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Identification of Biomarkers by Proteomics for Prenatal Screening for Neural Tube Defects

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Neural tube defect (NTD) is a serious congenital defect, but current methods for identifying NTD are limited. We used proteomic analysis of maternal serum to identify NTD-specific proteins whose levels differed between women with NTD fetuses (n = 50) and those with healthy fetuses (n = 40). Three NTD-specific protein peaks (8,130.6, 15,941.7, and 3,960.3 m/z) were identified using MALDI-TOF-mass spectrophotemetry, and were included in a diagnostic model developed using Biomarker Patterns software. The model used cut-offs for the relative intensity of the three peaks to indicate if a case had or did not have NTD. The model identified 48 of the 50 NTD cases and 36 of the 40 control cases correctly, resulting in the sensitivity of 96.0% (48/50) and the specificity of 90.0% (36/40). The diagnostic model was also tested on 105 clinical cases at high risk for NTD, as determined by having high alpha-fetoprotein levels, resulting in the sensitivity of 100% (101/101) and the specificity of 75.0% (3/4). Using the International Protein Index database, we identified proteins with a molecular mass of 8,130.6 Da as ADP-ribosylation factor 1 and a protein similar to cold agglutinin FS-1 antibody light-chain. The 15,941.7-Da peak corresponded to vitamin K3 protein, and the identity of the 3,960.3-Da protein was unclear. Thus, this study developed a diagnostic model consisting of the three peaks which may be indicators of NTD. This new assay may be at least as accurate for diagnosing NTD compared with the commonly used clinical test that assesses alpha-fetoprotein levels.

Keywords: congenital disorder; MALDI-TOF mass spectrometry; neural tube defect; prenatal diagnosis; prenatal screening

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Introduction

Neural tube defects (NTDs) are complex congenital malformations of the central nervous system occurring secondarily to lack of closure of the neural tube. The worldwide incidence ranges from 1.0 to 10.0 per 1,000 births (Salih et al. 2014). In China, the prevalence of NTDs is about 6 per 1000 births which is considerably higher than that in the United States (about 5 in 10,000 births) and in Western Australia (about 15 per 10,000 births) (Moore et al. 1997; Boulet et al. 2008; Bower et al. 2009). Folate supplementation both before and during the initial weeks of pregnancy reduces the risk of NTDs, with a protective rate of about 50-80% (MRC Vitamin Study Research Group 1991; Czeizel et al. 2004; Chen 2008).

About 95% of NTDs occur spontaneously in women without family history of the disease, indicating the importance of prenatal screening to detect NTDs (Chen 2008). Ultrasound is used to detect NTDs (Lepage et al. 2012); however, the accuracy of detecting NTDs by ultrasounds is affected by a number of factors including prevalence, position of the fetus, maternal obesity, and the technical skill of the person(s) performing the ultrasound (Chan et al. 1995). Elevated levels of alpha-fetoprotein (AFP) in the amniotic fluid and maternal serum are also used during the second trimester to screen for NTDs in fetuses; however, the specificity and sensitivity of AFP is limited (Chen 2008; Salih et al. 2014).

In this study, proteomic analysis of maternal serum was conducted to identify NTD-specific protein peaks as biomarkers for NTD. The aim was to develop a non-invasive and safe method with high specificity for prenatal detection of NTDs. We used nano-magnetic beads and MALDI-TOF-MS for this study to identify proteins that have different expression patterns in NTD compared with non-NTD fetal samples. This technology has high sensitivity and specificity and has been widely used for screening and identification of biomarkers for various diseases and tumor including breast cancer, colorectal cancer, and oral cancer with (Mengel-Jorgensen et al. 2004; Villanueva et al. 2004, 2007; Cheng et al. 2005; Lopez et al. 2005; de Noo et al. 2006; Hortin 2006).

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Materials and Methods

This prospective, controlled study enrolled patients from Huzhou Maternity & Child Hospital, Zhejiang Province, China between 2012 and 2013. The study was performed according to the Declaration of Helsinki. The protocol was approved by the institutional review board of the Huzhou Maternal & Child Care Hospital and all subjects gave their written informed consent.

Study subjects

Eligible subjects were women in their second trimester of pregnancy and whose fetus was diagnosed with NTD in accordance with the standards of the Chinese Ministry of Health (i.e., by ultrasound and pathology). Also included were healthy pregnant women (controls) and women at high risk for NTD fetuses for blinded validation of the biomarkers. The high risk women were identified through analysis of AFP levels. A multiple of the median (MoM) was calculated by measuring AFP in maternal serum and comparing this value to the median AFP value in an unaffected population. High-risk subjects were defined as having AFP levels ≥ 2.5 MoMs at 15 and 21 weeks of pregnancy (Wald and Cuckle 1987).

Biomarker analysis

Serum preparation: Peripheral blood samples were collected and placed at 4°C immediately following collection, and subsequently centrifuged at 4°C at 4,000 r/min for 5 min to collect serum, followed by additional centrifugation at 14,000 r/min for 5 min at 4°C to remove cell debris. Serum aliquots were stored at −80°C and each sample was used only once.

NTD-related protein identification: Proteins were isolated using weak cation exchange type nano-magnetic beads (Saierdi Biotech Co., Ltd., Beijing, China). Serum samples were thawed on ice prior to use. Samples (10 μL) were added to 20 μL of 9 mol/L buffer (9 mol/L Urea, 2% CHAPS, 50 mmol/Tris-HCl, 1% DTT, pH 9.0) (Sigma Aldrich, St. Louis, MO, USA) and incubated at room temperature for 10 min before being mixed with 360 μL of weak cation exchange binding buffer.

Weak cation exchange magnetic beads were washed twice and equilibrated with binding buffer. A diluted serum sample of 100 μL was added and incubated at room temperature for 15 min after mixing. The magnetic processor was used to remove unbound sample and the weak cation exchange beads were then washed two times with binding buffer. Following the addition of 10 μL of washing buffer, the beads were placed in the magnetic processor for 5 min. The supernatant (5 μL) was mixed with 5 μL of sinapinic acid saturated solution, and then each sample (1 μL each) was transferred to an Aurum chip (Au-chip, Ciphergen Biosystems, Austen, TX, USA) for analysis.

The Au-chips were scanned using Protein Biological System IIc mass spectrometer (Ciphergen Biosystems), according to the manufacturer's instruction. Standardization was done by using Ciphergen NP20 chip (Ciphergen Biosystems) which includes all-in-one standard proteins for calibration to ensure the error of molecular quality was within the range of 0.1%. The internal standard peak at 6,634.0 m/z was used for calibration of the protein fingerprints of the raw data to minimize the error of molecular weight to less than 0.01%, and to obtain the maximum energy setting at 40-50%. The settings were as follows: laser intensity 175, detection sensitivity 8, optimization range of m/z 2,000-20,000 (maximum 50,000) and each spot on the chip was scanned 90 times. Ciphergen ProteinChip® 3.2.2 software was used to collect data. Data process modeling included standardization of parameters for proteome maps. The serum m/z peak of 6,634.0 was used for calibration, followed by "baseline reduction" in order to minimize errors caused by factors such as chip differences and fluctuations of the instrument. Biomarker Wizard 3.1 software was used to calculate the P values of the differences of the proteins with the same m/z between fetal NTD group and the control group.

The protein peaks with a signal-to-noise ratio greater than 5 and an occurrence rate higher than 90% was selected as valid protein peaks. Furthermore, to ensure small protein peaks were not missed, all peaks with the signal-to-noise ratio of > 2 and molecular quality deviation < 0.1% were also marked as valid protein peaks. After further optimization of the parameters (including the Gini, Testing, Advance and Costs, etc.), the diagnostic model was determined. The diagnostic model was used for blinded analysis in the NTD high-risk pregnant women, and the sensitivity and the specificity of the results were analyzed by blinded analysis.

NTD-high risk women were identified by evaluating serum levels of AFP, the level of which was determined by time-resolved fluoroimmunoassay employing europium-labeled antigen as a tracer. Multicalc software (Perkin Elmer Wallac, Tirku, Finland) calculated the risk of NTD by inclusion of the factors of maternal age, gestational age and weight. AFP levels ≥ 2.5 MoMs indicated high risk.

Diagnosis of NTD

NTD was diagnosed using ultrasound (Toshiba Nemio with a probe frequency of 3.5-7.0 MHz). The presence of anencephaly, spina bifida aperta was evaluated. In addition, autopsy of NTD fetuses was used to evaluate the presence of NTD.

Statistical analyses

P values of the proteins with the same m/z but with different expression levels between the NTD fetal group and control group were calculated according to the principles of analysis of variance (ANOVA). Biomarker Patterns Software 5.0 (Ciphergen Biosystems, Fremont, CA, USA) was used for linear analysis of the differences between the differential protein peaks with the same m/z in NTD fetal group and control group. Mann-Whitney U test was used to compare the protein peaks between the NTD fetal group and control group. $P < 0.05$ indicates statistically significant. The Biomarker Patterns Software identified the best markers for diagnosis of fetal NTD and for establishment of the proteomics-based diagnostic model.

Results

Model development

The study included 90 women in their second trimester of pregnancy: 50 women with NTD fetuses and 40 women with healthy fetuses (Table 1). The mean age in each group was about 28 years.

After standardization of the original protein fingerprints between groups, 58 protein peaks ($P < 0.05$) were detected within the m/z range of 2,000-50,000 using Biomarker Wizard software. Thirty-five of these peaks differed significantly between the fetal NTD and control groups ($P < 0.001$, Table 2). The 35 peaks were further

Table 1. Demographic distribution between the NTD and the normal groups.

	NTD (n = 50)	Normal (n = 40)
Age (years)	27.6 ± 4.4	28.0 ± 4.0
Gestation weeks of total subjects	19.1 ± 2.0	18.0 ± 1.3
Diagnosis result[1]		
Anencephalus	46 (92.0%)	–
Myelomeningocele	1 (2.0%)	–
Encephalocele	3 (6.0%)	–

NTD, neural tube defect.
[1]Diagnosed by prenatal ultrasound.

Table 2. Comparison of the differential protein peaks between 50 serum samples of pregnant women with NTD fetuses and 40 serum samples of normal control group (mean ± SD).

m/z	NTD	Normal	P value
6,115.6	1.01 ± 1.48	8.62 ± 2.0	1.29E-08
2,955.8	3.18 ± 2.84	14.82 ± 3.27	2.19E-08
5,909.1	11.85 ± 18.33	83.94 ± 13.53	2.5E-08
8,130.6	55.35 ± 35.82	6.27 ± 2.28	2.16E-07
8,588.5	7.22 ± 6.33	1.14 ± 0.38	1.02E-06
4,466.8	5.60 ± 5.09	0.67 ± 0.88	2.00E-06
13,562.4	0.74 ± 0.73	0.08 ± 0.12	2.00E-06
3,960.3	7.04 ± 6.21	17.98 ± 6.49	8.00E-06
8,939.4	19.57 ± 15.17	6.31 ± 1.35	1.43E-05
7,921.6	13.73 ± 15.11	1.93 ± 0.85	1.58E-05
8,335.4	7.07 ± 5.77	0.79 ± 0.51	2.64E-05
2,013.8	8.80 ± 12.74	17.53 ± 6.98	2.91E-05
8,355.6	6.31 ± 4.97	1.11 ± 0.47	4.79E-05
4,064.7	15.29 ± 12.92	0.78 ± 1.22	9.39E-05
15,941.7	5.93 ± 7.71	19.58 ± 9.35	9.39E-05
16,152.9	1.88 ± 2.00	5.43 ± 2.44	0.000113
7,983.7	14.27 ± 13.72	36.78 ± 15.87	0.000124
2,038.1	2.58 ± 2.94	5.28 ± 2.35	0.00015
7,481.8	3.51 ± 4.77	0.16 ± 0.38	0.00015
16,068.6	1.64 ± 0.87	2.97 ± 1.36	0.000258
9,412.2	4.97 ± 2.99	8.96 ± 3.31	0.000281
15,109.3	1.08 ± 0.74	0.56 ± 1.18	0.000307
6,849.3	11.02 ± 7.78	4.98 ± 3.92	0.000336
2,262.6	3.70 ± 4.46	6.94 ± 3.24	0.000366
8,707.9	3.80 ± 2.07	2.26 ± 1.54	0.000474
4,620.6	5.25 ± 5.94	0.99 ± 1.21	0.000517
2,744.5	5.42 ± 6.51	11.6 ± 5.51	0.001893
22,845.78	1.05 ± 1.11	1.83 ± 0.57	0.001893
4,652.4	9.08 ± 5.14	13.07 ± 2.73	0.002208
9,714.9	2.41 ± 1.81	3.96 ± 1.14	0.002382
22,262.2	0.52 ± 0.42	0.18 ± 0.08	0.002382
23,436.2	1.79 ± 2.12	3.13 ± 1.03	0.004301
2,193.7	3.17 ± 1.30	4.57 ± 1.63	0.005325
10,060.18	2.28 ± 4.04	0.24 ± 0.20	0.005325
4,305.3	13.30 ± 6.43	5.86 ± 4.60	0.009216

NTD, neural tube defect; SD, standard deviation.

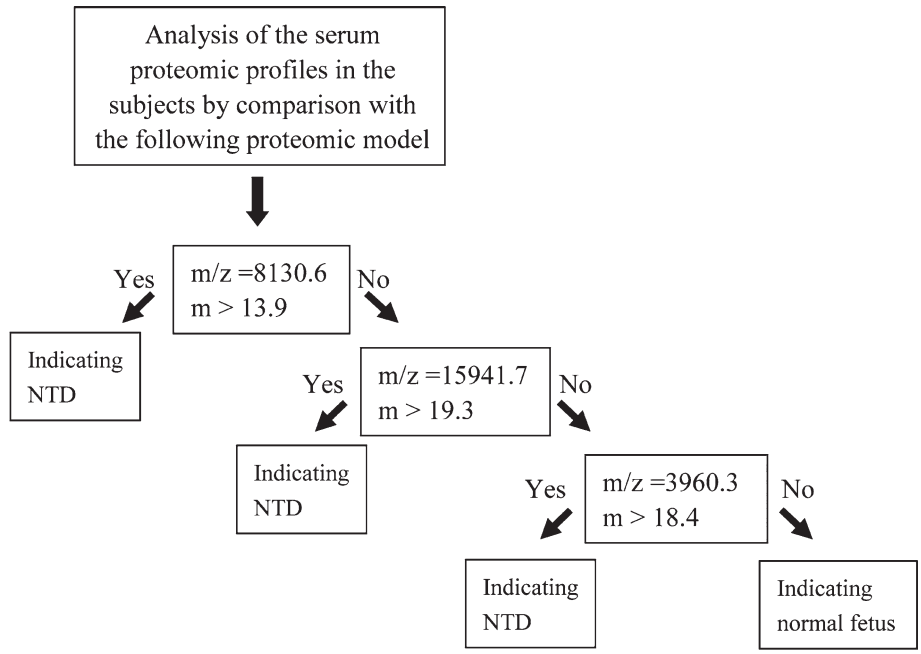

Fig. 1. The diagnostic model.
The diagnostic model was constructed with three peaks that differentiated between cases with NTDs and those without NTDs: 8,130.6, 15,941.7 and 3,960.3 m/z. In this model, the fetus is diagnosed with NTD fetus if its peak at 8,130.6 m/z is > 13.9, its peak at 15,941.7 m/z is > 19.3 or its peak at 3,960.3 m/z is > 18.4, otherwise the fetus is normal. m: relative intensity of a peak in the histogram.

Table 3. Diagnostic model screening results of 105 cases at high-risk for NTD.

	N	NTD Positive	NTD Negative
Screened by diagnostic model	105	4	101
Diagnosed with NTD through secondary screening by prenatal ultrasound	105	3	0
Diagnosed with NTD during follow-up visits	105	n.a.	0
Total of subjects with NTD		3	0

n.a., not applicable; NTD, neural tube defect.

analyzed by Biomarker Patterns software. We thus constructed a decision tree model by random arrangement of the 35 differential proteins and by selecting the best differential protein, according to the software rules (the sensitivity and specificity obtained after combination of the weighted score of each differential proteins). The final diagnostic model consisted of three differential protein peaks: 8,130.6, 15,941.7, and 3,960.3 m/z (Fig. 1). In the model, if the peak at 8,130.6 m/z had the relative intensity of > 13.9, the case was diagnosed as having an NTD fetus. If not, then the peak 15,941.7 m/z was examined and if the peak's relative intensity was > 19.3, the case was diagnosed as positive for NTD. If the relative intensity was < 19.3, then the relative intensity of peak 3,960.3 m/z was examined. If the 3,960.3 m/z peak's relative intensity was > 18.4, then the case was considered positive for NTD. For the mothers with an NTD fetus, the number of abnormal expression proteins could have been one, two, or three.

Using this model, 48 of the fetuses were correctly diagnosed with NTD, and two were incorrectly identified, resulting in the sensitivity of 96.0%. Of the 40 normal control samples, 36 samples were correctly diagnosed and 4 were identified incorrectly, resulting in the specificity of 90.0%.

Clinical validation

The diagnostic model was used to screen an additional 105 women at high risk of carrying fetuses with NTD. The model identified four cases as NTD and 101 cases as normal (Table 3). Three out of the four potential NTD cases were confirmed by ultrasound as NTD fetuses and verified by pathological autopsy following termination of the pregnancy; one case was anencephaly and two were spina bifida. No abnormality was observed in the fourth case by ultrasound or after birth.

Among the 101 cases which were identified as normal by the diagnostic model, none were identified to have NTD by further screening (Table 3), while three fetuses were found to have abnormalities by ultrasound. One had multiple abnormalities (abnormalities in two or more

organs); this baby was delivered alive and was found to have heart defects and syndactyly. There were two still births, but the autopsy indicated that they did not have NTD. Three additional abnormal cases were identified in this group during follow-up visits; one fetus was spontaneously aborted, one baby was born with a cleft palate, and a third went full-term but the baby was small. Therefore, the sensitivity and the specificity for the protein based diagnostic model in a clinical setting was 100% (101/101) and 75% (3/4), respectively.

Protein identification

Proteins were extracted from serum samples for identification using mass spectrometry. The three peaks of interest were compared with the International Protein Index (IPI) database. Three proteins (IPI database accession numbers: IPI00975633, IPI00215914, and IPI00979320) were found to have a molecular mass of 8,130.6 Da. For these three proteins, IPI00975633 is of unknown function, IPI00215914 was identified as ADP-ribosylation factor 1, and IPI00979320 was identified as a protein that is similar to cold agglutinin FS-1 light-chain (L-chain). Cold agglutinins are human autoantibodies, usually of the IgM class, which agglutinate red blood cells at low temperature. One protein (IPI00827839) was identified with a molecular mass of 15,941.7 Da and corresponded to a fragment of vitamin K3 (VK3) protein. No corresponding IPI database proteins were identified for the 3,960.3 protein peak.

Discussion

NTD is a serious congenital defect and has the highest incidence among all birth defects, accounting for about 20% of the total number of birth defects (Onda et al. 2000). In this study, we used weak cation exchange nano-magnetic beads, MALDI-TOF-MS, and decision-tree modeling to identify proteins that may be associated with NTD. To build the model, we compared protein profiles from 50 women with NTD fetuses and 40 women with healthy fetuses. In our final diagnostic model, we found three protein peaks: (8,130.6, 15,941.7, and 3,960.3 m/z) that were associated with NTD. Using the final model, 48 of the 50 NTD fetuses were correctly diagnosed, resulting in the sensitivity of 96.0%. Of the 40 normal controls, 36 were diagnosed correctly, resulting in the specificity of 90.0%. The model was validated in 105 women who were at high risk for carrying fetuses with NTD. Of the 105 cases, four cases were predicted to have NTD, three of which were confirmed by ultrasound to have NTD. Of the remaining 101 cases, none were found by ultrasound and follow-up visits to have NTD. Therefore, in a clinical setting the sensitivity and specificity of our model was 100% and 75%, respectively. Our findings indicate that this model has higher sensitivity and specificity for screening NTDs than AFP. In clinical practice, amniotic fluid AFP testing with the inclusion of acetylcholinesterase, which is measured if the AFP levels are abnormally high, has the sensitivity of 22% to 77% (Wang et al. 2009; Flick et al. 2014) and the specificity of about 97.7% (Wang et al. 2009).

The findings in this study are consistent with an earlier study from our group that used surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) technology to characterize possible proteins that would be diagnostic of NTD (Shen et al. 2012). By a proteomic analysis of amniotic fluid from 8 women with NTD fetuses and 16 women with normal fetuses, we identified five protein peaks that showed a significant difference between groups (3,484, 8,126, 15,112, 16,139 and 30,891 m/z) (Shen et al. 2012). We compared these five protein peaks to the peaks identified in the current study and the 8,126, 15,112 and 16,139 m/z differential peaks from our prior study are consistent with the 8,130.6, 15,109.3 and 15,941.7 m/z differential peaks of this study. The reproducibility of these three peaks strengthens their role as diagnostic markers for NTDs. All three protein peaks were detected only in the amniotic fluid of the NTD cases, suggesting that they may be associated with the cause of NTD and not only fetal markers. Several candidate proteins with matched m/z values have been suggested via the IPI database search. However, none of these proteins have previously been associated with NTD; hence, the role of these proteins in NTD is unclear. Future studies are required to investigate this issue.

Currently, NTD is screened for by measuring AFP levels in the amniotic fluid or maternal serum (about 2.5 times normal) and by ultrasound (Chen 2008). However, elevated AFP levels are not specific to NTD. Elevated AFP is also present in fetal omphalocele, gastroschisis, nephrotic syndrome, stillbirth, fetal hydrops, and in pregnant women with liver disease or fetal blood contamination (Chen 2008). In addition, the levels of AFP in the amniotic fluid and maternal serum can be normal if the spina bifida is covered with healthy skin. As mentioned above, AFP screening suffers from having poor sensitivity (Wang et al. 2009; Flick et al. 2014). Ultrasound is used both as a screening test and as a follow-up test after a positive result of AFP screening. Although published sensitivity and specificity of ultrasound detection of spina bifida are high (79-96% and 90-100%, respectively), these data are obtained from centers with special expertise (Roberts et al. 1983; Wilson et al. 2014). The sensitivity and specificity may be lower in centers with older technology or less expertise (Roberts et al. 1983). In addition, domestic prenatal diagnosis for NTDs is generally limited to B-mode ultrasound diagnosis which can be negatively impacted by other factors such as fetal position and mother weight.

This study investigated small molecular weight peptides present in serum samples. For this type of analysis, the use of weak cation exchange magnetic beads is better for separating and acquiring a spectrum of the peptides than anion exchange beads. Several studies have shown that magnetic beads-based weak cation exchange chromato-

graph gives reliable and reproducible enrichment of small proteins in serum, plasma, and urine (Fiedler et al. 2007; Shin et al. 2007; Xiao et al. 2011; Chen et al. 2012). In addition, the parameters of the signal-to-noise ratio > 5 and the signal-to-noise ratio > 2 used in this study were optimal values on the instrument performance, and were the default values for the machine. Generally, if there is no special requirement for screening, these parameters are used in studies using PBS II/C protein fingerprint spectrometer. Another study also used the same default parameters as our study (Li et al. 2012).

At our hospital, which has a fully implemented screening program for NTD, we found that in general 0.5% to 1% of the pregnant women will be at a high risk for NTD. Ultrasound is usually performed to diagnose high risk cases and most NTD fetuses can be diagnosed through this technology. However, among the 105 cases in this study that were identified as high-risk pregnancy by elevated AFP levels in the maternal serum, only 3 cases were confirmed to have NTD and thus, from a strict point of view, the rest were all false positives. Importantly, more than 90% of the NTD high-risk pregnant women had to bear unnecessary psychological burden of possibly having a baby with NTD throughout their pregnancy. Additional prospective studies are necessary to confirm and extend the findings of this study. In addition, the involvement of the three identified protein peaks (either direct or indirect involvement) would be of interest as it may give insight into the causes of NTDs.

In conclusion, we have identified three protein biomarkers by weak cation exchange and the MALDI-TOF-MS that showed high specificity and sensitivity for prenatal detection of NTD. Prior proteomic analysis using MALDI-TOF-MS has identified biomarkers that may be useful in diagnosing fetal chromosomal abnormalities (Kolialexi et al. 2011). Only a few studies have used mass spectrometry technology to identify potential markers for NTDs (Onda et al. 2000; Fan et al. 2011; Shan et al. 2012). The present study is the first to use weak cation exchange magnetic beads and MALDI-TOF-MS to screen maternal serum for NTD-specific protein peaks. In the future, we will use a variety of biochemical methods to isolate and gain insight into the functions of the proteins identified in this study. The diagnostic model described in this paper is potentially a non-invasive technology, with high specificity and sensitivity, to screen for NTD. However, before this model can be used to screen for NTDs in the clinic, side-by-side blinded testing of large numbers of screening samples should be performed in a low risk population. In addition, samples from pregnancies with open spina bifida should be tested, rather than anencephaly.

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Conflict of Interest

The authors declare no conflict of interest.

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