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The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

Regulation of Nod-like receptor expression in the liver of ewes during early pregnancy

L. Zhang, Y. Ren, Y. Li, Y. Meng, H. Fang and L. Yang*

Hebei University of Engineering, School of Life Sciences and Food Engineering, No. 19 Taiji Road, Handan 056038, China

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* Corresponding author:
e-mail: yangling@hebeu.edu.cn

ABSTRACT. Multiple factors are involved in the regulation of maternal-foetal tolerance, and nucleotide-binding and oligomerisation domain (NOD)-like receptors (NLRs) were shown to play roles in innate immune signalling pathways. The objective of this study was to investigate the effects of early pregnancy on the expression of NLRs in the liver of ewes. Livers from ewes were collected on day 16 of the oestrous cycle, and days 13, 16 and 25 of gestation (n = 6 for each group). Real-time quantitative PCR, Western blot and immunohistochemical analysis were used to analyse mRNA and protein expression of NLRs, including NOD1, NOD2, major histocompatibility complex class II transactivator (CIITA), neuronal apoptosis inhibitor protein (NAIP), NLR family, pyrin domain-containing 1 (NLRP1), NLRP3 and NACHT, LRR and PYD domains-containing protein 7 (NLRP7). The data showed that NOD1, CIITA, NLRP1, NLRP3 and NLRP7 were upregulated in maternal liver on days 13 to 25 of pregnancy, and NOD2 expression was upregulated on days 13 and 16 of pregnancy, but downregulated on day 25 of pregnancy. In addition, NOD2 and NLRP7 proteins were located in the endothelial cells of the proper hepatic arteries and portal veins, and in hepatocytes. NAIP expression was the highest at day 16 of pregnancy. In conclusion, this study reported for the first time that early pregnancy modulated the expression of NLRs, suggesting that NLRs were involved in the regulation of maternal hepatic immune functions during early pregnancy in sheep.

Introduction

There are numerous factors, including nutrition, genetics, anatomy, hormonal regulation, environmental influences and many others that are involved in regulating the balance between maternal and foetal tolerance, maternal health and foetal protection during pregnancy in humans and animal models (Bonney, 2017). During normal pregnancy, there are highly dynamic cooperative interactions between the maternal and foetal immune systems rather than broad maternal immune suppression, contributing to significant systemic physiological and immunological adaptations (Abu-Raya et al., 2020). The "crosstalk" between the mother and the conceptus in cattle was shown to be initiated before implantation, resulting in alterations in maternal endocrine status and immune system function (Velázquez et al., 2019). Therefore, both the foetal and maternal immune systems must control their responses to allow the establishment of pregnancy.

Interferon-tau (IFNT) secreted by the conceptus was demonstrated not only to prevent pulsatile prostaglandin F2α (PGF2α) secretion and luteolysis, but also modulated the innate immune system to allow development of the semi-allogenic conceptus during early pregnancy in ruminants (Rocha et al., 2021). In ruminants, IFNT, progesterone, pregnancy-

associated glycoproteins and chaperonin 10 were shown to regulate gene expression of the innate immune system in the uterus and peripheral immune cells, as well as other tissues throughout the body during pregnancy, i.e. they modulated maternal immune functions both locally and systemically (Ott et al., 2020). During early pregnancy in sheep, IFNT and progesterone was shown to regulate gene and protein expression of interferon-stimulated genes (ISGs), progesterone receptor and progesterone-induced blocking factor in maternal bone marrow (Yang et al., 2017a; Zhang et al., 2017), thymus (Zhang et al., 2018; 2020b), spleen (Yang et al., 2018a; 2018b; Wang et al., 2019) and lymph nodes (Yang et al., 2017b; 2019b; Zhang et al., 2020a).

Nucleotide-binding and oligomerisation domain (NOD)-like receptors (NLRs) are a subgroup of cytosolic pattern recognition receptors that participate in innate immune signalling pathways. The main proteins in the NLR family include major histocompatibility complex (MHC) class II transactivator, CIITA, neuronal apoptosis inhibitor protein (NAIP), NOD1, NOD2, and nucleotide-binding oligomerisation domain, leucine-rich repeat, and pyrin domain-containing (NLRP) (Zheng, 2021). NOD1 and NOD2 play roles in host defence and inflammatory diseases by stimulating the production of pro-inflammatory cytokines through the activation of nuclear factor-kappa B (NF-κB) family proteins (Caruso et al., 2014). CIITA is the master regulator of MHC II, which is essential for the adaptive immune response as MHC II presents processed antigens to CD4 T cells (Oda et al., 2022). *NLRP* genes were found to exert important effects on both the mammalian innate immune system and the mammalian reproductive system, and several *NLRP* genes were specifically expressed in the mammalian reproductive system (Tian et al., 2009). Macrophages, neutrophils and peripheral blood mononuclear cell express NAIP, which is involved in caspase-1-dependent proteolytic cleavage of the proinflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Kay et al., 2020). Serum total cholesterol and uric acid activated NLRP3 inflammasome, which was implicated in the release of IL-1β, leading to severe inflammation in the syncytiotrophoblast layer (Stødle et al., 2018).

During pregnancy, liver size and function change in women and rodents, and these alterations are regulated by reproductive status and pregnancy hormones (thyroid hormone, oestrogen and progesterone) (Bartlett et al., 2021). Pregnancy is characterised by the occurrence of various immune modulations, with the liver primarily mediating local and systemic immune tolerance through specialized liver resident nonconventional antigen-presenting cells (Bremer et al., 2016). An increase in the expression of two forms of prolactin receptor transcripts was observed in the liver on day 19 of gestation in rats, affected by prolactin itself, growth hormone, oestrogens, glucocorticoids or progesterone and other unidentified factors (Jahn et al., 1991). Insulin-like growth factor binding protein 3 (*IGFBP-3*) mRNA expression was downregulated, but *IGFBP-4* mRNA expression level was reported to be upregulated in the liver during pregnancy in rats (Rosato et al., 2002). During early pregnancy in sheep, expression levels of ISGs (Yang et al., 2020b), prostaglandin synthases (Yang et al., 2020a), T helper cytokines (Yang et al., 2019a), melatonin receptor 1, CD4, gonadotropin-releasing hormone and its receptor, prolactin and its receptor were shown to be altered in maternal liver (Bai et al., 2020; Cao et al., 2021; Feng et al., 2022). In addition, early pregnancy modulates protein expression involved in the toll-like receptor, NF-κB and complement pathways in maternal liver in sheep (Gao et al., 2021; Feng et al., 2021; Fang et al., 2022). Moreover, expression levels of NLR family proteins, including NOD1, NOD2, CIITA, NAIP, NLRP1, NLRP3 and NLRP7 were reported to be altered in maternal thymus and inguinal lymph nodes, which contributed to maternal immunoregulation and pregnancy maintenance in sheep (Zhang et al., 2022; Zhao et al., 2022).

It has been hypothesised that early pregnancy affects the expression of NLR family proteins in sheep liver. The objective of the present study was to analyse the expression of NOD1, NOD2, CIITA, NAIP, NLRP1, NLRP3 and NACHT, LRR and PYD domains-containing protein 7 (NLRP7) in maternal liver during early pregnancy in sheep, which would be useful to elucidate changes in maternal liver function during early pregnancy in ruminants.

Material and methods

Animals and experimental design

Humane animal care and handling procedures were followed throughout the experiment, and all experiments were approved by the Hebei University of Engineering Animal Care and Use Committee (HUEAE 2019-017). A total of 24 Small-tail Han ewes (approximately 18-month-old) were randomly divided into four groups (n = 6 for each group), and used to determine the expression of NLR family

proteins in the liver during pregnancy. The ewes used in this study had a cycle length of 16–17 days. They were run with either entire (3 pregnant groups) or vasectomized rams (one non-pregnant group) and were checked twice daily with raddle marks to determine the day of oestrus (day 0). Livers from ewes (pregnant groups) were collected at the time of slaughter on days 13, 16 and 25 post-fertilisation, while non-pregnant ewes were slaughtered on day 16 of the oestrous cycle. Pregnancy was confirmed by the presence of an embryo in the ovine uterus. Hepatic cross sections (0.5 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde in PBS (pH 7.4) for subsequent immunohistochemical analysis, and the remaining tissues were frozen in liquid N_2 and stored at −80 °C for real-time quantitative PCR (RT-qPCR) and Western blot analysis.

RNA extraction and PCR assay

Total RNA was prepared from liver samples kept at −80 °C using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Ultraviolet spectrophotometry was used to measure the amount of RNA, and its quality and quantity were verified by electrophoresis in a 1% agarose gel. Complementary deoxyribonucleic acid was synthesised using the FastQuant RT kit with DNase (Tiangen Biotech Co., Ltd, Beijing, China) according to the manufacturer's instructions. The primer sequences of the *NLR* and *GAPDH* genes were designed and synthesised by Shanghai Sangon Biotech Co., Ltd (Shanghai, China) (Table 1), and the amplified products were sequenced to check reaction specificity. The Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to perform RT-qPCR with the SuperReal PreMix Plus kit (Tiangen Biotech Co., Ltd, Beijing, China). The following program was used to carry out PCR reactions: 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 10 s), annealing (59 to 62 °C for 20 s) and extension (72 °C for 25 s), followed by one cycle of final extension (72 °C for 7 min). Annealing temperatures were: 60.5 °C for *NOD1* and *CIITA*, 62 °C for *NOD2*, 59.5 °C for *NAIP*, 60 °C for *NALP1*, 59 °C for *NLRP3*, and 61 °C for *NLRP7*. The *GAPDH* gene was amplified in parallel with the target genes. The $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001) was used to calculate relative expression values for the target genes, with *GAPDH* as a normalisation control. Relative levels of mRNA transcripts were normalised to data from ewes on day 16 of the oestrous cycle.

Western blot analysis

Total proteins of liver samples were prepared on ice using lysis buffer for the radioimmunoprecipitation assay (cat no.: BL504A; Biosharp, Hefei, AH,

Table 1. Primers used for real-time quantitative PCR

Gene	(Forward/Reverse) Sequence 5'→3'	Size, bp	Accession no.
NOD1	F: CCTTGGCTGTCAGAGATTGGCTTC R: GCTTCTGGCTGTATCTGCTCACTG	94	XM_042248630.1
NOD2	F: TGCCATCCTCGCTCAGACATCTC R: CAGCCACACTGCCCTCTTTGC	117	XM_042231601.1
CIITA	F: GCACCTCCTTCCAGTTCCTTGTTG R: CCTGTCCCAGTCCCTGAGATCG	119	XM_042239890.1
NAIP	F: TTGTCCAGCAGTGTCAGCATCTTC R: ATTTCCACCACGCTGTCATCATCC	82	XM_012096791.3
NLRP1	F: AAGGAGGTGACCGAGATGCTGAG R: TGCCGCTTGAGTGAGGATGTATTG	143	XM_012185551.4
NLRP3	F: CTCTGGTTGGTCAGTTGCTGTCTC R: GGTCAGGGAATGGTTGGTGCTTAG	81	XM_042250402.1
NLRP7	F: GCCTGCTACTCGTTCATCCATCTC R: CCCTTCCTCCTCCTGCTCTTCC	90	XM_004015893.5
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	176	NM_001190390.1

NOD1 – nucleotide binding oligomerization domain containing 1, *NOD2* – nucleotide binding oligomerization domain containing 2, *CIITA* – class II major histocompatibility complex transactivator, *NAIP* – NLR family apoptosis inhibitory protein, *NLRP1* – NLR family pyrin domain containing 1, *NLRP3* – NLR family pyrin domain containing 3, *NLRP7* – NACHT, LRR and PYD domains-containing protein 7, GAPDH – glyceraldehyde-3-phosphate dehydrogenase

China). Proteins were quantified using the bicinchoninic acid protein assay kit (Tiangen Biotech Co., Ltd, Beijing, China) according to the manufacturer's instructions. Equal protein quantities (10 μg per well) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 5% stacking and 12% separating gels, and subsequently electro-transferred onto methanol-activated polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked by incubating in 5% skim milk at room temperature for 2 h, followed by overnight incubation with primary antibodies at a dilution of 1:1000 at 4 °C. Primary antibodies, including mouse anti-NOD1 monoclonal antibody (cat no.: sc-398696; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NOD2 monoclonal antibody (cat. no.: sc-56168; Santa Cruz Biotechnology), mouse anti-CIITA monoclonal antibody (cat. no.: sc-13556; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-NAIP polyclonal antibody (cat. no.: ab25968; Abcam, Cambridge, UK), mouse anti-NLRP1 monoclonal antibody (cat. no.: sc-390133; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NLRP3 monoclonal antibody (cat. no.: sc-134306; Santa Cruz Biotechnology, Santa Cruz, CA, USA,), and mouse anti-NLRP7 monoclonal antibody (cat. no.: sc-377190; Santa Cruz Biotechnology, Santa Cruz, CA, USA,) were used to detect respective proteins. After washing the membranes, horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no.: BL001A, 1:10000 for anti-mouse IgG-HRP; or anti-rabbit IgG-HRP, cat. no.: BL003A) were used to detect primary antibodies. Protein signals were detected using a pro-light HRP chemiluminescence detection reagent (Tiangen Biotech Co., Ltd, Beijing, China); immunospecific bands were digitally quantified using Quantity One V452 (Bio-Rad Laboratories, Hercules, CA, USA). Anti-GAPDH antibody (cat. no.: sc-20357, 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used to normalise the expression levels of the target proteins under the same incubation conditions as above.

Immunohistochemical analysis

Liver paraffin-embedded 5-μm sections were deparaffinised by multiple xylene washes, and rehydrated in a series of increasing ethanol dilutions; some sections were stained with haematoxylin and eosin. Heat-induced epitope retrieval was carried out in boiling 0.01 M citric buffer for 10 min, followed by the addition of 3% hydrogen peroxide solution to block endogenous peroxidase activity. After blocking non-specific binding sites with 5% normal goat serum in PBS, sections were incubated overnight with mouse anti-NOD2 monoclonal antibody (cat. no.: sc-56168; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-NLRP7 monoclonal antibody (cat. no.: sc-377190; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:200 in a hydration container at 4 °C. Primary antibodies were replaced with an antiserum-specific isotype at the same protein concentration for negative controls. After washing with PBS, the DAB kit (Tiangen Biotech Co., Ltd., Beijing, China) was used to visualise bound antibodies in hepatic tissue according to the manufacturer's instructions. Finally, the nuclei were stained with haematoxylin. Quantitative analysis of antigen expression intensity (DAB chromogen stain) for NOD2 and NLRP7 was performed using images captured with a digital camera DP12 under a light microscope (Nikon Eclipse E800, Nikon Corporation, Tokyo, Japan). Staining intensities and staining cell densities were analysed on images by 4 independent observers, and immunostaining intensities of hepatic samples from individual ewes were assessed in a blinded fashion. Staining intensities were analysed by rating immunoreactive intensity on a scale of 0 to 3, as previously described (Kandil et al., 2007).

Statistical analysis

The experiment used a completely randomized design, with an ewe as the experimental unit. Relative mRNA and protein expression data for *NOD1*, *NOD2*, *CIITA*, *NAIP*, *NLRP1*, *NLRP3* and *NLRP7* were analysed using MIXED procedure in SAS (Version 9.1; SAS Institute, Cary, NC, USA). Duncan's method was used to compare relative expression levels across groups and control the "experimentwise" type I ± error equal to 0.05. Data are presented as least squares means (± standard error of the mean). Differences between means at the $P < 0.05$ level were considered significant.

Results

Relative mRNA expression levels of *NOD1, NOD2, CIITA, NAIP, NLRP1, NLRP3* and *NLRP7* in liver

RT-qPCR analysis (n = 3 for each group) showed that *NOD1, CIITA* and *NLRP3* mRNA levels were elevated, but *NOD2* mRNA expression was downregulated on day 25 of pregnancy compared to the other three groups ($P < 0.05$; Figure 1).

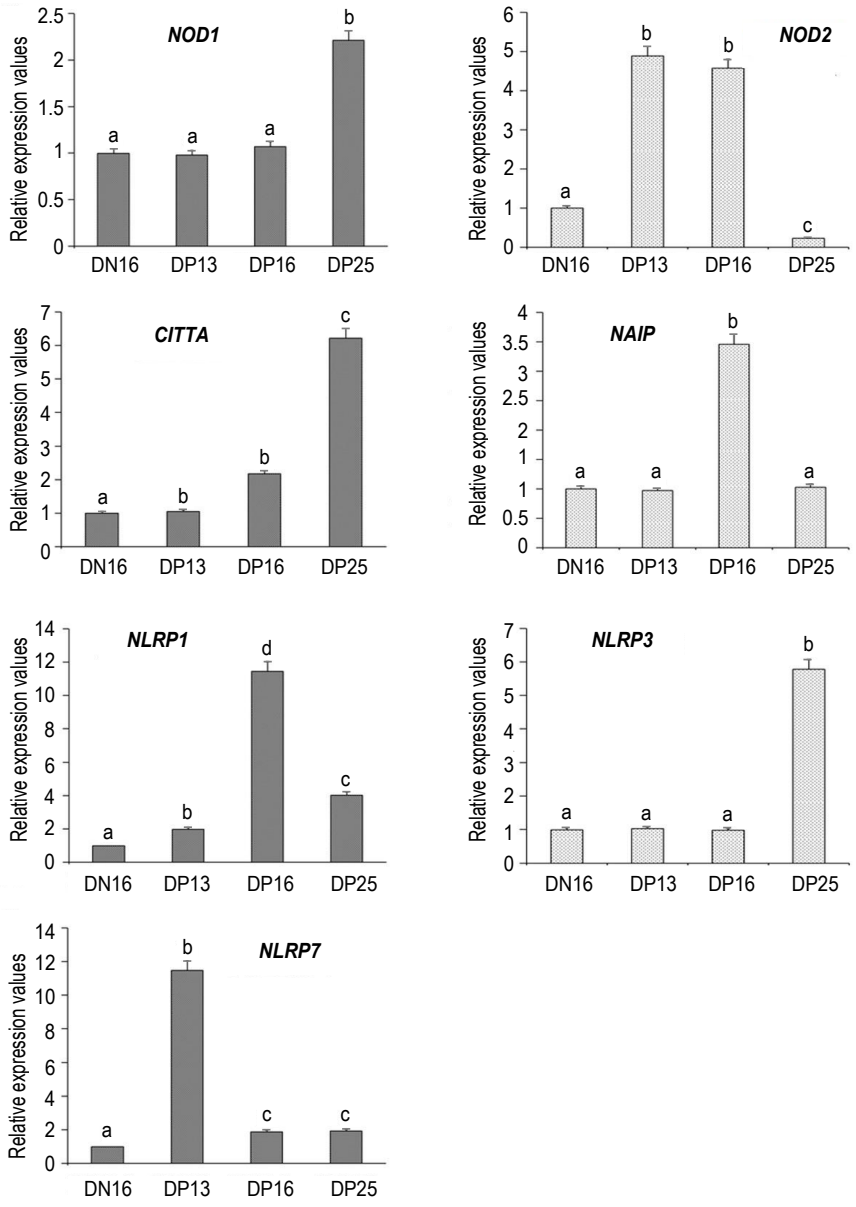

Figure 1. Relative values of *NOD1*, NOD2, *CIITA*, *NAIP*, *NLRP1*, *NLRP3* and *NLRP7* mRNA expression in liver as measured by real-time quantitative PCR in ewes. Note: DN16 – day 16 of non-pregnancy, DP13 – day 13 of pregnancy, DP16 – day 16 of pregnancy, DP25 – day 25 of pregnancy. Significant differences ($P < 0.05$) are indicated by different letters within the same colour bar. Nucleotide-binding oligomerisation domain (NOD)-like receptors include NOD1, NOD2, major histocompatibility complex class II transactivator (CIITA), neuronal apoptosis inhibitor protein (NAIP), NOD-like receptor family, pyrin domain-containing 1 (NLRP1), NLRP3 and NACHT, LRR and PYD domains-containing protein 7 (NLRP7).

However, *NOD2* mRNA expression level was higher on days 13 and 16 of pregnancy compared to day 16 of the oestrous cycle ($P < 0.05$; Figure 1). *NAIP* and *NLRP1* expression levels were the highest on day 16 of pregnancy, while *NLRP1* expression level was higher on day 25 of pregnancy than on day 16 of the oestrous cycle and day 13 of pregnancy ($P < 0.05$; Figure 1). The expression level of *NLRP7* mRNA was the highest on day 13 of pregnancy among the four groups, and lower on day 16 of the oestrous cycle than on days 16 and 25 of pregnancy ($P < 0.05$; Figure 1).

Expression of NOD1, NOD2, CIITA, NAIP, NLRP1, NLRP3 and NLRP7 proteins in the liver

Western blot analysis (n = 3 for each group) indicated that NOD1 and NLRP3 protein levels on day 25 of pregnancy were increased compared to the other three groups ($P < 0.05$; Figure 2), between which there was no significant difference ($P > 0.05$; Figure 2). However, NOD2 protein level was higher on days 13 and 16 of pregnancy compared to day 16 of the oestrous cycle ($P < 0.05$; Figure 2), but this protein was not detected on day 25 of pregnancy.

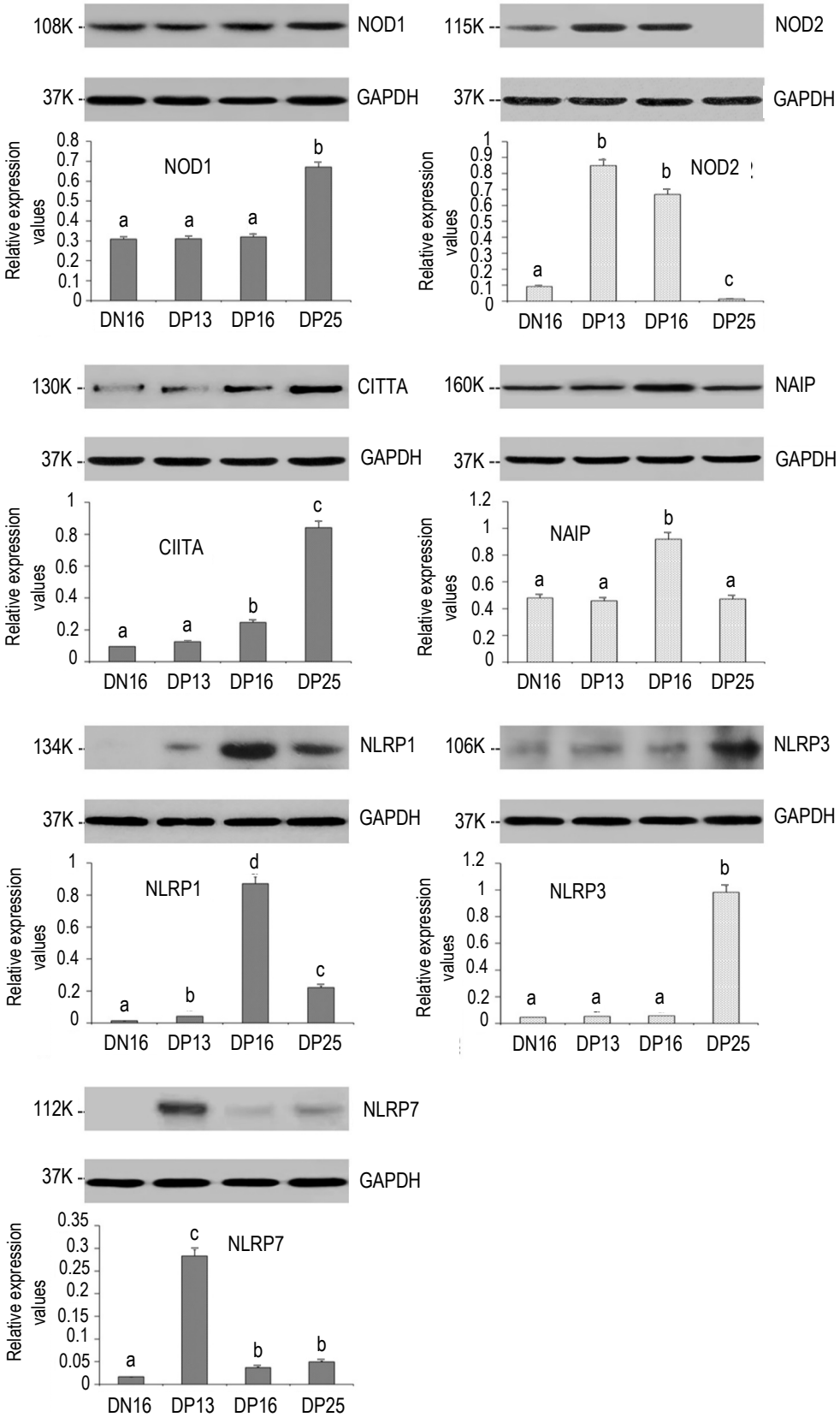

Figure 2. Expression of NOD1, NOD2, CIITA, NAIP, NLRP1, NLRP3 and NLRP7 proteins in liver analysed by Western blot in ewes. Note: DN16 – day 16 of non-pregnancy, DP13 – day 13 of pregnancy, DP16 – day 16 of pregnancy, DP25 – day 25 of pregnancy. Significant differences ($P < 0.05$) are indicated by different superscript letters within the same colour bar. Nucleotide-binding oligomerisation domain (NOD)-like receptors include NOD1, NOD2, major histocompatibility complex class II transactivator (CIITA), neuronal apoptosis inhibitor protein (NAIP), NOD-like receptor family, pyrin domain-containing 1 (NLRP1), NLRP3 and NACHT, LRR and PYD domains-containing protein 7 (NLRP7).

CIITA protein level was higher on day 25 of pregnancy compared to other three groups, but it was downregulated on day 16 of the oestrous cycle, and day 13 of pregnancy compared to day 16 of pregnancy ($P < 0.05$; Figure 2). Moreover, NAIP and NLRP1 protein expression levels were the highest on day 16 of pregnancy in all four groups ($P < 0.05$; Figure 2), but NLRP1 protein was not detected on day 16 of the oestrous cycle. NLRP7 protein was upregulated on day 13 of pregnancy compared to other three groups ($P < 0.05$; Figure 1), but was not detected on day 16 of the oestrous cycle.

Immunohistochemistry of NOD2 and NLRP7 proteins in the liver

NOD2 and NLRP7 proteins were localised in the endothelial cells of the proper hepatic arteries and portal veins, and in hepatocytes (Figure 3). Staining intensity of NOD2 protein was scored as 0, 2, 3, 3, and 0 for the negative control, while that for

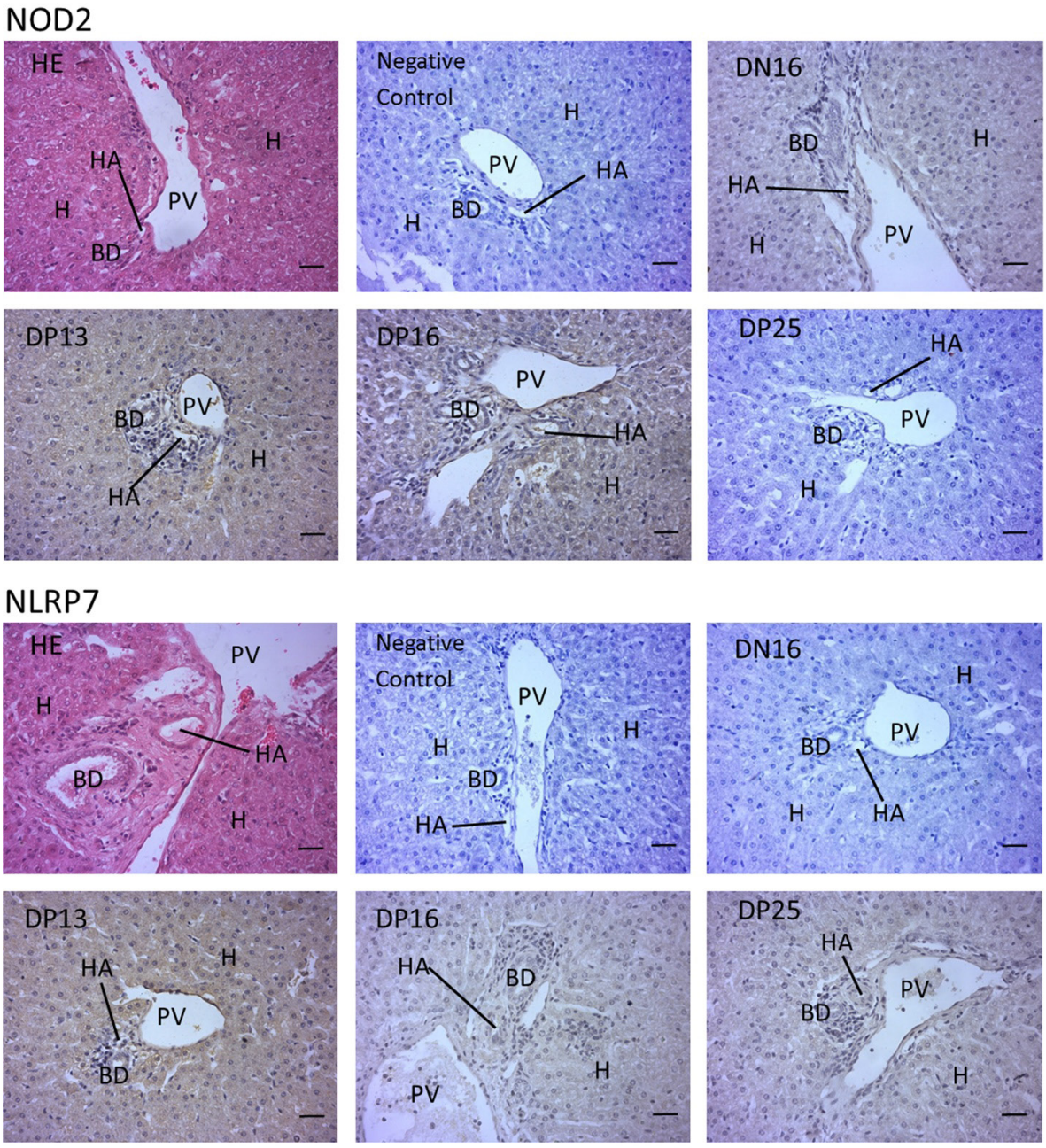

Figure 3. Immunohistochemical localisation of nucleotide-binding oligomerisation domain containing (NOD) 1 and NACHT, LRR and PYD domains-containing protein 7 proteins in liver. The liver is divided into lobes, and each lobe is composed of hepatic lobules. The portal triad is a component of the hepatic lobule and consists of the proper hepatic artery (HA), hepatic portal vein (PV), and small bile ductile (BD). Note: HE – staining with haematoxylin and eosin, H – hepatocyte; DN16 – day 16 of non-pregnancy, DP13 – day 13 of pregnancy, DP16 – day 16 of pregnancy, DP25 – day 25 of pregnancy; Bar – 50 μm.

NLRP7 protein was scored as 0, 0, 3, 2, and 2 for the negative control, for liver samples from day 16 of the oestrous cycle, and liver samples from days 13, 16, and 25 of pregnancy, respectively (Figure 3). Staining intensity was as follows: 0 = negative, 2 = strong, 3 = stronger.

Discussion

NOD1 plays an indirect role in metabolic processes by shaping cellular stress responses or enhancing inflammation (Zangara et al., 2021). In addition, *NOD1* transcription in the endometrium is higher during the first trimester of pregnancy than during the second and third trimester of pregnancy in cattle (Silva et al., 2012). In addition, NOD1 expression level was shown to be lower in the decidual stromal cells derived from unexplained recurrent pregnancy loss compared to normal early trimester pregnancy, and it has been reported to play a significant role in maintaining pregnancy by controlling infection and regulating immune responses (Zhang et al., 2019). Moreover, NOD1 has been found to affect the crosstalk between decidual stromal cells and the trophoblast, which plays a key role in regulating trophoblast invasion, and maintaining pregnancy during the early trimester (Ryu et al., 2017). On the other hand, *NOD1* mRNA and protein were shown to be highly expressed in hepatocytes, and hepatocytic NOD1 was associated with enhanced innate immune responses to both self and foreign antigens (Scott et al., 2010). In this study, NOD1 expression was upregulated on day 25 of pregnancy, and it could be associated with the high stimulus of foetal antigens, and thus required for early pregnancy maintenance in ewes.

NOD2 has been shown to interact with protein factors that affect and modulate signal transduction pathways involved in NOD2 signalling, which are crucial for NF-κB activation (Boyle et al., 2014). *NOD2* has also been found to be strongly associated with inflammatory bowel disease, which is genetically linked to premature rupture of foetal membranes (Strauss et al., 2018). Furthermore, treatment with muramyl dipeptide was demonstrated to induce first trimester trophoblast cells to a pro-inflammatory cytokine response via NOD2 activation (Costello et al., 2007). *NOD2* deficiency had a beneficial effect on cholestatic liver injury and fibrosis, involving increased renal excretion of bile acids, which in turn contributed to reduced bile acid concentrations in hepatocytes (Wang et al., 2014). NOD2 was also shown to be expressed in immune cells and hepatocytes and was upregulated during liver injury in mice and humans (Body-Malapel et al., 2008). Our results revealed that *NOD2* mRNA and protein were downregulated in the maternal liver on day 25 of pregnancy, and NOD2 protein expression was localised in the endothelial cells of the proper hepatic arteries and portal veins, and in hepatocytes. Therefore, the downregulation of NOD2 expression could be associated with the attenuation of maternal inflammatory responses and could favourably affect pregnancy establishment.

As a transcriptional activator and general transcription factor, CIITA regulates MHC transcription, contributing to the normal transcription of *MHC* class I and II genes, and plays a critical role in immune responses (Devaiah and Singer, 2013). In addition, CIITA protein has been localised in the basal layer of endometrial luminal epithelial cells and endothelial cells in blood vessels, and has been shown to be upregulated on days 15 and 30 of pregnancy compared to day 15 of the oestrous cycle in pigs (Yoo et al., 2020). Furthermore, *CIITA* was shown to be expressed in the inner cell mass side of bovine embryos that plays an important role in embryo development at the blastocyst stage in cattle (Nagatomo et al., 2015). Moreover, increased CD74 (type II membrane glycoprotein and MHC class II chaperone) expression in hepatocytes with *Ikkβ* (inhibitor of NF-κB kinases β) deletion was associated with increased expression of CIITA, implicated in antigen processing, host defence and liver tolerance in mice (Koch and Leffert, 2011). In the present study, CIITA expression level in the maternal liver was lower on day 16 of the oestrous cycle and increased from day 13 to 25 of pregnancy. Therefore, the upregulation of CIITA expression during early pregnancy could be associated with liver tolerance to foetal antigens, and could support embryonic development in sheep.

NAIP is involved in caspase-1 dependent processing and release of pro-inflammatory IL-1β and IL-18, which play key roles in immune defence (Rauch et al., 2017). In addition, NAIP protein is located in the cytoplasm of villous cytotrophoblast, syncytiotrophoblast, villous mesenchymal and villous endothelial cells of the human placenta, and shows greater immunoreactivity in first trimester placentas compared to term placentas (Ka and Hunt, 2003). The long non-coding RNA AK002210 was shown to modulate apoptosis, migration and invasion of trophoblast cell through regulation of *NAIP* expression in these cells (Zhao et al, 2020). Expression of *NAIP* mRNA has been characterised in murine embryogenesis, but *NAIP* deletion resulted in type I spinal muscular atrophy in mice (Ingram-Crooks et al., 2002). On the other hand,

NAIP mRNA transcripts were shown to be expressed in macrophage-rich tissues, including liver, involved in the modulation of macrophage function (Diez et al., 2000). This study found that the expression of *NAIP* mRNA and protein was enhanced in the maternal liver on days 16 and 25 of pregnancy. Therefore, increased NAIP expression could be associated with the regulation of maternal hepatic functions and could have been beneficial for pregnancy maintenance.

NLRP1 acts as a molecular decoy to protect other innate immune receptors and also plays a key role in monitoring cellular homeostasis (Taabazuing et al., 2020). In addition, NLRP1 is expressed in lymphocytes and monocytes/macrophages, as a response to altered homeostasis, which is crucial for mucosal homeostasis and antitumor activity (Fernandes et al., 2020). NLRP1 was also shown to be downregulated in trauma patients, but its expression was related with restoring the imbalanced immune response after injury (Relja et al., 2015). As an innate immune sensor, NLRP1 has been shown to induce the production of plasma IL-18, which is involved in the prevention of obesity and diet-induced metabolic dysfunction (Murphy et al., 2016). The liver participates in the absorption of digestion products and modification of the body's supply of intermediate metabolites in cattle (Huntington, 1990), and our data showed that early pregnancy enhanced the expression of *NLRP1* mRNA and protein in maternal liver, with a peak on day 16 of pregnancy. Therefore, upregulation of NLRP1 expression may be related to hepatic functions associated with nutritional metabolism and could be essential for normal pregnancy.

Traumatic injuries reduce the release of monocytic IL-1β, which plays an important role in host immunity, but NLRP3 has been shown to restore the ability of monocytes to produce active IL-1β (Kany et al., 2018). In addition, NALP3 in resting vascular endothelial cells was implicated in endothelial response to danger signals, but did not participate in endothelial cell activation through the IL-1β/IL-1R pathway (Wei et al., 2015). Endoplasmic reticulum stress has been associated with the embryo decidualisation and implantation, and NLRP3 has been shown to be involved in these processes and to contribute to a tolerogenic microenvironment sustaining pregnancy (Soczewski et al., 2020). NLRP3 participates in the regulation of metabolism and inflammation by inducing central pro-inflammatory cytokine IL-1β, which is involved in insulin resistance and obesity (Haneklaus and O'Neill, 2015). Our results demonstrated that NLRP3 expression was upregulated from day 13 to 25 of pregnancy. Therefore, increased NLRP3 expression could be related to the modulation of hepatic metabolism and immune responses during early pregnancy.

NLRP7 participates in innate immune processes in an inflammasome-dependent or independent manner, and is expressed in trophoblast cells, contributing to immune tolerance by regulating key factors associated with immune tolerance during normal pregnancy (Abi Nahed et al., 2022). In addition, NLRP7 has been shown to be expressed in decidual macrophages in the first trimester of pregnancy, and has been associated with decidualization and macrophage differentiation to maintain endometrial haemostasis and reproductive success in humans (Tsai et al., 2019). Moreover, NLRP7 was shown to serve as an innate immune sensor, it was expressed in decidual stromal cells of human first-trimester endometrium to support embryo implantation and proper placental development, but mutations and genetic variants of the *NLRP7* gene could cause infertility (Huang et al., 2017). NLRP7 expression was also detected in trophoblasts, where it was implicated in trophoblast proliferation, migration and invasion, but deregulation of NLRP7 resulted in foetal growth restriction due to abnormal placental development (Abi Nahed et al., 2019). However, there was an upregulation of NLRP7 observed in tumour cells, hydatidiform moles (CHM) and gestational choriocarcinoma, and NLRP7 was reported to be the major gene responsible for recurrent complete CHM and abnormal pregnancy (Reynaud et al., 2021). In this study, the expression of NLRP7 mRNA and protein was the highest on day 13 of pregnancy, and decreased on days 16 and 25 of pregnancy; NLRP7 protein was localised in the endothelial cells of the proper hepatic arteries and portal veins, as well as in hepatocytes. Therefore, peak expression of NLRP7 on day 13 of pregnancy may have contributed to the initiation of maternal immune tolerance and embryo implantation, but downregulation of NLRP7 on day 16 and 25 of pregnancy could be essential for maintaining pregnancy in ewes.

During early pregnancy in sheep, IFNT and progesterone signalling induces upregulation of NOD1, CIITA, NLRP1, NLRP3 and NLRP7 expression, but downregulates NOD2 expression, and modulates NAIP expression in maternal liver through blood circulation, i.e. proteins associated with regulation of maternal hepatic functions and pregnancy maintenance (Figure 4).

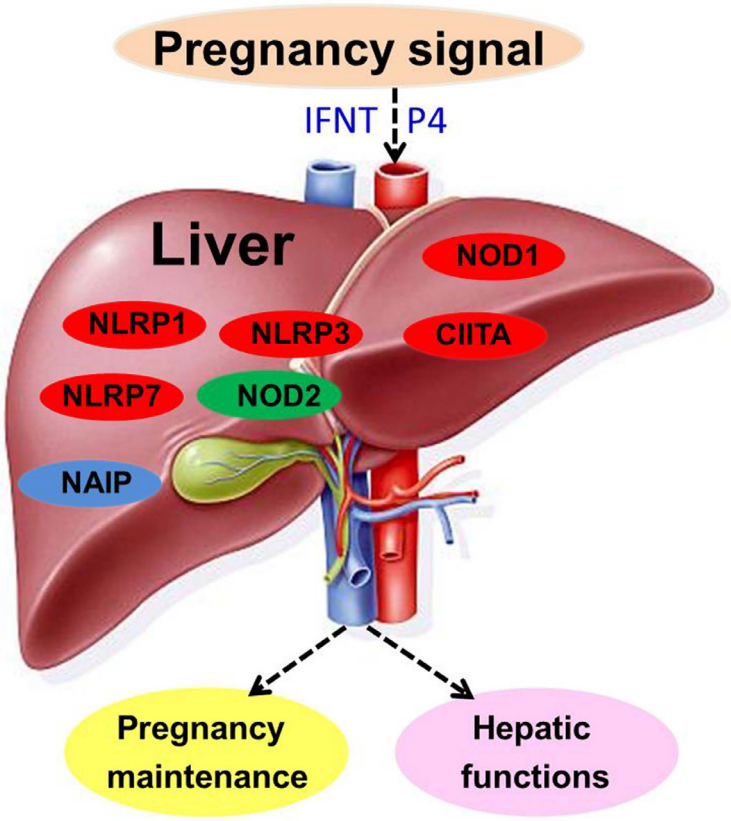

Figure 4. Diagram of Nod-like receptors in maternal liver during early pregnancy in sheep. Early pregnancy signals, including (interferon-tau, IFNT) and progesterone (P4), modulate the expression of Nod-like receptors associated with maternal peripheral tolerance and pregnancy establishment. Nucleotide-binding oligomerisation domain (NOD)-like receptors include NOD1, NOD2, major histocompatibility complex class II transactivator (CIITA), neuronal apoptosis inhibitor protein (NAIP), NOD-like receptor family, pyrin domain-containing 1 (NLRP1), NLRP3 and NACHT, LRR and PYD domains-containing protein 7 (NLRP7). Red, stimulator; Green, changed and negative regulator; Blue, changed.

Conclusions

Early pregnancy induced the upregulation of NOD1, CIITA, NLRP1, NLRP3 and NLRP7 in maternal liver, increased NOD2 and NAIP expression levels, but subsequently downregulated it on day 25 of pregnancy. In addition, NOD2 and NLRP7 protein expression was restricted to the endothelial cells of the proper hepatic arteries, portal veins and hepatocytes. Therefore, the present study reported for the first time that early pregnancy modulated the expression of the NLR protein family in sheep liver, which could be beneficial for the regulation of maternal hepatic immune function during early pregnancy in sheep.

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Conflicts of interest

The Authors declare that there is no conflict of interest.

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