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Original Article

Serum Zn and Ca correlate with false positive non-invasive prenatal test results

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Abstract: Objectives: The precise cause of false-positive results in non-invasive prenatal testing (NIPT) associated with suspected placental chimerism - where the placenta exhibits chromosomal abnormalities absent in the fetus, is not well understood. It is also unclear whether serum minerals such as zinc (Zn) and calcium (Ca), which are essential for maternal and fetal health, influence these false positives. Methods: In this retrospective analysis, 16,270 pregnant women were evaluated for serum mineral concentrations at 14 weeks of gestation, while NIPT was conducted between 12 and 22 weeks of gestation. Positive NIPT results prompted amniotic fluid karyotyping to confirm the diagnosis. Binary logistic regression analysis and restricted cubic spline (RCS) methods were used to assess the association between individual serum mineral levels and false-positive NIPT results. Results: The false-positive NIPT group exhibited altered serum mineral levels compared to the normal group, with increased sodium (Na), potassium (K), and Zn and decreased Ca, phosphorus (P), and iron (Fe). Multifactor regression analysis confirmed these trends. RCS analysis revealed a non-linear relationship between Zn and Ca levels and NIPT false-positive rates. Specifically, false-positive rates decreased with increasing Zn levels up to 13.1 μmol/L but rose beyond this threshold. Conversely, false-positive rates increased with higher Ca concentrations up to 2.31 mmol/L and then declined. These associations remained significant after adjusting for confounding factors. Additionally, a strong correlation was observed between NIPT false positivity and restrictive placental chimerism, highlighting a significant link between maternal serum Zn and Ca levels and the occurrence of restrictive placental chimerism. Conclusion: This study uncovers a non-linear relationship between maternal serum Zn and Ca levels and NIPT false positives, which are suspected to be linked to restrictive placental chimerism. Moreover, moderate Zn and Ca supplementation may contribute to the development of a more precise nutritional management program for pregnancy.

Keywords: Non-invasive prenatal testing, Zn, Ca, restricted cubic spline

Introduction

Noninvasive prenatal testing (NIPT) is an effective screening tool for detecting chromosomal abnormalities, including trisomies 21, 18, and 13, as well as abnormalities in sex chromosomes, rare autosomal anomalies, and subchromosomal copy number variations [1-3]. However, its diagnostic accuracy is limited by the potential for false-positive and false-negative results, with restricted placental chimerism being a key contributing factor [4]. False-positive NIPT outcomes can cause significant distress for pregnant women and often necessitate invasive diagnostic procedures, increasing the risk of miscarriage. Additionally, placental chimerism has been associated with adverse pregnancy outcomes such as intrauterine growth restriction (IUGR), preterm labor, and preeclampsia. These conditions not only endanger the pregnancy but can also have long-term consequences for maternal and infant health.

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For instance, IUGR is linked to a higher risk of developmental delay and chronic health conditions in neonates, while preeclampsia increases the likelihood of preterm delivery and low birth weight [4]. Investigating the factors underlying false-positive NIPT results due to placental chimerism may provide valuable insight for reducing birth defect risks and improving maternal and infant health outcomes.

Minerals such as zinc (Zn) and calcium (Ca) play essential roles in maintaining maternal and fetal health during pregnancy [5]. Zinc is crucial for placental development, immune regulation, and oxidative stress management. Its deficiency has been linked to an increased risk of gestational diabetes, preterm labor, and fetal growth abnormalities, including impaired cardiovascular development [6, 7]. Additionally, inadequate zinc levels may contribute to obstetric complications such as preeclampsia [8]. Similarly, calcium is vital for bone and tooth formation, enzyme activity in coagulation, acid-base balance, and nerve and muscle function. Inadequate calcium intake during pregnancy can lead to gestational hypertension, an increased risk of postpartum hemorrhage, and potential adverse effects on fetal nervous system and bone development [9]. Research indicates that maintaining adequate levels of these minerals supports maternal and fetal well-being, underscoring the importance of a balanced intake in preventing pregnancy complications. However, the influence of serum Zn and Ca levels on NIPT false positives - largely attributed to placental chimerism - remains poorly understood.

In this study, we conducted a regression analysis on 16,270 pregnant women who underwent mid-pregnancy non-invasive prenatal testing (NIPT). False-positive results, identified through amniocentesis or follow-up evaluations as chromosomally normal fetuses, were suspected to result from placental chimerism. Additionally, these women underwent serological monitoring for various minerals, including sodium, potassium, chloride, calcium, phosphorus, magnesium, copper, iron, and zinc. We applied binary logistic regression analysis and restricted cubic spline (RCS) methods to assess whether variations in serum mineral levels were associated with false-positive NIPT results linked to suspected placental chimerism. The study aimed to identify factors contributing to placental chimerism and to establish a robust scientific foundation for pregnancy nutrition guidelines, emphasizing the importance of individualized micronutrient supplementation.

Patients and methods

Study population

The study recruited 16270 pregnant women who underwent NIPT between August 2015 and December 2021 at Suzhou Municipal Hospital, with successful test results obtained. Inclusion criteria required a biochemical test before 15 weeks of gestation, and all samples were analyzed for fetal chromosome abnormalities using single nucleotide polymorphism (SNP) arrays or clinical follow-up results. Women with unsatisfactory test results were excluded. Data on maternal age, height, weight, gravida, parity, conception via *in vitro* fertilization (IVF), and gestational age at biochemical testing were collected. The study received approval from the Ethics Committee of Reproductive Medicine at Suzhou Municipal Hospital.

cfDNA testing

10 ml of peripheral blood was collected and collected into EDTA anticoagulation tubes, and plasma was separated within 4 hours using the using the following protocol: centrifugation at 4°C and 1600 g for 10 minutes, removal of the supernatant plasma, and centrifugation again at 16,000 g for 10 minutes to obtain cell-free plasma. cfDNA was extracted from plasma using the TIANamp Micro DNA Purification Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. cfDNA sequencing was performed using the Illumina NextSeq CN500 platform (Illumina, San Diego, CA, United States), the Ion Torrent Proton system (Thermo Fisher Scientific, Waltham, MA, United States), and the BGISEQ-500 sequencing platform (BGI, Shenzhen, China). Sequencing data were aligned to the hg19 human genome reference sequence. Unmapped, low-quality, and duplicate reads were removed. All chromosomes were divided into equal size segments, termed bins, with a bin size of 20 kb selected for all subsequent analyses. A locally weighted scatterplot smoothing (LOESS) regression was applied to the guanine and cyto-

sine (GC)-corrected read counts to remove GC-related bias [10]. The Z-score foreach chromosomes was calculated using the following formula: Z-score of the percentage of chromosome of interest in the test sample = [(percentage of chromosome of interest in the test sample)-(mean percentage of chromosome of interest in the reference control)]/(standard deviation of the percentage of chromosome of interest in the reference control), A Z-score threshold of 3 was used as the cutoff [11].

SNP array analysis

SNP array analysis was performed using the Affymetrix CytoScan platform (Affymetrix, Santa Clara, CA, USA) following a previously described procedure [12]. Genomic DNA (250 ng) was digested, ligated, PCR amplified, purified, fragmented, labelled and hybridized to the Affymetrix 750 K array, which contains 550,000 copy number variant (CNV) markers and 200,000 SNP markers. After the array was washed, stained, and scanned, raw data were analyzed using Chromosome Analysis Suite (ChAS) 3.2 (Affymetrix, Santa Clara, CA, USA). CNVs were interpreted according to the standards and guidelines for the interpretation and reporting of postnatal constitutional copy number variants published by the American College of Medical Genetics, with a minimum length requirement of 50 kb and at least 20 contiguous markers [13].

Biochemical analysis

Serum levels of sodium (Na), potassium (K), chlorine (Cl), Ca, phosphorus (P), magnesium (Mg), copper (Cu), iron (Fe), and Zn in serum were measured using the Beckman AU5800 Automatic Biochemical Analyzer (Beckman Coulter, USA). Stringent quality control was maintained using standard reference materials from the China National Center for Standard Materials (CNCSM).

Statistical analysis

Descriptive data are presented as medians and interquartile ranges for non-normally distributed continuous variables [14]. The nonparametric Wilcoxon rank-sum test was used to compare differences between the two groups, while the Chi square test was used to assess differences in IVF status. Univariate and multivariate logistic regression models were applied to evaluate the association between NIPT false positives, gestational age at biochemical testing, and trace element levels. The linear relationship between plasma Zn and Ca levels and NIPT false positives was analyzed using restricted cubic spline (RCS) curves. All statistical analyses were performed using IBM SPSS software version 26.0 (IBM Corp, Armonk, NY, USA). A two-sided *p* value of < 0.05 was considered significant.

Results

Basic characteristics of the pregnant women

Among the 16,270 cases tested by NIPT, 16,005 were found to be normal, while 265 samples (1.63%) were identified as false positives (**Figure 1**). As shown in **Table 1**, compared to the normal group, the NIPT false-positive group had a lower number of previous maternal pregnancies ($P < 0.05$). However, no significant differences were observed in key maternal characteristics, including age, height, weight, or parity between the normal group and the NIPT false positive group (all $P > 0.05$).

Differences in plasma trace element levels

Biochemical tests were performed on the peripheral blood of pregnant women to analyze trace element levels, revealing that the pregnant women in the NIPT false positive group had a shorter gestational age at the time of biochemical testing (**Table 1**; **Figure 2A**). The differences in trace elements between the NIPT false-positive group and normal groups were assessed using the nonparametric Wilcoxon rank-sum test. The results indicated that plasma levels of Na, K, and Zn were significantly higher in the NIPT false-positive group compared to the normal group (**Table 1**; **Figure 2B**, **2C**, **2G**, all $P < 0.05$). Additionally, plasma levels of Ca, P, and Fe were significantly lower in the NIPT false-positive group compared to the normal group (**Table 1**; **Figure 2D-F**, all $P < 0.05$).

Multiple factors affecting false positive results

To evaluate the correlation between plasma trace elements and NIPT false positives, a multivariate logistic regression model was employed to account for potential confounding

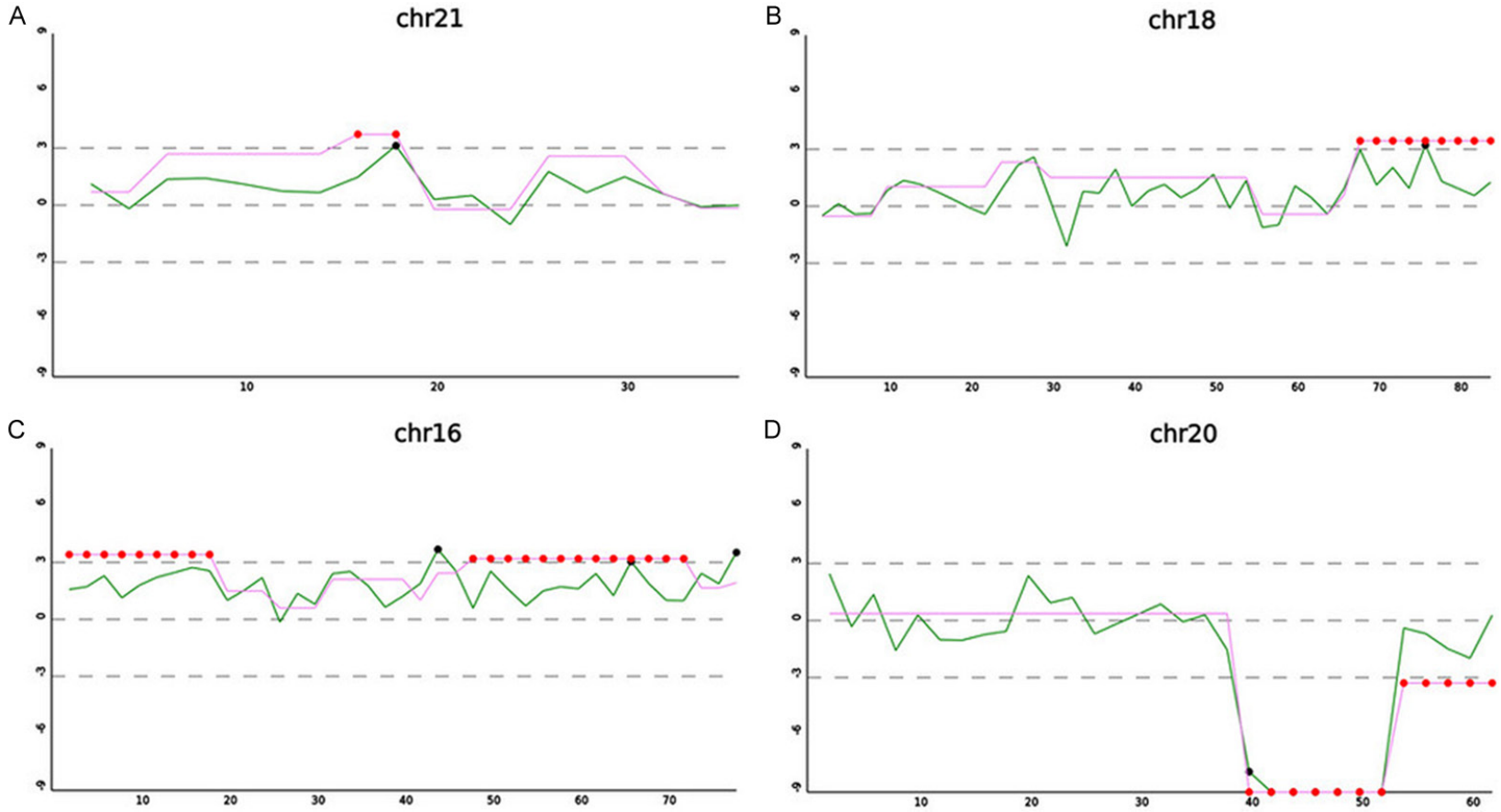

Figure 1. NIPT testing suggested abnormalities on chromosome 21 (A), chromosome 18 (B), chromosome 16 (C) and chromosome 20 (D), whereas SNP array analysis was normal. NIPT: Noninvasive prenatal testing.

Table 1. Sample characteristics of NIPT false positive group and normal group

Variable	NIPT false positive group M (P_{25}, P_{75})	Normal group M (P_{25}, P_{75})	Z	p
Age (year)	31.0 (28.0, 34.0)	31.0 (28.0, 35.0)	-0.335	0.738
Height (cm)	160.0 (158.0, 164.5)	161.0 (158.0, 165.0)	-1.171	0.242
Weight (kg)	58.0 (52.4, 65.2)	57.5 (52.7, 63.0)	-1.604	0.109
Gravida	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	-2.372	0.018
Parity	0.0 (0.0, 1.0)	0.0 (0.0, 1.0)	-0.625	0.532
Gestational week	12.3 (11.6, 13.0)	12.7 (12.1, 13.3)	-6.397	< 0.001
Na (mmol/L)	139.0 (137.8, 140.1)	138.2 (136.6, 139.8)	-5.260	< 0.001
K (mmol/L)	4.0 (3.9, 4.2)	4.0 (3.8, 4.2)	-3.562	< 0.001
Cl (mmol/L)	103.3 (102.0, 104.7)	103.0 (101.7, 104.3)	-3.311	0.001
Ca (mmol/L)	2.3 (2.2, 2.3)	2.3 (2.2, 2.4)	-6.058	< 0.001
P (mmol/L)	1.2 (1.2, 1.3)	1.3 (1.2, 1.4)	-5.227	< 0.001
Fe (μmol/L)	20.1 (16.1, 24.2)	21.2 (17.5, 25.0)	-2.365	0.018
Zn (μmol/L)	14.2 (12.4, 16.1)	13.1 (11.6, 14.7)	-6.577	< 0.001

Z: Mann-Whitney test. NIPT: Noninvasive prenatal testing.

variables. As shown in **Figure 3**, the analysis revealed that a higher rate of NIPT false positive was associated with increased plasma levels of Na (OR (95% CI): 1.092 (1.034, 1.152), P = 0.001), K (OR (95% CI): 2.082 (1.366, 3.171), P = 0.001), and Zn (OR (95% CI): 1.144 (1.099, 1.192), P < 0.001). Conversely, a lower rate of NIPT false positives was associated with decreased plasma levels of Ca (OR (95% CI): 0.034 (0.011, 0.105), P < 0.001), P (OR (95% CI): 0.132 (0.052, 0.336), P < 0.001), and Fe (OR (95% CI): 0.970 (0.950, 0.990), P = 0.004). Furthermore, an increase in gestational age at the time of biochemical testing was associated with a reduction in the NIPT false positive rate (OR (95% CI): 0.625 (0.547, 0.714), P < 0.001).

Univariate and multivariate RCS analyses of Zn and NIPT false positives

Restricted cubic spline (RCS) models were used to assess the relationship between plasma Zn levels and NIPT false positives. Univariate RCS analysis demonstrated a nonlinear relationship, with smoothed spline plots indicating that the rate of NIPT false positives increased as Zn levels rose (**Figure 4A**, p for overall < 0.001, p for nonlinearity = 0.001). The reference Zn level was determined to be 13.1 μmol/L. Additionally, a multivariate RCS model, adjusted for gestational age at biochemical testing and plasma levels of Na, K, Ca, P, and Fe, yielded consistent results (**Figure 4B**, p for overall < 0.001, p for nonlinearity = 0.001).

Univariate and multivariate RCS analyses of Ca and NIPT false positives

Next, the relationship between plasma Ca levels and NIPT false-positive rates was analyzed and visualized using RCS models. Univariate RCS analyses indicated a significant nonlinear relationship between plasma Ca levels and NIPT false positives (**Figure 5A**, p for overall < 0.001, p for nonlinearity < 0.001). The reference Ca level was 2.31 mmol/L. The results showed a decreasing trend, suggesting that the NIPT false-positive rate declined as plasma Ca levels increased. These findings were further supported by multivariate analyses, which produced consistent results (**Figure 5B**, p for overall < 0.001, p for nonlinearity < 0.001).

Discussion

In this study, a large sample of 16,270 pregnant women who had undergone NIPT testing was retrospectively analyzed. Among these, 16,005 tested normal, while 265 had false-positive results, allowing for the investigation of possible sources of placental chimerism and maternal serum mineral levels. For the first time, it was found that levels of Na, K, and Zn were higher, while Ca, P, and Fe were lower in cases suspected of placental chimerism. Multifactorial logistic analysis confirmed these findings. Moreover, through restricted cubic spline (RCS) analysis focusing on zinc and calcium, we observed that these minerals influ-

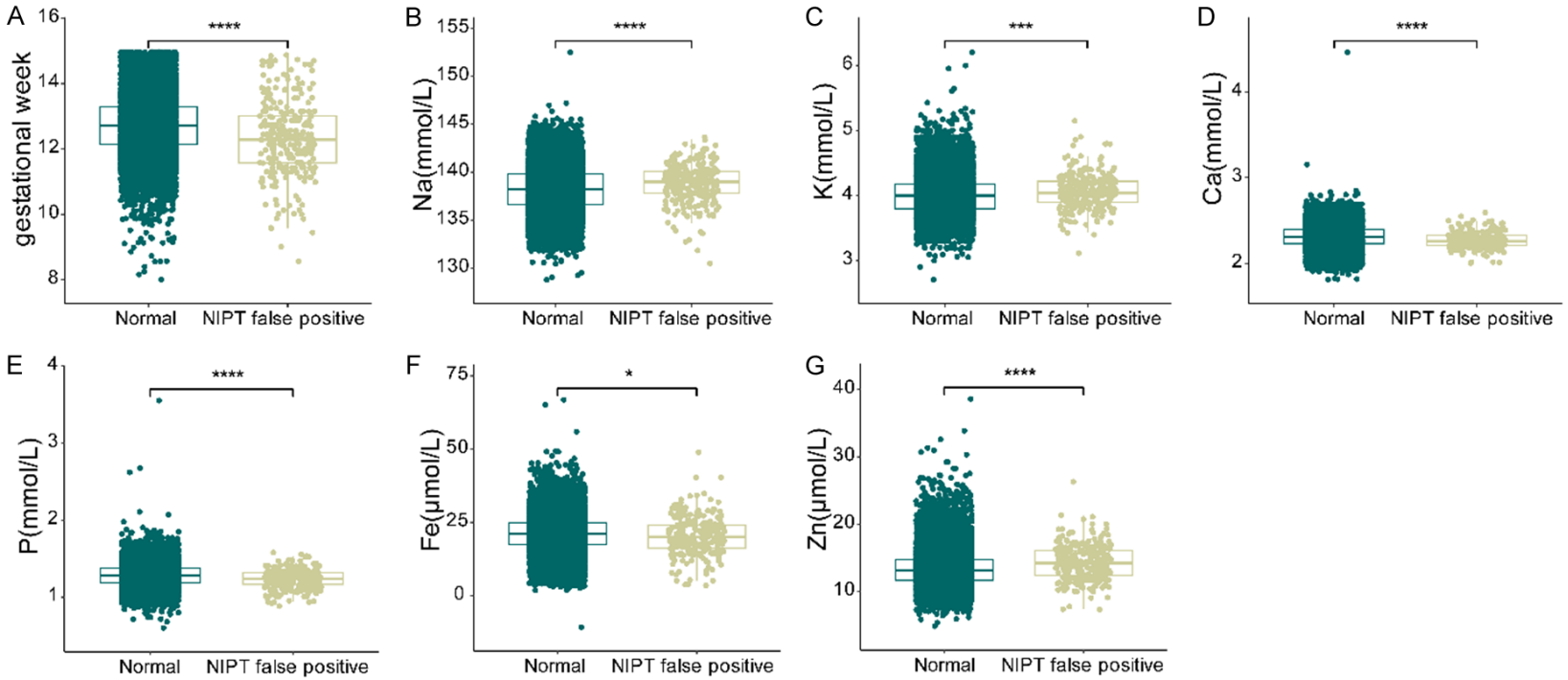

Figure 2. Differences in trace elements levels between the NITP false positive and normal groups. A. Gestational week of biochemical testing; B. Na; C. K; D. Ca; E. P; F. Fe; G. Zn. *P < 0.05, ***P < 0.001, ****P < 0.0001. NIPT: Noninvasive prenatal testing.

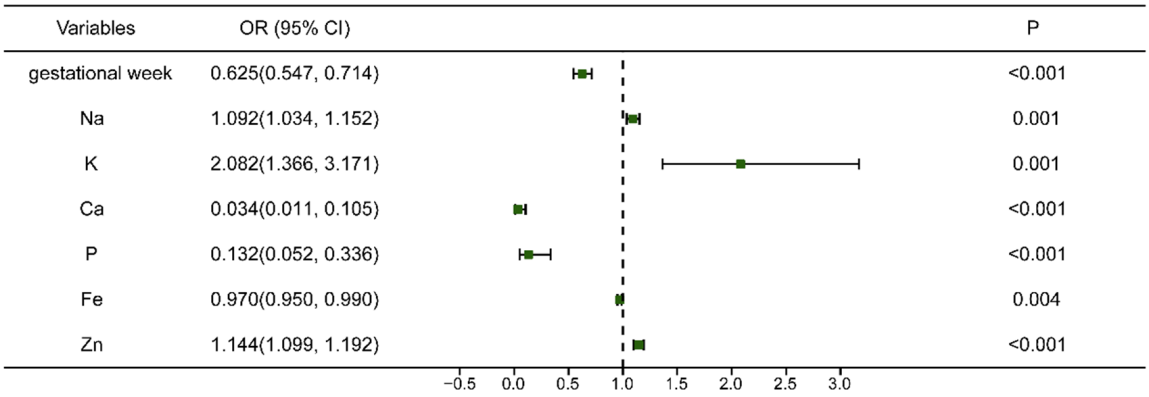

Figure 3. Multivariate logistic regression of the correlation between plasma trace elements and NIPT false positives. NIPT: Noninvasive prenatal testing.

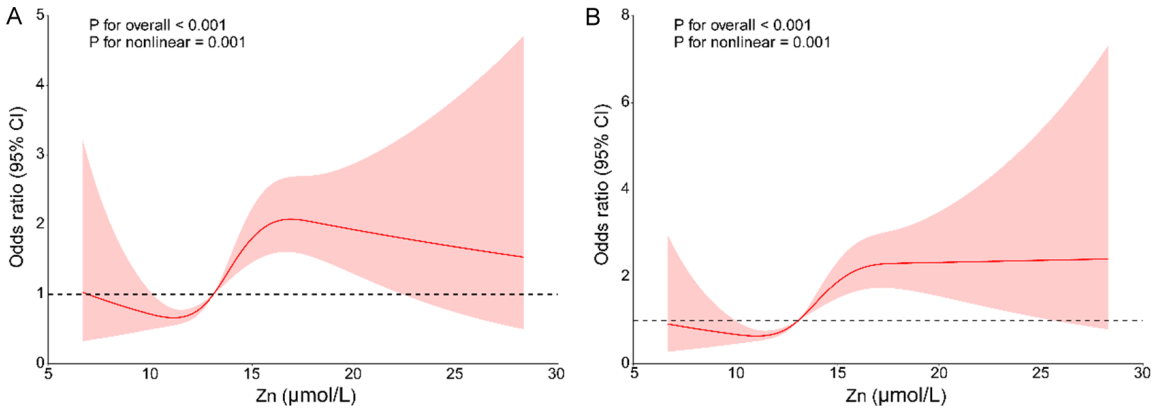

Figure 4. RCS models of the relationship between plasma Zn levels and NIPT false positives. A. Univariate RCS analyses; B. Multivariate RCS analyses. RCS: restricted cubic spline, NIPT: Noninvasive prenatal testing.

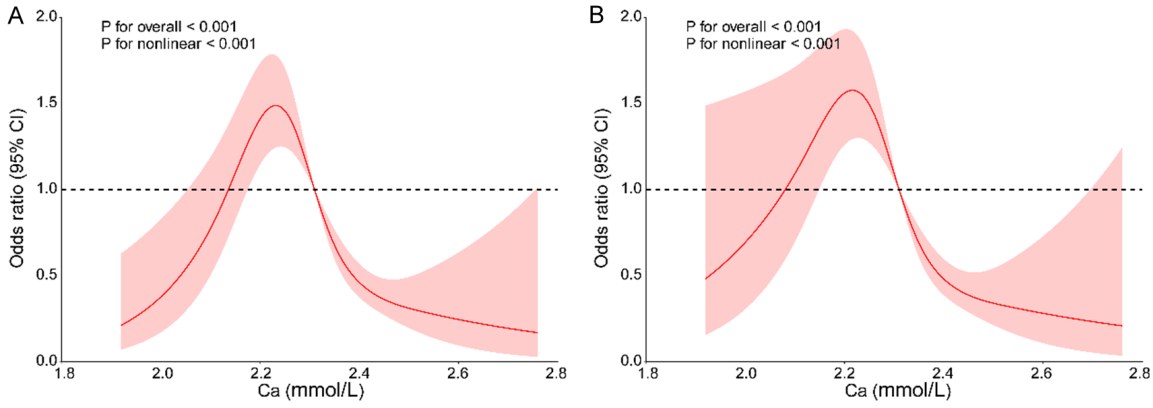

Figure 5. RCS models of the relationship between plasma Ca levels and NIPT false positives. A. Univariate RCS analyses; B. Multivariate RCS analyses. RCS: restricted cubic spline, NIPT: Noninvasive prenatal testing.

enced the rate of false positives in NIPT tests, exhibiting adverse effects at zinc levels of 13.1 μmol/L and Ca levels at a threshold of 2.31 mmol/L. This indicates that varying levels of zinc and calcium levels in pregnant women's serum might significantly affect the develop-

ment of placental chimerism, leading to false-positive results in NIPT tests.

Zinc is a vital trace element, second only in abundance to iron in the body, which lacks the ability to store it [15]. Thus, a diet rich in zinc is crucial for maintaining metabolic health, particularly for expectant mothers [16]. During pregnancy, the need for zinc intensifies as both the fetus and placental tissues require more of it. However, intestinal absorption of zinc does not increase, highlighting the need for greater dietary and supplemental intake by the mother. Insufficient zinc during pregnancy can lead to adverse outcomes, including miscarriage, premature labor, stillbirths, and defects in fetal neural tube development [17-19]. Furthermore, maternal zinc deficiency may also be passed to newborns, resulting in symptoms such as hair loss, reduced appetite, diarrhea, weakened immune function, and skin inflammation [20]. Given these risks, zinc supplementation is crucial for the health of both mother and child.

Our research indicates that lower zinc levels, specifically below 13.1 μmol/L, correlate with a reduced false positive rate in NIPT tests, thereby decreasing the likelihood of detecting placental chimerism. In contrast, higher zinc levels above 13.1 μmol/L are associated with an increase in false positives and a higher risk of chromosomal anomalies in the placenta, which may contribute to placental chimerism. Additionally, some studies suggest that excess intracellular zinc can interfere with the normal meiotic process of oocytes, disrupting the of spindles and chromosomes organization, which may influence chromosome segregation [21]. Further research is needed to determine whether elevated zinc levels affect the cytokinesis and contribute to division failure in placental cells.

Calcium plays an essential role in numerous biological functions throughout pregnancy, including the development of bones and teeth, signal transduction, muscle contractions, enzyme regulation, and blood coagulation [22-24]. Insufficient calcium levels may pose risks for the mother and increase the likelihood of pre-eclampsia, a major contributor to fetal growth restriction and preterm labor, thereby elevating the risk of neonatal mortality [25]. Additionally, calcium is believed to play a crucial role in regulating mitosis, a process essential for proper growth, development, and tissue repair, as it ensures accurate chromosome distribution in newly formed cells. Errors in this regulation can lead to genetic imbalances or aneuploidy [26]. Moreover, our findings suggest that the false-positive rate of the NIPT assay is higher when calcium concentrations fall below 2.31 mmol/L and lower at higher levels, indicating a possible decrease in placental chimerism or chromosomal abnormalities associated with increased calcium intake.

Our study identified elevated serum Na, K, and Zn levels alongside decreased Ca, P, and Fe levels in pregnant women with NIPT false positives, likely influenced by placental chimerism. Additionally, the observed non-linear relationships between maternal serum Zn and Ca levels and the rate of NIPT false positives suggest that variations in ion concentrations may play a key role in the development of placental chimerism. These findings provide insight into developing individualized nutritional guidance during pregnancy to improve NIPT accuracy and maternal-fetal health outcomes.

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Disclosure of conflict of interest

None.

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