

# Serum paraoxonase as an indicator for fatty liver in sheep

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## Abstract

**Introduction:** A model of fatty liver in postpartum sheep was established to measure blood paraoxonase 1 (PON1) and other biochemical indicators, which were used to predict fatty liver in sheep. **Material and Methods:** Sheep were assigned into two experimental groups: a fatty liver group (T, n = 10) and a healthy control group (C, n = 5). PON1 enzyme activity towards paraoxon as a substrate was quantified spectrophotometrically. The results were analysed by *t*-test and Pearson correlation coefficient. Disease was predicted by binary logistic analysis, and diagnostic thresholds were determined by receiver operating characteristic (ROC) analysis. **Results:** The activity of serum PON1 in group T was significantly decreased ( $P < 0.05$ ) when compared with C group, and liver lipid content and the levels of serum BHBA, NEFA, and TG were significantly increased ( $P < 0.05$ ). Thresholds were lower than 74.0 U/mL for PON1, higher than 0.97 mmol/L for  $\beta$ -hydroxybutyrate, higher than 1.29 mmol/L for non-esterified fatty acids, higher than 0.24 mmol/L for triglycerides, and lower than 71.35 g/L for total protein. **Conclusion:** This study verified that PON1, BHBA, NEFA, TG, and TP could be used to predict the risk of fatty liver in sheep.

**Keywords:** sheep, fatty liver, paraoxonase 1, biochemical indices.

## Introduction

Fatty liver is a nutritional and metabolic disease of ruminants that affects animal production, reproductive performance, and immune function, and can be fatal due to liver failure in severe perinatal cases (14). Many studies have examined fatty liver in dairy cows in China and in other countries, but there are few studies on fatty liver in perinatal sheep (15). With increasing intensification of ruminant farming, perinatal diseases are becoming increasingly prominent. Changes in body condition, energy metabolism, hormone secretion, and feeding behaviour in ruminants during the transition period lead to reduced dry matter intake, resulting in a state of negative energy balance, ketosis, and fatty liver, which can cause serious economic losses in animal husbandry.

The gold standard for diagnosis of fatty liver is still a liver biopsy in a living animal (7). As an

invasive method, it has several disadvantages. It is not suitable for early detection and clinical diagnosis. The hepatic biochemical index does not have a high sensitivity and specificity for the diagnosis of fatty liver. Early monitoring and diagnosis of fatty liver are difficult because of its long duration and slow development. Thus, there are technical problems for establishing practical rapid early monitoring and diagnosis of fatty liver to prevent and control the disease.

Paraoxonase 1 (PON1) is a calcium-dependent enzyme that is synthesised in the liver and secreted into the blood (17). PON1 is associated with high density lipoproteins (HDL) and shows antioxidant activity, hydrolysing and reducing lipid peroxides (5). Research has demonstrated that the activity of PON1 is specific for the diagnosis of fatty liver in dairy cows (11). Therefore, the aims of this study were to establish a model of fatty liver in postpartum sheep, and measure blood PON1 and other biochemical

indicators that could be used to predict fatty liver in sheep.

## Material and Methods

**Feeding and management.** In total, 15 pregnant multiparous small-tailed han sheep were used: 10 were assigned to the experimental group (T) and 5 into control group (C). Feed was administered twice a day at 09:00 and 17:00 for 16 days after calving. In group T, feeding was restricted after calving to 200 g forage (peanut straw) and 30 g concentrate (50% hominy, 29% sorghum, 10% soybean meal, 5% sunflower cake, 1% limestone powder, 1% salt, 1% disodium hydrogen phosphate, and 3% mixed additives) for 16 days. In group C, sheep were fed 1 kg forage and 150 g concentrate (50% hominy, 29% sorghum, 10% soybean meal, 5% sunflower cake, 1% limestone powder, 1% salt, 1% disodium hydrogen phosphate, and 3% mixed additives). Postpartum ewes were fed for three days in pens to allow them to nurse their lambs, and the ewes were free for at least 6 h/day of field activity. During the experimental period the ewes were free for nursing and had unlimited access to drinking water.

**Sample collection.** Blood samples (10 mL) were collected in both groups from the jugular vein on days 8 and 16 postpartum, and then centrifuged at 1400 g for 10 min at room temperature. The resulting supernatant was transferred to Eppendorf tubes (1 mL serum/tube) and stored at  $-80^{\circ}\text{C}$ . Liver biopsy was performed by rapid puncture (1 s) using a needle with 1 mm internal diameter with a stylet. The liver biopsy specimens were frozen immediately after collection in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for TG analysis.

**Biochemical parameter assays.** PON1 enzyme activity towards paraoxon as a substrate was quantified spectrophotometrically as described by Gan *et al.* (12). Serum concentration of PON1 was assayed using the PON1 ELISA (Haling Biological Technology, China). The following parameters were assayed: triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL),  $\beta$ -hydroxybutyrate (BHBA), non-esterified fatty acids (NEFAs), glucose (GLU), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), total protein (TP), albumin (ALB), globulin (GLO), alanine aminotransferase (ALT), aspartate aminotransferase (AST),  $\gamma$ -glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), cholesteryl ester (CHE), and lactate dehydrogenase (LDH). The blood biochemical parameters were detected using a kit (Roche Diagnostics, China) and a fully automatic biochemical analyser (Synchron DXC800; Beckman Coulter, USA). The content of liver lipid was detected by

colorimetric assay kit (Applygen Technologies, China).

**Data analysis.** Experimental data were analysed by *t*-test and Pearson correlation coefficient; fatty liver disease was predicted by binary logistic analysis; and diagnostic thresholds were determined by receiver operating characteristic (ROC) analysis. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR), and negative likelihood ratio (-LR) were calculated with the corresponding 95 % confidence intervals.

## Results

**Comparison of biochemical parameters.** Table 1 shows the biochemical parameters in healthy sheep and sheep with fatty liver. The activity and content of serum PON1, body weight, and the levels of LDL-cholesterol (LDL-C), TP, HDL-cholesterol (HDL-C) were significantly reduced ( $P < 0.05$ ), and the level of liver lipid, BHBA and NEFA significantly increased on day 8 postpartum in group T, compared with group C. In comparison with group C, body weight as well as the levels and activity of PON1, GLU, LDL, and HDL decreased significantly ( $P < 0.05$ ) on day 16 postpartum in sheep from group T. Liver fat content and the levels of AST, BHBA, NEFA, TG, ALB/GLO ratio (A/G), TBIL, and IBIL increased significantly ( $P < 0.05$ ) in group T.

**Correlation between PON1 and biochemical parameters in serum and liver.** The Pearson correlation coefficients of the regression lines between serum PON1 and other biochemical analytes on days 8 and 16 postpartum are presented in Table 2. On day 8 postpartum, the coefficients showed that PON1 concentration had a significant positive correlation with body weight, activity of PON1, LDL-C, and HDL-C concentration, and PON1 concentration was negatively correlated with the level of liver lipid and BHBA. PON1 activity was positively correlated with body weight, PON1, LDL-C, TP, and GLO concentration, and PON1 activity was negatively correlated with the level of liver lipid, AST, BHBA, and LDH. On day 16 postpartum, PON1 concentration had a significantly positive correlation with body weight, activity of PON1, and level of GLU, LDL-C, GLO, and HDL (Table 2). PON1 concentration was negatively correlated with liver lipid level, AST, NEFA, TBIL, IBIL, and LDH. PON1 activity was positively correlated with body weight, level of PON1, LDL-C, GLO, and TBIL; PON1 activity was negatively correlated with liver lipid level, AST, BHBA, NEFA, TG, A/G, DBIL, IBIL, and LDH.

**Table 1.** Mean ( $\pm$  standard error) and P values for blood biochemical parameters on postpartum days 8 and 16 in group C and T

Parameter	8 day postpartum		16 day postpartum	
	Group C	Group T	Group C	Group T
PON1 (nmol/L)	83.24 $\pm$ 9.74	68.83 $\pm$ 7.35*	76.79 $\pm$ 10.99	43.7 $\pm$ 3.62*
PON1 (U/mL)	91.94 $\pm$ 7.08	63.02 $\pm$ 15.06*	84.4 $\pm$ 4.1	43.60 $\pm$ 4.23*
Body weight (kg)	43.76 $\pm$ 1.31	37.68 $\pm$ 5.18*	41.62 $\pm$ 2.42	30.74 $\pm$ 4.67*
Liver lipid (%)	1.03 $\pm$ 0.19	6.31 $\pm$ 2.45*	1.22 $\pm$ 0.40	10.56 $\pm$ 3.63*
AST(U/L)	127.0 $\pm$ 19.58	151.0 $\pm$ 20.5	122.8 $\pm$ 14.67	219.22 $\pm$ 28.74*
BHBA (mmol/L)	0.78 $\pm$ 0.33	1.52 $\pm$ 0.37*	0.48 $\pm$ 0.28	1.63 $\pm$ 1.05*
GLU (mmol/L)	3.71 $\pm$ 0.26	3.27 $\pm$ 0.71	4.47 $\pm$ 0.94	3.47 $\pm$ 0.55*
NEFA (mmol/L)	0.91 $\pm$ 0.9	1.69 $\pm$ 0.43*	0.88 $\pm$ 0.62	2.27 $\pm$ 0.68*
T.CHOL( mmol/L)	2.49 $\pm$ 0.65	2.63 $\pm$ 0.67	2.24 $\pm$ 1.06	2.53 $\pm$ 0.59
TG (mmol/L)	0.25 $\pm$ 0.1	0.31 $\pm$ 0.09	0.16 $\pm$ 0.08	0.27 $\pm$ 0.08*
LDL-C (mmol/L)	1.25 $\pm$ 0.35	0.9 $\pm$ 0.27*	1.06 $\pm$ 0.15	0.68 $\pm$ 0.15*
GGT (U/L)	39.4 $\pm$ 11.84	55.2 $\pm$ 11.84	51.6 $\pm$ 17.33	39.4 $\pm$ 11.84
ALP (U/L)	19.3 $\pm$ 21.87	29.4 $\pm$ 28.06	42.2 $\pm$ 29.13	19.3 $\pm$ 21.87
TP (g/L)	75.74 $\pm$ 4.04	70.87 $\pm$ 3.12*	73.88 $\pm$ 5.38	68.46 $\pm$ 7.56
GLO (g/L)	39.08 $\pm$ 4.05	36.09 $\pm$ 4.55	40.42 $\pm$ 2.36	34.34 $\pm$ 6.25
A/G (g/L)	0.96 $\pm$ 0.21	1.05 $\pm$ 0.28	0.84 $\pm$ 0.1	1.07 $\pm$ 0.19*
TBIL ( $\mu$ mol/L)	3.68 $\pm$ 1.95	5.45 $\pm$ 1.94	2.54 $\pm$ 0.99	5.05 $\pm$ 2.05*
DBIL ( $\mu$ mol/L)	1.76 $\pm$ 0.59	2.32 $\pm$ 0.74	1.34 $\pm$ 0.46	1.96 $\pm$ 0.74
IBIL ( $\mu$ mol/L)	1.92 $\pm$ 14	3.13 $\pm$ 1.28	1.2 $\pm$ 0.79	3.09 $\pm$ 1.44*
HDL-C (mmol/L)	1.55 $\pm$ 0.29	1.2 $\pm$ 0.17*	1.32 $\pm$ 0.22	1.05 $\pm$ 0.16*
LDH (U/L)	508.6 $\pm$ 97.6	631.3 $\pm$ 180.8	496.0 $\pm$ 45.01	634.5 $\pm$ 142.33
ALT (U/L)	27.0 $\pm$ 7.11	22.9 $\pm$ 7.06	25.2 $\pm$ 6.46	24.0 $\pm$ 6.43
CHE (U/L)	812.0 $\pm$ 23.62	810.1 $\pm$ 22.5	800.0 $\pm$ 10.86	806.6 $\pm$ 14.39
ALB (g/L)	36.66 $\pm$ 4.94	35.48 $\pm$ 4.08	34.26 $\pm$ 3.57	35.12 $\pm$ 3.19

\* P < 0.05; PON1 – paraoxonase-1; AST – aspartate aminotransferase; BHBA – hydroxybutyric acid; GLU – glucose; NEFA – non-esterified fatty acids; T.CHOL – total cholesterol; TG – triglyceride; LDL-C – low-density lipoprotein cholesterol; GGT –  $\gamma$ -glutamyl transpeptidase; ALP – alkaline phosphatase; TP – total protein; GLO – globulin; A/G – albumin/globulin ratio; TBIL – total bilirubin; DBIL – direct bilirubin; IBIL – indirect bilirubin; HDL-C – high-density lipoprotein cholesterol; LDH – lactate dehydrogenase; ALT – alanine aminotransferase; CHE – cholesteryl ester; ALB – albumin

**Table 2.** Correlation between PON1 and biochemical parameters in plasma and liver of sheep

Parameter	8 day postpartum		16 day postpartum	
	PON1 content	PON1 activity	PON1 content	PON1 activity
PON1 content	1.0	0.581*	1.0	0.953**
PON1 activity	0.581*	1.0	0.953**	1.0
Body weight (kg)	0.724**	0.679*	0.864**	0.921**
Liver lipid (%)	-0.692*	-0.865**	-0.848**	-0.899**
AST	-0.199	-0.577*	-0.785**	-0.843**
BHBA	-0.62*	-0.784**	-0.529	-0.659*
GLU	0.106	0.247	0.653*	0.55
NEFA	-0.368	-0.43	-0.729**	-0.861**
T.CHOL	-0.079	0.034	0.105	-0.072
TG	-0.374	0.258	-0.488	-0.657*
LDL-C	0.579*	0.557*	0.784**	0.865**
GGT	-0.056	0.167	0.136	0.286
ALP	-0.218	0.03	0.437	0.399
TP	0.502	0.573*	0.477	0.383
GLO	0.29	0.573*	0.587*	0.593*
A/G	-0.177	-0.441	-0.529	-0.621*
TBIL	-0.439	0.115	-0.602*	0.755**
DBIL	-0.393	-0.449	-0.462	-0.671*
IBIL	-0.438	-0.166	-0.608*	-0.74**
HDL-C	0.583*	0.456	0.706**	0.711**
LDH	0.600	-0.565*	-0.623*	-0.642*
ALT	0.158	0.139	0.065	-0.048
CHE	-0.206	-0.174	-0.33	-0.36
ALB	-0.023	0.023	0.019	-0.159

**Table 3.** Cut-off point, sensitivity, specificity, NPV, PPV, +LR, -LR, and area under the ROC

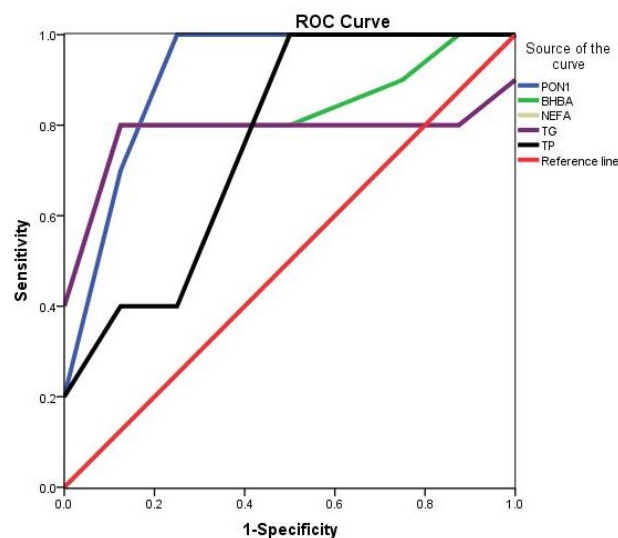
Parameter	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	-LR	AUC
PON1	74.0	80	80	89	67	4	0.25	0.925 P = 0.013
BHBA	0.97	100	80	91	100	5	0	0.85 P = 0.04
NEFA	1.29	90	80	90	80	4.5	0.125	0.8 P = 0.079
TG	0.24	90	80	90	80	4.5	0.125	0.8 P = 0.079
TP	71.35	60	100	100	56	-	0.4	0.775 P = 0.107

NPV – negative predictive value; PPV – positive predictive value; +LR – positive likelihood ratio; -LR – negative likelihood ratio; ROC – receiver operating characteristic; PON1 – paraoxonase-1; BHBA – hydroxybutyric acid; NEFA – non-esterified fatty acids; TG – triglyceride; TP – total protein

### Risk prediction and diagnosis of fatty liver.

SPSS version 19.0 was used to define three binary logistic regression models to simulate the above parameters in order to predict fatty liver in sheep. Model I consisted of eight parameters of liver enzymes, including PON1 activity, PON1 concentration, AST, ALP, CHE, ALT, LDH, and GGT. Model II had seven parameters of lipid metabolism, including BHBA, GLU, NEFA, TG, TC, LDL-C, and HDL-C. Model III was composed of seven parameters of liver function, including TP, ALB, GLO, A/G, TBIL, DBIL, and IBIL.

NEFA, TG, and TP was 100%. The specificity of PON-1, BHBA, NEFA, TG, and TP was 87.5%, 80%, 80%, 80% and 62.5 %, respectively. The area under the curve of PON-1, BHBA, NEFA, TG, and TP was 0.925, 0.85, 0.8, 0.8, and 0.775, respectively. These results indicate that the PON1 activity, concentration of BHBA, NEFA, TG, and TP can predict fatty liver in sheep. The activity of PON1 can be used as an index of liver fat deposition. The concentration of BHBA, NEFA, and TG can be used as a lipid mobilisation index. TP can be used as an indicator of liver function. PON1 was the most sensitive to hepatic lipid deposition, followed by BHBA, NEFA, TG, and TP.



**Fig. 1.** Receiver operating characteristic (ROC) plots for PON1 activity, BHBA, NEFA, TG and TP for diagnosis of ketosis in sheep (PON1: paraoxonase-1; BHBA: hydroxybutyric acid; NEFA: non-esterified fatty acids; TG: triglyceride; TP: total protein). The NEFA line and the TG line coincide together, and part of the BHBA line coincides with the TG line

Table 3 and Fig. 1 present the sensitivity, specificity, NPV, PPV, +LR, and -LR which were calculated at the corresponding 95% confidence intervals for early warning of fatty liver in sheep. The values were determined by Youden index and showed PON1 activity <74 U/ml, BHBA>0.97 mmol/L, NEFA >1.29 mmol/L, TG >0.24 mmol/L, and TP <71.35 g/L. The sensitivity of PON-1, BHBA,

### Discussion

The liver is important for blood glucose metabolism because it is the predominant site of gluconeogenesis in the organism (3). Currently, the gold standard for diagnosis of fatty liver is liver biopsy (7), which can cause serious injury and whose application is not worthwhile in clinical practice. Therefore, it is important to find a new method for early monitoring and diagnosis of fatty liver. This study indicated that serum PON1 activity was negatively correlated with the degree of liver lipid deposition in sheep with fatty liver. The same observation was reported by Farid *et al.* (11). Two mechanisms may explain this decrease in serum PON1 activity. First, the intake of energy and glucose in postpartum sheep is mostly used for the synthesis of milk fat to meet the demands of lactation, and there is little direct absorption of glucose and galactose by the ruminant intestinal tract. Moreover, restricted feeding and other factors reducing sources of propionate in gluconeogenesis do not meet the energy needs of the organism, resulting in negative energy balance and mobilisation of body lipids (21). At the same time, concentrations of BHBA, NEFA, and liver lipid increase, which intensifies the risk of ketosis and fatty liver (9). Large amounts of fatty acids accumulated through body fat mobilisation produce a great number of reactive oxygen species (ROS) in the liver

mitochondria and endoplasmic reticulum during  $\beta$ -oxidation (25). It is reported that the ketone body, acetoacetate, can generate superoxide radicals that can then form hydroxyl radicals (16). The various kinds of antioxidative factors that take part in antioxidant responses lead to oxidation and anti-oxidation in a dynamic equilibrium in which ROS are maintained within a certain range (13). Markers of oxidative stress are increased in cows with subclinical ketosis (22) and in hyperketonaemic compared with normoketonaemic people with type 1 diabetes (16). Excess ROS and free radical depletion of antioxidant factors lead to the oxidative stress state with disorder of the dynamic balance between oxides and antioxidants (8). Lipid peroxides can create many intermediates exerting cytotoxicity, which in turn may lead to necrosis and death of liver cells, and inflammation or inflammatory cell infiltration in the liver parenchyma (1). This leads to observed reduction of PON1 activity when fatty liver occurs. Similarly, our study confirmed that the activity and concentration of PON1 was visibly reduced when fatty liver occurred. This may be related to oxidative stress that is inversely proportional to PON1 activity (4). NEFAs are hepatotoxic and can enhance toxicity of cytokines, such as the tumour necrosis factor, mitochondrial swelling and permeability, liver cell degeneration, necrosis, and apoptosis (19). Table 2 shows that NEFA concentration and activity of PON1 were significantly negatively correlated ( $P < 0.01$ ). This further proves the relationship between PON1 and NEFA. In contrast, some liver NEFAs are re-esterified to TG, phospholipid, and cholesteryl ester, and together with cholesterol, phospholipid, and apolipoprotein binding, they form VLDL that is transported into the blood (6). VLDL in the blood is exchanged with HDL-C, hydrolyses a lot of TG, and is converted to LDL-C through further metabolic action (10). The liver is the main area of lipoprotein synthesis; fat deposition can cause liver damage in sheep, and excessive unsaturated fatty acids in liver peroxidation can damage liver cells (8), resulting in significantly decreased blood lipoprotein. PON1 that is transferred from the liver into blood is mainly used as an important component of HDL-C and LDL-C in antioxidation (24). The N-domain of PON1 combines with apolipoprotein A1 of HDL-C, and the interaction of PON1 and APOA1 helps to maintain the activity of PON1. PON1 is an antioxidant enzyme which is involved in peroxide hydrolysis similarly to other antioxidant enzymes in the body. In our study PON1 activity decreased gradually, which in turn reduced direct participation in the hydrolysis of lipid peroxide in blood. Peroxide causes oxidative modification of the structure and function of LDL-C and HDL-C, which further affects the activity of PON1 in blood. Table 2 shows that there was a significant positive correlation between the levels of both HDL and LDL and the activity and concentration of PON1 on postpartum days 8 and 16.

PON1 activity and concentration were significantly negatively correlated with liver lipid content on days 8 and 16 postpartum, indicating reduction of hepatic synthesis of PON1 with liver fat deposition increase, and serum PON1 decrease. At the same time, the activity of AST was significantly increased, and there was a significant negative correlation between PON1 and AST in this study. AST is present in the cytoplasm or mitochondria of many organs, especially the liver, heart, and skeletal muscles, showing the highest activity in the liver, myocardium, and skeletal muscles. When fatty liver occurs, cell membrane permeability is increased, AST is released into the cytoplasm, and the activity of the enzyme is increased in blood. Therefore, AST is used as a marker of hepatic lipid deposition in the diagnosis of fatty liver (23). An increase in bilirubin concentration in serum is also positively correlated with liver injury. Liver lipid content is significantly correlated with TBIL concentration (20). In the present study, IBIL level decreased significantly on day 16 postpartum, indicating some degree of liver damage. IBIL is not soluble in water, but is transported at a ratio of 1:1 with ALB in blood. Under normal conditions, the ALB content is higher than the content of IBIL, and the combination of ALB with bilirubin is strong, which could prevent the bilirubin from passing through the cell membrane and inducing cytotoxicity. When the IBIL concentration exceeds that of ALB in the blood, damage to peripheral tissues may occur through the blood-brain barrier and liver sinusoids (18). In the present study, blood level of IBIL was lower than that of ALB, showing that an increase in IBIL did not cause damage to the peripheral tissues, but it did damage liver function (2).

Fatty liver is a nutritional and metabolic disease that is harmful to the health and production performance of animals. Therefore, early diagnosis of fatty liver is necessary for effective prevention and control. In our study, dualistic regression analysis and receiver operating characteristics were used to establish an early warning model of fatty liver in sheep: PON1 activity should not be less than 74.00 U/ml, BHBA should not be higher than 0.97 mmol/L, NEFA should not be higher than 1.29 mmol/L, TG should not be higher than 0.24 mmol/L, and TP should not be higher than 71.35 g/L. If there is one or more indicators above or below the standards, it indicates that the sheep have an increased risk of fatty liver. At this time, immediate access to diet, body condition, and other feeding behaviour status are essential to ensure adequate energy supply.

In summary, this study verified that activity of PON1, and levels of BHBA, NEFA, TG, and TP could be used to predict the risk of fatty liver in sheep.

**Conflict of Interests Statement:** The authors declare that there is no conflict of interests regarding the publication of this article.

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