Early warning for inactive ovaries based on liver function index, serum MDA, IL-6, FGF21 and ANGPTL8 in dairy cows

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ABSTRACT
Postpartum inactive ovaries (IOs) in dairy cows reduce the economic returns of the dairy industry. It is related to energy metabolism disorder, inflammatory response and oxidative stress. The objective of this study was to investigate the association of liver function index (LFI) and serum cytokines at 21 days postpartum with IO and to predict the risk of IO in dairy cows. The blood of 60 parturient cows was collected through caudal root vein at 3, 21, 28 and 55 days postpartum. Ultrasonography was performed at 50 and 55 days postpartum to determine follicular development. With the median LFI as the standard, it was divided into high LFI (n = 30) and low LFI group (n = 30). A cohort study was used to analyse the risk of LFI to IO and t-test was used to compare the blood biochemical indicators of different LFI groups. Then, 12 cows (oestrus = 6 and IO = 6) were slaughtered 55 days postpartum. The differences of LFI, cytokines and biochemical indexes were compared, and data were analysed by t-test, Spearman’s correlation analysis, binary logistic regression analysis and receiver operating characteristic analysis. The results show that growth and development of follicles of low LFI dairy cows were impaired, the risk of IO increased by 2.67 times. Cows with lower LFI had energy metabolism disorders, increased inflammation and oxidative stress and decreased ability to resist oxidative stress at 21 days postpartum. LFI and serum MDA, IL-6, FGF21 and ANGPTL8 at 21 days postpartum can predict IO in dairy cows.

HIGHLIGHTS
• The risk of inactive ovaries (IOs) in cows with low liver function index (LFI) will increase by 2.67 times, serum MDA, IL-6, FGF21 and ANGPTL8 at 21 days postpartum can predict IOs in dairy cows at 50–55 days postpartum.
• A high predictive risk of IOs in dairy cows occurred when the LFI was less than –3.18, MDA more than 3.62 mmol/L, IL-6 more than 23.68 ng/L, FGF21 less than 812.41 ng/L and ANGPTL8 less than 695.79 ng/L at 21 days postpartum.

Introduction
The new definition of inactive ovaries (IOs) is the presence of a follicular wave on the surface of the ovary, but the growth of the follicle stops before the follicle deviates (Butler 2003). Usually, cows have no oestrus and no corpus luteum on the surface of the ovary during a postpartum period at 45–60 days. There are many reasons for postpartum IO in dairy cows, including nutritional imbalance and mismanagement (McDougall 2006). After calving, the dairy cows nutrition and energy requirements increase significantly because milk yield rapidly rises and feed intake only increases slowly. The mobilisation of body fat by dairy cows increases to release non-esterified fatty acids (NEFAs) to meet the energy demand of early lactation. Once the levels of NEFA exceed the utilisation capacity of the liver, they are converted into ketone bodies and cause ketosis. The resulting inflammation leads to inadequate release of low-density lipoprotein, hepatic triacylglycerol deposits, hepatocyte damage and promote fatty liver (Rukkwamsuk et al. 1999; Gathercole et al. 2013). Inflammatory inducers act on specific
immune cells in tissues to trigger the production of inflammatory mediators, including pro-inflammatory cytokines (PICs) such as tumour necrosis factor-α, interleukin-6 (IL-6) and IL-1, and lipid mediators such as prostaglandin, thromboxane, leukotriene, liposomes and vasoactive amines (Luster et al. 2005). In the liver, PICs increase the synthesis of acute phase proteins (+APP) such as haptoglobin (HP), serum amyloid A (SAA), ceruloplasmin, C-reactive protein, globulin and reduce the synthesis of other proteins, causing various liver cytokine disorders (Bertoni et al. 2015). Inflammation can also hinder follicular growth due to the oestriadiol disorder (Sheldon et al. 2002). The increase of cellular reactive oxygen species (ROS) can also induce granulosa cell apoptosis through the ROS-JNK-p53 pathway (Yang H et al. 2017); therefore, abnormal energy metabolism, inflammation and oxidative stress are also closely related to IO.

The basic definition of cytokines classifies these mediators as soluble hormone-like proteins, which are produced by a variety of cells under the stimulation of various inducers (Simpson et al. 1997). The cellular sources and biological targets of these proteins are not limited to cells of the immune system, but also endothelial, stellate, Ito, fat storage cells and liver cells, that along with myofibroblasts can produce and respond to many different cytokines.

The liver is an important organ for cytokine metabolism and can produce and remove cytokines (Simpson et al. 1997). Liver function indicators are often used to detect liver damage in dairy cows, which has important significance for the prevention and monitoring of other diseases and the improvement of production and reproductive performance. Bertoni and Trevisi (2013) reported that the liver function index (LFI) as a comprehensive index may evaluate cow health and inflammation during early lactation. It may be calculated using the levels of albumin (ALB), total cholesterol (TC) and total bilirubin (TBIL) in the blood of cows at day three and 28 post-partum. The value of LFI ranges from −12 to 5 and the higher the LFI, the better the liver function and metabolic health of the dairy cow (Zhou et al. 2016), so it is a simple practical indicator for evaluating liver function and other related diseases.

The liver plays a significant role in energy metabolism, glucose homeostasis regulation and follicular development (Jung and Kang 2010; Roh et al. 2016). Interleukin-6 also called muscle-derived IL-6 is a cytokine secreted by skeletal muscle and fibroblast growth factor 21 (FGF21) and angiopoietin-like protein 8 (ANGPTL8) are cytokines secreted by the liver. IL-6 can also be produced by ovarian epithelial cells and granulosa cells and the mRNA of IL-6 and protein in granulosa cells is affected by IL-1 (Yang M et al. 2017). It is closely related to ovulation disorders, such as human polycystic ovary syndrome (PCOS) and bovine follicular cysts (Goodarzi et al. 2011). The relationship between FGF21 and follicular development is still unclear. Owen et al. (2013) found that the increased expression of FGF21 by the liver can affect follicular development by altering the secretion of follicle-stimulating hormone. Xu et al. (2017) found that when the FGF21 gene was silenced, the follicle development of mice was hindered and the oestrus delayed. Studies in mice also showed that FGF21 derived from liver and adipose tissue regulated oestrus and follicular development (Singhal et al. 2016). Polycystic ovary syndrome is often accompanied by insulin resistance and ANGPTL2 might participate in the development of PCOS through the PI3K/Akt signalling pathway (Wang et al. 2020). At the same time, ANGPTL1 and ANGPTL6 genes can also affect the degree of lipid metabolism or insulin resistance in animals, affecting the development of ovarian follicles in PCOS patients with insulin resistance (Boztosun et al. 2012). Among the ANGPTL family, ANGPTL8 was recently reported to regulate follicle development by gene expression.

As LFI is a comprehensive indicator reflecting inflammation in the body, so when it is low the excessive secretion of +APP, such as HP and SAA may affect release of other cytokines, such as FGF21 and ANGPTL8, resulting in metabolic disorders and affecting follicular development in the later stages. This study proposes the hypothesis that dairy cow LFI and cytokines including IL-6, FGF21 and ANGPTL8 at 21 days postpartum could be new indicators for predicting postpartum IO in dairy cows that facilitate an early diagnosis of the diseases and control its progression.

Materials and methods

Animals and groups

Chinese Holstein dairy cows were randomly selected as a laboratory animal from a large-scale farm with 6000 dairy cows. The total mixed ration (TMR) of tested dairy cows complied with the United States National Research Council (NRC 2001) and with the Chinese Feeding Standard for dairy cows. The composition and ingredients of TMR diet included 1.03 kg cottonseed, 1.50 kg soybean husk, 2.50 kg alfalfa, 1.30 kg soybean meal, 2.00 kg corn flake, 1.00 kg molasses,
morning, centrifuged at 3000 xg for 10 min, the supernatant was collected into 1.5 mL tube, recently-fuged at 12,000 xg for 10 min and then this supernatant was divided and stored at −80°C. B-ultrasonography was performed on postpartum day 50 and 55 for all cows to calculate follicular growth rate (mm/d). At 55 days postpartum, six cows with no other diseases or abnormal clinical manifestations were selected for slaughter and the thigh muscle tissue from the right hind leg 20 cm below the ischial tuberosity, liver and ovarian cortical tissue were collected. After freezing in liquid nitrogen, samples were stored at −80 °C for cytokine gene and protein detection.

Serum biochemical index detection

The serum biochemical index combined indicators for energy metabolism and the LFI. The energy metabolism indicators included β-hydroxybutyric acid (BHBA), NEFA, glucose (GLU) and TC (mmol/L). The LFI included ALB (g/L) and TBIL (μmol/L). Serum BHBA was measured using a serum ketone metre and ketosis reagent strips with 93.8% sensitivity, 100% specificity and a 93.8% Youden index (Yicheng, Beijing, China). Other biochemical parameters were measured using commercial kits (Mindray Biomedical Electronics Co. Ltd., Shenzhen, China) and instruments from our previous research.

Quantification of mRNA expression

Total RNA was extracted from muscle, liver and ovarian cortical tissue samples with Trizol RNA extraction reagent (Invitrogen Corporation, Carlsbad, CA), mRNA transcription and cDNA synthesis were performed with Reverse Transcriptase AMV (New England Biolabs, Ipswich, MA) using an oligodT Primer. The quantitative real-time polymerase chain reaction (RT-PCR) was performed by FastStart Universal SYBR Green Master under the following conditions of 94°C for 3 min, followed by 40 cycles at 94°C for 15 s, 60°C for 1 min and 72°C for 25 s on a BioRad iCycler IQTM RT-PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA). The calculated IL-6, FGF21, ANGPTL8 and β-actin mRNA expression levels were quantified with the 2^ΔΔCt method. The primers used are shown in Table 1 (Sangon Biotech Company, Shanghai, China).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used to detect serum and tissue inflammation factors and
IL-6, HP SAA; cytokines IL-6, FGF21 and ANGPTL8; oxidative stress indicators malondialdehyde (MDA), glutathione peroxidase (GSH-PX), glutathione (GSH), total antioxidant capacity (T-AOC) and restraining ability to hydroxyl free radicals (RAHFR). After cleaning tissue blocks with PBS 0.01 mol/L at a pH of 7.0–7.2, they were transferred to a homogeniser on ice and 10 mL PBS was added for grinding, followed by centrifugation at 5000 rpm for 5 min. The concentration of each standard product was assessed and two repeats were made. The required ELISA kits were purchased from Lengton Company (Shanghai, China).

Radioimmunoassay procedures

Growth hormone, oestradiol and progesterone concentrations were measured using commercial kits with the same lot number (Xinfan Biotechnology Co. Ltd., Shanghai, China) and the kit manufacturer's validated radioimmunoassay procedures. The intra-assay coefficients of variation were less than 10% and the inter-assay coefficients of variation less than 15%.

Statistical analysis

The test data were statistically analysed using IBM SPSS 23.0 (IBM, Armonk, NY). The \( \chi^2 \) test of the cohort study was used to analyse the proportion of IO cows in the LO-LFI and HI-LFI groups and to clarify the correlation between the decline in LFI of the cows and the occurrence of IO. The independent sample t-test was used to analyse the significance of differences in clinical background information, reproductive performance indexes, follicular growth status, serum indexes and cytokine genes in tissues between LO-LFI and HI-LFI groups and E and IO groups and the data were expressed as mean ± standard error. Spearman’s correlation analysis, binary logistic regression analysis and receiver operating characteristic (ROC) analysis were used to predict the occurrence of IO in dairy cows. Receiver operating characteristic curves with a threshold \( p < .05 \) was used to determine the early warning risk index of IO.

Results

Risk assessment of IO in dairy cows by LFI

As shown in Tables 2 and 3, the proportion of IO in dairy cows in the LO-LFI group of 36.67% was significantly higher than that in the HI-LFI group at 10% \( (p < .05) \). The RR was 3.67, greater than 3 and the 95% confidence interval \((1.10–12.20)\), excluding 1, made it statistically significant \( (\chi^2 = 4.49, \ p = .03) \). According to the corresponding correlation strength in Table 4, an RR between 3.0 and 9.0 is strongly positively correlated. The results show that low LFI can increase the risk of postpartum IO in dairy cows by 2.67 times, which makes it an important risk factor.

Clinical and reproduction information of LO-LFI and HI-LFI cows

As shown in Table 5, the body condition score (BCS) at 7 days postpartum, insemination times \( (p < .05) \), the body condition score loss (BCL), first oestrus days, pregnancy days and calving interval of the LO-LFI group were all significantly higher than those of the HI-LFI group \( (p < .01) \). The oestrus times, oestrus rate, conception rate \( (p < .05) \), average daily milk production and follicular growth rate from 50 to 55 days after delivery \( (p < .01) \) of the LO-LFI group were significantly lower than those of the HI-LFI group. There was no significant difference in age, parity or BCS on the 50th day after delivery, first mating days and follicular diameter at 50 and 55 days between the two groups \( (p > .05) \).
The results showed that the loss of BCS increased and the average daily milk yield was lower in the early lactation period of LO-LFI cows. Postpartum low LFI can prolong the first oestrus and pregnancy days, calving interval, increase insemination times, reduce oes- 
lactation period of LO-LFI cows. Postpartum low LFI and the average daily milk yield was lower in the early

### Table 5. Clinical information of the 60 cows with LO-LFI and HI-LFI.

| Project                          | LO-LFI (n = 30) | HI-LFI (n = 30) | p Value |
|----------------------------------|-----------------|-----------------|---------|
| Age                              | 3.47 ± 0.36     | 3.45 ± 0.32     | .967    |
| Parity                           | 2.33 ± 0.32     | 2.23 ± 0.36     | .836    |
| 7 d BCS                          | 3.45 ± 0.06     | 3.23 ± 0.08     | .032    |
| 50 d BCS                         | 2.83 ± 0.08     | 2.95 ± 0.07     | .264    |
| BCL                              | 0.62 ± 0.08     | 0.28 ± 0.08     | .004    |
| Average daily milk production, kg/d | 37.62 ± 1.43   | 41.72 ± 1.21    | .033    |
| First oestrus days, days         | 56.77 ± 3.58    | 44.70 ± 2.56    | .008    |
| Oestrus times, times             | 0.83 ± 0.14     | 1.23 ± 0.11     | .029    |
| Inseminations times, times       | 2.53 ± 0.21     | 1.87 ± 0.16     | .015    |
| First mating days, days          | 65.70 ± 1.87    | 63.17 ± 1.60    | .308    |
| Pregnancy days, days             | 118.73 ± 5.29   | 92.33 ± 4.37    | <.001   |
| Calving interval, days           | 401.73 ± 6.08   | 367.50 ± 4.50   | <.001   |
| Oestrus rate, %                  | 10.00           | 36.67           | .015    |
| Conception rate, %               | 51.03           | 66.57           | .048    |
| 50 d follicle diameter, mm       | 7.00 ± 0.850    | 6.60 ± 0.94     | .755    |
| 55 d follicle diameter, mm       | 10.47 ± 0.92    | 12.40 ± 0.72    | .104    |
| Follicle growth rate, mm/d       | 0.60 ± 0.11     | 1.16 ± 0.11     | .004    |

BCS: body condition score; BCL: body condition score loss; HI-LFI: high liver function index; LO-LFI: low liver function index.

### Table 6. Serum biochemical index of 60 HI-LFI and LO-LFI dairy cows at 21 and 55 days postpartum.

| Project                          | LO-LFI (n = 30) | HI-LFI (n = 30) | p Value |
|----------------------------------|-----------------|-----------------|---------|
| HP, ng/L                         | 31.41 ± 1.35    | 24.54 ± 1.36    | <.001   |
| SAA, ng/mL                       | 32.38 ± 1.30    | 27.45 ± 1.29    | .009    |
| SOD, U/mL                        | 102.54 ± 6.65   | 121.78 ± 6.58   | .047    |
| MDA, mmol/mL                     | 6.62 ± 0.36     | 4.58 ± 0.32     | .035    |
| GSH-PX, U/mL                     | 112.15 ± 2.69   | 120.98 ± 2.80   | <.01    |
| GSH, μmol/mL                     | 63.54 ± 3.14    | 108.28 ± 3.23   | <.001   |
| T-AOC, mmol/L                    | 0.43 ± 0.02     | 0.55 ± 0.02     | <.001   |
| RHFR, mmol/L                     | 627.89 ± 7.05   | 653.28 ± 9.60   | .037    |
| BHBA, mmol/L                     | 1.48 ± 0.12     | 1.05 ± 0.10     | .008    |
| NEFA, mmol/L                     | 0.76 ± 0.04     | 0.63 ± 0.04     | .025    |
| Glu, mmol/L                      | 2.78 ± 0.15     | 3.50 ± 0.17     | .002    |
| IL-6, ng/L                       | 28.23 ± 2.89    | 19.98 ± 2.58    | .038    |
| FGF21, ng/L                      | 713.10 ± 21.85  | 796.88 ± 25.43  | .015    |
| ANGPTL8, ng/L                    | 607.70 ± 35.94  | 721.20 ± 37.87  | .034    |
| IGF-1, ng/mL                     | 69.88 ± 6.23    | 89.69 ± 6.11    | .027    |
| Growth hormone, ng/mL, 55 days   | 43.84 ± 1.12    | 48.43 ± 1.19    | .009    |
| Oestradiol, pg/mL, 55 days       | 15.74 ± 1.04    | 18.79 ± 0.81    | .024    |
| Progesterone, ng/mL, 55 days     | 1.67 ± 0.21     | 2.37 ± 0.27     | .045    |

HP: haptoglobin; SAA: serum amyloid A; SOD: superoxide dismutase; MDA: malondialdehyde; GSH-PX: glutathione peroxidase; GSH: glutathi- 
one; T-AOC: total antioxidant capacity; RHFR: restraining ability to hydroxyl free radicals; BHBA: β-hydroxybutyrate; NEFA: non-esterified fatty 
acid; GLU: glucose; IL-6: interleukin-6; FGF21: fibroblast growth factor 21; ANGPTL8: angiopoietin-like protein 8; IGF-1: insulin-like growth factor 1; 
HI-LFI: high liver function index; LO-LFI: low liver function index.

### Clinical information and serum biochemical indexes of E and IO cows at 21 postpartum days

As shown in Table 7, average daily milk production, serum FGF21, ANGPTL8, T-AOC and SOD (p < .05); and 
LFI (p < .01) of the IO group were significantly lower 
than those of the E group. Serum MDA (p < .05), and 
IL-6 and SAA (p < .01) of the IO group were significantly 
higher than those of the E group. There was no signifi- 
cant difference in age and parity between the two 
groups (p > .05). The results showed that at 21 days 
postpartum, liver function and antioxidant stress cap- 
acity were decreased and inflammatory response and 
oxidative stress were increased, characteristic of IO.

### Expression of cytokine genes and proteins in the 
tissues of E and IO cows

As shown in Figure 1, the mRNA expression of IL-6 in both muscle (p < .01) and ovarian tissue (p < .05), hepatic 
FGF21 (p < .01) and hepatic ANGPTL8 (p < .05) in the 
IO group was significantly lower than the E group. The protein expression of IL-6 in both muscle (p < .05) 
and ovarian (p < .01) tissue and hepatic FGF21 and 
ANGPTL8 in group IO was significantly lower than 
group E (p < .05). The mRNA and protein expressions 
of FGF21 and ANGPTL8 in ovarian tissue had no signi- 
ficant difference between the two groups. The results 
showed that the level of myogenic IL-6 was 
lower during IO, which was opposite to that in serum. 
The levels of FGF21 and ANGPTL8 in the liver were
low and consistent with those in serum, but the transcription and expression in the ovaries did not change.

**Association between serum biochemical indexes and IO in postpartum cows at 21 days**

As shown in Table 8, LFI ($p < .01$), serum FGF21, ANGPTL8 and T-AOC were significantly positively correlated with IO ($p < .05$). There was a significant negative correlation between serum IL-6 ($p < .01$), SAA, MDA and IO ($p < .05$). The results showed that these indexes were significantly correlated with follicular growth and could be used for the early warning assessment of the risk of IO in dairy cows.

### Risk assessment of postpartum IO in dairy cows

As shown in Table 9 and Figure 2, according to Youden’s index, there were several factors that offered the best early warning value of cows with IO. The LFI needed to be less than $-3.18$ and had a sensitivity of 80%, a specificity of 90.1%, the area under the curve 0.80, and an AUC value of 0.91.

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**Table 7. Clinical information and serum biochemical indexes of 12 E and IO cows at 21 postpartum days.**

| Project      | E (n = 6) | IO (n = 6) | $p$ Value |
|--------------|-----------|------------|-----------|
| Age          | 3.44 ± 0.45 | 3.45 ± 0.31 | .986      |
| Parity       | 2.41 ± 0.35 | 2.35 ± 0.38 | .910      |
| Average daily milk production, kg/d | 42.22 ± 1.49 | 37.47 ± 1.36 | .040      |
| LFI          | 1.35 ± 0.92 | -6.35 ± 1.64 | .002      |
| IL-6, ng/L   | 19.42 ± 2.43 | 29.68 ± 1.53 | .005      |
| FGF21, ng/L  | 893.34 ± 49.53 | 749.15 ± 30.23 | .032      |
| ANGPTL8, ng/L| 798.69 ± 60.24 | 628.12 ± 46.65 | .049      |
| SAA, ng/L    | 10.57 ± 1.79 | 28.69 ± 1.62 | <.001     |
| SOD, U/mL    | 164.37 ± 11.91 | 121.09 ± 12.64 | .032      |
| MDA, nmol/mL | 3.36 ± 0.55 | 5.45 ± 0.75 | .048      |
| GSH, µmol/mL | 113.69 ± 20.68 | 66.97 ± 13.85 | .088      |
| T-AOC, mmol/mL| 0.33 ± 0.06 | 0.16 ± 0.04 | .040      |
| GSH-PX, U/mL | 668.91 ± 22.36 | 605.29 ± 15.52 | .042      |

LFI: liver function index; IL-6: interleukin-6; FGF21: fibroblast growth factor 21; ANGPTL8: angiopoietin-like protein 8; SAA: serum amyloid A; SOD: superoxide dismutase; MDA: malondialdehyde; GSH: glutathione; T-AOC: total antioxidant capacity; GSH-PX: glutathione peroxidase; RAHFR: restraining ability to hydroxyl free radicals.

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**Table 8. Association between serum biochemical indexes and inactive ovarian in 12 dairy cows.**

| Project      | Mean (n = 12) | $r$-value | $p$ Value |
|--------------|---------------|-----------|-----------|
| LFI          | -2.61 ± 1.03  | 0.532     | .001      |
| IL-6, ng/L   | 22.49 ± 3.78  | -0.615    | .003      |
| FGF21, ng/L  | 802.95 ± 82.31| 0.573     | .016      |
| ANGPTL8, ng/L| 714.65 ± 77.67| 0.651     | .022      |
| SAA, ng/L    | 16.79 ± 1.25  | -0.507    | .031      |
| MDA, nmol/mL | 4.01 ± 0.81   | -0.614    | .011      |

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**Figure 1.** Cytokine mRNA abundance and protein levels in tissues. (A, D) Interleukin 6 (IL-6); (B, E) Fibroblast Growth Factor 21 (FGF21); (C, F) Angiopoietin Like Protein 8 (ANGPTL8); *$p < .05$, **$p < .01$. E: oestrus; IO: inactive ovary.
AUC of 0.855 and the confidence interval of 0.724–0.987 to give good diagnostic significance. Serum MDA needed to be less than 3.62 mmol/L and had a sensitivity of 70.3%, a specificity of 90.9%, an AUC of 0.701 and the confidence interval of 0.589–0.813, indicating low diagnostic significance. However, when combined, the sensitivity was 87.9%, the specificity was 91.3%, the AUC was 0.898–1, which was of high diagnostic significance to predict IO in dairy cows.

As shown in Table 10 and Figure 3, there were several indicators for the prediction of IO cows as determined by the Youden index. Serum IL-6 needed to be less than 23.68 ng/L and it had a sensitivity of 81.5%, a specificity of 89.2%, an AUC of 0.820, and the confidence interval of 0.688–0.952. Serum FGF21 needed to be less than 812.41 ng/L and had a sensitivity of 80.6%, a specificity of 90.1%, an AUC of 0.715 and the confidence interval of 0.491–0.939. Lastly, serum ANGPTL8 had to be less than 695.79 ng/L, with a sensitivity of 81.3%, a specificity of 86.1%, an AUC of 0.776 and the confidence interval of 0.712–0.840 for good diagnostic significance. When the three serum cytokines were combined for prediction, a sensitivity of 86.3%, a specificity of 90.9%, an AUC of 0.947 and the confidence interval of 0.867–1 meant high diagnostic significance.

**Discussion**

The use of LFI can provide a good assessment of inflammatory phenomena and their consequences (Bertoni and Trevisi 2013) and could be used to identify cows at risk in the transition period, assisting improved farm management (Trevisi et al. 2012). At present, there have been no reports on the relationship between reduced LFI and IO in peripartal cows. In a retrospective comparison, cows with lower LFI showed lower milk yield, lower DMI, slightly higher BCL and higher serum concentrations of NEFA and BHBA (Trevisi et al. 2010) and the results of this study are similar. This means that the LO-LFI group has a higher fat mobilisation and a greater risk of liver steatosis. Zhou et al. found that the mating times with high LFI was 1.6, which was less than that of cows with low LFI. The success rate of first breeding was 53% and the number of days without pregnancy was 93 days, while the success rate of first breeding of cows with low LFI was only 32% and the number of empty days was 110 days, which was significantly longer (Zhou et al. 2016). As a variable, LFI is an index of inflammatory conditions, with inflammation having a particularly important effect on liver function. It increases the synthesis and release of +APP and at the same time reduces the blood concentration of −APP, including ALB, TC and proteins involved in bilirubin clearance, which are closely related to LFI (Powanda 1980; Bertoni et al. 2008, 2015). The reduction of apolipoprotein encourages the accumulation of triglycerides in the liver, leading to more severe lipid deposition (Ametaj et al. 2005). This means that in high-yielding cows, there are more negative effects on oxidative stress and when they occur at the same time, it reduces reproductive function (Bertoni et al. 2008). Similarly, it was also found in this study that the incidence of postpartum IO in cows with high LFI was 11.2% and that in cows with low LFI was 39.47%. Low LFI increased the risk of postpartum IO by 1.67 times, suggesting that low LFI was an important risk factor for postpartum IO in cows.

The cytokine IL-6 is multifunctional and one of the positive acute phase proteins produced by the body’s immune cells, muscles and other tissues. When the postpartum LFI is low, it indicates that the body is in a state of inflammation and the levels of +APP including IL-6, HP, SAA, are high, as shown in this study.
where IL-6, HP and SAA of cows in the LO-LFI group were significantly higher than those in the HI-LFI group. At the same time, considering that inflammation is the cause of the increase in ROS, the oxidative stress related indicators in the study, including MDA, GSH-PX, GSH, T-AOC and RAHFR, were significantly different between the LO-LFI and HI-LFI. At 55 days postpartum, both inflammation and oxidative stress indexes of cows in group IO were higher than those in group E, so it was suspected that the low LFI status of cows at 21 days postpartum lasted until 55 days postpartum and played a key role in IO and that LFI, 21 days postpartum inflammation indicators, and oxidative stress indicators can be used as one of the indicators to predict IO. This was subsequently verified by the data from this study.

The results of studies on the effect of IL-6 on the development of follicular cells are inconsistent. Follicular granulocyte cells also secrete IL-6, which not only suppresses steroidogenesis induced by follicle-stimulating hormone (Alpizar and Spicer 1994; Salmassi et al. 2001), but also promotes the luteinising hormone receptor expression (Imai et al. 2014) and cumulus-oocyte complexes expansion (Liu et al. 2009). In this study, IL-6 mRNA and protein content of the ovarian tissues of IO cows were significantly decreased, due to the negative effects of decreased IL-6 content in ovarian tissues on follicular development. In this study, IL-6 gene and protein expression levels were decreased in both muscle and ovarian tissues. There was a higher level of IL-6 in the serum because it comes from a wide variety of sources and increased levels of IL-6 in the serum reflect the increased production of IL-6 due to the body’s inflammatory response. The decrease of IL-6 content in tissues and the increase of IL-6 content in serum of IO cows therefore not only reflect that the body is in an inflammatory state but also suggest that IL-6 plays a significant role in the development of follicles.

FGF21 can be highly expressed in liver and adipose tissue. Its expression in liver tissue of dairy cows increases significantly up to 30 times from late pregnancy to the beginning of lactation (Schlegel et al. 2013). It can rely on βKlotho to regulate metabolism, and is decreased in the liver when nutrient supply is inadequate (Ding et al. 2012). It also acts on the supra-chiasmatic nucleus in the hypothalamus to suppress the vasopressin-kisspeptin signalling cascade, inhibiting the pro-oestrus surge in luteinising hormone during starvation (Owen et al. 2013) and directly acts on gonadotropin-releasing hormone (GnRH) neurons to modulate GnRH secretion via ERK1/2 pathway (Hua et al. 2020). A prior study observed that female mice with FGF21 over-expression were infertile due to central suppression of kisspeptin signalling and GnRH release (Owen et al. 2013). As mice engineered to transgenically overexpress FGF21 had super-physiologic levels of FGF21, high doses of FGF21 at physiological levels may not have adverse effects on reproduction. However, in this study, the expression of FGF21 mRNA in the ovarian tissues of IO cows did not significantly change, indicating that FGF21 in the ovary of IO cows does not directly participate in the follicular development process, but affects follicular development through energy or hormone metabolism.

The liver produces a variety of cytokines that can regulate carbohydrate and fat metabolism, such as ANGPTL8 which plays a significant role in triglyceride metabolism (Li and Teng 2014; Abu-Farha et al. 2016). Studies have shown that ANGPTL1 and ANGPTL2 can play a coordinated role during the maturation of follicles and are highly expressed in human PCOS oocytes caused by insulin resistance (Liu et al. 2016). Immunohistochemistry showed that ANGPTL4 could...
be differentially expressed in mouse ovarian tissue, indicating that it was closely related to follicular development, that the ANGPTL family played a significant role in the process of ovarian follicular development (Scott et al. 2012) and ANGPTL8 is an important new cytokine. In this study, there was no significant change in the mRNA expression of ANGPTL8 in the ovarian tissue of IO cows, while the content of ANGPTL8 in liver tissue decreased, indicating that it was not directly involved in the development of ovarian follicles and affected follicle development by participating in the regulation of energy metabolism during IO in dairy cows. However, the role of ANGPTL8 in regulating energy metabolism or follicle development in early lactation in dairy cows remains unclear.

The health status of dairy cows during the perinatal period has an important influence on their reproductive performance. Although biomarkers of energy state or liver function state can be used for the risk warning of ovarian diseases, these are all single indicators for the early warning of ovarian diseases. Liver function, inflammation and oxidative stress of perinatal cows all have a significant impact on the health of cows, but there is still a lack of analysis of these comprehensive indicators in the early detection of IO. To improve this, serum LFI, inflammation and oxidative stress indexes at 21 days postpartum were selected for analysis in this study and Spearman’s correlation analysis, binary logistic regression model fitting analysis and ROC analysis defined this as occurring when MDA exceeded 3.62 ng/L, LFI was less than –3.18, IL-6 exceeded 23.68 ng/L, FGF21 was less than 812.41 ng/L and ANGPTL8 was less than 695.79 ng/L. The sensitivity, specificity and AUC were 87.9%, 91.3% and 0.964, respectively, in the combined early warning analysis of LFI and MDA, and the sensitivity, specificity and AUC of the combined early warning analysis of IL-6, FGF21 and ANGPTL8 were 86.3%, 90.9% and 0.947, respectively. It therefore suggests that the combined early warning improves the specificity and value of diagnosis and has a better early warning effect. This study identified potential markers for IO in dairy cattle, but the scale was small. Similar research is needed in large-scale farms to improve the prediction accuracy of the early detection of dairy cow IO.

Conclusions
This study confirmed the association between postpartum LFI; cytokines IL-6, FGF21 and ANGPTL8; inflammation factors IL-6, HP and SAA; oxidative stress factors MDA, GSH-PX, GSH, T-AOC and RAHFR; energy metabolism indicators BHBA, NEFA and GLU; hormones such as growth hormone, oestradiol and progesterone; and dairy cow IO. The potential risk early warning indexes of LFI, MDA, IL-6, FGF21 and ANGPTL8 and their judgement thresholds were calculated, which has facilitated an early diagnosis of the diseases and control its progression.

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Ethical approval
According to the requirements of the Veterinary Medical Ethics Committee of the Ministry of Agriculture of China, this experiment was conducted in a large intensive cattle farm in the central region of Heilongjiang Province. All experiments on animals were conducted according to the standards approved by the Animal Welfare and Research Ethics Committee at Heilongjiang Bayi Agricultural University (No. 20200127).

Disclosure statement
The authors declare that they have no competing interests.

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Data availability statement
The raw data generated in this study can be obtained by reasonable request to the corresponding author.

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