



Decreased placental TLR3 is associated with hepatitis B virus vaccine responsiveness in infants born to HBsAg-positive mothers

Lina Wu^{1,2,3}, Gan Fu^{1,3}, Xiuyang Xu^{1,3}, Linzhu Yi^{1,3}, Tian Yao^{1,3}, Huili Wan², Junli Li², Bo Wang⁴, Shuying Feng⁴, Yongliang Feng^{1,3}, Hongxing Wang^{5,6}, Frank Tacke⁷, Raymond Kwok⁸, Keita Kai⁹, Suping Wang^{1,3}

¹School of Public Health, Shanxi Medical University, Taiyuan, China; ²Department of Pathology, the First Hospital, Shanxi Medical University, Taiyuan, China; ³Center of Clinical Epidemiology and Evidence Based Medicine, Shanxi Medical University, Taiyuan, China; ⁴Department of Obstetrics and Gynaecology, the Third People Hospital of Taiyuan City, Taiyuan, China; ⁵Institute of Special Medical Sciences, School of Forensic Medicine, Shanxi Medical University, Taiyuan, China; ⁶Division of Neuropsychiatry and Psychosomatics, Department of Neurology, Beijing Psychosomatic Disease Consultation Center, National Center for Neurological Disorders, National Clinical Research Center for Geriatric Diseases, Xuanwu Hospital, Capital Medical University, Beijing, China; ⁷Department of Hepatology and Gastroenterology, Charité Universitätsmedizin Berlin, Campus Virchow-Klinikum and Campus Charité Mitte, Berlin, Germany; ⁸Department of Medicine, Khoo Teck Puat Hospital, Singapore, Singapore; ⁹Department of Pathology, Saga University Hospital, Saga, Japan

Contributions: (I) Conception and design: L Wu, Y Feng, H Wang, S Wang; (II) Administrative support: S Wang; (III) Provision of study materials or patients: B Wang, S Feng; (IV) Collection and assembly of data: G Fu, X Xu, L Wu, H Wan, J Li; (V) Data analysis and interpretation: L Wu, L Yi, T Yao, Y Feng, H Wang, S Wang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Suping Wang, PhD. School of Public Health, Shanxi Medical University, 56 South Xinjian Road, Taiyuan 030001, China. Email: supingwang@sxmu.edu.cn.

Background: Although hepatitis B vaccination has a significant impact on the reduction hepatitis B virus (HBV) infection, babies born to hepatitis B surface antigen (HBsAg) positive mothers bear a high risk of being poor responsive to the vaccine with unilluminated mechanism. Toll-like receptor 3 (TLR3) plays a vital role in placental immunity, which affects the immune response of these babies. This study investigated the role of placental TLR3 in the immune responses of babies born to HBsAg-positive mothers to the HBV vaccine.

Methods: One hundred pairs of HBsAg-positive mothers and their newborns were recruited. Maternal blood samples were collected before delivery, and placental tissues were collected after delivery. Newborns were administered standard passive and active immunoprophylaxis and followed up until the age of 1. Infant blood samples were collected at 1 year of age. Mothers and infants were tested for HBV serological markers and HBV DNA by electrochemiluminescence immunoassay and fluorescence quantitative polymerase chain reaction, respectively. Placental TLR3 was detected by immunohistochemistry and score in a semi-quantitative fashion, circulating cytokines in infants were detected by enzyme-linked immunosorbent assay. Infants with anti-HBs ≥ 100 and < 100 mIU/mL were classified into the high-responsiveness group and the non- or hypo-responsiveness group.

Results: The TLR3 protein was expressed in all placentas. Compared with the high-responsiveness group, the expression of TLR3 in the non- or hypo-responsiveness group was significantly decreased ($\chi^2=10.39$, $P=0.001$). A non-conditional logistic regression model showed that the increased expression of placental TLR3 protein decreased the odds of HBV vaccine non- or hypo-responsiveness in the babies of HBsAg-positive mothers [OR =0.25 (95% CI: 0.11–0.58)], and this association remained significant after accounting for maternal factors, such as HBeAg and HBV DNA, as well as infant cytokines, including IL-6, IL-12, TNF- α , IFN- α , and IFN- γ [OR =0.15 (95% CI: 0.05–0.44)].

Conclusions: Decreased placental TLR3 expression is associated with impaired responsiveness to HBV vaccination in babies born to HBsAg-positive mothers.

Keywords: Hepatitis B virus vaccine; toll-like receptor 3 (TLR3); HBsAg-positive mother; placenta; non- or hypo-response

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Introduction

Hepatitis B virus (HBV) infection is a highly prevalent global public health issue (1). According to the World Health Organization (WHO), approximately 296 million people are living with chronic HBV infection and approximately 887,000 HBV-related deaths worldwide each year (2,3). Although HBV infection can be safely and effectively prevented with the available vaccines (4), epidemiological studies demonstrate that 5–10% of the population exhibits poor immune responses to the vaccines (5). Babies born to hepatitis B surface antigen (HBsAg)-positive mothers experience a high rate of non- or hypo-responsiveness to the vaccine (approximately 10–40%) (6-8) and therefore remain vulnerable to HBV. Moreover, nearly 90% of infant infections remain chronic (9), and 15–25% of infected infants die from HBV-related liver disease in their lifetime (10). Consequently, improving the immune response rate of babies born to HBsAg-positive mothers

could be key to preventing HBV infection. Identifying the related causes of their poor immune response to the vaccine may facilitate to achieving this aim.

Numerous factors, such as mutations in the HBV genome, vaccine type, and host immunity, contribute to the reaction to vaccination in infants born to HBsAg-positive mothers (11-14). However, the mechanisms through which these factors, which partly are associated with HBsAg-positive mothers, influence infant immunity and are involved in HBV vaccine responsiveness remain largely understudied. It has recently been reported that sustaining low levels of pathogen stimulation alters neonatal cytokine responses by causing a defense response in the placenta (15), which might help understanding the response to the HBV vaccine in infants born to HBsAg-positive mothers. The fetal-placental unit, as an actively developing immune system, can be modified by the maternal immune response and is closely related to the neonatal immune response (16,17). Throughout pregnancy, persistent exposure of HBsAg-positive mothers' placentas to maternal HBV-associated intrauterine microenvironments may influence placental immune function and consequently alter infant immunity, affecting the HBV vaccine response.

Toll-like receptors (TLRs), which are important pattern recognition receptors, play vital roles in innate immunity. The placenta performs pregnancy-specific immune functions through the expression of TLRs (18-20). In principle, placental immune mechanisms are intended to protect pregnant mothers and fetuses from pathogenic microorganisms, while the mothers need to be kept immunologically tolerant to the semi-allogenic fetus (21). HBV infects trophoblasts *in vivo* and *in vitro* (22) and suppresses the expression of placental TLRs to disrupt the placental immune response in HBV-positive mothers (23). TLR3 plays a vital role in the response to HBV (24). The placenta expresses a high level of TLR3 (25-27). Our previous study found that both the mRNA and protein expressions of placental TLR3 decreased in HBsAg-positive mothers suggesting that TLR3 may be a protective factor against HBV infection in the placenta (28). However, the

Highlight box

Key findings

- HBsAg-positive maternal microenvironment may affect placental toll-like receptor 3 (TLR3) protein expression. The decreased placental TLR3 expression is associated with impaired responsiveness to HBV vaccination in babies born to the HBsAg-positive mothers.

What is known and what is new?

- It is known that babies born to HBsAg-positive mothers bear a high risk of being poor responsive to the HBV vaccine with unilluminated mechanism. The placenta, as a special organ that connects mother and child, has a profound effect on babies' immune function, but its role in responsiveness to HBV vaccination in the babies born to the HBsAg-positive mothers is unknown.
- At present study, we found that TLR3 plays a vital role in placental immunity and affects the immune response of the babies born to HBsAg-positive mothers to HBV vaccine.

What is the implication, and what should change now?

- Placental TLR3 may be a potential target for improving hepatitis B vaccine response in babies born to HBsAg-positive mothers.

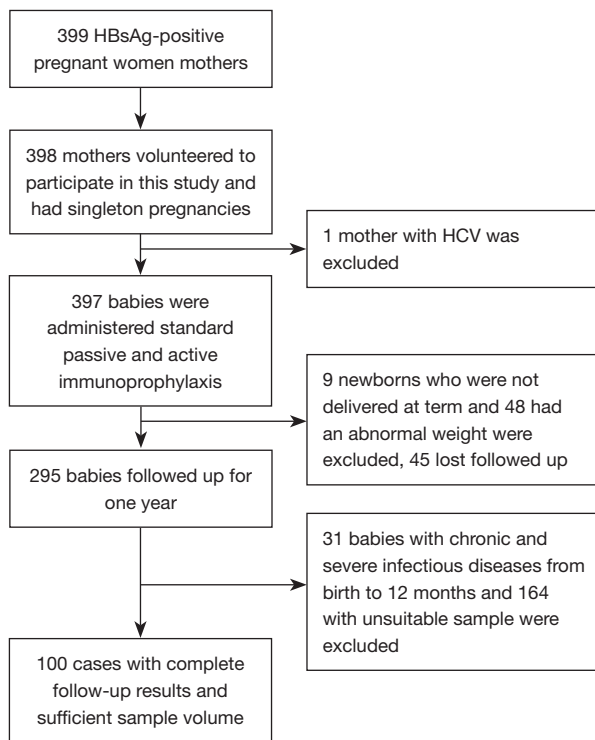


Figure 1 Study flowchart for identifying participants. HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus.

role of placental TLR3 in the immune response to the HBV vaccine in infants born to HBsAg-positive mothers has not been analyzed in detail.

Therefore, in the present study, we aimed to first detect the expression of TLR3 in the placentas of HBsAg-positive mothers with different HBV serological markers and then analyze the infant HBV serological markers and cytokines involved in the response to the HBV vaccine. Finally, we investigated the potential role of placental TLR3 in the immune response to HBV vaccination in infants born to HBsAg-positive mothers under these multiple factors. We present this article in accordance with the MDAR reporting checklist (available at <https://tp.amegroups.com/article/view/10.21037/tp-23-266/rc>).

Methods

Participants and study design

HBsAg-positive mothers and their children were recruited from the Third People Hospital of Taiyuan City, Shanxi Province, China, from June 2011 to July 2013. All neonates were administered hepatitis B immune globulin (HBIG)

within 24 h after birth and HBV vaccine at 0, 1 and 6 months of age. Follow-up was performed until the infants reached the age of 1. All mothers who volunteered to participate in this study had singleton pregnancies and no history of infection with human immunodeficiency virus (HIV), hepatitis C virus (HCV), or other viruses at enrollment. All newborns were delivered at full term (gestational weeks 37–42) and had an average weight of 2.5–4.0 kg. Infants with chronic and severe infectious diseases from birth to 12 months were excluded. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Ethics Committee of Shanxi Medical University (No. 2010032). All mothers and legal guardians of the children signed informed consent forms for participation in this study. The study flowchart is shown in *Figure 1*.

Specimen collection

After delivery, face-to-face interviews were conducted by well-trained interviewers with standardized questionnaires to collect general demographic information about the mothers and their children. Before delivery, peripheral blood samples were collected from the elbow veins of all mothers. The placentas were collected within 30 min after delivery. Then, tissue samples were fixed in 4% paraformaldehyde, dehydrated with an alcohol gradient, cleared with xylene, and embedded in paraffin. We collected femoral venous blood samples from the infants. All blood samples were pretreated and stored at -80°C until use in subsequent experiments.

Determination of HBV serological markers

The HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc levels of the mothers and their babies were measured by electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. Six months after completing the active-passive hepatitis B vaccination regimen, children with anti-HBs titers <10 mIU/mL were defined as non-responders, those with anti-HBs titers ≥ 10 and <100 mIU/mL were defined as hypo-responders, and those with anti-HBs levels ≥ 100 mIU/mL were defined as high responders (11). Since HBs titers ≥ 100 mIU/mL are associated with prolonged and effective seroprotection (29), we grouped cases with anti-HBs titers <100 mIU/mL into a non- or hypo-responsiveness group and cases with anti-HBs

levels ≥ 100 mIU/mL into a highly responsiveness group.

Quantification of serum HBV-DNA

A fluorescence quantitative polymerase chain reaction (FQ-PCR) kit was used to detect the serum HBV DNA load. The procedure and evaluation of the results were carried out according to the instruction manual (DAAN Gene Co. Ltd., Sun Yat-sen University, Guangdong, China).

Detection of TLR3 in placental tissue

For immunostaining, the placental tissue was sectioned into 4- μ m-thick samples and deparaffinized. Thereafter, the slides were subjected to a heat-induced epitope retrieval step for 2 min before incubation with primary antibodies. The antibodies specific for TLR3 (ab62556) were rabbit polyclonal antibodies obtained from Abcam (USA), which were used at a dilution of 1:400. We used EnVision Plus to detect these antibodies, which were developed in 1:1,000 DAB for 5 minutes and then counterstained with hematoxylin. Appropriate positive and negative control cases were stained in parallel. Blinded evaluator performed the evaluation of immunohistochemistry.

The results were scored according to the integral composed of staining intensity (1, weak; 2, moderate; and 3, strong) and positive cell percentage (0, negative; 1, <25%; 2, 25–50%; 3, >50%) and graded into the following categories: 0 [0], 1 [1–2], 2 [3–4], and 3 [>4]. The expression of TLR3 was divided into increase and decrease relative to the integral of the median in the control group.

Measurement of cytokines

Serum cytokines, including IL-6, IL-12, TNF- α , IFN- α , and IFN- γ , were assessed by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, USA) according to the manufacturer's instructions.

Statistical analysis

Database creation was performed using Epidata 3.1. Statistical analysis was performed using the SAS version 9.4 software package. Descriptive data are presented as the mean \pm standard deviation (SD) or absolute numbers (n) with percentages based on the data type and distribution. Statistical significance was tested by a *t*-test

or nonparametric test for continuous data and by the chi-square test for categorical data. All statistical tests were two-tailed, with $P < 0.05$ considered indicative of statistically significant results. A logistic regression model was utilized to examine the association between placental TLR3 expression and responsiveness to the HBV vaccine in babies born to HBsAg-positive mothers.

Results

General information on HBsAg-positive mothers and children

All 100 pairs of HBsAg-positive mothers and their children were collected at the end of the 1-year follow-up. Among these children, there were 14 cases of non-response, 36 cases of hypo-response, and 50 cases of high response. The average age of the 100 mothers was 27.71 ± 4.33 years, and the average gestational time was 39.09 ± 1.13 weeks. On average, the 100 infants were 11.20 ± 3.39 kg in weight and 76.84 ± 4.12 cm in height. There were no significant differences between the non- or hypo-response and high-response HBsAg-positive mothers in age, gestational time, mode of delivery, history of antiviral drug use, HBV serological markers, and height and weight of the infants. *Table 1* shows the general information of the 100 HbsAg-positive mothers and children in the non- or hypo-responsiveness and high-responsiveness groups.

Expression of the placental TLR3 protein in HBV vaccine non- or hypo-responders and high responders

Immunohistochemistry was performed to investigate the TLR3 protein expression in the placenta to analyze the role of the placental TLR3 protein in HBV vaccine non- or hypo-responsiveness and high responsiveness. The TLR3 protein was detected in all samples. Cytoplasmic expression of the TLR3 protein was mainly found in trophoblastic cells but was also detectable in part decidual cells, villous mesenchymal cells, and villous capillary endothelial cells (as shown in *Figure S1*). TLR3 expression was decreased in 72% of non- or hypo-responsive cases. However, reduced expression of TLR3 accounted for 40% of cases in the high-responsiveness group. The chi-square test showed that the decreased expression of placental TLR3 was significantly more common in the non- or hypo-responsiveness group, as compared to the high-responsiveness group.

Table 1 General information of the HBsAg-positive mothers and their children in non- or hypo-responsiveness and high-responsiveness groups

Characteristics	Total (n=100)	Non- or hypo-responsiveness (n=50)	High responsiveness (n=50)	t/ χ^2 /Z	P
Mothers					
Age (year)	27.71±4.33	28.16±4.74	27.24±3.85	-0.99	0.32 [†]
Gestational week	39.09±1.13	39.24±1.14	38.94±1.11	1.11	0.27 [†]
Mode of delivery				1.02	0.31 [‡]
Vaginal delivery	43 (43.00)	24 (48.00)	19 (38.00)		
Cesarean section	57 (57.00)	26 (52.00)	31 (62.00)		
Taking antiviral drugs				1.78	0.18 [‡]
Yes	17 (17.00)	6 (12.00)	11 (22.00)		
No	83 (83.00)	44 (88.00)	39 (78.00)		
Serum HBeAg				0.65	0.42 [‡]
Positive	44 (44.00)	20 (40.00)	24 (48.00)		
Negative	56 (56.00)	30 (60.00)	26 (52.00)		
Serum HBV DNA				3.35	0.07 [‡]
Positive	41 (41.00)	16 (32.00)	25 (50.00)		
Negative	59 (59.00)	34 (64.00)	25 (50.00)		
Serum HBV DNA (IU/mL)				0.75	0.69 [‡]
<200	63 (63.00)	32 (62.00)	31 (64.00)		
200–200,000	10 (10.00)	6 (8.00)	4 (12.00)		
>200,000	27 (27.00)	12 (30.00)	15 (24.00)		
Placental TLR3 protein				10.39	0.001 [‡]
Decrease expression	56 (56.00)	36 (72.00)	20 (40.00)		
Increase expression	44 (44.00)	14 (28.00)	30 (60.00)		
Infants					
Gender				0.36	0.55 [‡]
Male	54 (54.00)	28 (56.00)	26 (52.00)		
Female	46 (46.00)	22 (44.00)	24 (48.00)		
Height (cm)	76.84±4.12	77.18±4.43	76.48±3.79	-0.84	0.40 [†]
Weight (kg)	11.20±3.39	11.55±4.54	10.84±1.48	-1.03	0.30 [†]
Cytokines (pg/mL)					
IL-6	30.14 (246.99)	26.09 (178.18)	30.23 (637.08)	0.61	0.54 [§]
IL-12	0.92 (0.44)	0.92 (0.45)	0.92 (0.45)	0.08	0.94 [§]
TNF- α	2.51 (7.14)	2.51 (7.21)	2.89 (7.08)	-0.19	0.85 [§]
IFN- α	0.65 (0.31)	0.58 (0.17)	0.65 (0.69)	0.56	0.57 [§]
IFN- γ	0.78 (1.24)	0.71 (1.22)	0.93 (1.41)	0.71	0.48 [§]

[†], one-way ANOVA (the one-way analysis of variance), described by $\bar{x}\pm s$; [‡], chi-square test, described by n (%); [§], Wilcoxon rank-sum test, described by median (interquartile range). HBeAg, hepatitis B virus e antigen; HBV, hepatitis B virus; TLR3, toll-like receptor 3; IL-6, interleukin-6; IL-12, interleukin-12; TNF- α , tumor necrosis factor- α ; IFN- α , interferon- α ; IFN- γ , interferon- γ .

Table 2 Association between placental TLR3 expression and hepatitis B vaccine non- or hypo-responsiveness in the babies of HBsAg-positive mothers

Factors	Unadjusted		Adjusted	
	OR (95% CI)	P	OR (95% CI)	P
TLR3	0.25 (0.11–0.58)	0.001	0.15 (0.05–0.44)	0.001

TLR3, toll-like receptor 3; HBsAg, hepatitis B surface antigen; OR, odds ratio; CI, confidence interval.

Expression of inflammatory cytokines in HBV vaccine non- or hypo-responsive and highly responsive infants

We assessed infant serum cytokines related to the immune response to the hepatitis B vaccine, including IL-6, IL-12, TNF- α , IFN- α , and IFN- γ , in non- or hypo-responders and high responders. Although there was no significant difference between the two responses, the expressions of IL-6, TNF- α , IFN- α , and IFN- γ showed a trend towards lower levels in the non- or hypo-responders relative to that in the high responders (as shown in *Table 1*).

Association between placental TLR3 expression and HBV vaccine non- or hypo-responsiveness

Potentially relevant factors determining non- or hypo-response, such as mother HBeAg, mother HBV DNA, and infant cytokines, were included in a logistic regression model. As shown in *Table 2*, placental TLR3 was introduced into the model. Increased expression of the placental TLR3 protein decreased the odds of HBV vaccine non- or hypo-responsiveness in the babies of HBsAg-positive mothers [OR =0.25 (95% CI: 0.11–0.58)], and this association remained after accounting for maternal factors such as HBeAg and HBV DNA, and infant cytokines including IL-6, IL-12, TNF- α , IFN- α , and IFN- γ [OR =0.15 (95% CI: 0.05–0.44); *Table 2*].

Discussion

Infants of HBsAg-positive mothers suffer high rates of non- or hypo-responsiveness to HBV vaccination, which predisposes them to poor outcomes after HBV infection. Therefore, it is imperative to clarify the possible mechanism of non- or hypo-responsiveness in this population. By comprehensively analyzing data from HBsAg-positive mothers, placentas, and babies, we found that decreased placental TLR3 protein expression was associated with

HBV vaccine non- or hypo-responsiveness.

Placental structure and function can be affected by viral factors in the mother (20–32). TLR3 is a vital molecule for the placenta to perform pregnancy-specific immune functions but can be impaired by HBV (33). Our previous study found that both the mRNA and protein expressions of placental TLR3 decreased in HBsAg-positive mothers relative to the normal controls (28). Furthermore, we also found that the expression of TLR3 was significantly decreased in the placentas of HBsAg-positive mothers who delivered infants with non- or hypo-responsiveness to the HBV vaccine. Given the crucial role of the placenta in protecting fetal growth and influencing the long-term health of the offspring (34), these results indicated that long-term exposure to a HBsAg-positive maternal intrauterine microenvironment may significantly affect placental TLR3 expression and impair fetal-placental unit immune function, possibly influencing the baby's immune response to the vaccine. This may be related to HBV polymerase, a key enzyme related to HBV replication, in HBsAg-positive mothers. Studies have shown that HBV polymerase has an inhibitory effect on TLR3 signal (33).

As a pregnancy-specific component of the immune system, placental immunity is not only sensitive to maternal factors but also affects the baby's immune system development (35), which is closely related to non- or hypo-responsiveness (36). Although the differences observed in the present study were not significant, serum cytokines indicating an immune response such as IL-6, TNF- α , IFN- α , and IFN- γ showed numerically lower levels in the non- or hypo-responders. These results might suggest to some extent that the immune response of babies with non- or hypo-responsiveness might be somewhat inadequate. This is consistent with current research showing that decreased levels of cytokines such as IL-6 and TNF are involved in insufficient responses to the HBV vaccine (37,38). TLR3 signals induce the activation of NF- κ B and IRF3 in the nucleus, which bind to the sites of relevant gene promoters to induce I-IFN production (39) and enhance the expressions of inflammatory cytokines such as IL-6, IL-8, IL-12, and TNF- α (39,40). Nevertheless, the mechanism underlying the effect of placental TLR3 on infant cytokine expression needs to be elucidated further.

Our study was constrained by a relatively small sample size due to ethical concerns about performing invasive sampling techniques on babies, which resulted in sample collection difficulties and an inability to perform more extensive analysis on the small sample volume. Further

studies are needed to confirm our findings and to explore the molecular mechanism of placental TLR3 in the HBV vaccine response.

In summary, the non- or hypo-responsiveness to HBV vaccination in babies born to HBsAg-positive mothers involves many factors. Our current study observed a striking association between reduced placental TLR3 expression and reduced response to HBV vaccination in these babies, indicating that the HBsAg-positive maternal microenvironment may affect trophoblast cell TLR3, which may subsequently impair the baby's immune response to the HBV vaccine. The underlying molecular mechanisms as well as implications for strategies overcoming hypo-responsiveness of these babies to HBV vaccination require further studies.

Conclusions

The findings presented in this study indicate that the microenvironment of HBsAg-positive mothers could affect the placental TLR3 expression, leading to impaired baby immune response to HBV vaccine and ultimately contributing to an inadequate response to the vaccine.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://tp.amegroups.com/article/view/10.21037/tp-23-266/rc>

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AbbVie, BMS, Boehringer, Madrigal, Intercept, Falk, Ionis, Inventiva, Merz, Pfizer, Alnylam, NGM, CSL Behring, Novo Nordisk, Novartis. FT has participated on a data safety monitoring board for Pfizer. and has received support for attending scientific meetings from Gilead. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and approved by the Ethics Committee of Shanxi Medical University (No. 2010032). All mothers and legal guardians of the children signed informed consent forms for participation in this study.

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