From the

Large Animal Clinic for Internal Medicine

Faculty of Veterinary Medicine

University of Leipzig

Relationship Between Metabolic Parameters and TNFα in the Peripartal Period in Ewes

Inaugural-Dissertation

to obtain the degree of a

Doctor medicinae veterinariae (Dr. med. vet.)

from the Faculty of Veterinary Medicine

University of Leipzig

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from Cairo, Egypt

Leipzig, 2011

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Day of the defence: 07.06.2011

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List of abbreviations

a.p.	after parturition
AUC	Area under curve
b.p.	before parturition
BHB	β-hydroxybutyrate
BUN	Blood urea nitrogen
CK	Creatin kinase
DGA	Days gestational age
DGKC	Deutsche Gesellschaft für Klinische Chemie
EC	Erythrocyte Count
EDTA	Ethylenediamine tetraacetic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related kinase
Fe	Iron
FFA	Free fatty acids
GGT	Gamma-glutamyl-transferase
GH	Growth hormone
GIR	Glucose Infusion Rate
GLDH	Glutamat-dehydrognase
GM-CSF	Granulocyte macrophage-colony-stimulating factor
GnRH	Gonadotropin Releasing hormone
GST	Glutathione-S-transferase
Hb	Haemoglobin concentration
HK	Haematocrite
IL	Interleukin
IVGTT	Intravenous glucose tolerance test
LC	Leukocyte Count
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular haemoglobin

MCHC	Mean corpuscular haemoglobin concentration
MCP-1	Monocyte Chemotactic Protein 1
MCV	Mean corpuscular volume
MPII	Mean Plasma Insulin Increment
NPNL	Non-pregnant, non-lactating
OPL	Ovine placental lactogen
OvTNFα	Ovine tumour necrosis factor-alpha
PE	Pre-eclampsia
PKA	Protein kinase A
PT	Pregnancy toxaemia
rhTNFα	Recombinant human TNFα
rOvTNFα	Recombinant Ov-TNFα
THC	Thrombocyte count
TNFα	Tumor necrosis factor alpha
UN	Under nutrition
VCAM	Vascular adhesion molecule

1 Introduction

Pregnancy toxaemia (ketosis) is a metabolic disorder of pregnant ewes which occurs during the late gestation. This happens as a result of the inability of the pregnant ewe to maintain an adequate energy balance for the fast growing maternal fetal unit (MARTENIUK and HERDT 1988).

The disease is characterized by hyperketonaemia, hypoglycaemia, insulin resistance, and hyperlipidaemia (MYATT and MIODOVNIK 1999). Carrying multiple fetuses or a single fetus of a larger size predisposes ewes to pregnancy toxaemia (ANDREWS et al. 1996). The disease is associated with a higher rate of mortality and cause severe economic losses (ROBERT et al. 1989).

The requirements for glucose considerably increase in the pregnant animal fetus, and uterus utilizes glucose as a major energy source (LINDSAY 1973). Energy deficient mobilization of lipid reserves results in a doubling of the plasma FFA, giving rise to fatty liver and increased ketone bodies in blood and urine (CHAIYABUTER et al. 1982).

Also in the late gestation period of clinically-healthy sheep, there is increased secretion of ketone bodies in urine. There is also a higher increased concentration of BHB in blood (FÜRLL et al. 1998).
Several studies have focused on factors associated with the marked metabolic changes occurring in late pregnancy, such as hypoglycaemia and hyperketonaemia. Recent studies (CONNOLLY et al. 2004) have indicated that the period of late pregnancy is probably accompanied by distinct hormonal changes that modify metabolic responses and change homeostasis adaptations of the whole body.

Some studies have shown that hyperketonaemia significantly depresses hepatic glucose production (SCHLUMBOHM and HARMEYER 2004), and can probably no

longer be regarded as a secondary disease sign. Instead, it appears that hyperketonaemia plays an essential role in the pathogenesis of disease.

In late gestation of multiparous ewes, concentrations of ketone bodies in blood are often higher than they are during early lactation. Aside from hyperketonaemia, elevated concentration of FFA in plasma and hypoglycaemia represent typical features of pregnancy toxaemia (BICKHARDT et al. 1989, HENZE et al. 1994, HENZE et al. 1998).

A key role for plasma cytokines has been hypothesized in pathogenesis of pregnancy toxaemia in sheep because a general inflammatory-response occurs with activation of leukocytes and elevation of cytokine production, along with abnormal activation of the clotting system in pregnancy toxaemia (CONRAD et al. 1998 and SACKS et al. 1999).

Uteroplacental hypoxia in pregnancy toxaemia induces local expression and abnormal production of several inflammatory cytokines, including IL-1ß and TNFα in the placenta (REDMAN and SARGENT 2005). Peripheral blood mononuclear cells exposed to hypoxia also produce IL-1α and TNFα (BENYO et al. 1997). Inflammatory and immunoregulatory cytokines in pregnancy toxaemia may originate from various sources, including peripheral mononuclear cells and fatal tissues, such as fetal villous tissues (DUDLY et al. 1996). Cytokines-including IL-1β, TNFα and MCP-1 are associated with immune responses and implicated in the pathogenesis of pregnancy toxaemia. This is because of their roles in endothelial damage (AKOUM et al. 1995, CONRAD and BENYO 1997, CONRAD et al. 1998, REDMAN and SARGENT 2005, YARIM 2007).

The objective of the present study was to investigate the different metabolic parameters in apparently healthy pregnant ewes in late gestation, and to characterize the metabolic changes during this period. This was in order to check the interaction between the TNFα and fat metabolism, and to analyse TNFα concentration, which may serve as markers to early diagnosis of pregnancy toxaemia in sheep.

2 Review of literature

2.1 Metabolic condition of ewes in the late pregnancy

In late-pregnant sheep, energy and nitrogen demands of the rapidly growing fetus are mostly met by placental intake of glucose and amino acids. The resulting increase in maternal requirements for these nutrients is partly met by increased voluntary feed intake and partly by a series of metabolic adaptations. These include increased hepatic gluconeogenesis from endogenous substrates (STEEL and LENG 1973, WILSON et al. 1983), and mobilization of FFA from adipose tissues (VERNON et al. 1981). Development of gestational insulin resistance appears to be a key factor in these adaptations, whereby glucose utilization is consequently reduced in maternal non-uterine tissues (ODDY et al. 1985, HAY 1988, PETTERSON et al. 1993).

Adipose tissue insulin responsiveness has also been reported to be reduced during late gestation as a result of down-regulation of insulin binding. In comparison, post-receptor sensitivity/signalling of biolysis and FFA mobilization are apparently unaffected (PETTERSON et al. 1994). An adaptive increase in insulin secretion is normally observed in the mid to late pregnant sheep and rats (LETURQUE et al. 1980, VERNON et al. 1981, OSGERBY et al. 2002, BONE and TAYLOR 1976), which can be due to an increased pancreatic β-cell proliferation (BONE and TAYLOR 1976, NIEUWENHUIZEN et al. 1997).

BLONDEAU et al. (1999) have demonstrated that the ability to increase insulin secretion during mid to late pregnancy (necessitated by the peripheral insulin resistance) can be impaired in four and eight months old female rats. If the rats previously have been exposed to UN in early pre and postnatal life. This group has further shown that β-cell proliferation in the head of the pancreas was decreased in the pregnant 8 month-old rats exposed to early life UN, which explains the absence of an insulin secretion up-regulation in mid to late pregnancy (AVRIL et al. 2002). It is, therefore, reasonable to assume that malnutrition during very early life may have long-lasting negative consequences for the capacity of the endocrine pancreas to cope with physiological conditions, where the glucose-insulin homeostasis is challenged.

2.2 Metabolic disorders subacute and acute pregnancy Toxaemia (Ovine Ketosis)

GAAL and FEKETE (1982) described two outbreaks of ketosis: The first lead to the deaths of 118 seemingly healthy ewes in a single flock of 760 animals that were pregnant with twins. They were four-five years old, mostly 10-14 days before expected parturition in the November and December. The second caused symptoms in eight ewes and death in four ewes (all pregnant with twin), three weeks before parturition in mid-March. They were among a smallholding flock of 30 animals. Until two to three weeks before lambing the large flock that had been kept in crowded pens with no exercise, they were fed rations that had a 30% excess in energy and 9% deficiency in protein; thereafter the rations were 13-15% deficient in energy and 60-65% deficient in protein. During the last quarter of pregnancy, the small flock, while adequately housed and exercised, had received rations that were 35% deficient in dry matter and 27% deficient in energy but with a 53% excess in protein content. In both flocks, the only pathological finding in affected animals was fatty degeneration of peripheral hepatic lobules. Blood and urine biochemical values were measured in selected symptomatic and asymptomatic animals. The most significant changes (raised liver enzymes, lowered glucose and total cholesterol and urinary pH) occurred in the large flock.

BICKHARDT (1988) recorded that ketosis in sheep is briefly summarized, and that differences between healthy and affected sheep (such as decreased feed intake, impaired rumen function, ataxia, increased heart-rate, increased plasma bilirubin and decreased plasma potassium) are described. Pathogenesis of sheep ketosis is unclear, but the main treatment goals are stimulation of feed intake, restoration of rumen function, and daily treatment with a mineral mix enriched with calcium lactate and potassium chloride. Mortality of ketosis was decreased to 34% by these measures applied over the previous five years, while five years before that, application of an intravenous glucose infusion decreased mortality to 44%.

SINGH et al. (1992) induced pregnancy toxaemia in 16 sheep in the third trimester of pregnancy by protamine zinc insulin along with semistarvation. The clinical symptoms included the following: anorexia, dullness, depression, weakness, drowsiness, stiffness of body, nervous symptoms, partial blindness, and the odor of ketones on the breath.

At the terminal stages, animals developed partial or complete blindness, convulsion, dyspnoea, lateral recumbency and fatal coma. The total period of illness extended from three to nine days. A significant decrease was observed in blood glucose, total serum protein, and albumin. A significant increase developed in the levels of ketone bodies, total volatile fatty acids, blood urea nitrogen creatinine, and cholesterol.

HENZE et al. (1994) studied the concentrations of hormones, metabolites and electrolytes in the plasma of 45 ewes which were repeatedly examined during gestation (day 80 to 149), and on the third day of lactation. Healthy single pregnant (n=8) and twin pregnant ewes (n=12) were compared with ewes with subclinical (BHB > 1.0 mmol/l, n=6), or clinical (BHB>1.6 mmol/l, n=19) ketosis. The concentrations of BHB, glucose, calcium, sodium, and growth hormone in the plasma of the healthy animals were higher postpartum than antepartum, while the concentrations of inorganic phosphorous, cortisol, and total oestrogen were lower postpartum than they had been during pregnancy. In the pregnant ewes, the concentrations of BHB, bilirubin, total oestrogen and growth hormone in the plasma increased, while the concentration of insulin decreased with increasing numbers of fetuses. In comparison with the healthy twin pregnant ewes, the animals with subclinical and clinical pregnancy ketosis showed an increase in values of bilirubin, glutamate dehydrogenase, somatotropin, total oestrogen, cortisol, and cortisol/insulin ratios, as well as decreased values of glucose, insulin, and potassium. Individual differences between animals within their groups were observed for the examined hormones as well as for glucose, calcium, sodium and potassium (analysis of variance). Aside from multiple pregnancy low insulin values, and high cortisol/insulin ratios (due to individual disposition), enhanced ketogenesis and disorder of glucostasis.

HENZE et al. (1998) studied clinical signs and selected blood chemical parameters for 214 ewes with pregnancy toxaemia (ketosis). 134 animals survived and 80 animals died. The animals that died were affected earlier in pregnancy (143±7 days) and the duration of disease was shorter (10±13 days) compared to animals that survived (146±8 and 14±9 days, respectively). The animals that died had more severe clinical signs and higher values of BHB (4.3±3.6 vs. 3.5±2.6 mmol/litre) and cortisol (72±98

vs. 52±80 nmol/l). They also had lower values of insulin (37±12 vs. 66±42 pmol/l) and potassium (4.1±1.0 vs. 4.4±1.0 mmol/litre) at the onset of disease, than did those which survived. Glucose levels did not differ between the two groups. Survival rates were 53.6% for 56 animals treated with glucose and fructose infusions, 62.7% for 126 animals treated orally with glucose precursors and electrolytes, and 86.7% for 15 animals treated orally with glucose precursors and electrolytes and s.c. with insulin. It is concluded that insulin deficiency plays an important role in the pathogenesis of ketosis in sheep.

LACETERA et al. (2001) determined the effects of moderate feed restriction, single or twin pregnancy, and subclinical pregnancy toxaemia (PT) on immune responses of ewes. 16 Sardinian ewes were used. Six weeks before lambing, ewes were assigned to 1 of 2 groups (n = 8/group) matched for number of fetuses, body condition score, and plasma glucose, FFA, and BHB concentrations. Feed intake was restricted for one of the groups. Cell-mediated immunity was evaluated in vivo and in vitro. Humoral immunity was evaluated in vivo by determining production of IgG antibodies against keyhole limpet hemocyanin (KLH). Four ewes developed subclinical PT (plasma BHB concentration > 0.86 mmol/L without any clinical signs of disease). Whether feed was restricted and type of pregnancy (single vs twin) did not have any significant effects on cell-mediated and humoral immune responses. Ewes with subclinical PT had significantly lower proliferation in vitro of phytohemagglutinin-stimulated peripheral blood mononuclear cells and significantly lower values for KLH-specific IgG than did healthy ewes. Plasma BHB and FFA concentrations were negatively correlated with in vitro proliferation of peripheral blood mononuclear cells; plasma FFA concentration was negatively correlated with values for KLH-specific IgG. Results suggest that subclinical PT may be associated with impairments in cell-mediated and humoral immune responses in sheep.

ROUBIES et al. (2003) investigated the incidence of subclinical ovine ketosis was performed in two large sheep flocks in an agricultural region of central Greece. 64 animals were allocated in two study groups: group C consisted of 29 non-pregnant females and group S of 35 end-term pregnant ewes. The trial lasted six weeks, including

the immediate pre- and postpartal period. Blood samples were collected on weekly intervals and used for determining the concentrations of BHB, acetoacetate [acetoacetic acid] (AcAc), glucose, total (TB) and conjugated (CB) bilirubin, calcium (Ca), inorganic phosphorus (P), sodium (Na), potassium (K), magnesium (Mg), and the activity of sorbitol dehydrogenase [L-iditol dehydrogenase] (SDH), and gamma-glutamyl transferase (gamma GT). All the animals remained clinically healthy throughout the trial and parturition was uneventful. 18 ewes of group S (51.4%) delivered one lamb, 12 (34.3%) twins, 4 (11.4%) triplets and 1 (2.9%) 4 lambs. Although the overall evaluation of the biochemical analysis did not support the existence of subclinical ketosis, blood glucose remained significantly lower ($p<0.05$) in group S sheep, with its lowest value (2.3±0.34 mmol/litre) recorded 1 week prior to parturition. Sporadic cases of subclinical hyperketonaemia may occur, despite optimal flock management.

KOLB and KASKOUS (2004) mentioned that pregnancy ketosis develops in sheep and goats with multiple pregnancies when there is an insufficient intake of metabolisable energy and low resorption of glucoplastic compounds, such as amino acids and propionate, during the last weeks of pregnancy. Prevention requires the administration of approximately 60 g propionate or propylene glycol for every animal per day, via the feed. Indicators of the severity of the disease are the concentration of ketone bodies, and a decrease in the concentration of insulin in the blood plasma. The reactions at the development of the disease and recommendations for treatment are included.

EL-DIN et al. (2005) determined the clinical and biochemical changes in pregnant toxaemic native ewes from Sharkia governorate to help diagnose the disease during its early stages. The ewes were divided into three groups according to clinical findings: Group one, moderately affected (n=20); group two, severely affected (n=20); and Group 3, control (n=10). Two blood samples were taken from each of the healthy control and the diseased animals. The first blood samples were mixed with sodium salt of EDTA for haematological studies. Total red blood cell count, total white blood cell count, haemoglobin concentrations and packed cell volume were significantly higher ($p\leq0.01$) in groups one and two compared to the healthy control. The other blood samples were collected in clean centrifuge tubes, in which serum was separated for bio-

chemical examinations. Total ketone bodies, alanine aminotransferase, alkaline phosphatase and free fatty acids were significantly higher in groups one and two compared to the control group. However, their glucose, calcium, total protein, albumin, globulin, total cholesterol and triglycerides were significantly lower. Urine examination indicated the presence of ketone bodies, glucose, and protein in pregnant toxaemic ewes. It is concluded that high concentrations of ketone bodies in the blood and urine, in addition to hypoglycaemia are markers for pregnancy toxaemia in ewes and early interference is recommended to save the animal's life.

CAL et al. (2006) evaluated the metabolic disorders related to pregnancy toxaemia in ewes under experimental starvation. 14 Corriedale ewes aged from four to six years were synchronized. Mating was considered as day 0 of gestation. At the 100th day of gestation, which was ultrasonographically confirmed, the ewes were randomly divided into two groups. The first and second groups were fed in natural and artificial grasslands, respectively. At 142 days of gestation, half of each group was starved for 48 hours. Glycaemia, uraemia, cortisol, ketonuria and body weight were recorded. Starvation during late gestation was not severe enough to produce metabolic alterations considered as characteristic of pregnancy toxaemia. Ewes with better energy storage underwent a lower risk of developing the condition. Among the studied variables, urinary ketones were the earliest indicators of starvation-induced metabolic changes.

SCHLUMBOHM and HARMEYER (2008) studied six ewes they were sequentially subjected to two types of hypoglycaemic stress, firstly by fasting for 14 hours and secondly through induction of moderate hyperketonaemia. Glucose kinetics was assessed in each animal during the dry non-gestational period, during late gestation, and during early lactation. Application of these stress factors was associated with variation of plasma glucose concentrations from 4.9 to 0.87 mmol/l. The plasma glucose concentration was always directly related to the glucose production rate. The greatest stress-induced reductions in glucose concentrations and glucose production rate were seen during late gestation in twin-bearing ewes. The decline in the glucose production rate after an overnight fast and during induced hyperketonaemia was greater in twin-bearing ewes than in single-bearing ewes (59% and 43%, respectively, $p<0.05$). The

stress conditions resulted in the lowest levels of glucose concentration and glucose turnover rates in the stressed, hyperketonaemic, and late gestation twin-bearing ewes. This illustrates that the glucose homoeostatic system of ewes bearing twins is significantly more susceptible to hypoglycaemic stress than that of ewes bearing single lambs. These findings also show that the primary cause of hypoglycaemia in late gestation twin-pregnant ewes is an increased susceptibility to a stress related reduction in glucose production rate. This metabolic condition leaves the twin-pregnant ewe predisposed for the development of pregnancy toxaemia.

AL-MUJALLI et al. (2008) investigated the pregnancy toxaemia in ewes presented at the Veterinary Teaching Hospital, King Faisal University, Saudi Arabia between 1992 and 2003. 27 ewes presented in the clinic in 2004 with the suspicious of pregnancy toxaemia were subjected to clinical and laboratory examinations. Blood samples for glucose level determination were taken from six ewes proved to have pregnancy toxaemia. Blood samples were collected from six healthy nonpregnant and six healthy pregnant ewes as control. The incidence of pregnancy toxaemia in 12 years ranged between 6.5-37%. The highest incidence was reported in 1998 (37%), while the lowest incidence was reported in 2003 (6.5%). The clinical manifestations showed by the six animals affected with toxaemia were mainly nervous manifestations. The level of blood glucose in ewes with pregnancy toxaemia was 1.02±0.14 mmol/l, while in healthy nonpregnant and pregnant animals were 2.95±0.65 mmol/l and 2.7±0.14 mmol/l, respectively. It is concluded that the incidence of the disease between 1992 and 2003 is low to moderate, and the disease has a significant effect on blood glucose level.

GUPTA et al. (2008) studied haemogram in subclinical ketosis in 514 goats and 76 sheep in an organized farming system. In normal physiological states TEC, Hb and PCV showed a decrease in values during pregnancy and lactation states. This indicates anemia in the last month of pregnancy. TLC levels were increased in both pregnancy and lactation as compared to dry period. TEC, Hb, PCV and MCV did not show any significant changes in sub-clinical ketotic cases in either species of animals under investigation compared to healthy group animals. TLC values also showed non-

significant changes in ketotic goats and sheep during pregnancy and lactation, though a mild-leucopenia was recorded. The study indicated that haemogram did not significantly change in sub-clinical ketosis in sheep and goats.

2.3 The importance and dynamic of the different biochemical parameters: glucose, insulin, free fatty acids, β-hydroxybutyrate of ewes in the late pregnancy

2.3.1 Glucose

BICKHARDT et al. (1989) determined glucose metabolism in 17 healthy ewes during different metabolic stages and in eight spontaneously ketotic sheep by the IVGTT with low doses of glucose (0.8 mmol/kg). The endogenous turnover rate (R) was calculated by using the total clearance (Cl), derived from the IVGTT. The values of R, determined in healthy ewes by this technique are in agreement with values presented in the literature, which were derived from kinetic studies with 3H- or 14C-labeled glucose. There were no differences in the mean values of R and Cl in non-pregnant, highly pregnant and ketotic ewes. The plasma concentration of glucose (C) was directly correlated with R in highly pregnant healthy and ketotic ewes; there was no correlation in the group of non-pregnant and early pregnant sheep. In highly pregnant ketotic ewes the mean value of C was lower than in healthy ewes but there was no significant correlation between the plasma concentration of glucose and that of BHB. It is concluded from the study, that the control of glucostasis fails frequently during late pregnancy in ewes and that ketosis of sheep is related closer to the excessive lipid mobilization than to disturbance of glucostasis.

PETTERSON et al. (1993) measured the whole-body kinetics of glucose metabolism in nonpregnant and late-pregnant ditocous ewes which were fed either to maintain zero energy balance in maternal tissues (fed) or at 50% of this level (underfed) for several weeks. Whole-body kinetics of glucose metabolism was measured under basal conditions, and the hyperinsulinemic, euglycemic clamp technique was used to define insulin-dose response profiles for several indices of whole-body glucose utilization, and for endogenous glucose production. Pregnancy increased and undernutrition decreased basal glucose entry rate (GER), glucose metabolic clearance rate (GMCR) and insulin-independent glucose utilization (IIGU). The consistent increment in IIGU of pregnant

over nonpregnant ewes was comparable to previous estimates of uterine glucose uptake. Pregnancy resulted in higher plasma concentration for 50% maximal responses (ED50) to insulin of GER, GMCR, and steady-state glucose infusion rate (SSGIR) to maintain euglycemia and insulin-dependent glucose utilization (IDGU). These changes were especially large in underfed pregnant ewes. Effects on the maximal response to insulin of these variables (Rmax) were relatively small (GMCR, IDGU) and nonsignificant (GER, SSGIR). Maximum insulin-induced suppression of endogenous glucose production was significantly lower due to undernutrition; neither Rmax nor ED50 for this response was affected by pregnancy. Insulin resistance in late-pregnant ewes is primarily due to decreased insulin sensitivity in (presumably) peripheral tissues, implying an alteration of receptor function or of early postreceptor signal transduction.

SCOTT et al. (1995) determined cerebrospinal fluid (CSF) and plasma glucose concentrations of spontaneous ovine pregnancy toxaemia cases within 24 hours of the onset of clinical signs, were significantly lower ($p<0.01$) compared to inappetent ewes and healthy ewes at a similar stage of gestation. When the data from the three groups were combined a highly significant correlation ($p<0.001$, $r=0.91$) was present between the plasma and CSF glucose concentrations. The data suggest that plasma glucose concentration accurately reflects CSF glucose concentration across the range of energy states in pregnant ewes and may provide support for the postulate that the neurological signs of ovine pregnancy toxaemia result from cerebral hypoglycaemia, as reflected by CSF glucose concentration. No cerebral glucose estimations were undertaken in this series.

EHRHARDT and BELL (1997) analysed glucose transport concentration in six twin pregnant ewes on days 75, 110, and 140 postcoitus the glucose transporter (GT) concentration (pmol/mg protein) of D-glucose-inhibitable binding sites, measured by [3H]cytochalasin B binding analysis, increased 3.4 times from mid- to late pregnancy. Concurrently, an abundance of GLUT-1 and GLUT-3 protein, measured by immuno blotting with specific polyclonal antibodies, increased 2.3 and 2.9 times, respectively, while abundance of GLUT-1 and GLUT-3 mRNA, measured by Northern blotting, increased 1.8 and 3.9 times, respectively. GLUT-4 protein was undetectable at all

stages of pregnancy. Quantitative immuno blotting indicated that GLUT-1 accounted for 86.8±1.6% at day 75 and 56.1±4.1% on day 140 of total cytochalasin B binding sites. Thus increases in GT concentration explain much of the gestational increase in glucose transfer capacity observed in vivo. The gestational decline in relative contribution of GLUT-1 to cytochalasin binding, together with the greater developmental increases in GLUT-3 mRNA and protein, further suggests that the relative importance of GLUT-3 increases with gestational age.

SCHLUMBOHM et al. (1997) investigated glucose turnover in nine ewes during three reproductive states in order to examine their importance for development of subclinical ketosis. The increase of insulin in plasma was measured after a continuous 60 min intravenous infusion of glucose (4.9 mmol. min^{-1}). Turnover of glucose and insulin clearance were quantified during a combined euglycemic, hyperinsulinemic clamp. Insulin was consecutively infused in doses of 5 and 10 mU. kg-1. Min-1 for about 2.5 hours, each. Plasma glucose concentration was adjusted to 5.3 to 5.8 mmol/l. The indicators of glucose and lipid metabolism which were examined in this study showed marked individual variation, particularly during late pregnancy. The marked changes of these indicators with reproductive stages as well as their great variation between individual sheep support the assumption that they are of significance for the development of pregnancy toxaemia in sheep.

2.3.2 Insulin

The endocrine system, especially the pancreas is directly involved in the development of ruminant ketosis. This review considers the role of the major pancreatic hormone insulin, in the pathogenesis of ruminant ketosis. However, since key information in ruminant animals is often lacking references to more abundant studies in nonruminant animals are used. Extrapolations from findings in diabetes mellitus are taken to fill gaps in our understanding of the roles of pancreatic hormones in the pathogenesis of ruminant ketosis. This is done, not because of the similarity in pathogenesis of these conditions, but rather because the alloxan diabetic animal serves as a model to study insulin. Insulin has long been associated with the ketoacidosis of diabetes mellitus. It is known to influence a) the mobilization of FFA from adipose tissue and b) utilization

of keton bodies by peripheral tissues. The lipogenic effect of insulin is well-recognized (BROCKMAN 1978). A fall in plasma insulin concentration triggers lipolysis in adipose tissue and elevates plasma FFA concentration. However, lipid mobilization is not a major ketogenic stimulus without a contribution from the liver (MAYES and FELTS 1967) Insulin inhibits ketogenesis when FFA levels remain high (BIEBERDORF et al. 1970). During starvation and/or ketosis, hepatic removal of FFA is increased (KATZ and BERGMAN 1969) and a greater proportion of FFA is converted to BHB by the liver than normally. Insulin also appears to be important in regulating the utilization of BHB. The uptakes of BHB and acetate by the sheep hind limb are impaired during alloxan diabetes and are restored by insulin (JARRET et al 1974).

HAY et al. (1988) tested the hypothesis that pregnancy in sheep alters the effects of insulin on glucose utilization and glucose production. Euglycemic, hyperinsulinemic glucose clamp experiments were performed in chronically catheterized, unstressed, fed or 24 hours fasted, nonpregnant sheep and fed, pregnant sheep. Endogenous glucose production rate for the whole sheep and glucose utilization rate of the uterine and nonuterine maternal tissues were measured in control and high-insulin periods by tracer technique using [6-3H]glucose. Control glucose utilization rate in the fed, nonpregnant sheep was significantly ($p<0.05$) greater than that in the fasted, nonpregnant sheep, 2.29±0.17 and 1.86±0.11 mg/min/kg, respectively, and also in the nonuterine maternal tissues of the pregnant sheep (1.71±0.18 mg/min/kg). Insulin stimulated glucose utilization 116.4±14.8% in the fed, nonpregnant sheep but only 82.8±11.0% in the fasted, nonpregnant sheep and 94.2±14.3% in the nonuterine tissues of the fed, pregnant sheep. Also, insulin suppressed endogenous glucose production to 53.2±5.6% in the fed, nonpregnant sheep, to 3.9±3.1% in the fasted, nonpregnant sheep, and to 9.0±3.7% in the fed, pregnant sheep. In the pregnant animals, uterine glucose uptake and uterine glucose utilization were not different and were not altered by changes in maternal insulin concentration. The results indicate that during late pregnancy glucose utilization is reduced and resistance to the effect of insulin to enhance glucose utilization is present in the nonuterine maternal tissues compared to nonpregnant, fed sheep. In contrast, the effectiveness of insulin to suppress glucose production in the pregnant sheep is greater than that in nonpregnant, fed sheep. These results also demonstrate

that differential changes in the effect of insulin can exist simultaneously between peripheral (glucose consuming) and central (glucose producing) tissues. The changes in glucose utilization and in insulin effect in the pregnant sheep are both qualitatively and quantitatively similar to those of the nonpregnant sheep when fasted, suggesting that similar substrate and/or hormonal factors may be involved.

PETTERSON et al. (1994) studied the development of insulin resistance in late pregnancy in ewes which were fed either to maintain zero energy balance in maternal tissues (fed) or at 50% of this level (underfed) for several weeks. Plasma concentrations of free fatty acids (FFA) and glycerol were measured under basal conditions and during infusion of various doses of insulin while maintaining euglycemia (hyperinsulinemic, euglycemic clamp technique). Pregnancy and undernutrition separately increased basal plasma FFA concentration in an additive manner; plasma glycerol was increased by pregnancy but unaffected by undernutrition. The molar ratio of FFA to glycerol was significantly greater in underfed ewes. Analysis of dose-response relations between plasma insulin and metabolites during insulin infusions showed that maximally insulin-suppressed concentrations of FFA and glycerol were significantly greater in pregnant than in nonpregnant ewes but were unaffected by undernutrition. Neither pregnancy nor undernutrition affected the maximally insulin-suppressed FFA to glycerol ratio, or the plasma insulin concentration for 50% maximal responses to insulin of plasma FFA, plasma glycerol, or the plasma FFA to glycerol ratio. Thus, even in ewes at or close to zero energy balance, pregnancy seems to reduce adipose responsiveness but not sensitivity to the antilipolytic effect of insulin. This is another manifestation of the normal development of insulin resistance in maternal tissues during late pregnancy

HENZE et al. (1998) examined 214 ewes suffering from pregnancy toxaemia (ketosis). Clinical signs during onset and course of disease and laboratory findings were compared between animals that survived and those which died. The latter onset of ketosis was earlier in pregnancy (day 143±7 vs. day 146±8) and duration of the disease was shorter (10±13 vs. 14 ± 9 days). The animals that died showed more severe clinical signs and higher values of BHB (4.3±3.6 vs. 3.5±2.6 mmol/l) and cortisol (72±98 vs.

52±80 mmol/l) as well as lower values of insulin (37±12 vs. 3.5±2.6 mmol/l) and potassium (4.1±1.0 vs. 4.4±1.0 mmol/1) at onset of the disease than those which survived (all of differences with $p<0.05$). Glucose levels did not differ between groups. Treated animals with glucose plus fructose infusions (n=56) or with oral application of glucose precursors plus electrolytes (n=126) had survival rates of 53.6% and 62.7%, respectively. Oral treatment with glucose precursors plus electrolytes and an additional subcutaneous insulin treatment (n=15) led to an enhanced survival rate of 86.7% ($p<0.05$). Low insulin levels in ketotic pregnant sheep and the therapeutic effect of insulin treatment support the hypothesis that insulin plays a causative role in the pathogenesis of ovine ketosis.

KULCSAR et al. (2006) diagnosed ketosis in a flock of Merino ewes that conceived from synchronised oestrus in the early autumn period. On day 140 of pregnancy the ewes were sampled for determination of BHB, AST, glucose, FFA, total cholesterol (TCH), insulin, T4, T3, cortisol, IGF-1 and leptin. The results were evaluated according to the number of fetuses born some days later and the presence of hyperketonaemia (BHB ≥1.60 mmol/l). In May, about three months after lambing, cyclic ovarian function was induced (Cronolone + eCG), and the ewes were artificially inseminated (AI) 48 h after the removal of gestagen-containing sponge. At the time of AI and 10 days later blood samples were collected again to check the plasma levels of the same constituents as previously (in samples taken at AI), and to monitor the ovarian response by assaying progesterone (in both samples). On day 140 of gestation significantly lower BHB levels were detected in dams with single (n=41) than in those with twin (n=57) pregnancies. Hyperketonaemia was found only in ewes bearing twins (n=27). These animals had higher FFA and cortisol, and lower TCH, insulin, IGF-1, leptin and T3 levels than their normoketonaemic twin-bearing flock-mates, and those with single pregnancy. The blood glucose concentrations varied within a wide range, and the means of groups did not exhibit any significant differences. The formerly hyperketonaemic individuals were characterized by lower leptin level three months after lambing, and they showed a poorer response to the cycle-induction procedure than the others. The non-responders had lower IGF-1 and leptin levels than those ovulated after this treatment. It was concluded that the subclinical form of ovine ketosis is character-

ized by complex endocrine alterations, reflecting an obvious form of negative energy balance. If attempts to induce cyclic ovarian function outside the breeding season are made soon after lambing, the ovarian response and fertility of these ewes may also be depressed.

HAYIRLI (2006) studied the changes in nutritional and metabolic status during the periparturient period predispose dairy cattle to develop hepatic lipidosis and ketosis. The metabolic profile during early lactation includes low concentrations of serum insulin, plasma glucose, and liver glycogen and high concentrations of serum glucagon, adrenaline, growth hormone, plasma BHB and FFA, and liver triglyceride. Moreover, during late gestation and early lactation, flow of nutrients to fetus and mammary tissues are accorded a high degree of metabolic priority. This priority coincides with lowered responsiveness and sensitivity of extra hepatic tissues to insulin, which presumably plays a key role in development of hepatic lipidosis and ketosis. Hepatic lipidosis and ketosis compromise production, immune function, and fertility. Cows with hepatic lipidosis and ketosis have low tissue responsiveness to insulin owing to ketoacidosis. Insulin has numerous roles in metabolism of carbohydrates, lipids and proteins. Insulin is an anabolic hormone and acts to preserve nutrients as well as being a potent feed intake regulator. In addition to the major replacement therapy to alleviate severity of negative energy balance, administration of insulin with concomitant delivery of dextrose increases efficiency of treatment for hepatic lipidosis and ketosis. However, data on use of insulin to prevent these lipid-related metabolic disorders are limited and it should be investigated.

2.3.3 β-hydroxybutyrate (BHB)

BICKHARDT et al. (1988) mentioned that the plasma concentration of BHB was negatively correlated with substrate concentrations of glucose metabolism, but positively correlated with the triglyceride content of the liver and with the plasma concentration of bilirubin. It is concluded that an increase of the plasma concentration of BHB is a sensitive indicator of the complex alteration of glucose and lipid metabolism in ketosis of sheep.

BICKHARDT (1989) estimated plasma glucose and BHB concentrations in 142 healthy and 119 ketotic ewes. Plasma glucose values were lower in twin pregnant

German Blackheaded Mutton and Merino sheep and in pregnant Heidschnucke than in non-pregnant ewes, or ewes in early pregnancy of these breeds. There were no such differences at different stages of the reproductive cycle in healthy East Friesian ewes. Plasma glucose concentrations did not vary between breeds. Plasma glucose concentrations varied three fold in ketotic ewes compared with healthy ones, but mean values were not significantly different. Plasma BHB concentrations differed significantly between healthy ewes of different breeds and were highest in German Blackheaded Mutton and Merino ewes in late pregnancy and during lactation. There were more German Blackheaded Mutton and Merino ewes in the ketotic group than would be expected from the breed distribution in the area. Plasma BHB concentrations were higher in ketotic than in healthy ewes. It is concluded that individual variation and breed were more important causes of ketosis than insufficient feed supply. Multiple pregnant German Blackheaded Mutton and Merino sheep were particularly susceptible.

HENZE et al. (1994) examined the concentrations of hormones, metabolites and electrolytes in plasma of 45 ewes during gestation (day 80 to 149) and on the 3rd day of lactation. Healthy single pregnant (n=8) and twin pregnant ewes (n=12) were compared with ewes with subclinical (BHB>1.0 mmol/l, n=6) or clinical BHB>1.6 mmol/l, n=19) ketosis. The concentration of BHB, glucose, calcium, sodium and growth hormone in the plasma of the healthy animals were higher postpartum than ante-partum, while the concentrations of inorganic phosphorous, cortisol and total oestrogen were lower postpartum than during pregnancy. In the pregnant ewes the concentrations of BHB, bilirubin, total oestrogen and growth hormone in the plasma increased, while the concentration of insulin decreased with increasing numbers of fetuses. In comparison with the healthy twin pregnant ewes the animals with subclinical and clinical pregnancy ketosis showed increased values of bilirubin, glutamate dehydrogenase, somatotropin, total oestrogen, cortisol and cortisol/insulin ratios as well as decreased values of glucose, insulin and potassium. Individual differences between animals within their groups were observed for the examined hormones as well as for glucose, calcium, sodium and potassium (analysis of variance). Beside multiple pregnancy low insulin values and high cortisol/insulin ratios, due to individual disposition, influence enhanced ketogenesis and disorder of glucostasis.

FRAT and OZPNAR (2002) examined 20 synchronized ewes from Sakiz, Turkey, aged 3-4 years. Blood samples were taken once in pre-pregnancy and at the 100th day of pregnancy. On the 120th day of pregnancy and on the 10th day of lactation, blood samples were collected every 2 hours for 24 hours from only ten ewes. Plasma cortisol levels were analysed by radioimmunoassay. Plasma glucose and BHB levels were determined spectrophotometrically. Plasma cortisol level slightly increased at the 100th day of pregnancy, decreased during late pregnancy and continued to decrease during lactation. Plasma glucose and BHB levels were not significantly different during pre-pregnancy, pregnancy and the early lactation periods. It is concluded that metabolic disorders such as pregnancy toxaemia does not occur in ewes fed a balanced ration sufficient in energy and protein levels even in a multiple lambing breed of ewes.

HARMEYER and SCHLUMBOHM (2006) examined the impact of pregnancy on ketone body disposal during a hyperketonaemic clamp by tracer isotope dilution techniques in seven 12 hours fasted sheep in three reproductive states, in the dry non-gestation period, late in gestation and during early lactation. After a sampling period of 60 minutes under basal conditions BHB racemate solution was continuously infused intravenously for 3 hours at rates of 14.3-24.3 micro mol/(kg min) to elevate the BHB concentration in blood plasma to values between 5 and 7 mmol/l. Two separate experiments were carried out with the same sheep in each of the three reproductive states. During pregnancy three ewes were pregnant with a single lamb and four ewes carried twins. Maximal BHB turnover rates fell significantly in late gestation by 26% relative to early lactation and by 22% when compared with the dry non-pregnant state. Reduction of maximal BHB disposal rate during late gestation was accompanied by a significant 297% ($p<0.005$) and a non-significant 49% increase in the basal BHB concentration in blood, a non-significant 10% and 4% decrease in the rate constant of BHB turnover and a non-significant 24% and 13% rise in the incremented increase of BHB concentration per unit BHB infusion, relative to the dry and the lowered FFA lactating period, respectively. Induction of hyperketonaemia significantly and glycerol concentrations in blood by 67% and 57%, respectively, compared to the pre-infusional concentrations. The magnitude of this effect was the same in all reproductive states and was explained as a direct antilipolytic action of BHB on adipose tissue. It is concluded that the reduced ability of the late gestating ewe to utilize BHB promotes hy-

perketonaemia. Since hyperketonaemia exerts several adverse effects, e.g. on energy balance and glucose metabolism it appears that the impairment of ketone bodies disposal in late pregnancy facilitates development of pregnancy toxaemia, especially in ewes carrying twins

RAMIN et al. (2007) determined the distribution of subclinical pregnancy toxaemia by evaluation of serum glucose, BHB, urea and cortisol concentrations in pregnant ewes. 809 blood samples were collected from the jugular veins of 497 pregnant, 242 lambed and 70 aborted ewes. They were apparently healthy without any signs of disease. The concentrations of BHB, glucose and urea were assessed by spectrophotometry and cortisol by ELISA. Mean serum glucose (mg/dl), BHB (mmol/litre), urea (mg/dl) and cortisol (ng/ml) concentrations in pregnant ewes were 35.1, 0.45, 33.1 and 22.4; in lambed ewes were 35.7, 0.44, 30.0 and 13.5 and in aborted ewes were 41.4, 0.4, 28.6 and 24, respectively. Mean urea and BHB concentrations in pregnant ewes were greater and glucose was lower than in lambed and aborted ewes. Cortisol concentrations in pregnant ewes were higher than in the lambed but lower than in aborted ewes. Mean comparison of parameters showed a significant difference ($p<0.01$) except for BHB. The BHB concentrations were similar in all groups. A negative correlation coefficient in urea concentration was observed between pregnant and lambed ewes ($r = -0.14$, $p<0.05$). There were also correlations between BHB and glucose concentrations in pregnant ewes ($r=-0.16$, $p<0.01$), between BHB and cortisol concentrations in lambed ewes ($r=0.25$, $p<0.01$) and between BHB and urea concentrations in aborted ewes ($r=0.28$, $p<0.05$). With regard to the references, the concentration of BHB>0.7 mmol/litre is considered as subclinical pregnancy toxaemia, the distribution and percentage of ewes in pregnant, lambed and aborted groups were 61 (12.3%), 17 (7%) and 6 (8.57%), respectively. Similarly, for glucose < 20 mg/dl were 76 (15.3%), 38 (15.7%) and 10 (14.3%) and for cortisol >52 ng/ml were 28 (8.54%), 1 (0.6) and 5 (10.2%), respectively. Significant differences were found for BHB and cortisol groups. The highest subclinical pregnancy toxaemia occurs among pregnant and aborted ewes. Mean comparison of parameters in the group with BHB > 0.7 mmol/litre showed a significant difference ($p<0.01$) for urea and cortisol concentrations and in the group with glucose < 20 mg/dl and cortisol >52 ng/ml showed differences just for BHB concentration. Thus, it is concluded that hypoglycaemia, uraemia and high cortisol con-

centration in late pregnancy could be considered as subclinical pregnancy toxaemia up to 12% that should be seriously considered in order for prevention or treatment.

MOGHADDAM and HASSANPOUR (2008) studied 134 ewes in the Tabriz area of Iran. The levels of glucose, BHB, blood urea nitrogen (BUN) and calcium were measured by spectrophotometry. The mean level of BHB in the prepartum period was significantly higher than in the postpartum period ($p<0.05$). The mean level of glucose in the prepartum period was significantly lower than the postpartum period ($p<0.05$). There were negative and significant correlations between BHB and glucose levels both pre- and postpartum periods ($r=-0.55$, $p<0.01$ and $r=-0.59$, $p<0.01$, respectively). The level of BUN in the prepartum period was greater than the postpartum period but was not significantly different. The level of calcium in the prepartum period was significantly higher than in the postpartum period ($p<0.01$), but there was no significant correlation between glucose and calcium levels in either periods. 20 ewes (14.9%, $p<0.05$) suffered from subclinical pregnancy toxaemia, as indicated by a BHB concentration more than 0.7 mmol/litre. In conclusion, hypoglycaemia, uraemia and hyperketonaemia in late pregnancy could be considered as subclinical pregnancy toxaemia.

2.3.4 Free fatty acids

SYMONDS et al. (1989) determined how the long-term metabolic adaptations to winter shearing of the pregnant ewe result in significant changes in the rates of lipid mobilization and utilization of FFA in comparison with unshorn controls. Continuous infusions of [1-^{14}C]palmitic acid, [2-^{3}H]glycerol and $NaH^{14}CO_3$ were used to measure whole-body lipid metabolism in fed (estimated metabolizable energy (ME) intake 9.54 MJ/d) and under-fed (estimated ME intake of 3 MJ/d), shorn and unshorn sheep over the final four weeks of pregnancy. Whole-body carbon dioxide, estimated heat production, total FFA entry and oxidation rates were all significantly higher in fed shorn ewes compared with unshorn controls, even though there was no difference in the arterial plasma FFA concentration. These differences may be mediated via an increase in the plasma concentrations of thyroid hormones in shorn animals. As a result of under-

feeding any significant differences in lipid metabolism between shorn and unshorn groups were removed. In all sheep the mean total FFA entry rate as measured using [1-^{14}C] palmitic acid was 3.4 times the value obtained using [2-^{3}H] glycerol. It is concluded that when sheep are fed on a diet from which no more than half the required ME for late pregnancy is obtained, and then lipolysis of body fat depots occurs via the incomplete breakdown of adipose tissue triglycerides. This effect is significantly greater in the fed shorn pregnant ewe which exhibits higher entry and oxidation rates of FFA.

PETTERSON et al (1994) measured Plasma concentrations of FFA and glycerol under basal conditions and during infusion of various doses of insulin while maintaining euglycemia (hyperinsulinemic, euglycemic clamp technique). Pregnancy and undernutrition separately increased basal plasma FFA concentration in an additive manner; plasma glycerol was increased by pregnancy but unaffected by undernutrition. The molar ratio of FFA to glycerol was significantly greater in underfed ewes. Analysis of dose-response relations between plasma insulin and metabolites during insulin infusions showed that maximally insulin-suppressed concentrations of FFA and glycerol were significantly greater in pregnant than in nonpregnant ewes but were unaffected by undernutrition. Neither pregnancy nor undernutrition affected the maximally insulin-suppressed FFA to glycerol ratio, or the plasma insulin concentration for 50% maximal responses to insulin of plasma FFA, plasma glycerol, or the plasma FFA to glycerol ratio. Thus, even in ewes at or close to zero energy balance, pregnancy seems to reduce adipose responsiveness but not sensitivity to the antilipolytic effect of insulin. This is another manifestation of the normal development of insulin resistance in maternal tissues during late pregnancy.

REGNAULT et al. (2004) designed experiments to examine if the pregnancy-associated rise in FFA concentration is associated with a reduced pancreatic sensitivity to glucose in vivo. they investigated the possible relationship of FFA concentrations in regulating maternal insulin concentrations during ovine pregnancy at three physiological states, non-pregnant, non-lactating (NPNL),and at 105 and 135 days gestational age (DGA, term 147±3 days).The plasma concentrations of insulin, growth hormone

(GH) and ovine placental lactogen (OPL) were determined by double antibody radio-immunoassay. Insulin responsiveness to glucose was measured using bolus injection and hyperglycaemic clamp techniques in 15 non-pregnant, non-lactating ewes and in nine pregnant ewes at 105 DGA and near term at 135 DGA. Plasma samples were also collected for hormone determination. In addition to bolus injection glucose and insulin area under curve calculations, the Mean plasma glucose increment, glucose infusion rate and mean plasma insulin increment and area under curve were determined for the hyperglycaemic clamp procedures. Statistical analysis of data was conducted with Students t-tests, repeated measures ANOVA and 2-way ANOVA. Maternal growth hormone, placental lactogen and FFA concentrations increased, while basal glucose and insulin concentrations declined with advancing gestation. At 135 DGA following bolus glucose injections, peak insulin concentrations and insulin area under curve (AUC) profiles were significantly reduced in pregnant ewes compared with NPNL control ewes ($p<0.001$ and $p<0.001$, respectively). In hyperglycaemic clamp studies, while maintaining glucose levels not different from NPNL ewes, pregnant ewes displayed significantly reduced insulin responses and a maintained depressed insulin secretion. In NPNL ewes, 105 and 135 dGA ewes, the Glucose infusion rate (GIR) was constant at approximately 5.8 mg glucose/kg/min during the last 40 minutes of the hyperglycaemic clamp and the Mean Plasma Insulin Increment (MPII) was only significantly ($p<0.001$) greater in NPNL ewes. Following the clamp, FFA concentrations were reduced by approximately 60% of pre-clamp levels in all groups, though a blunted and suppressed insulin response was maintained in 105 and 135 DGA ewes. Results suggest that despite an acute suppression of circulating FFA concentrations during pregnancy, the associated steroids and hormones of pregnancy and possibly FFA metabolism, may act to maintain a reduced insulin output, thereby sparing glucose for non-insulin dependent placental uptake and ultimately, fetal requirements.

2.4 TNFα and its role in fat metabolism and pregnancy toxaemia

GREER et al. (1994) determined if plasma concentrations of defined cytokines are increased in women with preeclampsia, and to correlate any increases with the elevated concentrations of the vascular cell adhesion molecule (VCAM)-1. Twenty

primiparous with preeclampsia were compared to 20 healthy primiparous. Plasma levels of cytokines, TNFα, interleukin (IL)-6, IL-8, IL-1 beta, IL-1 receptor antagonist (IL-1ra), granulocyte macrophage-colony-stimulating factor (GM-CSF), and VCAM-1, were measured by enzyme-linked immunosorbent assay. It is concluded that concentrations of IL-6 and IL-1ra were significantly higher ($p<0.01$) in preeclamptic women (2.56 and 251.85 pg/mL, respectively) compared to normal pregnant patients (2.06 and 142.00 pg/mL, respectively). There were no significant changes in concentrations of TNFα, IL-8, GM-CSF, and IL-1 β in preeclamptic patients (14.09, 50.52, 125.8, and 2.08 pg/mL, respectively) compared to normal patients (11.96 44.46, 121.3, and 2.01 pg/mL, respectively). Serum concentrations of VCAM-1 were increased in women with preeclampsia (preeclamptic group 841.9±49.7 ng/mL, control group 560.2±47.9 ng/mL; $t=3.673$, $p<0.001$). Interleukin-6 and IL-1ra concentrations correlated with VCAM-1 concentrations (IL-6: $r=0.539$, $z = 2.9$, $p<0.005$; IL-1ra: $r=0.451$, $z = 2.428$, $p<0.02$). In conclusion, increased cytokine concentrations may contribute to the endothelial damage that occurs with preeclampsia and may explain the mechanism underlying leukocyte activation in this disorder. The increased cytokine concentration may also be responsible for the endothelial adhesion that accompanies preeclampsia.

KUPFERMINC (1994) investigated whether markers for activation of the immune system are present in patients with preeclampsia by assessing maternal plasma and amniotic fluid for TNFα and IL-1 β. Twenty-one patients with severe preeclampsia composed the study group (group A). An ante partum comparison group was composed of healthy nulliparous patients not in labour and matched for gestational age (group B). Another control group consisted of term nulliparous patients in labour with uneventful pregnancies (group C). Maternal plasma samples were collected from all patients at recruitment and from patients in groups A and C immediately after delivery and again 20 to 24 hours post partum. Amniotic fluid was also collected from patients in groups A and C during labour. All samples were collectively assayed for tumor necrosis factor-alpha and interleukin-1 beta by specific enzyme-linked immunoassays. Before labour tumor necrosis factor-alpha was detected more frequently in the plasma of preeclamptic patients than in the plasma of patients in group B (12/16 vs 5/16, $p<0.05$) and in higher concentrations (median 35 pg/ml, $p<0.05$). Although tumor ne-

crosis factor-alpha was frequently detected in the plasma of patients in group C in early labour (16/20) concentrations were higher in the four preeclamptic patients first sampled in early labour (210 pg/ml vs 65 pg/ml, $p<0.05$). Similarly, amniotic fluid levels of tumor necrosis factor-alpha were increased in preeclamptic patients compared with control patients. At delivery tumor necrosis factor-alpha was more likely to be identified in the plasma of preeclamptic patients and was found in higher concentrations, but by 20 to 24 hours post partum measurements in the preeclamptic and control patients were similar. There were no differences in the frequency with which IL-1 β was detected or the concentration of IL-1 β in any of the samples. It is concluded that TNFα is increased in the plasma and amniotic fluid of patients with severe preeclampsia. These data are suggestive of a role for abnormal immune activation in the pathophysiological mechanisms of preeclampsia.

SEOW et al. (1995) cloned ovine tumour necrosis factor-alpha (OvTNFα) by reverse transcription-polymerase reaction using RNA isolated from lipopolysaccharide (LPS)-stimulated alveolar macrophages and primers based on the human TNFα cDNA sequence. An expression vector which carries the coding sequence of the mature form of ovine TNF was constructed. rOvTNFα was expressed as a glutathione-S-transferase (GST) fusion protein. It was cleaved with thrombin to yield rOvTNF free of the GST moiety. Growth at a lower temperature of 30ºC and use of Escherichia coli strains AM207, AM305, E392 and NM522 did not improve the recovery of rOvTNFα from the soluble fraction to a significant extent. Purification of recombinant proteins was achieved rapidly and easily by affinity chromatography using glutathione-Sepharose. Yields of pure rOvTNFα achieved in E. coli JM109 and AM207 were approximately 1 mg/litre. Both rOvTNFα and rhTNFα exerted cytotoxicity on L929 cells. However, rOvTNF- alpha but not rhTNF- alpha stimulated proliferation of ovine thymocytes. Maximum levels of TNFα mRNA expression by LPS-stimulated ovine alveolar macrophages were detected at approximately four hours after stimulation.

VINCE et al. (1995) investigated TNFα may play an important role in pre-eclampsia and contribute to endothelial activation. Plasma TNFα, IL-6 and both forms of soluble TNF receptors (p55 and p75 TNF-R) have been measured by ELISA in 31 pre-

eclamptic patients and 31 pregnant controls matched for age, parity and gestational age. Levels of IL-6, TNF-alpha and soluble TNF-R (p55 and p75) were significantly higher in pre-eclamptic patients, compared with age and gestation matched controls with a wide variation in levels between pre-eclamptic individuals. There was a correlation between levels of IL-6 and TNF or TNF-R and between TNF and TNF-R levels. However, when the pre-eclamptic patients were subdivided on the basis of the severity of their disease, the median values of plasma concentrations of IL-6, TNFα and TNF-R were all higher in the group with lower platelet counts. It was concluded that these new findings were consistent with the concept that the maternal syndrome of pre-eclampsia is associated with endothelial dysfunction and provide evidence that at least part of this dysfunction could arise from excessive release of TNFα into the circulation.

BENYO et al. (1997) investigated TNFα and interleukin-1 (IL-1) are inflammatory cytokines capable of eliciting endothelial cell dysfunction, whether the production of these inflammatory cytokines by cultured villous explants from the human placenta was affected by incubation in reduced oxygen (2% O_2). The term placenta produced TNFα, IL-6, and low levels of IL-1α and IL-1ß under standard tissue culture conditions. Hypoxia significantly increased TNFα, IL-1α, and IL-1ß production by 2-, 6-, and 23-fold, respectively, but did not affect IL-6 production. Further, cytokines were immunolocalized to the syncytiotrophoblast layer as well as to some villous core cells. Hypoxic regulation of placental TNFα and IL-1ß production also appeared to differ based on gestational age. Finally, treatment with either cobalt chloride or an iron chelator mimicked the hypoxic response, suggesting that stimulation of placental cytokine production may involve a heme-containing, O_2-sensing protein. These results suggest that placental hypoxia can lead to the elaboration of inflammatory cytokines, which may contribute to the pathophysiology of preeclampsia.

CONRAD et al. (1997) explored the hypothesis that TNFα and possibly other inflammatory cytokines are overproduced by the placenta in response to local ischemia/hypoxia contributing to increased plasma levels, and subsequent endothelial activation and dysfunction in the pregnancy disorder, preeclampsia. It is widely held that

inadequate trophoblast invasion and physiologic remodeling of spiral arteries initiate placental ischemia/hypoxia in preeclampsia. Furthermore, focal areas of placental hypoxia have been implicated in the production of "toxic" factor by the placenta, which circulate and cause maternal disease. Placental trophoblast cells and fetoplacental macrophages normally produce TNFα and interleukin-1 (IL-1), which are capable of producing endothelial cell activation and dysfunction. Hypoxia has recently been reported to increase TNFα and IL-1 production by term villous explants from the human placenta. Placental cells also express erythropoietin (EPO), which is the prototype molecule for transcriptional regulation by hypoxia in mammals. Interestingly, TNFα and IL-1 have DNA sequence homologous or nearly homologous to the hypoxia-responsive enhancer element of the EPO gene, thus providing a potential, but as of yet, untested molecular link between placental hypoxia and stimulation of cytokine production. Inflammatory cytokines overproduced by the placenta in response to hypoxia may then lead to increased plasma levels and endothelial activation and dysfunction in preeclampsia. The purpose of this short review is to critically evaluate the hypothesis that placental cytokines contribute to the pathogenesis of preeclampsia. Off note, the etiology of the disease presumably related to deficient trophoblast invasion is beyond the scope of this work.

CONRAD et al. (1998) measured plasma levels of immunoreactive TNFα and -β, interleukin (IL)-1 α and -β, and IL-6 and -10 in women with preeclampsia, in women with transient gestational hypertension, and throughout normal pregnancy. Enzyme-linked immunosorbent assays were used and subjected to extensive validation studies. The median concentration of plasma TNFα was increased two fold in women with preeclampsia compared with that in normal third-trimester pregnancy ($p<0.001$) and in women with gestational hypertension ($p<0.04$). The median concentration of plasma IL-6 was increased by threefold in women with preeclampsia compared with that in normal third-trimester pregnancy ($p<0.001$) and increased twofold compared with that in women with gestational hypertension ($p<0.1$). There were no significant differences observed in the levels of plasma IL-1 β and IL-10 between the preeclamptic and other subject groups. The level of IL-1 β, but not the levels of IL-10, TNFα, or IL-6, was significantly changed during normal pregnancy compared with the nonpregnant condi-

tion manifesting an overall decline ($p<0.04$). TNFα and IL-1 were not detected in any samples, possibly because of the low sensitivity of these particular immunoassays. It was concluded that elevated levels TNFα and IL-6 may contribute to the putative endothelial dysfunction of preeclampsia.

GASIC et al. (1998) investigated the hypothesis that TNFα stimulates lipolysis by blocking the action of endogenous adenosine. Adipocytes were incubated for 48 hours with TNFα, and lipolysis was measured in the absence and presence of adenosine deaminase. Without adenosine deaminase, the rate of glycerol release was two-three times higher in the TNFα-treated cells, but with adenosine deaminase lipolysis increased in the control cells to approximately that in the TNFα-treated cells. This suggests that TNFα blocks adenosine release, or prevents its antilipolytic effect. Both N^6-phenylisopropyl adenosine and nicotinic acid were less potent and efficacious inhibitors of lipolysis in treated cells. A decrease in the concentration of α-subunits of all three G_i subtypes was detected by western blotting without a change in $G_s$proteins or β-subunits. $G_{i2}\alpha$ was about 50% of control, whereas $G_{i1}\alpha$ and $G_{i3}\alpha$ were about 20% and 40% of control values, respectively. The time course of $G_i$down-regulation correlated with the stimulation of lipolysis. Furthermore, down-regulation of G_i by an alternative approach (prolonged incubation with N^6-phenylisopropyl adenosine) stimulated lipolysis. These findings indicate that TNFα stimulates lipolysis by blunting endogenous inhibition of lipolysis. The mechanism appears to be a G_i protein down-regulation.

JOHNSON et al. (1999) studied the hypothesis that TNFα secreted from the oocyte-cumulus cell complex stimulates follicular collagenase secretion and thereby contributes to ovarian wall degradation and ovulatory rupture. Pro-oestrous ewes were treated with GnRH-agonist (5 µg i.m. des Gly^{10}-Ala^6 ethylamide) to synchronize the onset of the gonadotropin surge; ovulation occurred approximately 24 hours later. There was an increase in TNFα (immunoassay) in antral fluid of preovulatory follicles at 18 hours after GnRH injection, which was related to tissue collagenolytic bioactivity (radiolabelled type 1 substrate digestion by enzymatic extract) and collagen (hydroxyproline) depletion. Intrafollicular injection of TNFα antibodies at 12 hours after

GnRH injection negated the rise in follicular collagenolytic bioactivity (and is known to block ovulation in the sheep). Collagenase secretion was enhanced when follicular tissues (0-h GnRH) were incubated (6 hours) with recombinant TNFα; this effect was inhibited by the transcriptional inhibitor actinomycin D. Secretion of TNFα by oocyte-cumulus cell complexes isolated from preovulatory follicles simulated the in vivo circumstance. Immunostaining indicated that TNFα was confined mainly to the oocyte before GnRH administration, accumulated in cumulus cells during the mid-to-late preovulatory period, and was expended with the imminent approach of ovulation. It is concluded that oocyte-cumulus cell complex is a source of soluble TNFα. This is thought to be the first study to show that up regulation of collagenase expression as a target mode of TNFα action in preovulatory follicles.

MOLDES et al. (2001) identified novel genes and proteins that could mediate the effects of TNFα on adipocyte metabolism and development, the authors have used a differential display technique comparing 3T3-L1 cells exposed or not to the cytokine. They have isolated a novel adipose cDNA encoding a TNFα-inducible 470-amino acid protein termed TIARP, with six putative transmembrane regions flanked by a large amino-terminal and a short carboxyl-terminal domain, a structure reminiscent of channel and transporter proteins. Commitment into the differentiation process is required for cytokine responsiveness. The differentiation process per se is accompanied by a sharp emergence of TIARP mRNA transcripts, in parallel with the expression of the protein at the plasma membrane. Transcripts are present at high levels in white and brown adipose tissues, and are also detectable in liver, kidney, heart, and skeletal muscle. Whereas the biological function of TIARP is presently unknown, its pattern of expression during adipose conversion and in response to TNFα exposure as a transmembrane protein mainly located at the cell surface suggest that TIARP might participate in adipocyte development, and metabolism and mediate some TNFα effects on the fat cell as a channel or transporter.

BENYO et al. (2001) compared TNFα, IL-1α, IL-1β, and IL-6 levels in placentas from patients with preeclampsia and normal term pregnancies. Because the placenta is a large heterogeneous organ, the authors analyzed multiple sites per placenta. On average, there was a three fold variation in cytokine protein levels across the eight sites

analyzed for each placenta. However, there were no significant overall differences among the normal term, preeclamptic, and preterm placentas from women without preeclampsia. There were also no significant differences in TNFα messenger ribonucleic acid between the normal term and preeclamptic placentas, although TNFα messenger ribonucleic acid levels were lower in placentas from preterm patients without diagnosis of preeclampsia than in the normal term placentas. In vitro, hypoxia stimulated the production of TNFα, IL-1α and IL-1β, but not that of IL-6, by placental villous explants from both groups of patients, and this was not exaggerated in preeclampsia. Finally, although peripheral and uterine venous levels of TNFα were elevated in preeclamptic women compared with normal term patients, the ratio of uterine to peripheral venous TNFα levels were not significantly different from 1.0 for either patient group. Taken together, these results suggest that sources other than the placenta contribute to the elevated concentrations of TNFα and IL-6 found in the circulation of preeclamptic women.

GONZALEZ et al. (2001) designed a multicenter prospective clinical study with the purpose of measuring TNFα and TNFα-receptors p55 and p75 (sTNFα-p55 and sTNFα-p75) in normal pregnancy and preeclampsia (PE). Blood samples were collected without anticoagulant from 30 pregnant women, matched for chronological and gestational age, 15 healthy pregnant (group A) and 15 women with PE (group B). TNFα was measured by enzyme linked immunosorbent assay and sTNFα-p55 and sTNFα-p75 were measured by amplified immuno enzymo metric assay. The statistical analysis was carried out using the paired t student test. The values of TNFα were 7.07±1.63 pg/mL (mean±SD) in group A and 16.43±2.92 in group B pg/mL ($p<0.05$).); concentrations of sTNFα-p55 were 2.69±0.17 ng/mL and 5.18±0.54 ng/mL in groups A and B respectively ($p<0.001$); sTNFα-p75 reached levels of 5.94±0.27 ng/mL in healthy pregnant and 9.02±0.66 ng/mL in patients with PE ($p<0.001$). TNFα, sTNFα-p55 and sTNFα-p75 were significantly higher in preeclamptic women than in normotensive controls. These findings indicate that the levels of TNFα, TNFα-p55 and TNFα-p75 were elevated in PE. Further research is needed to identify modifiable risk factors for the excessive synthesis and release of TNFα in pregnancy, and to assess

whether the lowering of TNFα concentrations in pregnancy alters the incidence and severity of PE.

COLDITZ (2002) mentioned that the immune system provides host defense by sensing non-self molecules that herald a threat from foreign organisms. A level of stimulation of this sense organ is necessary for health of the host. Following excessive stimulation, as may occur during disease challenge or exposure to a high microbial burden in an unhygienic environment, outputs of the immune system affect homeostatic pathways that regulate metabolism, nutrient partitioning, behaviour, thermoregulation and hypothalamic-pituitary-adrenocortical (HPA) activity. Important consequences of excessive immune activation include production of the pro-inflammatory cytokines IL-1β, IL-6, TNFα and IFNα/β , activation of the acute phase response, fever, inappetence, amino acid resorption from muscle, redirection of nutrients from accretion in meat, milk and wool towards liver anabolism of acute phase proteins, and stimulation of leptin production. Thus immunological resistance to disease in livestock may incur a production cost due to redirection of nutrients away from production tissues. Counter-balancing the cost of resistance is the metabolic cost of disease. The metabolic costs of resistance to internal parasites in sheep are considered. Genetic variation in the cost of disease resistance could occur due to (1) variation in sensitivity to input stimuli that trigger immune responses or (2) variation in the quantity or type of output signals generated following immune stimulation. Downstream sensitivity of tissues to outputs of the immune system could also differ. The ideal product to monitor the burden imposed on metabolic pathways by activity of the immune system would be unique to innate and acquired immune responses. Acute phase proteins and pro-inflammatory cytokines have attracted attention in this respect; however, non-immunological stimuli such as tissue injury and psychosocial stressors can also induce these products that are usually associated with the immune system. Immuno nutritional and therapeutic strategies to limit the production costs of excessive activation of the immune system are considered

RYDEN et al. (2002) recorded that TNFα is a pleiotropic cytokine with a proposed role in obesity-related insulin resistance. This could be mediated by increased lipolysis in adipose tissue resulting in elevated free fatty acid levels. The early intracellular signals entailed in TNFα-mediated lipolysis are unknown but may involve members of

the mitogen-activated protein kinase (MAPK) family. The authors investigated the possible contribution of MAPK in TNFα-induced lipolysis in human preadipocytes. TNFα activated the three mammalian MAPK, p44/42, JNK, and p38, in a distinct time- and concentration-dependent manner. TNFα also induced a concentration-dependent stimulation of lipolysis with a more than three fold increase at the maximal dose. Lipolysis was completely inhibited by blockers specific for p44/42 (PD98059) and JNK (dimetylaminopurine) but was not affected by the p38 blocker SB203580. Use of receptor-specific TNFα mutants showed that activation of MAPK is entirely mediated by the TNFR1 receptor. The results in human preadipocytes differed from those obtained in murine 3T3-L1 adipocytes in which all three MAPK were constitutively active. Thus, studies of intracellular signaling pathways obtained in different cellular contents should be interpreted with caution according to the authors. In conclusion, although TNFα activates all three known MAPK in human preadipocytes, only p44/42 and JNK appear to be involved in the regulation of lipolysis.

ZHANG et al. (2002) demonstrate that TNFα increases lipolysis in differentiated human adipocytes by activation of mitogen-activated protein kinase (MAPK), extracellular signal-related kinase (ERK), and elevation of intracellular cAMP. TNFα activated ERK and increased lipolysis; these effects were inhibited by two specific MAPK inhibitors, PD98059 and U0126. TNFα treatment caused an electrophoretic shift of perilipin from 65 to 67 kDa, consistent with perilipin hyperphosphorylation by activated cAMP-dependent protein kinase A (PKA). Coincubation with TNFα and MAPK inhibitors caused perilipin to migrate as a single 65-kDa band. Consistent with the hypothesis that TNFα induces perilipin hyperphosphorylation by activating PKA, TNFα increased intracellular cAMP ~1.7-fold, and the increase was abrogated by PD98059. Furthermore, H89, a specific PKA inhibitor, blocked TNF-α-induced lipolysis and the electrophoretic shift of perilipin, suggesting a role for PKA in TNFα-induced lipolysis. Finally, TNFα decreased the expression of cyclic-nucleotide phosphodiesterase 3B (PDE3B) by ~50%, delineating a mechanism by which TNFα could increase intracellular cAMP. Cotreatment with PD98059 restored PDE3B

expression. These studies suggest that in human adipocytes, TNFα stimulates lipolysis through activation of MAPK-ERK and subsequent increase in intracellular cAMP.

DANIEL et al. (2003) designed four studies to determine whether the following: (1) TNFα and the Lipopolysaccharide (LPS) binding ligand, CD14, are produced by sheep adipose tissue; (2) nutritional reserves and/or short-term fasting affect circulating concentrations of TNF; (3) there is a relationship between TNF and metabolic factors in sheep; and (4) inflammation alters circulating concentrations of leptin. Ewes were assigned in experiments one and two, based on ultrasonic assessments of last-rib subcutaneous fat measurements to fat (fat thickness >1 cm; mean=1.52±0.03 cm) or thin (fat thickness < 1 cm; mean = 0.25±0.03 cm) groups. Fat and thin ewes were assigned to fed or fasted groups for a total of four groups (fed-fat, fasted-fat, fed-thin, fasted-thin). Fed ewes had ad libitum access to feed, and fasted-ewes were prohibited feed 48 hours before initiation of sample collection. In Experiment one, subcutaneous fat samples were collected from just above the last rib for detection of TNFα and CD14 mRNA, and immunoreactivity. TNFα -like immunoreactivity in adipocytes was sparse and more pronounced in cells in fed-ewes than fasted-ewes, and localized to membranes between adjacent cells in nucleated regions. Immunoreactivity for CD14 was minimally observed but present in adipocytes and widely expressed in infiltrating monocytes and epithelial vascular cells. Leptin was detected in adipocytes. In Exp. two, plasma samples collected every six hours for 24 hours were analyzed for plasma concentrations of TNFα. Fat ewes had greater plasma concentrations of TNFα than thin ewes ($p= 0.039$). In Experiment three ewes were injected intravenous with interleukin-1β or TNFα. Blood samples were collected every 15 minutes for eight hours following injection. Plasma concentration of leptin was not affected by treatment ($p> 0.39$). In Experiment four ewes were injected intavenous with interleukin-1beta or TNF. Blood samples were collected every 15 minutes for eight hours following injection. Plasma concentration of leptin was not altered by LPS ($p>0.20$). The following results provide evidence: (1) of TNF-like immunoreactivity within fat tissue; (2) that elements within fatty tissues have CD14 that may allow adipocyte function to be directly affected by LPS; (3) that plasma concentrations of leptin are not altered by LPS treatment; and lastly (4) that circulating concentrations of TNFα are elevated with obesity in sheep.

RAHMAN et al. (2004) examined the expression of key cytokines and vascular surface molecules in the uterus of pregnant sheep around the time of implantation. Uterine tissues and uterine washings were collected from non-pregnant and pregnant sheep at 17-19 days post-coitus (dpc), 26-27 and 34-36 dpc. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of caruncular/placentomal tissues revealed that cytokines IL-2, IL-4 and IL-8, which were not detected in non-pregnant uteri, were induced more strongly at 26-27 dpc than in other stages of pregnancy tested. Cytokines LIF, IL-6, IL-10, TNFα were also most highly expressed at 26-27 dpc, expression of them being lower at other time-points during early pregnancy and non-pregnancy. The cytokines IL-1 beta, IFN- gamma and TGF- beta were expressed in all non-pregnant and pregnant tissues examined. Enzyme-linked immunosorbent assay (ELISA) performed on uterine washings clearly detected the presence of IL-1α protein at 26-27 and 34-36 dpc. Immuno histochemistry revealed that expression of vascular adhesion molecule VCAM-1 in endometrial endothelium was strongly induced at 26-27 dpc in the pregnant endometrium. Expression of CD5 on vascular endothelium was not induced in placentomal tissues until 26-27 dpc and was further increased by 34-36 dpc. These results demonstrate a dynamic change in a wide range of cytokines during early stages of pregnancy, with a critical period around 26-27 dpc. In addition, at 26-27 dpc, expression of the surface/adhesion molecules, CD5 and VCAM-1, is induced on vascular endothelium of the sheep endometrium, possibly as a direct consequence of the changed cytokine environment, and may be involved in directing the changes in leukocyte migration observed during pregnancy.

JIANG et al. (2004) examined 13 healthy pregnant and five non-pregnant ewes (control) after 15-40 days of parturition. The contents of IL-1β, IL-6 and TNFα were measured at 85, 105, 125 and 140 days after gestation and 150 days of parturition, using the liquid phase competition and equilibrium methods. The contents of serum IL-β and IL-6 at 125 day after gestation were significantly higher than those in non-pregnant sheep (control), and the contents of serum TNFα in the mid and late stages of pregnancy were also higher than in the control. During pregnancy, the level of three cytokines in serum increased gradually and reached its peak at 125 days. They then

subsequently decreased, and rose again at parturition, but still remained lower than the maximum at 125 days. It is suggested that there is a close relationship between cytokines and gestation, which play an important role in maintaining gestation. TNF probably played an important role in parturition, while IL-1β and IL-6 showed no effect.

GREEN et al. (2004) studied that both TNFα and hyperglycemia impair insulin sensitivity in vivo. This may be secondary to stimulation of adipose tissue lipolysis and consequent increased circulating FFA. Here we report that neither TNFα nor glucose alone has a pronounced effect on lipolysis in 3T3-L1 adipocytes. However, the combination of TNFα plus glucose markedly stimulates lipolysis. Glucose does not affect the ability of isoproterenol to stimulate lipolysis. Alternative substrates such as acetate, pyruvate, and lactate do not allow the TNFα effect. Mannose was almost as effective as glucose; fructose was marginally effective, but galactose was ineffective. The effectiveness of the sugars corresponded with production of lactate, it means that the cells readily produced lactate from glucose or mannose, slightly from fructose, and not at all from galactose. The ability of TNFα to phosphorylate extracellular signal-regulated kinase 1 (ERK1) and ERK2 and to downregulate perilipin (which has been implicated in the lipolytic effect of TNFα) was not affected by glucose. It is concluded that the lipolytic action of TNFα is influenced by glucose in 3T3-L1 adipocytes. The findings suggest that glucose metabolism is required for the lipolytic response to TNFα but not for early signaling events. These findings suggest novel mechanisms by which TNFα and hyperglycemia raise FFA levels and induce insulin resistance.

HUNG et al. (2004) demonstrated that hypoxia-reoxygenation (H/R) of placental tissues in vitro causes equivalent oxidative stress to that seen in preeclampsia. The aim was to determine whether H/R also increases production of TNFα, and whether conditioned media from samples exposed to H/R causes activation of human umbilical vein endothelial cells (HUVECs). Concentrations of mRNA encoding TNFα were significantly higher in placental tissues subjected to H/R compared to hypoxic or normoxic controls. Although there was no difference in the concentrations of TNFα protein in tissue homogenates, levels of TNFα protein in the medium were significantly higher after H/R compared to controls, indicating increased secretion. Furthermore, condi-

tioned medium from samples subjected to H/R caused increased expression of E-selectin by HUVECs, and the addition of anti-TNFα antibodies significantly reduced that activation. These results are consistent with the hypothesis that intermittent perfusion of the placenta, secondary to reduced trophoblast invasion, causes increased secretion of TNFα, and that this contributes to the activation of maternal endothelial cells that characterizes preeclampsia.

YARIM et al. (2007) determined plasma IL-1β, TNFα and MCP-1 concentrations in ewes with severe (*n*=6) and mild (*n*=4) naturally occurring pregnancy toxaemia and in uncomplicated pregnant ewes (*n*=10) using enzyme-linked immunosorbent assay (ELISA). All ewes with pregnancy toxaemia had significantly lower body temperature and respiratory rate than uncomplicated pregnant ewes ($p<0.05$). With the highest concentrations in severe cases, heart rate, proteinuria and serum uric acid levels as well as plasma IL-1β, TNFα and MCP-1 were significantly different among all three groups ($p<0.05$). The plasma concentrations of IL-1β in control ewes and ewes with mild and severe toxaemia were 15.81±3.90 pg/ml, 23.83±2.42 pg/ml and 34.55±8.03 pg/ml, respectively. The plasma concentrations of TNFα in control ewes and ewes with mild and severe toxaemia were 7.71±0.61 pg/ml, 16.13±3.63 pg/ml, and 22.85±3.64 pg/ml, respectively. The plasma concentrations of MCP-1 in control ewes and ewes with mild and severe toxaemia were 101.70±9.86 pg/ml, 134.75±6.24 pg/ml, and 157.67±9.69 pg/ml, respectively. Moreover, plasma IL-1β, TNFα and MCP-1 levels were positively correlated with clinical and well-establish biochemical parameters of pregnancy toxaemia, serum uric acid and proteinuria ($p<0.01$). Concomitant increase of plasma IL-1β, TNFα and MCP-1 concentrations along with serum uric acid, proteinuria, and worsening of the clinical signs indicates that such cytokines are involved in the aetiopathogenesis and in perpetuation of the local and systemic inflammatory reactions in pregnancy toxaemia in ewes. Hence plasma IL-1β, TNFα and MCP-1 may potentially serve as markers to monitor prognosis of pregnancy toxaemia in ewes.

PLOMGAARD et al. (2008) hypothesized that an acute elevation of TNFα in plasma would cause an increase in lipolysis, therefore increasing circulatory free fatty acid (FFA) levels. Using a randomized controlled, crossover design, healthy young male

individuals (n=10) received recombinant human (rh) TNFα (700 ng/m^{-2}·h^{-1}) for four hours, and energy metabolism was evaluated using a combination of tracer dilution methodology and arterial-venous differences over the leg. Plasma TNFα levels increased from 0.7±0.04 to 16.7±1.8 pg/ml, and plasma IL-6 increased from 1.0±0.2 to 9.2±1.0 pg/ml ($p< 0.05$) after four hours rhTNFα infusion. Here, we demonstrate that four hours rhTNFα infusion increases whole body lipolysis by 40% ($p< 0.05$) with a concomitant increase in FFA clearance, with no changes in skeletal muscle FFA uptake, release, or oxidation. Off note, systemic glucose turnover and lactate and catecholamine levels were unaffected by rhTNFα infusion. This study demonstrates that a relatively low dose of rhTNFα induces systemic lipolysis and that the skeletal muscle fat metabolism is unaffected.

LEWIS et al. (2009) studied that maternal cytokine levels of TNFα, interleukin (IL)-6, IL-8, and IL-10 were varied in women with pre-eclampsia (PE) compared to those from normal pregnancies. In this study, the authors determined whether these cytokine levels are correlated before and after delivery in patients with PE. Method of study: venous blood was obtained from 50 women diagnosed with severe PE at the time of admission and 24 hours after delivery. Plasma concentrations for TNFα, IL-6, IL-8, and IL-10 were measured by ELISA. Results: there were no statistical differences for maternal levels of TNFα, IL-6, IL-8, and IL-10 before and 24 hours postpartum. TNFα and IL-10, but not IL-6 and IL-8, levels were significantly correlated before and 24 hr after delivery: TNFα: y = 19.963 + 0.953*x; r (2) = 0.924; IL-10: y = 10.521 + 1.113*x; r(2) = 0.984, $P<0.001$, respectively. Furthermore, TNFα levels were correlated with IL-10 levels, but not with IL-6 and IL-8 levels. In Conclusion the correlation patterns of TNFα with IL-10 and TNFα with IL-6 and IL-8 suggest disparity in functional regulations between these cytokines in maternal circulation in PE.

ZHU et al. (2010) mentioned that the placenta is a source of cytokines which can induce maternal gestational insulin resistance and alter nutrient transport to the fetus. Obesity induces placental inflammation at term, but the impact of obesity on placental inflammation earlier in pregnancy has not been defined. Using sheep as an experimental model, the authors hypothesized that maternal obesity (MO) would induce inflam-

mation in the cotyledonary (COT) tissue of the placentome by mid-gestation. Non-pregnant ewes were randomly assigned to a control (C, 100% of NRC recommendations) or obese (OB, 150% of NRC) group from 60 days before conception to 75 day of gestation (dG), when ewes were necropsied and placental COT tissue collected for analyses. Free fatty acids content, triglyceride and cholesterol content were higher ($p<0.05$) in the fetal plasma of OB compared to C ewes on day 75. MO increased mRNA levels of toll-like receptor (TLR) 2 ($p<0.05$) and TLR4 ($p = 0.06$), macrophage markers cluster of differentiation (CD)11b ($P=0.06$), CD14 and CD68 ($P<0.05$), and proinflammatory TNFα ($p<0.01$), interleukin (IL)-6 ($p<0.05$), IL-8 ($p<0.01$) and IL-18 ($p = 0.06$), in COT tissue. Inflammatory c-Jun N-terminal kinase (JNK)/c-Jun and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB) signaling pathways were up-regulated ($p<0.05$) in COT of OB ewes. In conclusion, MO enhanced the placental inflammatory response in OB ewes at mid-gestation, possibly as a result of increased TLR4 and free fatty acids.

3 Animals, material and methods

3.1 Animals

Twenty nine pregnant apparently healthy ewes, sixteen from Merino breed and thirteen from blackhead breed were housed in Oberholz Farm, Faculty of Veterinary Medicine, Leipzig University, Germany.

Sheep were fed on Wilting Silage in the last five weeks of gestation and then the feed was supplemented with whole wheat.

3.2 Clinical examination

Rectal temperature, heart beat, visible mucous membranes, pulse and respiratory rates were examined in all pregnant ewes every time before the blood samples collection. The average numbers of lambs were 2.18 and 1.58 /ewe for Merino and Blackhead breeds respectively.

3.3 Collection of blood samples

Blood samples were collected from the jugular veins of 29 pregnant ewes in 5 ml Ethylenediaminetetraacetic Acid (EDTA) vacutainer tubes for haematological analysis and also into 10 ml plain tubes for serum biochemical analysis.

The blood samples collection began during the lambing season in January, five weeks before parturition and continued according to the following steps.

- 5 weeks before parturition.
- 3 weeks before parturition.
- 1 week before parturition.
- 4 weeks after parturition

3.4 Analysis of haematological parameters in blood samples

Packed cell volume (PCV), haemoglobin concentration (HB), Red blood cell count (RBCs) and white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) were estimated by using Hämatologieautomat Advia 120, Fa. Bayer Vital GmbH, Fernwald resp. Cellcounter Typ Alvet, Fa. möLAB GmbH, Langenfeld.

3.5 Determination of biochemical parameters

Parameter	Method	Equipment
Glucose	Hexokinase-Method	Hitachi912, Testkit Roche Diagnostics GmbH, Mannheim
BHB	UV-Method	
FFA	Kinetic UV-Test	
GLDH	UV-Test; optimized standard method of DGKC	
CK	NAC-activated, optimized standard method of DGKC	
GGT	according to Szast	
Albumin	Bromocresol green method	
TP	Biuret-Method	
Fe	Identification with ferrozine (without deproteinization)	
Cholesterol	CHOD-PAP-Method	
Insulin	Radioimmunoassay	Insulin – RIA –Kit, IBL Hamburg

3.6 Determination of haptoglobin by using haptoglobin assay

3.6.1 Haptoglobin assay principle

Free haemoglobin exhibits peroxidase activity, which is inhibited at low pH. Haptoglobin present in the specimen combines with haemoglobin and at low pH preserves the peroxidase activity of the bounds haemoglobin (Tridelta kits, Dublin, Ireland). Preservation of the peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin present in the specimen.

3.6.2 Components

- Haemoglobin 1*12 ml stabilised Haemoglobin
- Haemoglobin diluent 1*12 ml haemoglobin diluent
- Calibrator 1*0.5 ml haptoglobin calibrator (2mg/ml)
- Chromogen 1*11 ml chromogen reagent
- Substrate 1*8 ml substrate containing stabilised hydrogen peroxides
- Sample/calibrator diluent 1*12 ml phosphate buffer saline (PBS)

3.6.3 Additional materials required

- Serum collection equipment
- Automated analyser (A600 mm) or micro plate reader (A630mm)
- Accurate micropipettes and disposable tips to deliver 0-10 µl
- Test tubes and plastic pipettes (5-10 ml)
- Timer
- 96 well microtiter plate or strips

3.6.4 Sample and reagent preparation

3.6.4.1 Samples

Bloods samples were collected by venipuncture into serum collection tubes and stored at -20°C.

3.6.4.2 Haemoglobin

Accurately premix equal volume of haemoglobin and haemoglobin diluent prior to commencement of assay (reagent 1). Prepared reagent was stored at room temperature (25°C).

3.6.4.3 Chromogen/Substrate

Accurately premix chromogen and substrate in a ratio of 9:5 prior to commencement of assay (reagent 2). Refer to the following table for suggested volumes.

Number of Tests	Volume of Chromogen	Volume of Substrate
10	0.09 ml	0.5 ml
20	1.8 ml	1.0 ml
40	3.6 ml	2 ml
8	7.2 ml	4 ml

3.6.5 Manual methods (microplate or spectrophotometric)

3.6.5.1 Calibrator

Label five tubes with number C1-C5 corresponding to haptoglobin standards 2, 1, 0.5, 0.25 and 0 mg/ml respectively. Calibrators for manual test format are prepared by following the steps provided in the table.

Tube number	Volume calibrator	Volume diluent	Tube concentration
C1	50 µl of stock	-	2.0 mg/ml
C2	50 µl of stock	50 µl	1.0 mg/ml
C3	50 µl C2	50 µl	0.5 mg/ml
C4	50 µl C3	50 µl	0.25 mg/ml
C5	-	50 µl	0 mg/ml

Prepared calibrators are stable for 8 hours when stored at room temperature (20-25°C).

3.6.5.2 Test temperature

The test was performed at 37°C.

3.6.5.3 Procedure

Note: Addition of diluent haemoglobin (reagent 1) and chromogen/substrate solution (reagent 2) was made with a multichannel pipette.

- Transfer 7.5 µl of each prepared calibrator (0-2mg/ml) -along with test specimens- in duplicate to the blank micro plate.
- 100 µl of reagent 1 was added to each microwell. The microplate was shacked gently to insure mixing of calibrators specimens and haemoglobin.

- 140 µl of reagent 2 was added to each microwell and then incubated for 5 minutes at room temperature (20-25°C).
- Reads immediately at 630 nm.
- Calibration curve was generated by plotting absorbance (630 nm) versus haptoglobin concentration (ng/ml) to facilitate calculation of haptoglobin concentration in test specimens.

Specimen values were read directly from the curve. Multiply observed value by appropriate factor if specimen dilution was necessary.

3.7 Analysis of cytokine tumor necrosis factor α (TNFα) by using ovine TNFα ELISA assay

Protocol of ovine TNFα ELISA assay

- Each well was filled with 0.5 mg/ml antiovin TNFα (Serotec Nr.AHP 852Z) and 100 µl concentration 1µg/ml in Carbonat buffer pH 9.0 and incubated overnight at 4°C.
- The plate was washed three times with PBS-T (0.05%) to get rid of excess unbound antigen and the remaining free binding sites were blocked by the addition of 200µl/well with PBS-T 20 (0.05%) and incubated for one hour at Room temperature.
- The plate was washed three times with PBS-T (0.05 %).
- Addition of standard 0-160 ng/ml recombinant ovin TNFα (Serotec PBP005). 20µg stock solution 100µl/well diluted standard in PBS-T 20 (0.05%), 100 µl undiluted 1:10 in PBS-T 20 (0.05%) incubation at room temperature respectively.
- The plate was washed with PBS-T 0.05%.
- Secondary Antibody rabbit anti ovin TNFα 0.25 mg (Serotec Nr. AHP 852B). 100µl was added to all wells (Conc 1µg/ml in PBS 0.05%Tween) and incubated for one hour at room temperature.
- The plate was washed with PBS-T 20 (0.05%).

- 100 µl of streptavidin-HRP conjugat (Dianova) was added to all wells (1:5000 in PBS-T 20) and incubated for 30 minutes at room temperature.
- The plate was washed with PBS-T 0.05 %.
- 100 µl TMB substrate (Pharmingen) was added to all wells (1:1), mixing 5-10 minute and incubated at room temperature. The reaction was stopped by adding 100 µl/well of 1 M sulphuric acid.
- Reading was obtained at optical density 450 nm, reference 620 nm using TNF bov/ovin microplate ELISA reader.

3.8 Statistical analysis

The basis of statistical analysis for the present data was performed according to SPss15 software, GmbH Munich. Mann–Whitney test used to determine significant differences in all parameters between Black head and Merino sheep and $p<0.05$ was considered statistically significant.

The Bonferroni test is used to determine significant differences in all parameters between periods of sampling (5 weeks before parturition, 3 weeks before parturition, 1 week before parturition and 4 weeks after parturition). The mean difference is significant at the 0.05 level.

The Pearson correlation test was used to analyse the correlation between TNFα and other parameters. p-value <0.05 was considered significant.

4 Results

4.1 Clinical examination (observation)

All sheep showed normal clinical findings (temperature, mucous membrane, and pulse) in all periods of sampling.

4.2 Biochemical parameters

4.2.1 Glucose

4.2.1.1 Glucose concentrations in all sheep

The mean values of glucose concentration in 29 pregnant ewes began with high levels (4.2 ± 0.86 mmol/l) in the first period of sampling (5 w.b.p.) and then decreased (2.9 ± 0.73 mmol/l) during the third period of sampling (one week before parturition). It remained at around the same low level (2.8 ± 0.7 mmol/l) in the fourth period of sampling (four weeks after parturition). Figure 1 shows the variation of glucose concentration at different sampling periods for all sheep.

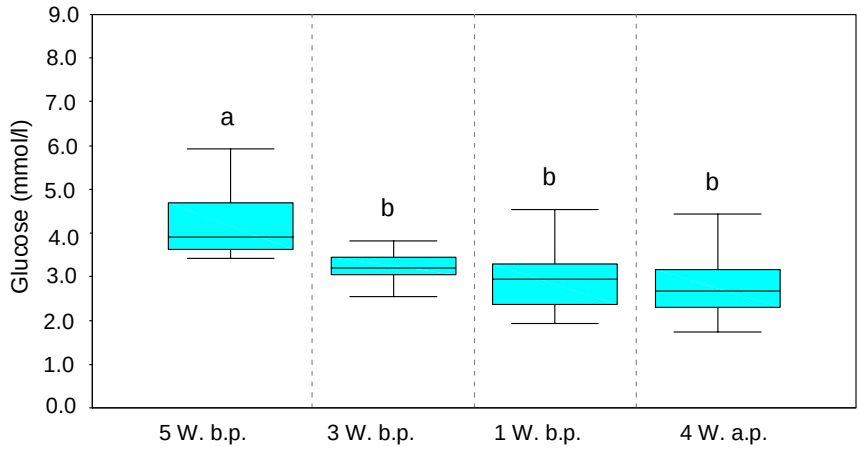

Figure 1: Glucose concentrations (mmol/l) in the serum of ewes around parturition

4.2.1.2 Glucose concentrations in Blackhead sheep

The mean values of glucose concentration began with 3.9 ± 0.79 mmol/l in the first sampling period and decreased in the third sampling period to 2.6 ± 0.74 mmol/l. In the last period of sampling it began to increase slightly, 2.9 ± 0.74 mmol/l was recorded, as seen in Figure 2.

4.2.1.3 Glucose concentrations in Merino sheep

The maximum mean value of glucose concentration was recorded in the first period of sampling (4.5 ± 0.86 mmol/l). This value decreased to 3.2 ± 0.66 mmol/l in the third sampling period and then to 2.7 ± 0.68 in the last period of sampling, as seen in Figure 2.

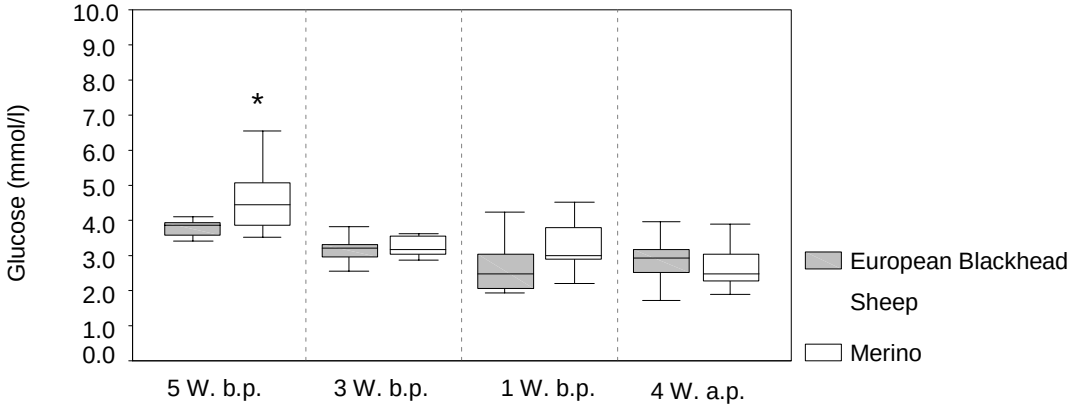

Figure 2: Glucose concentrations (mmol/l) in the serum of Blackhead and Merino ewes around parturition

Statistical analysis illustrated that there was significant difference ($p<0.05$) between Blackhead and Merino sheep in the first sampling period while there was no significant difference in the other periods of sampling. On the other hand, regarding time of sampling, there were significant differences between the periods of sampling one and periods, two, three, and four.

4.2.2 Insulin

4.2.2.1 Insulin concentrations in all sheep

The median values of insulin concentration in period 1 (5 w.b.p.) were 0.13 (0.08, 0.23) nmol/l (median, first, and third quartiles). Almost the same values were obtained in period 2 (3 w.b.p.) and period 3 (1 w.b.p.) recording 0.12 (0.09, 0.21) and 0.12 (0.09, 0.18) respectively. Then increased in period 4 (4 w.a.p.) to 0.22 (0.18, 0.36) nmol/l, as seen in figure 3.

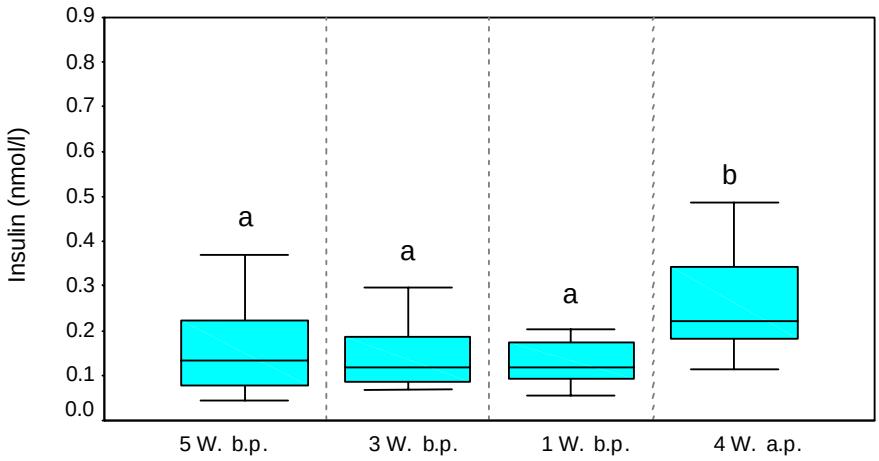

Figure 3: Insulin concentrations (nmol/l) in the serum of ewes around parturition

4.2.2.2 Insulin concentrations in Blackhead sheep

Before parturition, the median value of insulin concentrations were 0.17 (0.1, 0.23); 0.13 (0.1, 0.3); 0.16 (0.1, 0.26) nmol/l in periods 1, 2, and 3, respectively. After parturition, it increased to 0.25 (0.17, 0.36) nmol/l, as seen in figure 4.

4.2.2.3 Insulin concentrations in Merino sheep

Before parturition, the median value of insulin concentrations were 0.1 (0.06, 0.23); 0.11 (0.08, 0.17); and 0.11 (0.08, 0.15) nmol/l in periods 1, 2, and 3, respectively. After parturition, it increased to 0.21 (0.18, 0.36) nmol/l in period 4, figure 4.

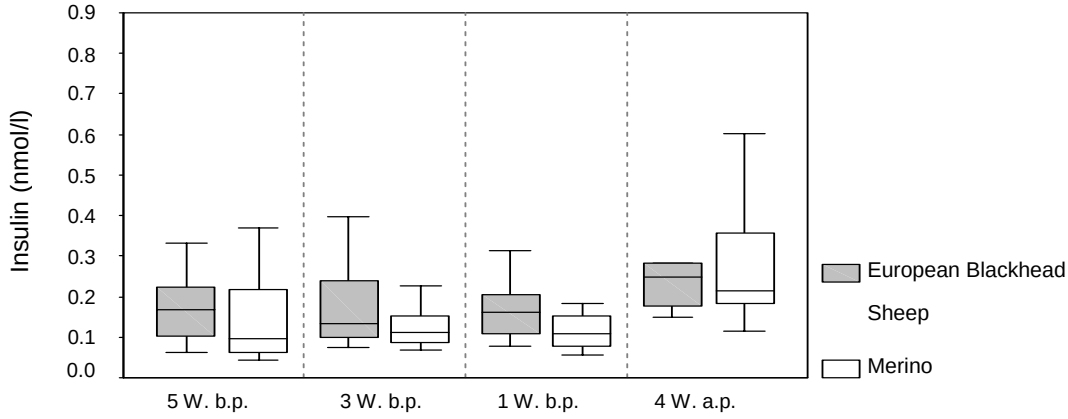

Figure 4: Insulin concentrations (nmol/l) in the serum of Blackhead and Merino ewes around parturition

Statistical analysis illustrated that there was a significant difference ($p<0.05$) between the period of sampling four (4 w.a.p.), and the other periods of sampling (before partu-

rition). On the other hand, there was no significant difference between Blackhead sheep and Merino sheep in all periods of sampling.

4.2.3 Free fatty acids

4.2.3.1 Free fatty acid concentrations in all sheep

The median values of FFA concentrations showed high levels before parturition. It started with 976 (544, 1160) µmol/l (median, first, and third quartiles) and 759 (568.7, 1313.2) µmol/l in the 1st and 2nd sampling period, respectively, before parturition. It then slightly decreased in the 3rd period to 523.5 (387.5, 931.2) µmol/l. After parturition (in the 4th sampling period), it dropped to 146 (89.5, 272.5) µmol/l, as seen in figure 5.

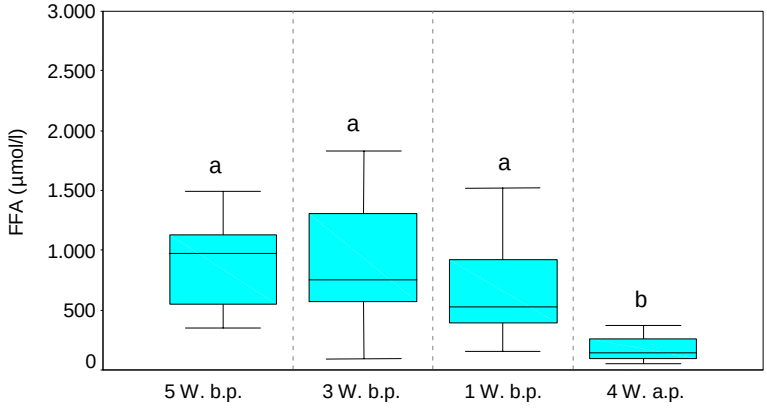

Figure 5: Free fatty acids concentrations (µmol/l) in the serum of ewes around parturition

4.2.3.2 Free fatty acid concentrations in Blackhead sheep

The median values of FFA concentrations were 671 (499.7, 1028) µmol/l and 621.5 (434.2, 761) µmol/l in the 1st and 2nd sampling periods, respectively. They then decreased in the 3rd period to 339 (224.7, 543) µmol/l. After parturition, the concentration decreased again to 191 (101, 272.5) µmol/l, as shown in figure 6.

4.2.3.3 Free fatty acid concentrations in Merino sheep

The median values of FFA concentrations for Merino sheep showed higher concentrations than that of Blackhead sheep in all of the sampling periods that took place before parturition. It began with 1041.5 (920.2, 1355) µmol/l and 920.5 (667.7, 1411.2)

µmol/l in the 1st and 2nd sampling periods respectively, and then gradually decreased to 678.5 (516, 1127.2) µmol/l in the 3rd sampling period. After parturition, in the 4th sampling period, it dropped to 122.5 (81.2, 280.5) µmol/l as seen in figure 6.

Statistical analysis showed that there were significant differences ($p<0.05$) between the 4th sampling period (four weeks after parturition) and the other sampling periods before parturition. On the other hand, there was a significant difference between Blackhead and Merino sheep in the 1st, 2nd, and 3rd periods of sampling (before parturition), while there was no significant difference in the 4th sampling period (after parturition).

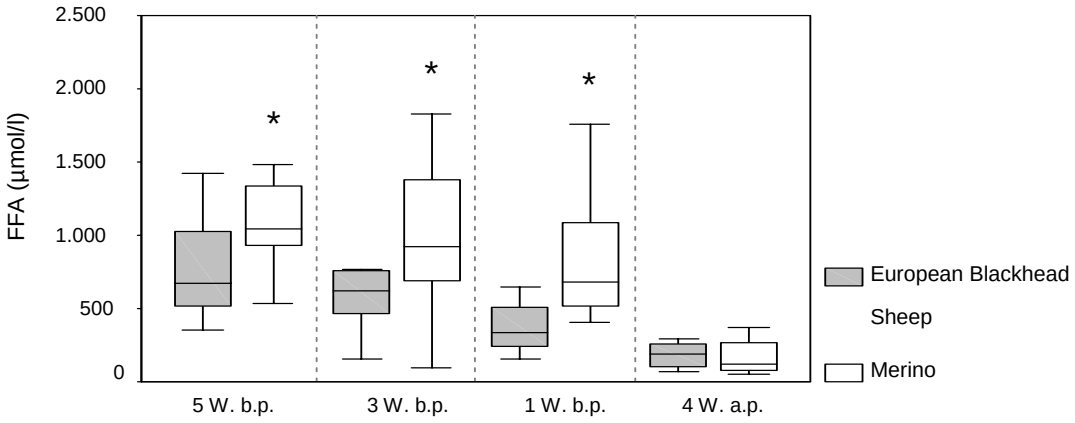

Figure 6: Fatty acid concentrations (µmol/l) in the serum of Blackhead and Merino ewes around parturition

4.2.4 β-hydroxybutyrate (BHB)

4.2.4.1 β-hydroxybutyrate concentrations in all sheep

Before parturition, the mean values of BHB were 0.38 ± 0.13, 0.28 ± 0.1 and 0.27 ± 0.22 mmol/l in the 1st, 2nd, and 3rd sampling periods. After parturition (in the 4th sampling period), 0.33 ± 0.11 mmol/l was recorded, as seen in figure 7.

4.2.4.2 β-hydroxybutyrate concentrations in Blackhead sheep

Before parturition, the mean value of BHB was 0.32 ± 0.12 mmol/l (1st sampling period). It then gradually decreased in the 2nd to 0.21 ± 0.12 mmol/l, and then again to 0.16 ± 0.05 in the 3rd sampling periods. After parturition, in the 4th sampling period, it increased to 0.30 ± 0.08 mmol/l as seen in figure 8.

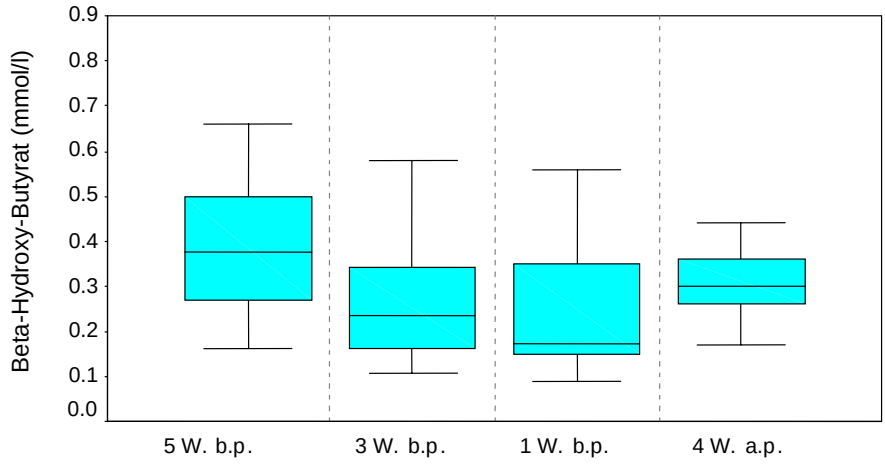

Figure 7: BHB concentrations (mmol/l) in the serum of ewes around parturition

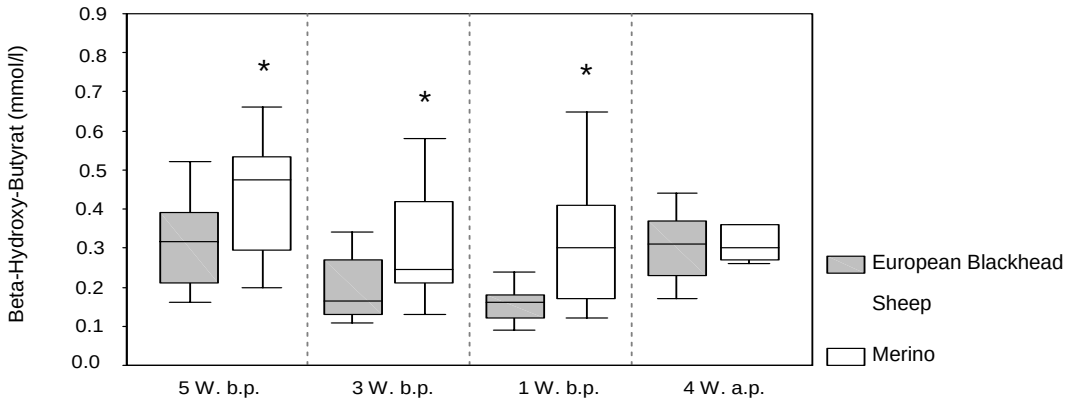

Figure 8: BHB concentration (mmol/l) in serum of Black and Merino ewes around parturition

4.2.4.3 β-hydroxybutyrate concentrations in Merino sheep

The mean value of BHB in the 1st sampling period was 0.43 ± 0.12 mmol/l (before parturition). It then decreased in the 2nd and 3rd sampling periods to 0.33 ± 0.22 mmol/l and 0.36 ± 0.26 mmol/l respectively. Four weeks after parturition (4th sampling period), it was 0.35 ± 0.13 mmol/l as seen in figure 8.

Statistical analysis showed that there were significant differences ($p<0.05$) between Blackhead and Merino sheep in the 1st, 2nd, and 3rd periods of sampling (before parturition), while there was no significant difference in the 4th sampling period (after parturition).

4.2.5 Tumor necrosis factor alpha (TNFα)

4.2.5.1 TNFα concentrations in all sheep

The median values of TNF α showed slightly high levels in all of the sampling periods before parturition; 30.4 (17.2, 78.5) (median, first, and third quartiles); 35.6 (13.6, 54.3); and 26.6 (13, 39.9) ng/ml in the 1st, 2nd, and 3rd sampling periods respectively. After parturition, during the 4th sampling period, this level decreased to 19.1 (9.9, 33.8) ng/ml, see figure 9.

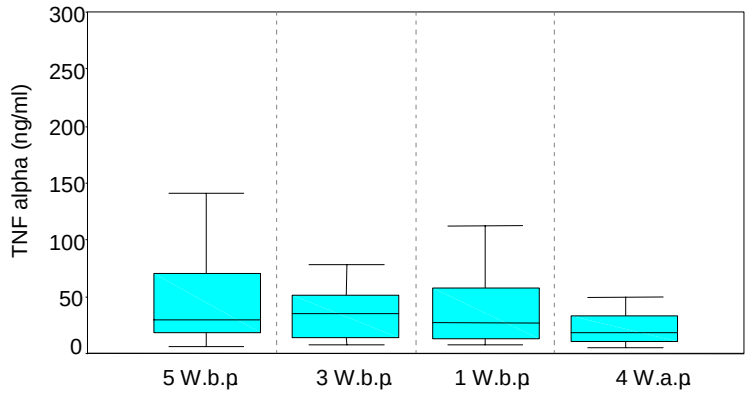

Figure 9: TNFα concentrations (ng/ml) in the serum of ewes around parturition

4.2.5.2 TNFα concentrations in Blackhead sheep

The median values of TNFα showed slightly high levels in all of the sampling periods before parturition; 26.2 (12.4, 43), 29.3 (12.7, 43.8), and 17.6 (11.5, 76.7) ng/ml in the 1st, 2nd, and 3rd sampling periods, respectively. After parturition, during the 4th sampling period this level decreased to 17.9 (8.4, 32.7) ng/ml, see figure 10.

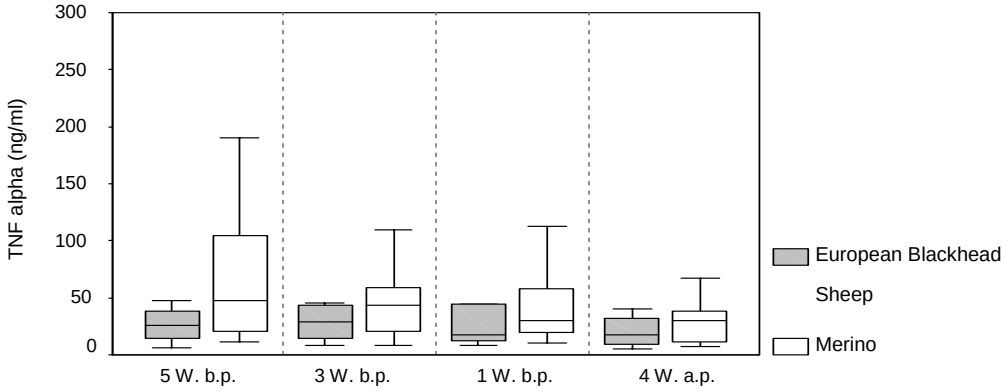

Figure 10: TNFα concentrations (ng/ml) in the serum of Blackhead and Merino ewes around parturition

4.2.5.3 TNFα concentrations in Merino sheep

The median values of TNF α showed a slightly high level in all the sampling periods before parturition; 47.7 (19.7, 109.3), 43.3 (15.7, 69.1), and 30.5 (16.3, 81.8) ng/ml in the 1st, 2nd, and 3rd sampling periods respectively. After parturition, during the 4th sampling period, this level slightly decreased to 30 (11.2, 39). ng/ml, see figure 10.

Statistical analysis illustrated that there was no significant difference between Blackhead and Merino sheep in all sampling periods. There was also no significant difference between the different sampling periods in regard to time.

4.2.6 Haptoglobin

4.2.6.1 Haptoglobin concentrations in all sheep

The mean values of haptoglobin concentrations were 0.15 g/l, 0.06 g/l, and 0.12 g/l in period 2 (3 w.b.p.), period 3, (1 w.b.p.), and period 4 (4 w.a.p.), respectively. These values showed lower concentrations before parturition in period 3 (1 w.b.p.). Figure 11 shows the variation of the haptoglobin concentration in 29 pregnant sheep.

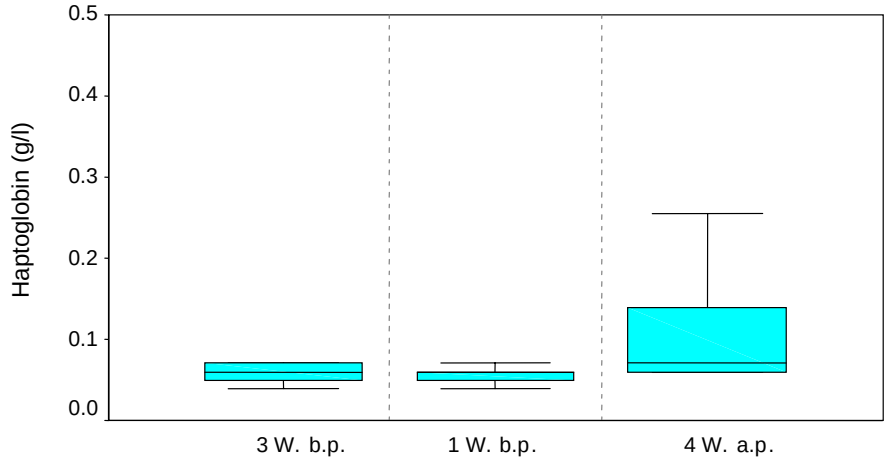

Figure11: Haptoglobin concentrations (g/l) in the serum of ewes around parturition

4.2.6.2 Haptoglobin concentrations in Blackhead and Merino sheep

Figure 12 shows the variation of Haptoglobin concentrations in Blackhead and Merino sheep.

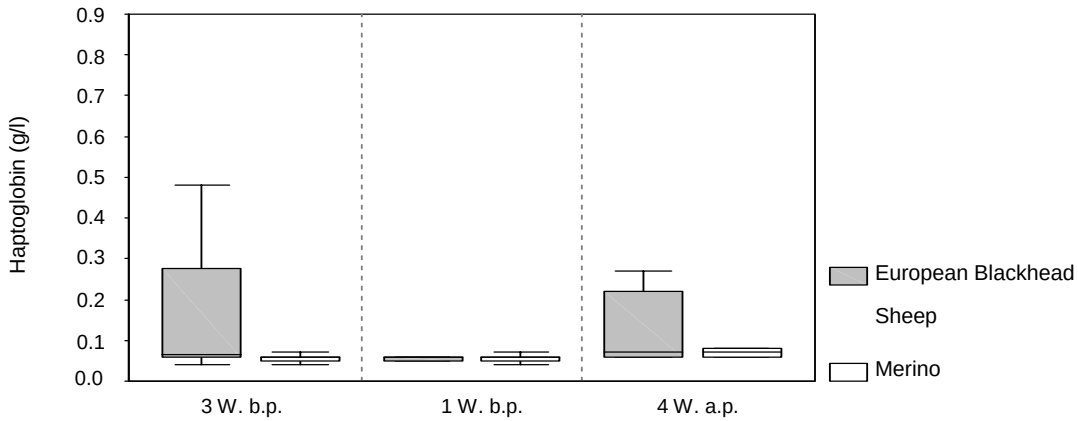

Figure 12: Haptoglobin concentrations (g/l) in the serum of Blackhead and Merino ewes around parturition

4.2.7 Albumin, R.R. 22-29 g/l (BICKHARDT 1992)

4.2.7.1 Albumin concentrations in all sheep

The mean values of Albumin concentrations were 38.51, 36.88 g/l, 30.64 g/l, and 25.41 g/l in period 1(5 w.b.p.), period 2 (3 w.b.p.), period 3, (1 w.b.p.), and period 4 (4 w.a.p.) respectively. These indicated that there was a slight increase in the Albumin concentrations in all the periods before parturition which then decreased to the normal concentrations after parturition. Figure 13 shows the variation in Albumin concentrations of all sheep during the investigation time.

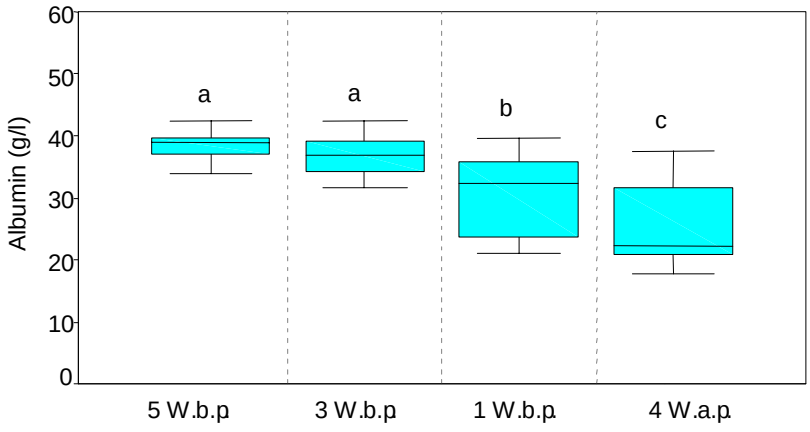

Figure13: Albumin concentrations (g/l) in the serum of ewes around parturition

4.2.7.2 Albumin concentrations in Blackhead and Merino sheep

Figure 14 shows the variation in Albumin concentrations in Blackhead in comparison to that of Merino sheep during the periods of study. The statistical analysis illustrated that there was no significant difference between Blackhead and Merino sheep in all periods of sampling.

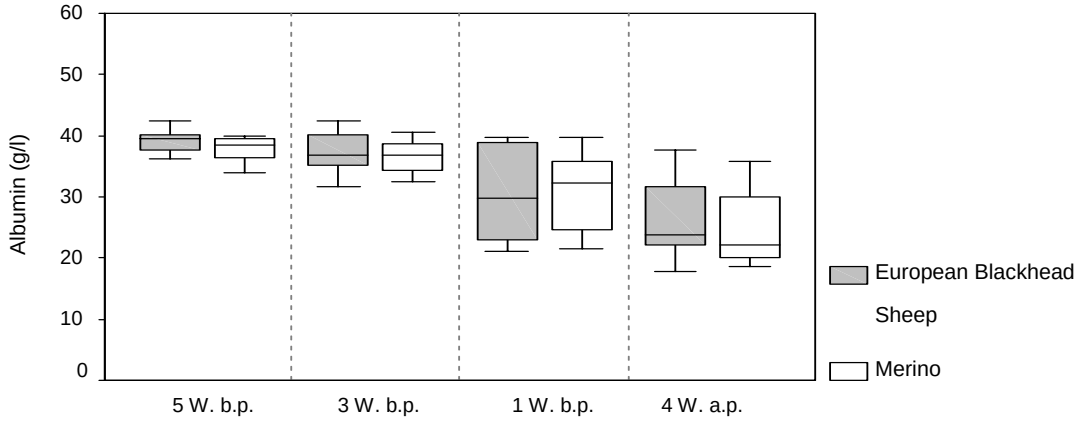

Figure 14: Albumin concentrations (g/l) in the serum of Blackhead and Merino ewes around parturition

4.2.8 Creatinkinase (CK), R.R 13- 230 U/l

4.2.8.1 Creatinkinase activity in all sheep

The mean values of CK in 29 pregnant ewes were 125.23 U/l, 116.97 U/l, 90.38 U/l, and 87.93 U/l in period 1(5 w.b.p.), period 2 (3 w.b.p.), period 3, (1 w.b.p.), and period 4 (4 w.a.p) respectively. These values showed that CK was within normal levels in all period of sampling, Figure 15 shows the variation in CK activities in all sheep during the investigation time.

4.2.8.2 Creatinkinase activity in Blackhead and Merino sheep

Figure 16 shows the variation in the activity of CK in Blackhead sheep in comparison to that of Merino sheep. The statistical analysis of CK showed that there was significant difference ($p<0.05$) between Blackhead and Merino sheep in the 1st sampling period while there was no significant difference in the other periods of sampling. On the other hand regarding time of sampling there was significant difference ($p<0.05$) between the 1st and 3rd sampling periods. Also there was significant difference between

the 1st and 4th sampling periods. Significant difference was shown also between the 2nd and 4th sampling periods.

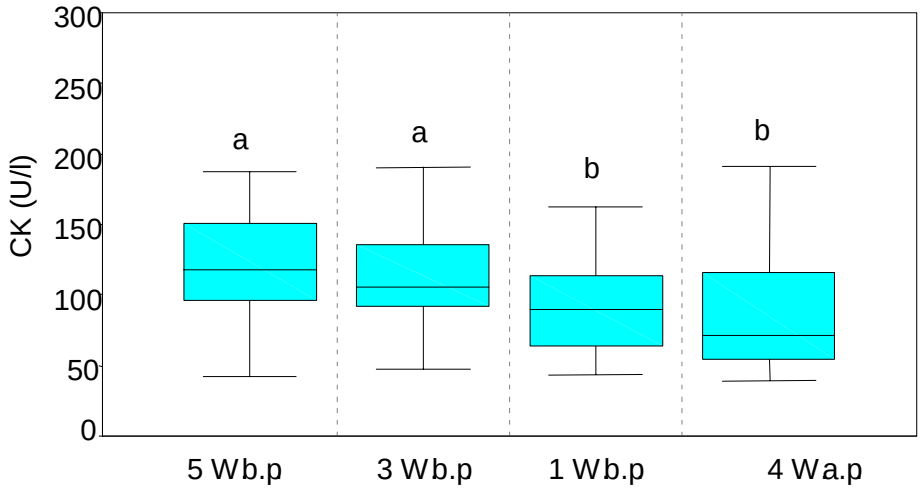

Figure15: CK activity (U/l) in the serum of ewes around parturition

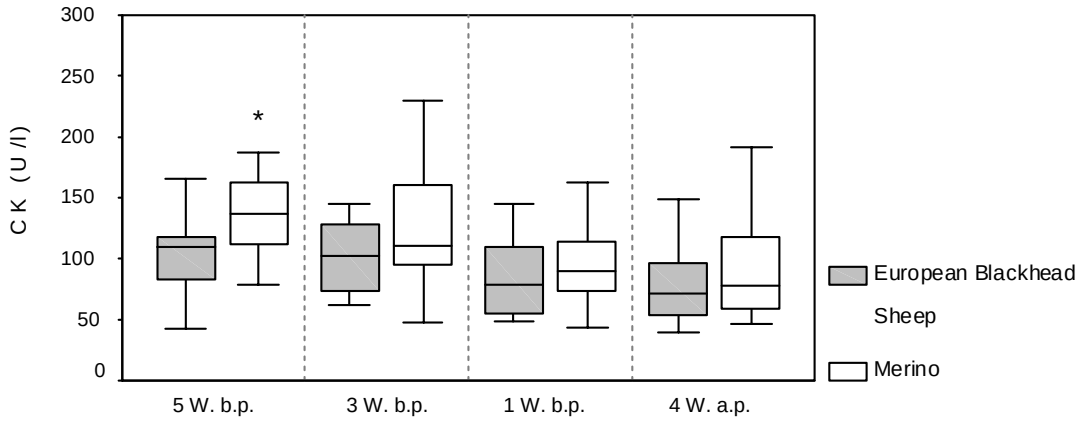

Figure 16: CK activity (U/l) in the serum of Blackhead and Merino ewes around parturition

4.2.9 Gamma Glutamyl Transferase (GGT), R.R 12-60 U/l (ROSSOW and BOLDUAN, 1994)

4.2.9.1 GGT activity in all sheep

The mean values of GGT in 29 pregnant ewes were 66.4 U/l, 64.94 U/l, 61.19 U/l, and 63.34 U/l in periods 1 (5 w.b.p.), 2 (3 w.b.p.), 3 (1 w.b.p.), and 4 (4 w.a.p.), respec-

tively. These values ranged within normal GGT activity in all sampling periods. Figure 17 shows t me.

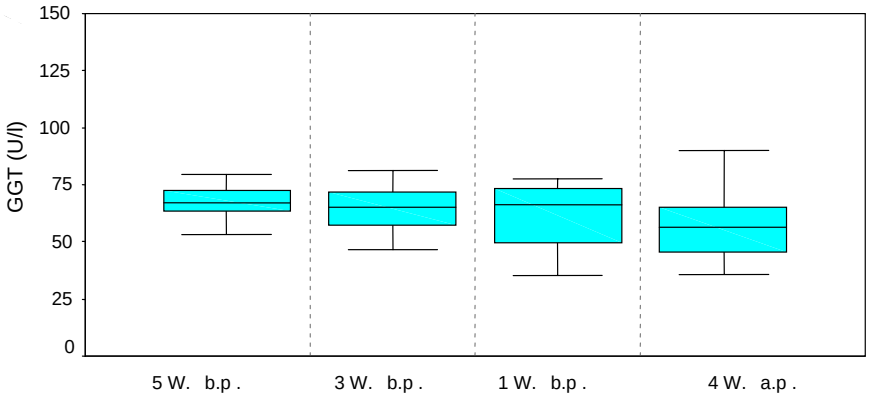

Figure 17: GGT activity (U/l) in the serum of ewes around parturition

4.2.9.2 GGT activity in Blackhead and Merino sheep

Figure 18 shows the variation of GGT activity of Blackhead sheep in comparison to Merino sheep. The statistical analysis of GGT showed no significant difference between Blackhead and Merino sheep in all periods of sampling.

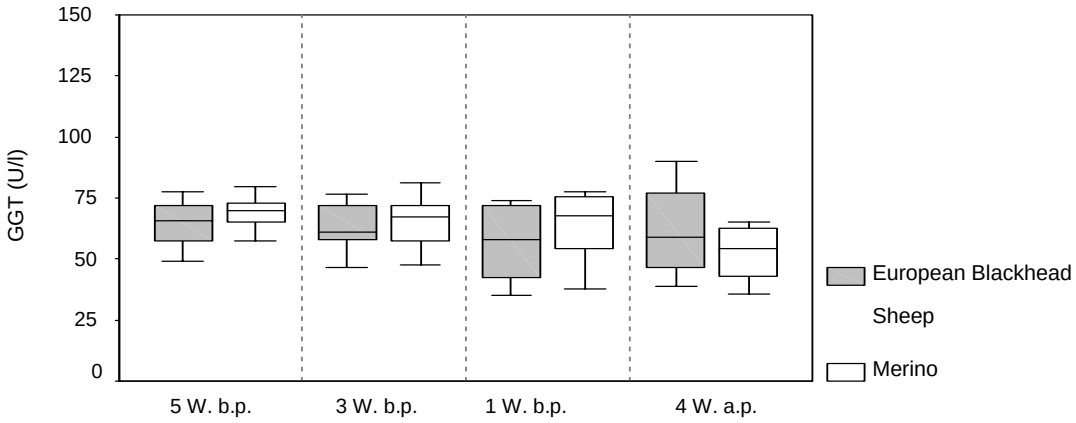

Figure 18: GGT activity (U/l) in the serum of Blackhead and Merino ewes around parturition

4.2.10 Glutamat-Dehydrogenase (GLDH), R.R. ≤ 19.8 U/l (ROSSOW and BOLDUAN, 1994)

4.2.10.1 GLDH activity in all sheep

In all periods of sampling in all sheep, the mean values of GLDH ranged within normal levels. GLDH activities were 6.5 U/l, 6.04 U/l, 7.02 U/l, and 21.32 U/l in period 1(5 w.b.p.), period 2 (3 w.b.p.), period 3, (1 w.b.p.), and period 4 (4 w.a.p.) respectively. Figure 19 shows the variation of GLDH activity of 29 pregnant ewes during the investigation periods.

4.2.10.2 GLDH activity in Blackhead and Merino sheep

Figure 20 shows the variation in GLDH activity in Blackhead sheep in comparison to that of Merino sheep. The statistical analysis showed that there was no significant difference between Blackhead and Merino sheep in all periods of sampling.

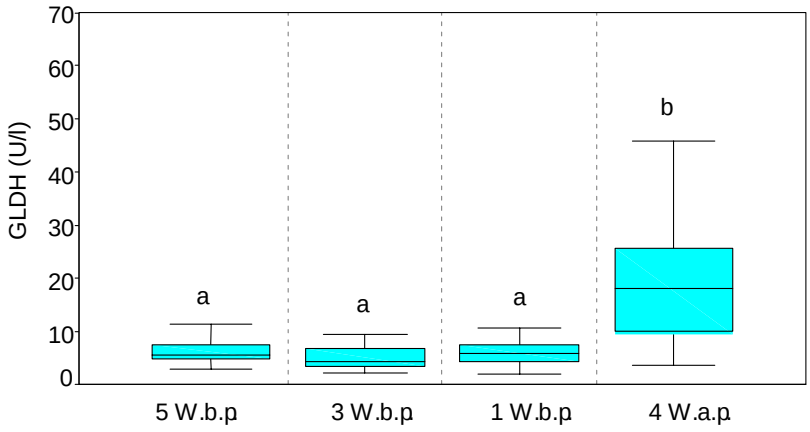

Figure19: GLDH activity (U/l) in the serum of ewes around parturition

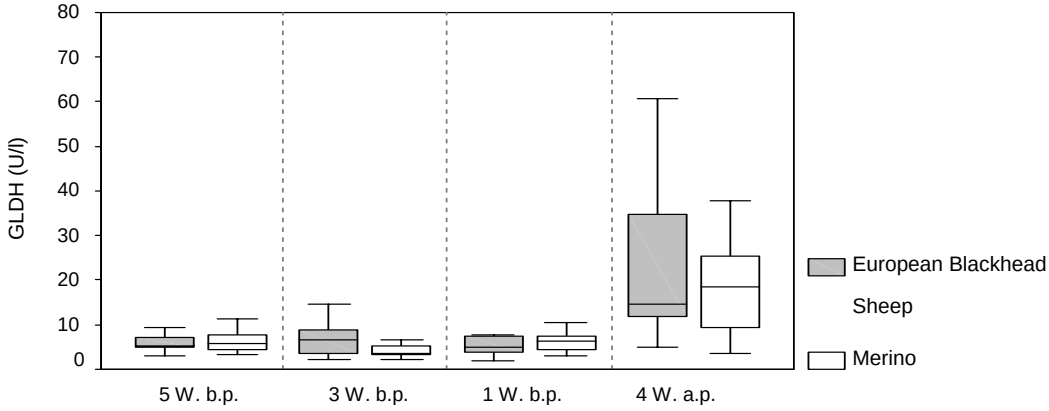

Figure 20: GLDH (U/l) in the serum of Blackhead and Merino ewes around parturition

4.2.11 Total protein, R.R. (60-79 g/l)

4.2.11.1 Total protein concentrations in all sheep

Figure 21 shows the variation of the total protein concentration of 29 pregnant ewes during the investigation time. The mean protein concentration were 7.19 g/l, 66.89 g/l, 52.9 g/l, and 47.67 g/l in period 1(5 w.b.p.), period 2 (3 w.b.p.), period 3, (1 w.b.p.), and period 4 (4 w.a.p.) respectively. These values indicate a slightly decrease in the protein concentration in the 3rd and 4th periods in comparison to the normal protein concentration.

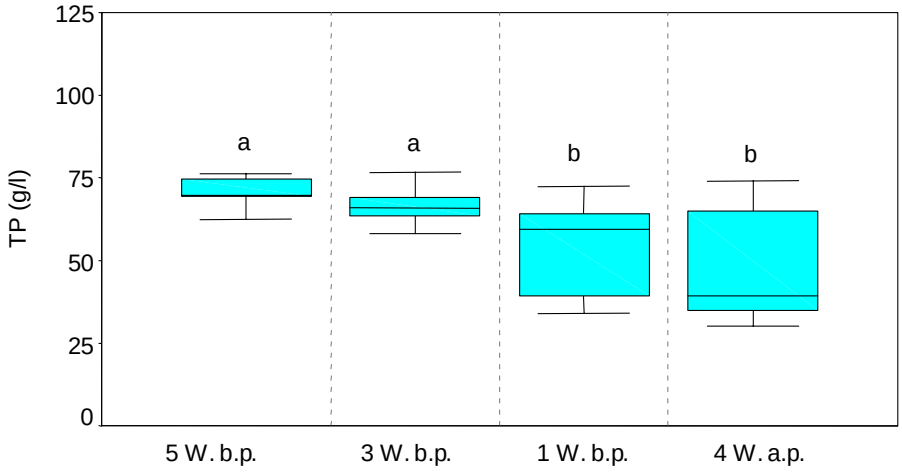

Figure 21: Total protein concentrations (g/l) in the serum of ewes around parturition

4.2.11.2 Total protein concentrations in Blackhead and Merino sheep

Figure 22 shows the variation of protein concentrations in Blackhead sheep in comparison to those of Merino sheep. The statistical analysis illustrated that there was no significant difference between Blackhead and Merino sheep in all sampling periods.

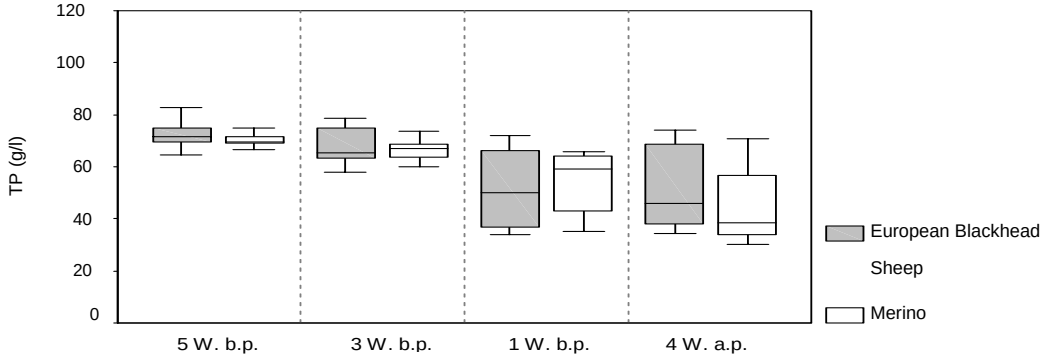

Figure 22: Total protein concentrations (g/l) in the serum of Blackhead and Merino ewes around parturition

4.2.12 Cholesterol, R. R. (1.5-2.5 mmol/l)

4.2.12.1 Cholesterol concentrations in all sheep

The mean values of cholesterol concentrations in all sheep were 1.93 mmol/l, 1.93 mmol/l, 1.6 mmol/l, and 1.3 mmol/l in period 1 (5 w.b.p.), period 2 (3 w.b.p.), period 3, (1 w.b.p.), and period 4 (4 w.a.p.) respectively. These concentrations indicate a slight decrease in the cholesterol concentration in period 3 (1 w.b.p.) and in period 4 (4 w.a.p.).

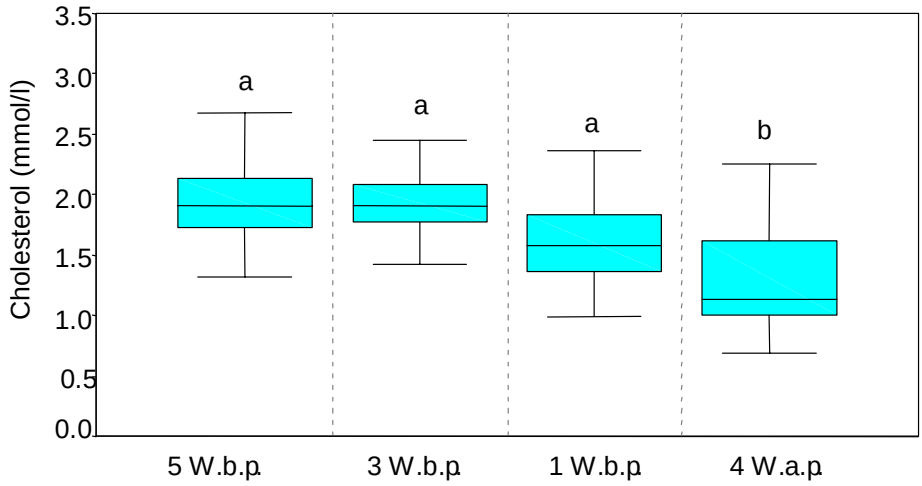

Figure 23: Cholesterol concentrations (mmol/l) in the serum of ewes around parturition

4.2.12.2 Cholesterol concentration in Blackhead and Merino sheep

Figure 24 shows the variations of cholesterol concentrations in Blackhead sheep in comparison to Merino sheep. The statistical analysis showed there was no significant difference between Blackhead and Merino sheep. On the other hand, there was a significant difference between period 3 from one side and periods 1 and 2 from the other side. Also there was significant difference between period 4 from one side and periods 1 and 2 from the other side.

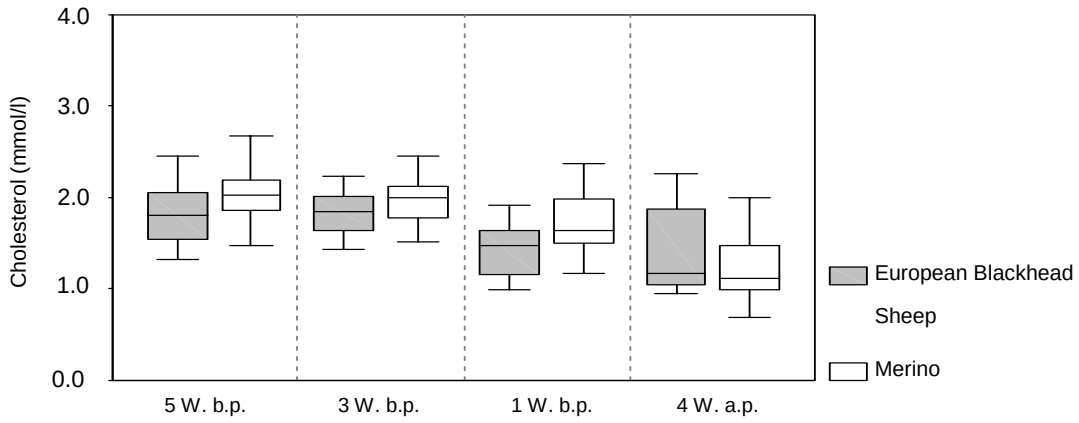

Figure 24: Cholesterol concentrations (mmol/l) in the serum of Blackhead and Merino ewes around parturition

4.2.13 Iron, R. R. (13-44 µmol/l, FÜRLL et al. 1981)

4.2.13.1 Iron concentration in all sheep

Figure 25 shows the variations in the iron concentrations of all pregnant sheep during the time of study. The mean values of iron concentrations were 47.2 µmol/l, 46.4 µmol/l, 40.7 µmol/l, and 16.5 µmol/l in period 1(5 w.b.p.), period 2 (3 w.b.p.), period 3, (1 w.b.p.), and period 4 (4 w.a.p.) respectively. These concentrations show a slight decrease in period 3 (1 w.b.p.), followed by a sharp decrease after parturition (4 w.a.p.).

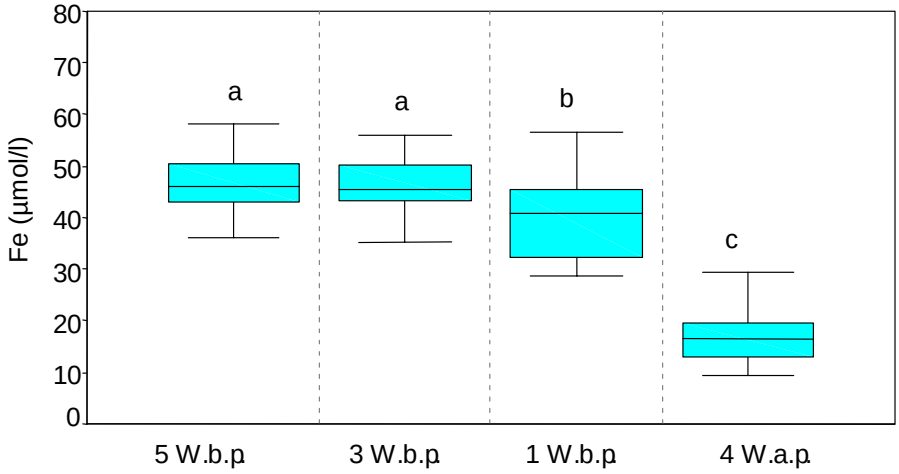

Figure 25: Iron concentrations (µmol/l) in the serum of ewes around parturition

4.2.13.2 Iron concentration in Blackhead and Merino sheep

Figure 26 shows the variation of the iron concentrations of Blackhead sheep in comparison to Merino sheep. The statistical analysis illustrated that there was no significant difference between Blackhead and Merino sheep in all sampling periods. However, regarding time of sampling, there were significant differences $p<0.05$ between the 1st, 3rd and 4th periods. There were also significant differences between the 2nd, 3rd, and 4th periods.

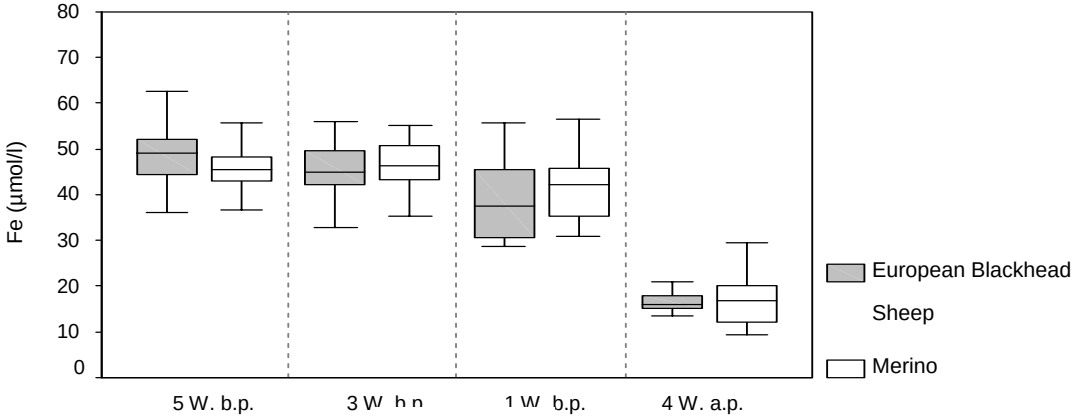

Figure 26: Iron concentrations (µmol/l) in the serum of Blackhead and Merino ewes around parturition

4.3 Haematological parameters

4.3.1 Haematological parameters in all sheep

Table 1 shows the mean values of different haematological parameters in 29 pregnant ewes before and after parturition.

Table 1: Haematological parameters of all sheep before and after parturition

Time	Hb (mmol/l)	Hk (l/l)	MCH (f mol)	MCV (f l)	MCHC (mmol/l)	THC (G/l)	LC (G/l)	RBC (T/l)
R.R.	5.6-9.3	0.27-0.4	0.6-0.7	28-40	19-23	280-650	5-11	7-11
5 w	8.47 ± 0.6	0.37 ± 0.17	0.78 ± 0.03	31.75 ± 1.9	24.5 ± 0.7	408 ± 143	5.5 ± 1.12	10.86 ± 0.69
3 w	7.9 ± 0.7	0.32 ± 0.03	0.76 ± 0.03	31.35 ± 1.8	24.3 ± 0.8	461 ± 162	5.4 ± 1	9.7 ± 2.5
1 w	7.57 ± 0.6	0.29 ± 0.02	0.8 ± 0.03	31.55 ± 1.5	25.3 ± 0.7	466 ± 154	5.8 ± 1.1	8.5 ± 2.5
4 w	6.9 ± 0.8	0.29 ± 0.03	0.7 ± 0.08	29.6 ± 6.3	23.4 ± 0.6	650 ± 148	6.08 ± 1.1	8.9 ± 2.4

4.3.2 Haematological parameters in Blackhead sheep

Table 2 shows the mean values of different haematological parameters in Blackhead sheep before and after parturition.

Table 2: Haematological parameters of Blackhead sheep before and after parturition

Time	Hb (mmol/l)	Hk (l/l)	MCH (f mol)	MCV (f l)	MCHC (mmol/l)	THC (G/l)	LC (G/l)	RBC (T/l)
R.R.	5.6-9.3	0.27-0.4	0.6-0.7	28-40	19-23	280-650	5-11	7-11
5 w	8.4 ± 0.6	0.4 ± 0.26	0.76 ± 0.04	30.3 ± 1.7	25 ± 0.7	448 ± 129.7	5.6 ± 1	11 ± 0.5
3 w	8.2 ± 0.5	0.33 ± 0.01	0.74 ± 0.03	30.03 ± 1.6	24.8 ± 0.7	520.7 ± 127.3	5.2 ± 1	9.4 ± 3.7
1 w	7.8 ± 0.58	0.3 ± 0.02	0.77 ± 0.03	30.3 ± 1	25.6 ± 0.8	490 ± 157	5.5 ± 0.6	9 ± 2.8
4 w	7.3 ± 0.65	0.3 ± 0.02	0.7 ± 0.03	29.7 ± 1.7	23.7 ± 0.6	641 ± 184.3	6 ± 1.2	9.4 ± 3.1

4.3.3 Haematological parameters in Merino sheep

Table 3 shows the mean values of different haematological parameters in Merino sheep before and after parturition.

Table 3: Haematological parameters of Merino sheep before and after parturition

Time	Hb (mmol/l)	Hk (l/l)	MCH (f mol)	MCV (f l)	MCHC (mmol/l)	THC (G/l)	LC (G/l)	RBC (T/l)
R.R.	5.6-9.3	0.27-0.4	0.6-0.7	28-40	19-23	280-650	5-11	7-11
5 w	8.5 ± 0.5	0.35 ± 0.02	0.7 ± 0.02	32.98 ± 1.1	24.2 ± 0.5	372.3 ± 148.5	5.5 ± 1.2	10.7 ± 0.7
3 w	7.7 ± 0.7	0.32 ± 0.036	0.7 ± 0.02	32.33 ± 1.2	24.09 ± 0.6	417.1 ± 175.2	5.6 ± 1	10 ± 1
1 w	7.3 ± 0.6	0.29 ± 0.02	0.8 ± 0.02	32.4 ± 1.1	25.1 ± 0.7	449 ± 157	6 ± 1.4	8.1 ± 2.4
4 w	6.6 ± 0.7	0.28 ± 0.03	0.7 ± 0.11	29.5 ± 1.2	23.1 ± 0.5	657 ± 115	6.2 ± 1.4	8.4 ± 1.4

All the haematological parameters ranged within normal levels in all periods of sampling (before and after parturition).

4.3.4 Statistical analyses of haematological parameters

4.3.4.1 Haemoglobin concentration (Hb)

There was significant difference ($p<0.05$) between Blackhead and Merino sheep in the 4th period (four weeks after parturition), while there was no significant difference in the other periods of sampling (before parturition). Furthermore, regarding time of sampling, there was a significant difference ($p<0.05$) between the 1st period (five weeks before parturition) and the other periods of sampling. There was no significant difference between the 2nd period (four weeks before parturition) and the 3rd period (one week before parturition). There was also a significant difference between the 4th period and the other periods of sampling.

4.3.4.2 Haematocrite (HK)

Statistical analysis showed that there were significant differences between Blackhead and Merino sheep in all periods of sampling. Furthermore, regarding time of sampling, there were significant differences between the 1st, 3rd, and the 4th periods. There was no significant difference between the 2nd period and the other sampling periods.

4.3.4.3 Mean corpuscular volume (MCV)

There were significant differences between Blackhead and Merino sheep in all period of sampling. Furthermore, regarding time of sampling, there was no significant difference between the varying periods of sampling.

4.3.4.4 Mean corpuscular haemoglobin (MCH)

There were significant differences ($p<0.05$) between Blackhead and Merino sheep in the 2nd, 3rd, and the 4th periods. There was no significant difference in the 1st period. Regarding time of sampling, there were no significant differences between the 1st, 2nd, and the 3rd periods. There were, however, significant differences between the 2nd and the 3rd period. There were also significant differences between the 4th period and the other periods of sampling (1, 2, and 3).

4.3.4.5 Mean corpuscular hemoglobin concentration (MCHC)

There were significant differences between Blackhead and Merino sheep in the 1st, 2nd, ands 4th periods. There was no significant difference in the 3rd period.

Regarding the time of sampling, there were significant differences between the 3rd periods and the 1st, 2nd, and 3rd sampling periods. There was no significant difference between the 1st and 2nd periods.

4.3.4.6 Thrombocytes count (THC)

There were no significant differences between Blackhead and Merino sheep in all periods of sampling. Regarding time of sampling, there was significant difference between the 4th periods and the other three periods of sampling.

4.3.4.7 Leukocytes (LC)

There was no significant difference between Blackhead and Merino sheep in all periods of sampling. Regarding the time of sampling, there was no significant difference between all periods of sampling.

4.3.4.8 Erythrocytes

There was significant difference between Blackhead and Merino sheep in the 3rd and the 4th periods. Regarding the time of sampling, there were significant differences between the 1st, 3rd, and the 4th periods.

Table 4: Degree of correlation between biochemical parameters and TNFα in Blackhead sheep before and after parturition

	Glucose (mmol/l)	BHB (mmol/l)	FFA (µmol/l)	Insulin (nmol/l)
BHB (mmol/l)	0.312**			
FFA (µmol/l)	0.442**	0.462**		
Insulin (nmol/l)	0.025	- 0.134	- 0.313	
TNFα (ng/ml)	- 0.079	0.278	0.320*	- 0.277

** Correlation is significant at the 0.01 level (2. tailed)

* Correlation is significant at the 0.05 level (2.tailed)

There was a significant positive correlation between the following:

- BHB and glucose
- FFA and BHB
- FFA and TNFα

There was, however, a negative correlation between fatty acids and insulin.

Table 5: Degree of correlation in Merino sheep between biochemical parameters and TNFα before and after parturition

	Glucose (mmol/l)	BHB (mmol/l)	FFA (µmol/l)	Insulin (nmol/l)
BHB (mmol/l)	0.309**			
FFA (µmol/l)	0.404**	0.544**		
Insulin (nmol/ml)	0.025	- 0.092	- 0.310*	
TNFα (ng/ml)	- 0.248	0.268	0.249*	- 0.170

** Correlation is significant at the 0.01 level (2. tailed)

* Correlation is significant at the 0.05 level (2.tailed)

There was a significant positive correlation between the following:

- β-hydroxybutyrate and glucose
- Free fatty acids and β-hydroxybutyrate
- Free fatty acids and TNFα

There was, however, a significant negative correlation between fatty acids and insulin.

5 Discussion

Method

The present study was directed in order to investigate the relationship between metabolic parameters and cytokine TNFα in late pregnant ewes. It was directed also to know whether TNFα plays a role in the pathogenesis of pregnancy toxaemia. The investigation was occurred in two sheep races (Merino and Blackhead), which are susceptible to pregnancy toxaemia (ketosis) in late gestation.

Characterization of metabolic parameters in the late pregnant ewes

The results of the glucose measurements showed a significant increase (4.2 ± 0.86 mmol/l) in period 1 (5 weeks before parturition), and declined with advancing gestation (2.9 ± 0.73 mmol/l) in period 3 (1 w. b. p.), see figure 1.

The obtained results were within normal levels of glucose (reference range 2.2-3.3 mmol/l, KRAFT and DÜRR 2005), and in agreement with those obtained by Al-MUJALLI et al. (2008), who recorded that blood glucose levels in healthy pregnant ewes was 2.7 ± 0.24 mmol/l, and in ewes suffering from pregnancy toxemia was 1.02 ± 0.14 mmol/l.

There are many other investigations showing low blood glucose levels in ketotic underfed ewes suffering from pregnancy toxemia (GALL and FEKETE 1982, BICKHARDT et al. 1989, SINGH et al. 1992, HENZE et al. 1994, ROUBIES et al. 2003, EL-DIN et al. 2005, SCHLUMBOHM and HARMEYER 2008).

These obtained findings illustrated the adaptation of maternal glucose metabolism during pregnancy, as glucose is a major limiting nutrient of fetal growth to meet the increasing fetal demands and maternal energy requirements of pregnancy (BATTAGLIA and MESCHIA 1988, Bell 1993, BELL and BAUMAN, 1997). It is believed that good nutrition in late pregnancy prevented sheep to supposed hypoglycemia and pregnancy toxemia (DURAK and ALTINER 2006).

The median values of insulin concentration in period 1 (5 w.b.p.) were 0.13 (0.08, 0.23) nmol/l (median, first, and third quartiles). Almost the same values were obtained in period 2 (3 w.b.p.) and period 3 (1 w.b.p.) recording 0.12 (0.09, 0.21) and 0.12 (0.09, 0.18) respectively. Then increased in period 4 (4 w.a.p.) to 0.22 (0.18, 0.36) nmol/l, see figure 3. These results agreed with those obtained by RICHTER (2000).

During the last third of gestation, the decreased insulin concentrations were postulated to be a result of the decreased response of pancreas to insulinotropic agents (LOMAX et al. 1979). Through the creation of an environment which supports minimizing peripheral glucose utilization and maximizing glucose extraction of the gravid uterus, reducing insulin secretion during pregnancy is proposed to be beneficial to fetal well being (CONNOLLY et al. 2004). Additionally, the sensitivity of peripheral tissues to insulin is reduced (HAY et al. 1988, PETERSON 1993).

In ketotic pregnant sheep, low insulin levels and therapeutic effect of insulin treatment support the hypothesis that insulin plays a causative role in the pathogenesis of ovine ketosis (HENZE et al. 1998).

The lipogenic effect of insulin is well recognized (BROCKMAN 1978). A drop in plasma insulin concentration triggers lipolysis in adipose tissue and elevates plasma free fatty acids concentration. Lipid mobilization is, however, not a major ketogenic stimulus without a contribution from the liver (MAYES and FELTS 1967).

Concerning the results of FFA, high concentrations were recorded in all periods of sampling before parturition; period 1 (5 w. b. p.), period 2 (3 w. b. p.), and period 3 (1 w. b. p.). The median values were 976 (544, 1160) µmol/l (median, first, and third quartiles); 759 (568.7, 1313.2) µmol/l; and 523.5 (387.5, 931.2) µmol/l, respectively. These results are comparable with R. R. < 600 µmol/l (KRAFT and DÜRR 2005). Free fatty acid concentration is significantly decreased in period 4 (4 w. a. p.), see figure 5.

The elevated results of FFA coincide with those recorded by SINGH et al. (1992), PETTERSON et al. (1994), EL-DIN et al. (2005), and KULSCAR (2006). The obtained finding explained that increased levels of cortisol (due to stress caused by pregnancy) lead to an increase of the FFA concentration (FLEMING 1997). Another reason for this situation may be due to increased mobilization of adipose tissue to supply FFA as an alternative maternal energy source to meet the energy requirements for a fast growing fetus (PETHICK et al. 1983, LLIOU and DEMARNE 1987, and BODEN 1996).

The mean values of BHB in all periods of sampling, period 1 (5 w. b. p.), period 2 (3 w. b. p.), period 3 (1 w. b. p.), and period 4 (4 w. a. p.) were 0.38 ± 0.13 mmol/l, 0.28 ± 0.1 mmol/l, 0.27 ± 0.22 mmol/l and 0.33 ± 0.1 mmol/l respectively. These values indicate normal levels of BHB before and after parturition, compared with subclinical ketosis (BHB > 1.0 mmol/l) or clinical ketosis BHB > 1.6 mmol/l (HENZE et al. 1994).

The results of haematological parameters (Hb, HK, MCH, MCV, MCHC, THC, and RBC) showed normal levels indicating that all pregnant ewes didn't suffer from anemia (GUPTA etal. 2008). The results of leukocytic count (LC) showed also normal levels and there was no significant correlation between leukocytic count and TNFα concentrations which may support that the measured TNFα increasement was due to the adipocytes. On the other hand, activation of Leukocytes occurs in case of pregnancy toxaemia accompanied with elevation of their TNFα production (SACKS et al. 1999).

Comparison between the Blackhead and Merino breed in the metabolic parameters in the late gestation

The results of glucose measurement illustrated that there was a significant difference ($p<0.05$) between Blackhead and Merino breed in period 1 (5 weeks before parturition), while there was no difference in the other periods of sampling, see figure 2. Measured insulin values showed that there was no significant difference between blackhead and merino breed in all periods of sampling, see figure 4.

The median values of FFA in Blackhead sheep were 671 (499.7, 1028) µmol/l (median, first, and third quartiles) in period 1 (5 w. b. p.); 621.5 (434.2, 761) µmol/l in period 2 (3 w. b. p.); and 339 (224.7, 543) µmol/l in period 3 (1 w. b. p.), see figure 6. For Merino sheep, the values of free fatty acids were 1041.5 (920.2, 1355) µmol/l in period 1 (5 w. b. p.); 920.5 (667.7, 1411.2) µmol/l in period 2 (3 w. b. p.); and 678.5 (516, 1127.2) µmol/l in period 3 (1 w. b. p.), see figure 6.

These finding showed significant increase ($p<0.05$) in the level of free fatty acid in Merino sheep than in Blackhead sheep before parturition, while there was no significant difference after parturition.
The obtained results of BHB revealed that there was a significant increase in the concentrations of BHB in Merino breed compared to Blackhead breed before parturition, while there was no significant difference after parturition, see figure 8.
The elevation of FFA and BHB concentrations in Merino breed before parturition can be explained by the increasing of the average numbers of Merino's lambs (2.18/ewe) in comparison to the average numbers of Blackhead's lambs (1.58 /ewe) which causing increase of the energy requirements for late pregnant multiparous Merino ewes to meet the fetal demands. This in turn results in the increase of lipolysis producing more FFA and BHB.

In Merino and Blackhead sheep there was a significant positive correlation between the following:

- BHB and glucose
- FFA and β-hydroxybutyrate
- FFA and TNFα

There was a significant negative correlation between FFA and insulin.
The obtained results indicate that these ewes did not suffer from subclinical pregnancy toxaemia, which was indicated by BHB concentration less than 0.7 mmol/l (MOGHADDAM and HASSANPOUR 2008).

Interaction between fat metabolism and TNFα

Classically, the immune, endocrine, neural, and metabolic systems were usually studied in isolation with the assumption that these systems operate more or less independently of each others. In recent years, however, the interconnected nature of all these control systems became well established (KELLY et al. 2000, DRACKLEY and ANDERSEN 2006). Thus, these systems work together to maintain homeostasis under the wide range of physical, pathological, and physiological states through which animals pass during their lifetimes, given the current understanding of the interdependency of all the detection and response systems in the body. It does not seem surprising that molecules traditionally thought of as products of the immune system (such as cytokines) may be produced by metabolic tissues such as adipose and muscle. They also can affect metabolic pathways traditionally thought to be only controlled via substrate and hormonal regulation of enzyme activity.

Adipose tissue produces a variety of adipocytokines that have been linked to insulin resistance in humans (WELLEN and HOTAMISLIGIL 2003). Of these, TNFα is one of the most studied (HOTAMISLIGIL et al.1993, GASIC et al.1998, RUAN et al. 2002). Fat ewes had greater plasma concentration of TNFα than thin ewes (DANIEL et al. 2003), resulting in increased lipolysis, and increased circulatory free fatty acids levels (GASIC et al. 1998, GREEN et al. 2004 and PLOMGAARD et al. 2008).

The measurements of TNFα showed elevated levels in all period of sampling before parturition. The median values of TNFα were 30.4 (17.2, 78.5) ng/ml (median, first, and third quartiles); 35.6 (13.6, 54.3) ng/ml; and 26.6 (13, 39.9) ng/ml in period 1(5 w.b.p.), period 2 (3 w.b .p.), and period 3 (1 w.b.p.) respectively. These values decreased to 19.1 (9.9, 33.8) ng/ml (4 weeks after parturition), see figure 9. There was a significant positive correlation between FFA and TNFα.

These finding may be confirmed by the results of investigation performed by JIANG et al. (2004), who recorded that the level of cytokine TNFα increased during pregnancy, and then subsequently decreased after parturition. This suggested that there is a

close relationship between cytokine TNFα and gestation, which play an important role in maintain gestation.

Cytokine TNFα may originate from various sources, including immune system cell macrophage, adipose tissue (GASIC et al. 1998), or placenta in which cytokine TNFα both induce maternal gestational insulin resistance and alter nutrient transport to the fetus. Maternal obesity enhanced the placental inflammatory response in ewes at mid gestation, possibly as result of increased free fatty acids (ZHU et al. 2010).

The role of TNFα in pathogenesis of pregnancy toxemia was examined by many investigators (GREER et al. 1994, KUPFERMINC 1994, VINCE 1995, BENYO 1997, CONRAD et al. 1997, CONRAD et al. 1998, HUNG et al. 2004, YARIM et al. 2007, LEWIS et al. 2009, and ZHU et al. 2010). They suggested that TNFα and other cytokines participate in the aetiopathogenesis of pregnancy toxemia via its effect on trophoblastic cell activation, which is a potential mediator of inflammatory response in pregnancy toxemia (ROBERTS et al. 1989). An excessive systematic inflammatory reaction and increased production of inflammatory cytokine occur in pregnancy toxemia.

From the recorded data, it can be concluded that fat mobilization occurred in the prepartum clinically healthy ewes with a significant increase in the levels of free fatty acids and also that there is an increase in the proinflammatory cytokine TNFα at late gestation, which predispose ewe to pregnancy toxemia, and can aid in the diagnosis of the disease. Further studies, based on ewes suffering from pregnancy toxemia, are needed to illuminate the potential effect of such cytokines in aetiopathogenesis of the disease.

6 Summary

Eman El-Ebissy

Relationship between Metabolic Parameters and TNFα in the Peripartal Period in Ewes

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Submitted in April 2011
74 pages, 26 figures, 5 tables, 104 references
Keywords: ewe, pregnancy toxaemia, fat metabolism, TNFα

Pregnancy toxaemia (ketosis) is a metabolic disease of ewes which occurs during the late gestation as a result of the inability of the pregnant ewe to maintain an adequate energy balance for the fast growing maternal fetal unit. As a result of energy deficiency mobilization of lipid reserves results in a doubling of the plasma free fatty acid (FFA) giving rise to fatty liver and increased ketone bodies β-hydroxybutyrate (BHB) in blood and urine. It is associated with a higher rate of mortality and causes severe economic losses.

The objective of this study was directed at investigating the relationship between metabolic parameters and cytokine TNFα, to check the interaction between the TNFα and fat metabolism in late pregnant ewes of different breeds, and whether TNFα play a role in the pathogenesis of pregnancy toxaemia, which may serve as marker to early diagnosis of the disease.

In this study, 29 pregnant and clinically healthy ewes (16 Merino, 13 Blackhead) were selected out of a flock of sheep. Blood samples were collected at 5, 3, and 1 week before parturition (b.p.) and also 4 weeks after parturition (a.p.). The average numbers of lambs were 2.18 and 1.58 /ewe for Merino and Blackhead breeds respectively. The blood samples were analyzed for the following:

- Concentration of metabolic parameters: glucose, insulin, free fatty acids (FFA), β-hydroxybutyrate (BHB), albumin, total protein (TP), iron (Fe), glutamat-dehydrogenase (GLDH), creatin kinase (CK), gamma-glutamyl-transferase (GGT), cholesterol, haptoglobin.
- Haematological parameters: Haematocrite (HK), haemoglobin concentration (HB), erythrocyte count (EC), leukocyte count (LC), mean corpuscular volume (MCV),

mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC).

- Cytokine TNFα by using ovine TNFα ELISA assay.

The results of glucose concentration of pregnant ewes showed significant increase (3.8 mmol/l) in five weeks b.p. and declined with advancing gestation (2.6 mmol/l) one week b.p. Insulin concentration remained constant with an average of 0.11 nmol/l b.p., and then significantly increased to 0.22 nmol/l four weeks a.p.

Maximal FFA concentrations were found at five weeks b.p. (976 µmol/l). The levels of FFA showed high levels b.p. compared with reference range (R.R. < 600 µmol/l), and the FFA levels significantly decreased postpartum (four weeks b.p.). while there was significant increasing ($p<0.05$) in the level of FFA in Merino sheep than in Blackhead sheep b.p. On the other hand there was no significant difference a.p.

The mean values of BHB in all periods of sampling, period 1(5 w.b.p.), period 2 (3 w.b.p.), period 3 (1 w.b.p.), and period 4 (4 w.a.p.) were 0.37 mmol/l, 0.23 mmol/l, 0.17 mmol/l and 0.3 mmol/l respectively. The mean of BHB indicated normal levels of BHB before and after parturition compared to subclinical ketosis (BHB > 1 mmol/l) and clinical ketoses (BHB > 1.6 mmol/l), and there was a significant difference ($p<0.05$) in the values of BHB between Blackhead and Merino breeds before parturition while there was no significant difference after parturition.

The concentration of TNFα showed elevated levels in all period of sampling before parturition. The TNFα values were 30.4 (17.2, 785.0) ng/ml (median, first, and third quartiles), 35.6 (13.6, 54.3), and 26.6 (13.0, 39.9) ng/ml in period 1(5 w.b.p.), period 2 (3 w.b.p.), and period 3 (1 w.b.p.) respectively. These values decreased to 19.1 (9.9, 33.8) ng/ml at 4 weeks after parturition.

Statistical analysis showed that there was a positive correlation between free fatty acids and TNFα. This correlation means that adipose tissue produces TNFα causing insulin resistance, which stimulates the lipolysis and leads to an increase of circulatory free fatty acids levels.

It is concluded that fat mobilization occurs in the prepartum clinically healthy ewes with a significant increase in the levels of FFA, and also there is an increase in the pro-inflammatory cytokine TNFα at late gestation which predisposes ewes to pregnancy toxaemia and can aid in the diagnosis of the disease.

7 Zusammenfassung

Eman El-Ebissy

Beziehungen zwischen Stoffwechselparametern und TNFα in der peripartalen Periode bei Mutterschafen

Medizinische Tierklinik der Universität Leipzig,
eingereicht im April 2011
74 Seiten, 26 Abbildungen, 5 Tabellen, 104 Literaturquellen
Schlüsselwörter: Mutterschafe, Trächtigkeitstoxikose, Fettstoffwechsel, TNFα

Die Trächtigkeitstoxikose (Ketose) ist eine Stoffwechselkrankheit der Mutterschafe, die in der Spätträchtigkeit infolge Imbalancen des Energiestoffwechsels der trächtigen Mutterschafe durch die schnell wachsenden Föten auftritt. Infolge des Energiemangels werden Fettreserven mobilisiert. Das führt zu einem Anstieg der FFS- (Freie Fettsäuren) Konzentrationen im Plasma, zur Entwicklung einer Fettleber sowie zum Anstieg der Ketonkörper einschließlich BHB (β-Hydroxybutyrat) in Blut und Harn. Dies geht mit einer höheren Mortalität sowie ökonomischen Verlusten einher.

Ziel der Studie war es, Beziehungen zwischen TNFα und Stoffwechselparametern in der Spätträchtigkeit von Mutterschafen, potentielle Unterschiede zwischen zwei Schafrassen sowie eine ätiologische Bedeutung von TNFα in der Pathogenese der Ketose zu prüfen.

Die Untersuchungen wurden an 29 klinisch gesunden Mutterschafen (16 Merinofleischschafe [MFS], 13 Schwarzköpfige Fleischschafe [SK]) durchgeführt. Blutproben wurden fünf, drei, und eine Woche vor (a.p.) sowie vier Wochen nach dem Lammen (p.p.) entnommen. Die durchschnittliche Zahl der Lämmer waren 2,18 und 1,58/Mutterschaf für MFS und SK Rasse bzw. In Serum bzw. Blut wurden untersucht:

- die Stoffwechselparameter: Glucose, Insulin, FFS, BHB, Albumin, Gesamteiweiß, Eisen (Fe), Glutamat-Dehydrogenase (GLDH), Creatinkinase (CK), Gamma-Glutamyl-Transferase (GGT), Cholesterol sowie Haptoglobin.
- Die hämatologischen Parameter: Hämatokrit (Hk), Hämoglobin (Hb), Erythrozyten (EC), Leukozyten (LC) sowie die erythrozytären Rechenwerte mittleres korpuskuläres Volumen (MCV), mittlere korpuskuläre Hämoglobinkonzentration der

Einzelerythrozyten (MCH) sowie die mittlere korpuskuläre Hämoglobinkonzentration (MCHC).

- das Zytokin TNFα mittels eines ovinen TNFα ELISA assay.

Die Glucosekonzentration der trächtigen Mutterschafe stieg signifikant bis auf 3,8 mmol/l zum Ablammen an und fiel auf 2,6 mmol/l eine Woche p.p. ab.

Die Insulinkonzentration blieb a.p. im Bereich von 0,11 nmol/l konstant, um bis vier Wochen p.p. signifikant auf 0,22 nmol/l anzusteigen

Die maximalen FFS-Konzentrationen wurden fünf Wochen a.p. (976 µmol/l) gefunden, d.h., sie lagen über dem Referenzwert von < 600 µmol/l. Nach dem Lammen fiel die FFS-Konzentration signifikant bis vier Wochen p.p. ab. Bei den MFS waren a.p. die FFS-Konzentrationen signifikant ($p<0.05$) höher als bei den SK; p.p. bestanden keine gesicherten Unterschiede.

Die BHB-Mittelwerte betrugen fünf Wochen a.p. 0,37 mmol/l, drei Wochen a.p. 0,23 mmol/l, eine Woche a.p. 0,17 mmol/l sowie vier Wochen p.p. 0,30 mmol/l, d.h., sie befanden sich sowohl vor wie auch dem Lammen im physiologischen Bereich. Es bestanden keine gesicherten Unterschiede zwischen MFS sowie SK.

Die TNFα-Konzentrationen waren mit 30,4 (17,2; 785,0) (Median; 1. und drittes Quartil) ng/ml fünf Wochen, mit 35,6 (13,6; 54,3) ng/ml drei Wochen und mit 26,6 (13,0; 39,9) ng/ml a.p. nichtsignifikant höher als mit 19,1 (9,9; 33,8) ng/ml vier Wochen p.p.

Die Korrelationsanalyse erbrachte mit r = 0,320 bei SK sowie r = 0,249 bei den MFS signifikant positive Korrelationen zwischen den TNFα- sowie FFS-Konzentrationen. Das unterstützt die These, dass das Fettgewebe via TNFα-Bildung eine Insulinresistenz mit der Folge gesteigerter Lipolyse und erhöhter FFS-Konzentrationen im Blut fördert.

Es kann geschlussfolgert werden, dass bei klinisch gesunden Mutterschafen am Ende der Trächtigkeit eine Fettmobilisierung mit gesteigerten FFS-Konzentrationen im Blut sowie gleichzeitig erhöhten Konzentrationen des proinflammatorischen Zytokins TNFα einsetzt. Diese Beziehungen können die Entstehung einer Trächtigkeitstoxikose begünstigen und zu diagnostischen Zwecken genutzt werden.

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Acknowledgements

I would like to take this opportunity to express my deep gratitude and heartfelt thanks to my supervisor Prof. Dr. habil. M. Fürll for giving me the opportunity to work on this scientific research, for his supervision and support throughout all stages of my Ph D programme till its completion. Thanks are due to his great efforts, planning, guidance in the course of scientific research and preparation of this thesis manuscript.

I'd like to express my deep thanks to Dr. G. Köller, Internal Medicine laboratory, Faculty of Veterinary Medicine at Leipzig University for his valuable help, and kind encouragement during this work.

I sincerely thank Dr. A. Richter, Faculty of Veterinary Medicine, Leipzig University for his help in statistical analysis of the data of this work.

Many thanks to Ms. K. Bauer, Institute for Immunology Faculty of Medicine at Leipzig University for her help in TNFα analysis.

I would also like to express many sincere thanks to prof. Dr. J. Gottschalk, professor of Physiology, Faculty of Veterinary Medicine at Leipzig University for help in insulin measurement.

Great gratitude to Dr.M. Hoops, Faculty of Veterinary Medicine at Leipzig University for both the facilities and help that he offered.

I'd like to express many thanks to Ms. A. Ruhland and Ms. C. Näther, laboratory of Internal Medicine, Faculty of Veterinary Medicine, Leipzig University for thier help in laboratory work during the period of study. Also many thanks to Ms. C. Thompson for her sincere help.

I’d like to express my deep thanks and great gratitude to Dr J. Jäger, Department of Veterinary Internal Medicine, Faculty of Veterinary Medicine, Leipzig University for her help and cooperation.

Many thanks and much gratitude to my colleagues in Internal Medicine Department, Faculty of Veterinary Medicine at Leipzig University.

I’d like to express my heartfelt gratitude to my husband, Dr. H. Khalil, for his help, cooperation, and kind encouragement.

Dedicated to my father’s soul, my mother, my husband, my sisters, my daughters Habiba and Hala, and to my son Abdallah.