.

The mass spectrometry conditions were as follows: positive ion mode of electrospray ion trap, electrospray voltage 3500 V, ionization temperature 350 °C, sheath gas (GS1) 50psi, aux gas 10psi. The cluster removal voltages and collision voltages were 30 V and 21 V for vitamin B1, 60 V and 31 V for vitamin B2, 45 V and 28 V for vitamin B3, 40 V and 21 V for vitamin B5, 48 V and 29 V for vitamin B6, 37 V and 23 V for vitamin B7, 46 V and 25 V for vitamin B9, 45 V and 55 V for vitamin B12, 25 V and 14 V for vitamin C, respectively. Mass spectrometry scan used a multiple reaction monitoring mode with the range from 150 to 500 m/z, and the rate of 1000 Da/s.

Chromatograms and process data were analyzed using TraceFinder™ modern data visualization software (version 4.1, Thermo). Via monitoring the paired Q1/Q3 ion mass, vitamin B1, B2, B3, B5, B6, B7, B9, B12, and C were distinctly separated and identified with the retention time of 1.47 min, 5.14 min, 1.81 min, 3.70 min, 2.92 min, 5.22 min, 4.30 min, 5.26 min, and 0.81 min, respectively (Supplementary Fig. 2).

Cut-off definitions of B vitamins during early pregnancy

The water-soluble vitamin level was determined using the isotope-dilution internal standard method. The chromatographic peak area ratio was calculated between the sample (or standard) and the internal standard. For each water-soluble vitamin, five standards with gradient concentration were used to draw a “peak area ratio-concentration” curve, which was used to quantify the concentration in sample.

The cut-off values for vitamin B9 and B12 deficiencies during early pregnancy adopted the BOND project and WHO recommendation^{24–27}, which were < 10 ng/mL and < 20 ng/dL respectively. While the cut-off values for vitamin B1, B2, B3, B5, B6, B7, B9, and B12, and C deficiencies adopted the lower reference limit provided by the kit, which were < 0.5 ng/mL, < 3 ng/mL, < 12 ng/mL, < 10 ng/mL, < 0.5 ng/mL, and < 6 µg/mL, respectively.

Statistical analysis

Statistical analysis was performed in the MedCalc software v20.1 (MedCalc, Belgium) and SPSS software v22.0 (SPSS, USA). The levels of water-soluble vitamins were presented as median (P_{25}, P_{75}) [min, max]. The Kruskal–Wallis test was employed to analyze the differences among groups, and POST-HOC analysis was conducted for pairwise comparisons using the adjusted P value (P_{adj}) to determine statistical significance. The deficiency rates were presented as n (%), and χ^2 test was used to compare the group difference. The Kaplan–Meier curves were used to depict the occurrence of miscarriage among the entire population of pregnant women, as well as within the OSA and CSA groups. The Cox proportional-hazards regression was used to examine the hazard ratio of each vitamin and establish a risk model. A significance level of $\alpha = 0.05$ was used for a two-tailed test.

Results

The baseline levels of nine water-soluble vitamins in three pregnant groups

The median, P_{25} , P_{75} , min, and max of eight vitamin levels in the three pregnant groups were listed in Table 1. There were significant differences in vitamin B1, B5, B6, B9, and B12 levels among three pregnant groups ($\chi^2 = 9.445 \sim 65.634$, all $P < 0.01$). Pairwise comparisons found that vitamin B1, B5, B6, B9, and B12 levels were lower in the CSA group than in the NFP group ($z = 2.915 \sim 8.008$, all $P_{adj} < 0.05$), while only vitamin B1, and B5 levels were lower in the CSA group than in the OSA group ($z = 2.656$ and 4.368 , both $P_{adj} < 0.05$). Additionally, vitamin B5, B6, and B9 levels were lower in the OSA group than in the NFP group ($z = 2.680 \sim 3.355$, all $P_{adj} < 0.05$). Except for vitamin B7, the levels of the other eight vitamins ranged widely within each pregnant group, with the exceptionally high individual outliers.

The prevalence of nine water-soluble vitamins deficiencies

According to the lower reference limit provided by the kit, or the BOND project and WHO recommendation, we analyzed the prevalence of nine water-soluble vitamins deficiencies. Vitamin B12 (76.7%), B6 (26.8%), B3 (22.8%), C (18.6%), B2 (8.0%) and B9 (7.8%) deficiencies exceeded 5% among overall pregnant women, moreover

Vitamin	NFP Group (n=608)	OSA Group (n=167)	CSA Group (n=143)	χ^2	P
VitB1 (ng/mL)	1.74 (1.27, 2.30) [0.41, 14.09]	1.75 (1.21, 2.38) [0.44, 7.69]	1.47 (1.14, 2.01) Δ° [0.44, 11.97]	9.445	0.009
VitB2 (ng/mL)	7.20 (4.50, 11.58) [1.48, 37.2]	8.20 (5.34, 13.93) [1.90, 31.08]	7.69 (4.44, 10.97) [1.13, 29.13]	4.977	0.071
VitB3 (ng/mL)	17.04 (12.54, 23.45) [4.48, 75.13]	17.81 (13.83, 22.31) [3.36, 66.21]	16.57 (10.71, 21.81) [4.73, 75.59]	5.185	0.053
VitB5 (ng/mL)	26.68 (21.83, 38.1) {9.61, 101.45}	23.96 (17.41, 42.31) Δ [7.07, 77.68]	17.68 (14.50, 30.16) Δ° [6.09, 71.10]	65.634	<0.001
VitB6 (ng/mL)	2.78 (2.08, 4.27) [0.75, 22.41]	2.69 (1.43, 3.95) Δ [0.83, 15.25]	2.26 (1.82, 3.00) Δ [0.62, 10.10]	28.653	<0.001
VitB7 (ng/mL)	0.59 (0.55, 0.65) [0.12, 1.91]	0.59 (0.55, 0.65) [0.20, 0.92]	0.59 (0.54, 0.67) [0.37, 1.64]	0.096	0.953
VitB9 (ng/mL)	23.50 (17.44, 29.02) [3.21, 40.86]	21.23 (18.25, 25.37) Δ [5.08, 35.28]	19.20 (14.15, 24.31) Δ [2.35, 35.57]	31.421	<0.001
VitB12 (ng/dL)	14.00 (11.49, 19.19) [2.04, 118.00]	13.72 (11.03, 20.20) [9.80, 70.29]	12.74 (10.78, 17.64) Δ [4.08, 75.46]	10.253	0.006
VitC (µg/mL)	8.80 (6.73, 11.35) [0.59, 27.91]	8.24 (6.52, 10.60) [1.37, 25.57]	8.29 (6.52, 11.25) [1.70, 24.21]	3.609	0.165

Table 1. Nine water-soluble vitamin levels in three pregnant groups [Median (P_{25}, P_{75})]. NFP, normal pregnancy; OSA, once spontaneous abortion; CSA, continuous spontaneous abortion. Δ vs. NFP group, $P_{adj} < 0.05$; \circ vs. OSA group, $P_{adj} < 0.05$. Results suggested that all eight vitamin levels ranged widely with notably high individual outliers, while only five B vitamins (B1, B5, B6, B9, and B12) existed significant differences in the median levels among the three pregnant groups.

vitamin B5 (7.7%) and B9 (17.5) deficiencies were also significant in CSA group (Table 2). Only vitamin B3, B5, B6 and B9 deficiencies were significantly different among the three groups ($\chi^2=12.191 \sim 37.561$, all $P < 0.001$). Pairwise comparisons found that B3 deficiency was higher in the CSA group than in the NFP and OSA groups ($\chi^2=9.202$ and 9.927 , both $P=0.002$), while B5 and B6 deficiencies were higher in the OSA and CSA groups than in the NFP group ($\chi^2=13.637 \sim 41.669$, all $P < 0.001$).

Univariate risk analysis of each water-soluble vitamin to RSA

The RSA occurrence in overall pregnant women was 6.54% (60/918), with a prevalence of 12.57% (21/167) in the OSA group and 27.27% (39/143) in the CSA group. Fetal survival curve showed that the RSA occurrence was higher in the CSA group than in the OSA group ($\chi^2=10.627$, $P=0.001$) (Fig. 1). The risk analysis of each water-soluble vitamin demonstrated that vitamin B5, B6, and B9 were the risk factors to RSA occurrence among both overall pregnant women ($HR=0.95$, 0.87, and 0.87, all $P < 0.001$) and the CSA group ($HR=0.87$, 0.71, and 0.85, all $P < 0.01$), while vitamin B6 and B9 were the risk factors to RSA occurrence among the OSA group ($HR=0.82$ and 0.91, all $P < 0.05$) (Table 3). These results suggest that the miscarriage rate in the CSA group significantly increased, which may be involved in more influencing factors including vitamin B5 deficiency.

Comprehensive risk assessment of water-soluble vitamin deficiency to RSA occurrence

Under the confounding effect of age and the interaction of each water-soluble vitamin (Table 4), vitamin B9 was an independent factor among overall pregnant women, OSA, and CSA groups ($HR=0.87$, 0.89, and 0.88, all $P < 0.001$); B6 was an independent factor among OSA, and CSA groups ($HR=0.83$ and 0.78, all $P < 0.05$); while B5 was an independent factor only among CSA group ($HR=0.93$, $P=0.005$). These results suggest that the risk of water-soluble vitamin deficiencies contributing to miscarriage varies among pregnant women with different SA history, indicating the need for tailored treatment approaches.

Vitamin	Overall (n=918)	NFP Group (n=608)	OSA Group (n=167)	CSA Group (n=143)	χ^2	P
VitB1	5 (0.5)	2 (0.3)	1 (0.6)	2 (1.4)	2.456	0.293
VitB2	73 (8.0)	55 (9.0)	6 (3.6)	12 (8.4)	5.367	0.068
VitB3	209 (22.8)	131 (21.5)	30 (18.0)	48 (33.6) Δ°	12.191	0.002
VitB5	17 (1.9)	1 (0.2)	5 (3.0) Δ	11 (7.7) Δ	37.561	<0.001
VitB6	246 (26.8)	126 (20.7)	69 (41.3) Δ	51 (35.7) Δ	35.114	<0.001
VitB7	20 (2.2)	12 (2.0)	5 (3.0)	3 (2.1)	0.645	0.724
VitB9	4 (0.4)	1 (0.2)	0 (0.0)	3 (2.1) Δ	10.869	0.004
VitB12	48 (5.2)	26 (4.3)	10 (6.0)	12 (8.4)	4.194	0.123
VitC	171 (18.6)	107 (17.6)	35 (21.0)	29 (20.3)	1.281	0.527

Table 2. The deficiencies of nine water-soluble vitamins in three pregnant groups [n(%)]. Note: NFP, normal pregnancy; OSA, once spontaneous abortion; CSA, continuous spontaneous abortion. Δ vs. NFP group, $P < 0.05$; \circ vs. OSA group, $P < 0.05$.

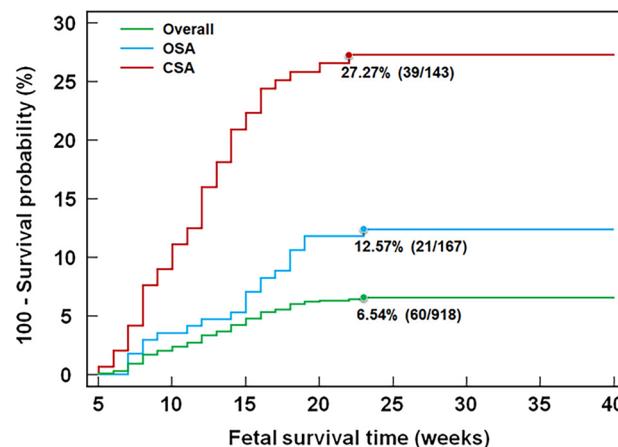


Fig. 1. The fetal survival curve in overall pregnant women, OSA and CSA groups. Note OSA, once spontaneous abortions; CSA, continuous spontaneous abortions. The default duration for fetal survival in any surviving fetus was set at 40 gestational weeks. The corresponding cumulative miscarriage rate in each group is highlighted using a dot.

Vitamin	Overall (n=918)			OSA vs. NFP (n=775)			CSA vs. NFP (n=751)		
	HR (95%CI)	Wald	P	HR (95%CI)	Wald	P	HR (95%CI)	Wald	P
VitB1	0.94 (0.73, 1.22)	0.229	0.632	1.12 (0.83, 1.51)	0.575	0.448	0.79 (0.53, 1.16)	1.452	0.786
VitB2	1.00 (0.96, 1.04)	0.001	0.983	1.01 (0.95, 1.08)	0.233	0.629	0.99 (0.95, 1.04)	0.040	0.841
VitB3	0.98 (0.95, 1.01)	1.926	0.165	0.96 (0.91, 1.02)	1.844	0.175	0.98 (0.95, 1.02)	0.565	0.452
VitB5	0.95 (0.92, 0.97)	15.881	<0.001	0.99 (0.96, 1.03)	0.028	0.867	0.87 (0.83, 0.91)	31.773	<0.001
VitB6	0.87 (0.74, 0.95)	11.595	<0.001	0.82 (0.67, 0.98)	6.336	0.019	0.71 (0.62, 0.89)	7.517	0.009
VitB7	1.18 (0.22, 6.39)	0.038	0.845	0.60 (0.02, 15.21)	0.098	0.597	1.37 (0.21, 9.18)	0.107	0.743
VitB9	0.87 (0.84, 0.90)	58.130	<0.001	0.91 (0.86, 0.96)	11.032	<0.001	0.85 (0.81, 0.89)	45.946	<0.001
VitB12	1.00 (0.98, 1.02)	0.002	0.963	0.99 (0.95, 1.04)	0.038	0.844	1.00 (0.97, 1.03)	1.198	0.274
VitC	0.99 (0.92, 1.06)	0.161	0.688	1.00 (0.89, 1.13)	0.001	0.999	0.97 (0.89, 1.06)	0.436	0.509

Table 3. The hazard ratio of each water-soluble vitamin to RSA occurrence. NFP, normal pregnancy; OSA, once spontaneous abortion; CSA, continuous spontaneous abortion. Significance values are shown in bold.

Vitamin	Overall (n=918)			OSA vs. NFP (n=775)			CSA vs. NFP (n=751)		
	HR (95%CI)	Wald	P	HR (95%CI)	Wald	P	HR (95%CI)	Wald	P
Age	1.06 (0.99–1.12)	2.977	0.084	1.07 (0.96–1.19)	1.612	0.204	1.06 (0.98–1.15)	2.066	0.151
VitB1	1.05 (0.91–1.22)	0.449	0.503	1.13 (0.88–1.44)	0.905	0.342	0.99 (0.82–1.19)	0.021	0.885
VitB2	1.01 (0.98–1.05)	0.445	0.505	1.02 (0.96–1.08)	0.255	0.614	1.00 (0.95–1.05)	0.000	0.988
VitB3	0.99 (0.96–1.01)	1.009	0.315	0.96 (0.91–1.02)	1.667	0.197	1.00 (0.97–1.04)	0.079	0.778
VitB5	0.98 (0.95–1.01)	1.896	0.169	1.01 (0.98–1.05)	0.402	0.526	0.93 (0.88–0.98)	7.843	0.005
VitB6	0.89 (0.71–1.10)	1.233	0.267	0.83 (0.67–0.99)	4.922	0.027	0.78 (0.66–0.94)	4.885	0.028
VitB7	1.83 (0.37–9.15)	0.540	0.463	0.50 (0.01–9.64)	0.142	0.707	1.59 (0.27 to 9.51)	0.257	0.612
VitB9	0.87 (0.84–0.91)	46.430	<0.001	0.89 (0.83–0.94)	14.076	<0.001	0.88 (0.84–0.93)	22.985	<0.001
VitB12	1.00 (0.98–1.03)	0.053	0.818	1.00 (0.96–1.05)	0.009	0.926	1.00 (0.97–1.04)	0.004	0.952
VitC	1.07 (1.00–1.15)	2.159	0.121	1.12 (0.99–1.26)	3.317	0.069	1.03 (0.95–1.12)	0.475	0.491

Table 4. The hazard ratio of water-soluble vitamin to RSA occurrence under a confounding effect. NFP, normal pregnancy; OSA, once spontaneous abortion; CSA, continuous spontaneous abortion. Significance values are shown in bold.

Predictive power of water-soluble vitamins on RSA occurrence

According to the above analysis, we utilized the data of overall pregnant women to establish a Cox proportional-hazards model for predicting the RSA occurrence based upon vitamin B5, B6, and B9 levels: Prognostic index (PI) = $-0.020 \times B5 - 0.143 \times B6 - 0.123 \times B9$ ($\chi^2 = 71.327$, $P < 0.001$; $C\text{-index} = 0.777$, 95% CI = 0.722 ~ 0.831) (See supplementary Table 1 for the details about the baseline cumulative hazard). The cumulative RSA risks at the average levels of vitamin B5 (29.92 ng/mL), B6 (3.45 ng/mL), and B9 (22.17 ng/mL) were observed to range from 0.1 ~ 3.5% (Fig. 2A). The performance of this model for predicting miscarriage in the OSA group was relatively low, with an AUC of 0.704, a sensitivity of 95.2%, and a specificity of 39.0% (Fig. 2B). In contrast, the model exhibited a relatively high performance for predicting miscarriage in the CSA group, with an AUC value of 0.825, a sensitivity of 92.3%, and a specificity of 60.6% (Fig. 2C). Overall, the model demonstrated quite high sensitivity in predicting RSA for both OSA and CSA groups, with no significant difference observed between the two groups ($\chi^2 = 1.134$, $P = 0.287$). However, the model was more suitable for predicting RSA occurrence in the CSA group, as it exhibited significantly higher AUC ($z = 2.056$, $P = 0.040$) and specificity ($\chi^2 = 14.549$, $P < 0.001$).

Correlation of water-soluble vitamins with RSA occurrence after supplementation therapy

After 4 weeks of vitamin supplementation, only in the CSA group, the pregnant women with miscarriage exhibited significantly lower levels in vitamin B5 ($z = -2.828$, $P = 0.005$) and B9 ($z = -2.453$, $P = 0.014$) compared to the pregnant women with non-miscarriage (Table 5). However, their B5, B6, and B9 levels were not associated with RSA occurrence (all $P > 0.05$).

Discussion

This study focused on pregnant women who had experienced one or more SA. Using UHPLC-MS/MS, their baseline water-soluble vitamin levels were measured at the time of clinically recognized pregnancy. Our study revealed significant heterogeneity in baseline levels of almost all water-soluble vitamins, and evident deficiencies in multiple B vitamins and vitamin C among pregnant women, as well as risks of baseline levels in vitamin B5, B6 and B9 to the RSA occurrence. The most notable highlight was the successful development of an RSA prediction model based on these risky vitamins, which demonstrated a sensitivity exceeding 90% for predicting RSA in either OSA or CSA groups. However, this model displayed a lower specificity of 39.0% in the OSA group

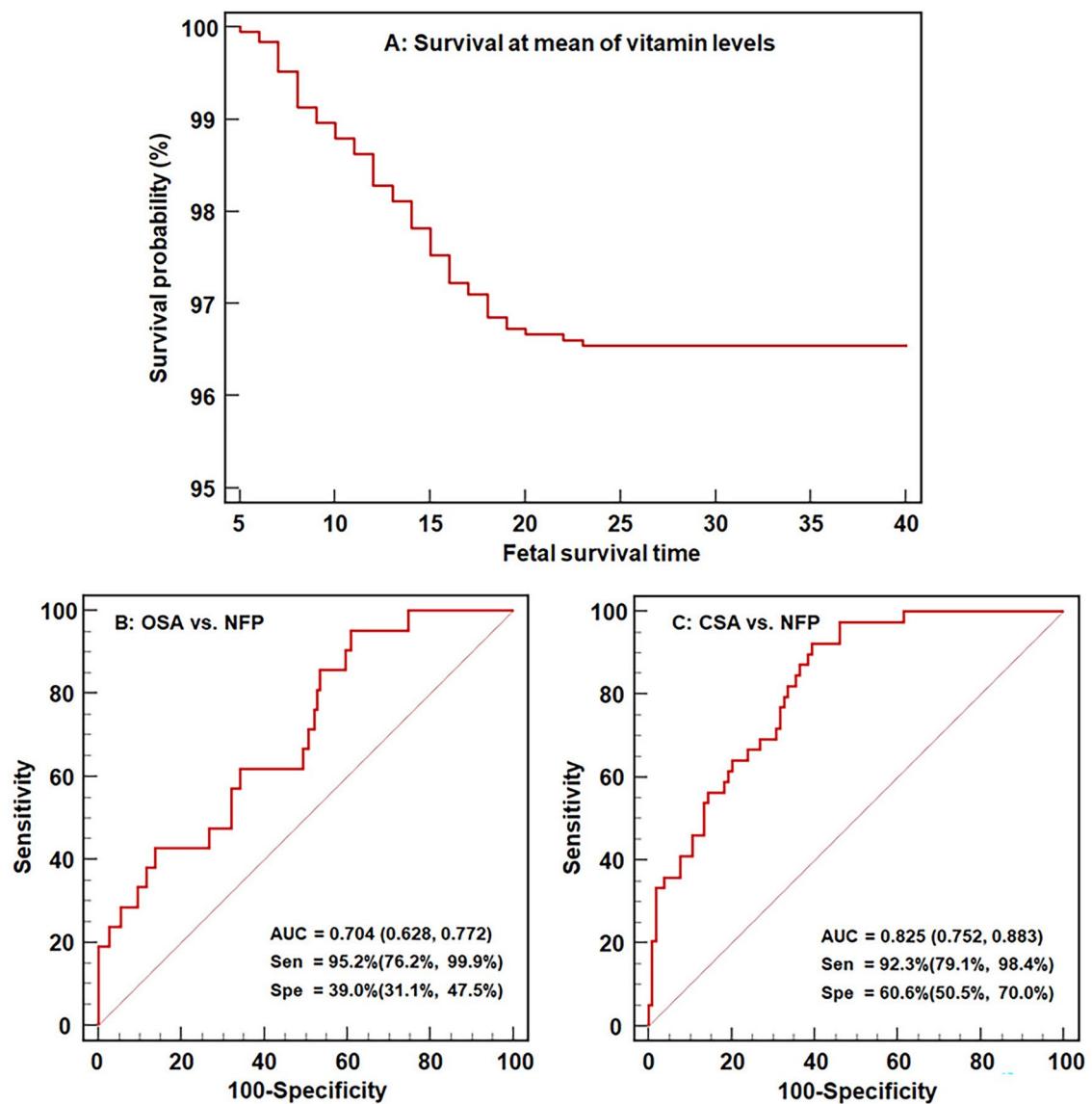


Fig. 2. The Cox proportional-hazards model based on baseline levels of vitamin B5, B6, and B9 to predict miscarriage risk. Note (A) Survival curve at mean baseline level of vitamin B5, B6, and B9, which cumulative RSA risks was 3.5% at 23 gestational weeks; (B) ROC curve of OSA vs. NFP, with sensitivity of 95.2% and specificity of 39.0% when maximum prediction accuracy; (C) ROC curve of CSA vs. NFP, with sensitivity of 92.3% and specificity of 60.6% when maximum prediction accuracy. NFP, normal first pregnancy; OSA, once spontaneous abortions; CSA, continuous spontaneous abortions.

Vitamin	Median (P_{25} , P_{75})		z	P	HR (95%CI)	Wald	P
	Non-miscarriage	Miscarriage					
OSA group							
VitB5	32.16 (24.78, 52.65)	32.17 (24.64, 58.88)	-0.130	0.896	0.99 (0.97, 1.02)	0.408	0.523
VitB6	5.33 (3.47, 7.21)	5.59 (2.97, 8.54)	-0.191	0.849	1.08 (0.98, 1.20)	2.435	0.119
VitB9	24.81 (20.99, 30.45)	25.46 (22.84, 29.66)	0.738	0.460	1.03 (0.97, 1.10)	0.951	0.330
OSA group							
VitB5	28.27 (22.09, 48.08)	24.33 (18.23, 30.27)	-2.828	0.005	0.97 (0.94, 1.01)	2.346	0.126
VitB6	3.61 (2.90, 4.84)	3.27 (2.74, 3.88)	-1.929	0.054	0.88 (0.67, 1.15)	0.891	0.345
VitB9	24.66 (19.39, 29.04)	19.65 (14.53, 27.04)	-2.453	0.014	0.98 (0.94, 1.02)	0.828	0.363

Table 5. Compensatory essential vitamin levels with RSA occurrence. OSA, once spontaneous abortion; CSA, continuous spontaneous abortion. Significance values are shown in bold.

compared to a higher specificity of 60.6% in the CSA group. The prediction of miscarriage using water-soluble vitamins has posed a significant puzzle in this research field. Our achievement on the initial attempt may be attributed to the use of a UHPLC-MS/MS technique for precise quantification, the detection of baseline levels in water-soluble vitamins, the distinctiveness of the pregnant women group being focus on, and subgrouping the RSA pregnant women by number of miscarriages. In addition, the value of compensatory essential vitamin levels in predicting RSA occurrence is limited.

Numerous studies have reported the relationship between vitamin B9 and adverse pregnancy^{28,29}. Vitamin B9, also known as folic acid, plays a crucial role in various cellular metabolic activities. Its deficiency can result in the occurrence of macrocytic anemia, mucositis, infertility, muscular weakness, cardiovascular disease, neurological disorders, cancer, other related conditions³⁰. Over the past two decades, numerous studies have consistently demonstrated that vitamin B9 supplementation significantly enhances pregnancy outcomes¹⁶⁻¹⁸, and continuous use of vitamin B9 during pregnancy also improved perinatal depression³¹. Despite some conflicting evidence³², vitamin B9 supplementation is now widely recommended and universally practiced among reproductive women for preconception planning or during pregnancy in many countries³³⁻³⁵. In this study, only 0.4% of the pregnant women exhibited vitamin B9 levels below the lower limit of reference (4.0 ng/mL), thereby confirming that a majority of pregnant women had initiated vitamin B9 supplementation prior to conception. However, owing to the heightened demand during pregnancy³⁶, there can still be a relative deficiency in vitamin B9. Referring to the recommended optimal serum folate threshold for neural tube defect prevention^{37,38} and the BOND project and WHO recommendation²⁴⁻²⁶, we set a threshold of 10 ng/mL, and identified significantly relative deficiencies of vitamin B9 during pregnancy, especially in the CSA group of 17.5% (25/143). Taken together, adequate pre-pregnancy supplementation of vitamin B9 may serve as a potent intervention in facilitating the growth and development of embryos and fetuses. It is noteworthy that recent evidence suggested excessive vitamin B9 as potential risks of adverse genomic and epigenomic alterations³⁹, thereby necessitating a consideration for continuous monitoring of maternal vitamin B9 levels during pregnancy.

Our study also revealed a significant insufficiency of vitamin B6 in pregnant women with different SA histories (ranging from 20.7 to 41.3%), and further it emerged as a crucial predictor for RSA. Vitamin B6, also known as pyridoxine, serves as a crucial cofactor regulating approximately 150 metabolic reaction processes of protein, glucose, lipids, neurotransmitters, and DNA⁴⁰. Numerous studies have demonstrated the involvement of vitamin B6, B9, and B12 in methionine metabolism, resulting in the accumulation of homocysteine^{41,42}. Elevated levels of homocysteine can lead to the occurrence of neural tube defect⁴³, preeclampsia^{44,45}, intrauterine growth retardation or fetal death^{44,45}, and gestational diabetes mellitus^{46,47}, as well as other factors contributing to miscarriages. Some scholar has suggested the preventive administration of vitamin B6 to reduce these complications⁴⁸. But its role in preventing miscarriage remains controversial^{48,49}. Our study findings indicated that vitamin B6 deficiency increase the risk for RSA in pregnant women with a history of at least one miscarriage, highlighting the importance of considering supplementation prior to planning subsequent pregnancies.

As for vitamin B5, also known as pantothenic acid, is essential for the synthesis of Coenzyme A, which plays a crucial role in various physiological processes such as energy metabolism from fats, carbohydrates, and proteins⁵⁰. In general, the abundant dietary sources and infrequent deficiencies of vitamin B5 contribute to limited research on this nutrient, as well as a dearth of significant findings. It has been reported that the levels of vitamin B5 in the blood significantly decrease during pregnancy⁵¹, which is associated with a higher risk of low birth weight in offspring⁵². Although clinical evidence is extremely lacking, vitamin B5 deficiency has been observed to be associated with miscarriage in an old animal study⁵³. Interestingly, our study revealed that vitamin B5 deficiency was extremely rare among overall pregnant women, but occurred to a certain extent in the CSA group (7.7%); so, it still posed a risk for the CSA group. Regarding the potential link between vitamin B5 deficiency and miscarriage in the CSA group, it could be attributed to factors such as fetal underdevelopment⁵³, as well as maternal depression^{54,55}, anxiety⁵⁵, and severe malnutrition⁵³. These conditions are commonly observed among reproductive women with continuous SA history¹. Moreover, it has been indicated that pregnant women should maintain only the average level of vitamin B5, as an increased intake of more than 5.6 mg/day may lead to genome instability and subsequent teratogenicity⁵⁴.

To the best of our knowledge, we successfully addressed the puzzle of utilizing B vitamins to predict RSA. Our prediction models, constructed using B5, B6, and B9, exhibited exceptional sensitivity and were deemed suitable for primary screening of RSA. Consequently, regular prenatal screening for B vitamins, particularly B5, B6, and B9, is imperative for ensuring pregnancy safety. However, the utilization of mass spectrometry for vitamin detection is not prevalent. Therefore, it is also imperative to explore alternative detection techniques that offer greater convenience compared to mass spectrometry.

The limitation of our study: (1) Our study retained only the NFP group as a control without women who experienced a miscarriage in their first pregnancy, which may have introduced bias into the conclusions. (2) The failure to investigate the exact etiology, which was unknown in most miscarriages, might introduce potential bias in the model applicability. (3) Although the proposed model demonstrated excellent sensitivity in predicting RSA, its unsatisfactory specificity resulted in significant false positives that cannot be disregarded, especially in OSA group.

Conclusion

Taken together, we developed for the first a Cox proportional-hazards model based on baseline levels of vitamin B5, B6, and B9 to predict miscarriage risk in pregnant women with a history of SA. On one hand, the model may facilitate the identification of pregnant women at high risk for RSA, thereby timely implementing individualized treatment and intervention. On the other hand, for the pregnant women at low risk for RSA identified by the

model, the excessive vitamin supplementation should be avoided to prevent vitamin toxicity-related adverse events.

Data availability

The datasets used and analyzed are available from the corresponding author on reasonable request.

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Author contributions

Y.Y., J.T. and Y.M. proposed the conceptualization and obtained funding support. B.W. and Z.L. wrote the main manuscript text. B.P. and Y.M. followed up the patients. B.W. and W.J. performed the investigation and conducted the data curation. Y.Y. graphed all figures and supplementary figures. All authors reviewed the manuscript. B.W., Z.L., and B.P. contributed equally to this work.

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Competing interests

The authors declare no competing interests.

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and approved by the Medical Ethics Committee of Mianyang Central Hospital, School of Medicine, University of Electronic Science and Technology of China (No. S2018085, and S2018093). All participants signed informed consents.

Additional information

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Correspondence and requests for materials should be addressed to Y.M., J.T. or Y.Y.

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