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AUTOIMMUNE PROCESSES IN THE PLACENTAS OF NEURAL TUBE DEFECT-AFFECTED PREGNANCIES

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AUTOIMMUNE PROCESSES IN THE PLACENTAS OF NEURAL TUBE DEFECT-AFFECTED PREGNANCIES

by

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Neural Tube Defects (NTDs) are a group of common congenital malformations that result from incomplete neural tube closure leading to abnormalities of the brain and/or spinal cord. Unfortunately, their etiology remains unknown, probably due to complex multifactorial interactions. The protective effect of dietary folates in preventing NTDs is well known, but this beneficial effect is limited to the 60 to 70% of cases; leaving 30% of the population without any known option for improving pregnancy outcomes.

The mechanism by which folates rescue NTD-affected embryos is poorly understood, but the ability of folate supplementation to overcome a significant percentage of NTDs and the critical role of 5-methyltetrahydrofolate in the remethylation of homocysteine (Hcy) to methionine in the placenta suggests that folate binding and/or transport might play a critical role during development. We hypothesized that maternal autoantibodies (AB) targeting placental folate receptor alpha (FRα) are blocking the receptor and limiting the ability of the FRα to bind folates, reducing intraembryonic folate levels. Furthermore, we hypothesized that AB binding to other relevant proteins required for trophoblastic growth and placentation can be involved in activating pathologic inflammatory pathways that can result in suboptimal uptake of nutrients and contribute to an abnormal closure of the neural tube. We developed a high throughput ELISA to evaluate whether mothers experiencing pregnancies complicated with NTDs are more likely to have placental AB to FRα than are mothers experiencing normal pregnancies. We optimized and simplified a protocol for AB elution from placental tissues and determined whether these antibodies were blocking the FRα from binding with available folates.

Although anti-FRα IgG antibodies were not associated to the blocking activity in this study, we found that the blocking activity was higher in the placentas from NTD-affected pregnancies compared to controls, that FRα IgM antibodies are most likely the type of antibody produced during gestation that is most relevant to the blocking activity and that it is unlikely that autoimmunity against other developmental proteins associated with NTDs is generating the NTDs.

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I. INTRODUCTION

a. **NEURAL TUBE DEFECTS**:

Neural Tube Defects (NTDs) are a group of common congenital malformations that result from incomplete neural tube closure leading to abnormalities of the brain and/or spinal cord; the most common examples being anencephaly and spina bifida (SB). Unfortunately, their etiology remains largely unknown, [1, 2], probably because it results from complex multifactorial interactions [3]. Numerous efforts to explain their etiology have been made, but to date, only unspecific or weak conclusions regarding the role of gene variants have been established. The protective effect of dietary folates with regards to the etiology of NTDs is well known, but this beneficial effect is limited to only 60 to 70% of cases; leaving 30% of the population without any known option for improving pregnancy outcomes [4-7]. The mechanism by which folates rescue NTD-affected embryos is poorly understood, but the ability of folate supplementation to overcome a significant percentage of NTDs and the critical role of 5-methyltetrahydrofolate (5MTHF) in the remethylation of homocysteine (Hcy) to methionine in the placenta suggests that folate binding and/or transport might play a critical role during development. The reason of a lack of folate-mediated protection is unclear, but one potential mechanism may be due to insufficient/defective folate binding and transport due to inhibition of the folate transporters [4-7].

The consequences of NTDs in terms of life expectancy and quality of life are severe for both the patients and their families. Prevalence of NTDs varies among the world. Shanxi is a province in northern China with one of the highest prevalence rates of NTDs, (20 per 1000 live births). It is also known to have high levels of air pollutants that originate from coal combustion from industrial processing, cooking, and heat production [10]. In the U.S., NTDs affect between 0.5 -1%of all live births, 10% of which have spina bifida (SB), which is the only NTD that survives beyond birth. The long–term survival of SB patients (with surgical intervention shortly after birth) to the third decade of life is only 50% to 60% [8]. SB patients demonstrate severe disabilities and detrimental psychosocial adjustment [9], and complications appear continuously. The financial impact is also severe – the medical cost of SB over a life time (analyzed in 1992 USD dollars) for individuals with NTDs born in 1988 was in average $294,000 for each infant [8,9].

b. FOLATES

Folates are essential B vitamins that are commonly present in green leafy vegetables (lat. Foliage which means leafs). In general, the molecular structure of folates consists of a p-aminobenzoic acid, a pteridine ring and a glutamic acid. The naturally occurring folates contain a reduced pteridine ring and a polyglutamate polypeptide which is hydrolyzed to a monoglutamic chain by the glutamate carboxypeptidase II before it is absorbed by the intestinal cell. Natural folates may carry a one-carbon unit (methyl, methylene, methenyl, or formyl) or can create bridges in the 5th or 10th positions. The bioavailability of folates varies between 10% to 85% in natural foodstuffs and 85% to 95% in the form of the biosynthetic and more oxidized form: folic acid. The folate bioavailability varies depending on the individual's genetics and enzymatic activity, but in general, it is considered to be around 50% [11]. Folates are implicated in various metabolic pathways including the one-carbon metabolism, as they are required for the synthesis of purines and pyrimidines, and assist in the re-methylation of homocysteine to methionine, which is a key step for biomethylation reactions. During pregnancy, folate demands can increase up to 10-fold [12]. The CDC recommends that all women of childbearing age consume 0.4mg of folic acid daily in the form of a supplement. The American Academy of Family Physicians and The American College of Obstetrics and Gynecology recommend supplementation of 4 mg/day for women with a history of neural tube defects [CDC Recommendations, folic acid, (2013)].

c. FOLATE TRANSPORTERS AND EMBRYOGENESIS:

The role of folate transporters is critical during embryogenesis, as demonstrated in previously published experimental studies [13, 14]. In humans, there is very little literature about placental and fetal folate homeostasis. Folate transport across the placenta in the first trimester when neural tube closure occurs, is performed by several folate receptors and transporters, FRα, FRβ, PCFT and RFC, present in the placental membranes, yolk sac, in fetal capillary endothelial cells, generating an increased concentration of this vitamin in the fetus [15]. Absorption of folates during neurulation appears to be mediated principally by two folate transporters, which are expressed highly in the neural tube, yolk sac and placenta: the reduced folate carrier (RFC1) encoded by the SLC19A1 gene, and the folate receptor alpha (FRα) [16, 17]. Considerable evidence suggests that the FRα is essential during morphogenesis of

the neural tube. During pregnancy in the mouse, Folr1, the mouse homologue of human FRα, is abundantly expressed in the yolk sac [18]. Furthermore, knockout Folr1 mice have NTDs among other associated severe structural malformations [14]. In the human first trimester placenta, FRα is highly expressed in the microvillus membrane of the syncytiotrophobast [15, 19], which is the major embryonic tissue in direct contact with the maternal immune system [20].

d. EMBRYONIC DEVELOPMENT OF THE IMMUNE SYSTEM

Hematopoiesis in the human embryo begins transitorily in the mesodermal blood islands of the yolk sac that first appear around the third week of gestation [21, 22]. Later, the hematopoietic tissue migrates to the bone marrow and thymus. Formation of the secondary lymphoid organs including spleen, lymph nodes and the mucosal immune system of the gut are formed. Between 17 to 20 weeks of gestational age, organogenesis of the thymus and other secondary lymphoid organs is occurring and T and B cells in the thymus and bone marrow respectively begin to develop [23].

Maternal antibodies (AB), especially immunoglobulin (Ig) G1 and IgG3, start crossing the placental barrier at 10 to 12 weeks of gestation and continue throughout pregnancy [24]. The immunoglobulin crossing is mediated by the neonatal Fc receptors present in syncytiotrophoblast cells. Placental endogenous IgG is associated with villous stromal cells, trophoblasts, fibrinoid deposits, and endothelial cells of fetal blood vessels [25, 26].

e. MATERNAL IMMUNITY DURING PREGNANCY

Autoimmune diseases have been associated with higher rates of adverse pregnancy outcomes including recurrent pregnancy losses, intrauterine growth retardation, severe preeclampsia, premature birth and placental pathology [27, 28]. An important part of the pathogenicity of by which autoimmunity is thought to negatively affect the pregnancies includes the presence of autoantibodies targeting different molecules during gestation. Experimental and *in vitro* studies of exposure of anti-phospholipid antibodies (AB) to rat embryos and their yolk sacs inhibited growth and increased the number of apoptotic events of giant cells in the ectoplacental cone, when compared to controls [29]. Exposure of anti-cardiolipin AB in mice produced lower rates of fecundity and number of embryos in each pregnancy. Additionally, an

increased rate of embryo resorption was observed, and decreased placental weight and embryonic weight when compared to mice used as controls [30].

f. FRα AUTOANTIBODIES

The possible role of the maternal immune response in the etiology of NTDs and other complex birth defects re-surfaced when Rothenberg and colleagues [31] demonstrated that there were high titers of serum antibodies to the folate receptors among mothers who had previously given birth to infants with spina bifida. These blocking AB were thought to restrict folate transport to the placenta and developing embryo during critical periods of the neural tube closure, increasing the risk for having a child with an NTD. Although this observation was confirmed in a larger study using serum samples obtained mid-gestationally from mothers carrying NTD affected pregnancies, [32] a more recent study by Molloy and colleagues [33] failed to find an association, although the collection of the samples was years after the pregnancy, and AB titers can change drastically over the time span of a few months. While the role of AB to the FR may be controversial, what is not debatable is that many of the known risk factors for NTDs such as maternal diabetes and obesity have important inflammatory features that have not been rigorously studied.

Unfortunately how this pathogenic autoimmune response arises has not been clearly established, but one mechanism is that existing, functional proteins become modified and are no longer recognized as self, generating autoantibodies that could be targeting both the modified and unmodified protein, compromising its function. A particularly compelling modification in the case of the FRs is homocysteinylation as it is inversely related to folate levels and several inflammatory responses are linked to it. Low folate bioavailability could be post-translationally modifying the folate receptors, changing or exposing new epitopes that previously were not exposed and generating an autoimmune response [34].

II. HYPOTHESIS

To date, little is known regarding the role of the placenta in the context of NTD-affected pregnancies in humans. The importance of this short-live organ is that it enables the embryo/fetus to survive during pregnancy. The functions of the placenta are widely varied, ranging from structural roles such as anchoring to the conceptus, maintaining the nutritional and waste metabolism of the embryo/fetus and assisting in important immunological processes such as avoiding the rejection by the maternal immune system and protecting the embryo/fetus against foreign microorganisms [35]. The main purpose of this study was to investigate the immunological profile of placentas in NTD-affected and normal pregnancies, to test the hypothesis that **maternal autoantibodies targeting placental FRα play a crucial role in the etiology of NTD by blocking the receptor and limiting the ability of the FRα to bind folates, reducing intraembryonic folate levels.** Furthermore, we hypothesized that **AB binding to other relevant proteins required for trophoblastic growth and placentation can be involved in activating pathologic inflammatory pathways. Increased inflammation can result in suboptimal uptake of nutrients and contribute to an abnormal closure of the neural tube**. Additionally we wanted to evaluate if gestational age had any influence in autoimmune processes in the placenta. We developed a high throughput ELISA to evaluate whether mothers experiencing pregnancies complicated with NTDs are more likely to have placental AB (Immunoglobulin G and M) to FRα than are mothers experiencing normal pregnancies. We optimized and simplified a protocol for AB elution from placental tissues and determined whether these antibodies were blocking the FRα from binding with available folates. We also created a high throughput assay to measure IgG antibodies to other relevant proteins in the placenta.

III. METHODS

a. SAMPLES

Access to 123 placental tissue aliquots kindly provided by Dr. Aiguo Ren collected from a population-based birth defect surveillance program in the Shanxi province in China. Cases were placentas from pregnancies complicated by an NTD including anencephaly, spina bifida and encephalocele (n=77), and controls were placentas from newborns without any external structural birth defect (n=44). Control placentas were matched to the case infant by geographic location, maternal ethnic group, sex, and date of conception [36]. Placentas were aliquoted and frozen at -80°C after delivery. Gestational age was only known for 118 placentas, and was calculated based on the last menstrual period. Mean gestational age for controls was of 40.3 weeks, and for cases 29.5 weeks. Comparisons of distributions between the gestational age of cases and controls were significantly different, $p \leq 0.001$.

b. IMMUNOGLOBULIN ELUTION FROM PLACENTAL TISSUES

Placental immunoglobulins (Ig) were eluted from 5g of frozen tissue that was then homogenized with 5mL of 10X phosphate buffered saline (PBS), (80mM Na2HPO4, 1.5M NaCl, 20mM KH2PO4, 30mM KCl, pH 7.4, G-Biosciences/Genotech: St. Louis, MO, USA) at 4°C with previous proteinase stabilization with Complete mini (Roche). After homogenization, samples were left at 4°C for 15 hours on a mechanical rocker, and then centrifuged at 12,000g for 20 minutes. The supernatant was collected and pellets were re-suspended in 5ml of 10X PBS, vortexed until the pellet was in suspension, and left for another 15 hours in the cold room over a mechanical rocker. After 15 hours of incubation, samples were centrifuged again for 20 minutes and the supernatant was collected. Based on Western blot analyses, recover of more than 90% of immunoglobulins was achieved when comparisons of the same volumes of homogenized starting material were performed. Supernatants and second pellets were then re-suspended in 10x PBS for a third time.

Immunoglobulin concentrations in each sample were measured via sandwich Enzyme Linked Immunosorbent Assay (ELISA); where samples were assayed in duplicates and interpolated to a

standard curve. Mean variation coefficients of the assays were 3.76% for IgG and 4.00% for IgM. Mean R^2 of the standard curves of both of the assays were above 0.99.

Protein concentrations of the eluates were assayed using the Bradford Assay (Thermo Fisher Scientific, USA) and measured using SpectraMax microplate reader. Samples were assayed in duplicates and interpolated to a standard curve of known concentration of bovine serum albumin. Mean variation coefficient of the assay was 2.56% and mean R^2: 0.99. The controls had a significantly higher concentration of total protein when compared to the cases, despite that placentas were weighed using a precision weight. Mean protein levels in the placental cases was 3.85mg/mL, and in the controls it was 4.73, p=0.006.

c. AUTOANTIBODIES TYPE IgG AND IgM REACTIVE AGAINST THE FRα

AB type IgG and IgM that reacted against the human FRα were measured through a high-throughput ELISA developed in our laboratory [32]. The recombinant human FRα was obtained from a baculovirus protein expression system. The proteins were printed to the 96-well high-binding ELISA plates from Immulon-Thermo Fisher Scientific, after suspending them in in 2.5% PBS-glycerol in 1.00µL volumes at room temperature (RT). After printing was completed, the arrays were covered and left overnight (16 hours). 50µL of the working solution of the samples were added to each of the wells after unbound protein was removed through three washes with TNT buffer (100mM Tris-HCl pH7.6, 150mM NaCl, 0.05% Tween-20). Plates were covered and left incubating at 4°C for 16 hours.

After the incubation period was completed, samples were washed 3 times with working concentrations of TNT and a secondary AB specific to IgG or IgM labeled with a horseradish peroxidase enzyme (HRP) diluted in TNT and added for one hour, covered, and in the dark at RT (Invitrogen). Following the HRP incubation, the placentas were washed 3X with TNT and 2X with TBS. Detection of the interaction between the printed proteins, AB, and secondary conjugate HRP-IgG or IgM was dobe by using Super Signal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.). The Quansys Biosciences Q-View™ Imager was used to take the pictures and the software to transform the pixel intensities. All samples were run in at least duplicates for the assays and mean variation coefficients

for anti FRα-IgG were 9.04%, and for anti FRα-IgM 9.55%. Mean R^2 of the curves used to interpolate the pixel intensities were 0.99.

To measure the amount of folic acid blocked from binding to the FRs a competitive binding assay between folic acid HRP and the placental eluate was optimized. As previously described [37], FA was removed from the eluates prior to the addition of the samples to the printed FR. The plate with the samples and the FR was incubated for 16 hours at 4°C in the dark, and then washed. A standard curve was constructed with known concentrations of folic acid and FA-HRP in a 1:1 dilution. Samples were developed using Femto and imaged through Quansys Biosciences Q-View™ Imager and Software. The mean variation coefficient of the assay was 10.28%. Pixel intensities of each sample were interpolated to a standard curve to relate intensities to concentrations of AB or folic acid. A similar process was performed to assay the other proteins.

d. MULTIPLEX ASSAY

A multiplex autoantibody assay including 9 candidate antigens and one positive control was designed and used to determine autoantibodies to other proteins involved in NTDs in the placental eluates. Proteins were printed in each week of 96-well plates by Quansys Biosciences. Candidate antigens were selected according to the following criteria: developmentally relevant human proteins involved in NTD-affected pregnancies: bone morphogenetic proteins (BMP) 1 and 2 and platelet derived growth factor soluble receptor alpha (PDGFRα); serum proteins that can become homocysteinylated: hemoglobin, transthyretin, albumin and fibronectin; proteins involved in inflammation, apoptosis and enhancement of autoimmunity (C-reactive protein - CRP), and yeast (*Candida albicans*) to be used as the positive control.

e. STATISTICAL ANALYSIS

Statistical analysis was performed using IBM-SPSS version 21. Box plots were constructed and outliers were removed from each variable to normalize the distributions of the results. Multiple linear regression analysis were done to evaluate the effect of the variables: gestational age, status (NTD vs. controls), and total placental protein for each dependent variable (total placental IgG, total placental IgM, FRα IgM and IgG autoantibodies, and blocking activity. An interaction term was also created between

gestational age and status to include the differences between gestational ages among cases and controls.

Data presented for the folate blocking activity only includes the samples whose blocking activity was detected by the assay: controls n=37, and cases n=46 from a stepwise linear regression analysis.

For the transcobalamin 1 (TCN1) IgG antibodies, data was squareroot transformed because after removal of the outliers, data failed to fit a normal distribution. To further support our findings, we performed a Mann-Whitney U Test to compare distributions between cases and controls and distributions were still significantly different between cases and controls ($p=0.04$).

IV. RESULTS

i. Table 1. Antibodies binding to FR, TCN and Laminin

Variable	CASES				CONTROLS				*p*
	n	**Mean**	**Median**	**SD**	**n**	**Mean**	**Median**	**SD**	
Gestational Age (weeks)	74	29.5	29	6.43	44	40.11	40	2.52	**≤0.001**
Blocking (nmol)	75	0.38	0.47	0.35	48	0.36	0.35	0.31	0.111
Total IgG (mg/mL)	75	0.43	0.38	0.26	48	0.57	0.49	0.26	**0.004**
Total IgM (mg/mL)	75	0.01	0.01	0.01	48	0.02	0.01	0.01	0.172
Total protein (mg/mL)	75	3.92	3.77	1.73	48	4.73	4.4	1.82	0.319
FRα IgG (µg/mL)	75	0.89	0.77	0.5	48	1.17	1.23	0.3	**≤0.001**
FRα IgM (µg/mL)	75	1.34	1.23	0.91	48	1.8	1.41	1.15	0.172
TCN1 IgG (µg/mL)	75	0.54	0.36	0.59	47	0.85	0.62	0.96	**0.001**
TCN1 IgM (µg/mL)	75	0.15	0.13	0.1	48	0.11	0.11	0.05	0.630
LAM IgG (µg/mL)	75	2.49	2.22	1.77	48	4.52	4.59	2.25	**≤0.001**

. **i. Table 1**. Means, medians, standard deviations (SD) and comparisons of medians through *Mann Whitney U-tests* between cases and controls of each of the variables listed.

ii. Table 2. Stepwise linear regression analysis for the dependent variables total IgG and IgM, FRα IgG and IgM, TCN1 IgG and IgM, and Laminin IgG AB. Linear regression analysis for the blocking activity

Model	R^2	B	*P*
Total IgG (mg/mL)	0.38		
Status		-018	0.119
Gestational Age		-0.01	0.500
Status*Gestational age		0.01	0.390
Total Protein		0.05	**≤0.001**
Total IgM (mg/mL)	0.22		
Status		-0.001	0.733
Gestational Age		≤0.001	0.440
Status*Gestational age		≤0.001	0.414
Total Protein		0.002	**≤0.001**
Blocking (nM)	0.27		
Status		0.12	**0.028**
FRα IgM AB		56.72	**0.017**
FRα IgG AB*		-296.19	**0.003**
Status*Gestational age		0.012	**0.027**
FRα IgG AB (mg/mL)	0.40		
Status		≤0.001	0.319
Gestational Age		$2.39x10^{-5}$	0.129
Status*Gestational age		$-1.21x10^{-5}$	0.499
Total Protein		$1.79x10^{-5}$	0.206
Blocking (pM)*		$-2.14x10^{-7}$	**0.005**
FRα IgM AB (mg/mL)	0.19		
Status		≤0.001	0.358
Gestational Age		$7.48x10^{-5}$	0.085
Status*Gestational age		$-4.21x10^{-5}$	0.352
Total Protein		7.3310^{-5}	0.069
Blocking (pM)*		$3.07x10^{-7}$	0.143
TCN1 IgG (mg/mL)	0.25		
Status		-0.001	0.870
Gestational Age		≤0.001	0.534
Status*Gestational Age		≤0.001	0.670
Total Protein		0.001	**0.013**
TCN1 IgM (mg/mL)	0.20		
Status		$9.77x10^{-5}$	**0.006**
Gestational Age		$4.84x10^{-6}$	0.409
Status*Gestational Age		$8.81x10^{-7}$	0.854
Total Protein		$7.30x10^{-6}$	0.068
Laminin IgG (mg/mL)	0.37		
Status		-0.704	0.332
Gestational Age		0.185	0.055
Status*Gestational Age		-0.118	0.243
Total Protein		0.037	0.670

* The blocking activity in this variable was analyzed in picoM to simplify the results. Total protein and immunoglobulin values are given in mg/mL, Status (case=1, control=0). Beta (β) coefficients are unstandardized.

a. Total Immunoglobulin G and M

In general, the control samples showed higher concentration of IgG and IgM antibodies as opposed to the cases (**Table 1**). Overall, the variation of the 37% of total IgG and the 21% of total IgM can be explained by the predictors run in the model for both variables. The total concentration of proteins was found to be a significant predictor of total IgG and total IgM as expected (**Table 2**).

b. Folate Blocking Activity

Only 57.33% of cases and 64.58% of the controls had detectable blocking activity. The mean blocking activity in the controls was 0.43 nM and median 0.42 nM, whereas in the cases, the mean blocking activity was 0.55nM and median 0.56nM. For this variable, the 27% of variation could be explained by the predictors in the model. The predictors for blocking activity were cases, ($p=0.028$), FRα IgM AB, which for every 56.72 increase of FRα IgM AB, the blocking activity increased 1nM. Contrary to what was expected, samples that showed lower FRα IgG AB were more likely to have significant blocking activity, (**Table 2**). To further support our findings, we performed a Mann-Whitney U Test to compare distributions between cases and controls and distributions were still significantly different between cases and controls ($p=0.04$).

c. FRα IgG antibodies

Median concentrations of FRα IgG antibodies were higher in the controls as opposed to the cases (1.23 and 0.77ug/mL respectively), **table 1**. The results show that the blocking activity is a negative predictor for IgG (Table 2). We also performed a stepwise linear regression analysis to observe which model fitted the best to predict these antibodies. $R^2=0.39$, $f(3,111)=23.48$, $p\leq0.001$. The predictors that were significant were **gestational age** ($\beta=2.02x10^{-5}$, $p\leq0.001$), and **blocking activity** remained significant ($\beta=-2.67x10^{-7}$, $p=0.006$). Finally, after analyzing only the samples that presented blocking activity, and excluding the control with very low gestational age, $R^2=0.37$, $f\ (2,82)=23.63$, $p\leq0.001$ we observed that the interaction term is no longer significant, only the predictors gestational age and blocking activity were significant predictors of IgG autoantibodies reacting against FRα. Gestational age $B=2.02x10^{-5}$, $t=5.77$, $p\leq0.001$, and blocking $\beta=-2.67x10^{-7}$, $t=-2.83$, $p=0.006$.

Figure 1 shows the box plots of the comparisons between cases and controls of the FRα IgG and IgM AB, and the blocking activity only in the samples that had detectable blocking activity.

d. FRα IgM antibodies

None of the covariates were significant predictors in the full model of IgM. However, when running a stepwise linear regression analysis, the predictors gestational age and total protein were found to be significant. R2=0.170, f(2,107)=10.93, p≤0.001. $\beta=3.22x10^{-5}$, p=0.001 for gestational age, and $B=8.30x10^{-5}$, p≤0.001 for total protein), for every week of gestational age, FRα IgM AB increase by 0.03µg/mL and for every mg/mL of protein, the FRα IgM AB will increase by 0.08 µg/mL.

Another stepwise linear regression analyzing the model with only the samples that only had detectable blocking activity was performed, and gestational age was the only significant predictor of FRα IgM AB. $R^2=0.33$, f(1,79)=9.63, p=0.003, gestational age ($\beta=3.08x10^{-5}$, t=3.10, *p*=0.003). Additionally, comparisons of means between cases and controls of the samples that only showed blocking activity were not significantly different (Mean=1.32µg/mL, SD=0.70 and 1.51µg/mL, SD=0.67) (**Figure 1**).

e. Transcobalamin 1 IgG antibodies

Concentrations of TCN1 IgG AB were higher in the controls as opposed to cases, and comparisons of medians between placentas from NTD-affected pregnancies and placentas from normal, term pregnancies show that the difference was statistically significant (**Table1**). Total protein was a significant predictor of TCN1 IgG AB (**Table 2**).

f. Transcobalamin 1 IgM antibodies

Interestingly, cases showed higher levels of antibodies binding to TCN1 as opposed to controls; however, comparisons of medians between groups were not significant. Cases and gestational age were found to be predictors of the concentration of IgM AB reactive to transcobalamin 1 (TCN1) (**Tables 1 and 2**). A functional assay was optimized to determine whether the antibodies were blocking the protein to bind with cobalamin, but no major blocking activity was observed.

g. Laminin IgG antibodies

Median laminin IgG AB were found to be significantly higher in the controls as opposed to the cases (**Table 1**). Advanced gestational age and controls were significant predictors of the model (**Table 2**).

v. Figure 2. Boxplots and graph comparing FR AB IgG, IgM and blocking activity

Figure 1. A

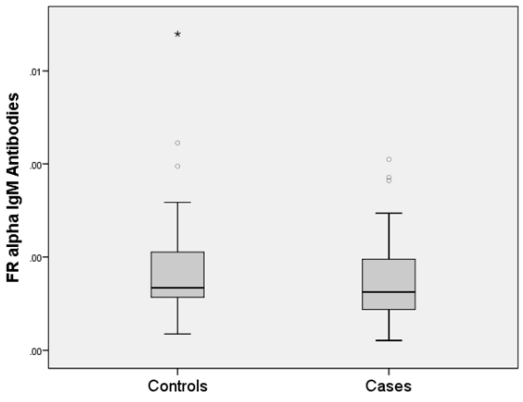
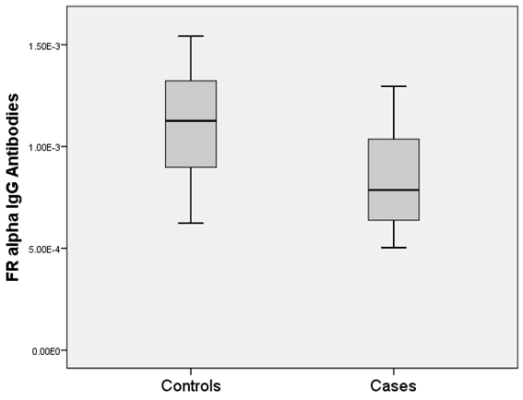
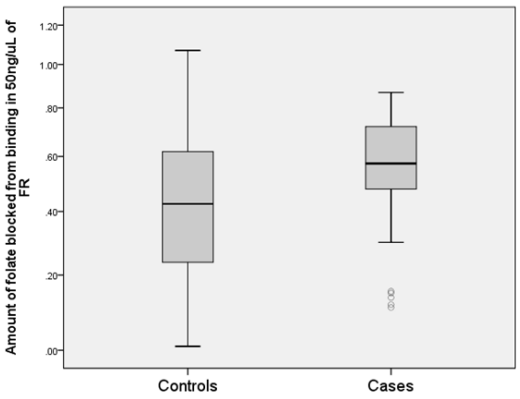
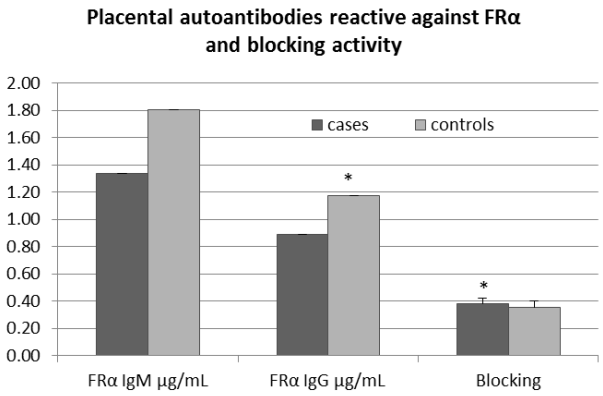


Figure 1. **B**





Graph 1



iii. **Figure 1.** Autoantibodies IgG, IgM and blocking activity in the samples that had detectable blocking activity. **Figure 1A and 1B** represent the boxplots for autoantibodies reactive against the FRα type IgM and IgG, respectively. **Figure 1C** represents the boxplots for the blocking activity. **Graph 1** shows the differences between cases and controls with the amount of blocking activity.

h. Multiplex Autoantibody assay

There were no significant differences between groups in the proteins included in the multiplex assay. **Table 3** shows the means and medians with the p-values of each of the proteins and figure 2 shows the differences between groups.

iv. Table 3. Multiplex autoantibody assay.

VARIABLE	CASES	CONTROLS	Mann-Whitney U Test (*p*)	Comparisons of Medians (*p*)
BMP2	3419	5056	0.258	0.172
BMP1	13351	15235	0.579	0.797
ALBUMIN	0	42.5	0.523	0.910
HEMOGLOBIN	652	860	0.582	0.797
TRANSTHYRETIN	3354	5200.5	0.156	0.319
CRP	11827	12332	0.883	0.441
PDGFRα	8436	8625	0.930	0.910
FIBRONECTIN	26958	26971	0.772	0.910
NOGGIN	7287	8021	0.312	0.319
YEAST	103331	102337	0.561	0.910

iv. Table 3. Median values of the pixel intensities of IgG autoantibodies binding to different molecules relevant during development.

v. Figure 2. Medians of autiantibodies reactive against some proteins relevant during development

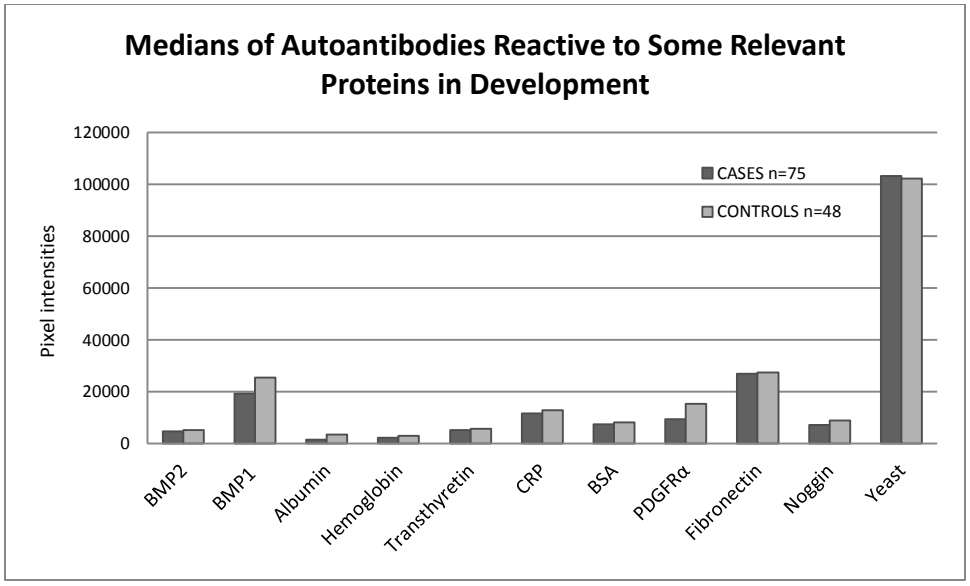


Figure 2: the median levels of autoantibodies binding to different proteins. Yeast was used as a positive control to evaluate the integrity of the samples.

V. DISCUSSION:

The role of autoimmune processes in the etiology of neural tube defects has been studied in maternal serum samples in the past, and the results appear to have marked variations among the populations studied. Rothenberg and colleagues (2004) found that the 75% of women who had an NTD affected pregnancy had serum autoantibodies to placental FRs, the women analyzed were mostly Caucasian (n=57), followed by Black (n=14) and Hispanic (n=6), and finally Asian (n=2). Using only two samples from the individuals that tested positive for folate receptor AB, they demonstrated that these samples showed blocking activity in a cell culture model [31] . In our lab, Cabrera *et. al.* 2008 showed that serum IgM and IgG AB binding to the FR were higher in women who were currently carrying a pregnancy complicated by a NTD compared to normal pregnancies, and that the mean blocking activity was significantly higher in NTD-affected pregnancies compared to controls in women from California from different ethnicities (Hispanic=54, White non-hispanic=34, Asian=11, Black=3, Other=3) [32]. Boyles found significantly increased blocking activity in pregnancies complicated by NTDs as opposed to controls in NTD affected pregnancies in a Norwegian population [38]; however, Molloy did not find a significant association of AB binding to the FR, or blocking the receptor in an Irish population [33].

In these placentas from pregnancies complicated by NTDs from the Shanxi province in China, we did not observe that a higher number of cases had blocking antibodies, but among those samples who had detectable blocking activity by our assay, the blocking activity was significantly higher in the placentas from NTD-affected pregnancies, as opposed to the placentas from normal pregnancies. We also observed that the FRα IgM ABs were more likely to be the antibodies that blocked folate uptake. In this study, most of the placentas had antibodies that were reactive against many of the proteins analyzed and in many cases, gestational age was a significant predictor of these antibodies.

We also observed that the control eluates in general, had higher concentrations of all antibodies and of protein, even though the placentas were controlled for weight when they were frozen tissue aliquots. The differences in protein levels between the eluates of the cases and controls may be explained by the differences between the gestational ages between them. Concentrations of FRα autoantibodies type IgG were found to be proportional to the gestational age of the placentas, and

gestational age was found to be an important predictor of FRα IgG AB regardless of the outcome status. Interpretation of this result is difficult, but one explanation is that increased concentrations of these antibodies could be secondary to a physiologic response to a more prolonged exposure to placental antigens, and maternal immunity against certain placental proteins at the end of the pregnancy could be involved in the mechanisms of labor induction. These results mimic the antibodies reactive against laminin type IgG, that were also higher in the controls, and gestational age was a predictor of the concentrations of these samples.

It is known that besides activation of innate immunity processes at the end of pregnancy, adaptive immunity actively participates in spontaneous human term parturition. Gomez-Lopez and collaborators found two different T-cell populations present in the chorionic decidua of normal labour and delivery births. Furthermore, chemokines and their respective receptors, cell adhesion molecules and cytokines were detected in these cells during birth suggesting that physiologic immune processes are actively participating in labor induction [39]. This suggests that since FRα ABs type IgG are not associated with blocking activity, their increasing concentrations as gestation advances can be a part of an inflammatory activation consistent with labor and parturition. Our results indicate that FRα AB type IgM were significant predictors of the blocking activity in the placentas, and the concentration appears to be increasing as gestation advances. It is well known IgM AB are part of the primary immune response. These pentameric molecules are the first line of antibodies produced in response to an acute antigenic exposure. Although they do not have the highest affinity to bind to antigens, IgM posseses the highest avidity compared to any other immunoglobulin (valence of 10), and is the most effective antibody in activating the complement cascade [40], since about 1,000 molecules of IgG are required to ellicit the same activation as one IgM pentamer of the classical complement pathway [41]. When placental cases and controls were assayed for other proteins relevant during gestation, we failed to find any significant results within the groups, indicating that in the Shanxi province the high NTD prevalence is probably not related to triggered autoimmune processes during pregnancy.

Shanxi is a province in northern China with one of the highest prevalence rates of NTDs in the whole world. It is also known to have high levels of air pollutants that originate from coal combustion from both industrial processing and that used for cooking and to produce heat. Recently [42], measurements

of polycyclic aromatic hydrocarbons (PAHs) were performed in the same placentas analyzed in this study, finding higher levels of PAHs, dichlorodiphenyltrichloroethane, and α-endosulfan in the cases compared to the control placentas, and that concentrations of PAHs that were above the median showed almost a 4-fold increased risk for NTDs.

PAHs are known to stimulate the aryl hydrocarbon receptor complex; which is a heteromeric transcription factor. Agonists of the aryl hydrocarbon receptor have shown to inhibit gene expression of immunoglobulins IgM, IgG and E, additionally activation of this receptor supresses differentiation of B cells into immunoglobulin producing cells [43]. It is also a regulator of cell proliferation and has beed documented to induce oxidative stress, and may play a role for cell migration and adhesion [44, 45]. Even though the placentas from the cases had a significantly lower gestational age, a study found that placentas from the same region in China, had higher concentrations of environmental pollutants, and disruption of immunologic processes might be a plausible explanation as to why the case placentas have such lower levels of immunoglobulins.

This study was specific for autoantibodies binding human FRα as opposed to other studies that do not discriminate the type of “placental folate receptors” [31]. One concern was that the placentas were collected 5 to 8 years before the study was performed, and that there were differences between gestational ages in both groups, challenged our ability to evaluate the integrity of the placentas. However, to evaluate integrity of the samples we measured the amount of reactivity against yeas in each sample under the premise that since yeast is an ubiquitous antigen, most likely all women should have antigens against it. Luckily our results indicate that there were high IgG antibody concentrations in both gruoups and the difference in results was not significant.

Another concern is that the blocking activity detected was very low, posing the question to how physiologically relevant these findings can be. Further studies should be conduced in placentas from earlier gestational ages to understand the possible pathophysiologic mechanisms underlying the disruption of an adequate closure of the neural tube and compare our results.

This study is the first to use a placental tissue as opposed to blood (serum) to evaluate the presence of autoantibodies binding to the FRs. It is also the first to propose a high-throughput and effective method for antibody elution from placenta.

Although anti-FRα IgG antibodies were not associated to the blocking activity, we found that the blocking activity was higher in the placentas from NTD-affected pregnancies compared to controls, that FRα IgM antibodies are most likely the type of antibody produced during gestation that is most related to the blocking activity and that it is unlikely that autoimmunity against other developmental proteins associated with NTDs is generating the NTDs.

VI. REFERENCES

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