Institut für Tierernährung
Bundesforschungsinstitut für Tiergesundheit des Friedrich-Loeffler-Instituts
&
Institut für Tierwissenschaften
Abteilung Physiologie und Hygiene der Rheinischen Friedrich-Wilhelms-Universität Bonn

---

Influence of body condition and dietary energy supply on lipolysis, ketogenesis and immune system of periparturient dairy cows with special regard to dipeptidyl peptidase-4

Inaugural - Dissertation
zur
Erlangung des Grades

Doktorin der Ernährungs- und Lebensmittelwissenschaften
(Dr. troph.)

der
Landwirtschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

von

Dipl. - Oecotroph. Kirsten Schulz

aus Güstrow

Referent: Frau Prof. Dr. Dr. Sauerwein

Korreferent: Frau PD Dr. Sarah Egert

Korreferent: Herr Prof. Dr. Dr. Dänicke

Tag der mündlichen Prüfung: 16.12.2015

Erscheinungsjahr: 2016

Abstract

The increased energy requirements and the decreased dry matter intake during late gestation and early lactation, in particular relative to the requirement, cause a catabolic state in the dairy cow, characterized by a distinct negative energy balance (NEB). During NEB increased concentrations in blood of non-esterified fatty acids (NEFA) are detectable. Subsequently, ketone bodies are produced in the liver. β-hydroxybutyrate (BHB) is the most stable ketone body in blood, therefore it is usually used to define a ketotic status, with 1.2 mM BHB in serum suggested as cut-off point for subclinical ketosis. The aim of the present dissertation was to induce subclinical ketosis in lactating cows to test potential therapeutical or prophylactic measures. Owing to a body condition score (BCS) of at least 3.0 six weeks before calving, a high energy diet *ante partum* (concentrate proportion of 60%) and a decreased energy supply *post partum*, 80% of the cows developed ketosis (81% subclinical, 19% clinical ketosis). The success of the animal model allowed for a well-controlled observation of different variables, related to metabolism and the immune system. Cows of subclinical ketosis had higher blood concentrations of NEFA, BHB and liver function enzymes, as well as higher liver lipid contents (e.g. 154 mg/g in the 2nd week *post partum*) than cows which were fed adequately and stayed metabolically healthy during the experimental trial. Moreover, subclinically ketotic cows had higher milk yields (Δ = 4.4 kg/day) and a fat:protein ratio in milk > 1.5, which also coincides a high lipolysis. Variables about immune function were slightly influenced by the ketotic metabolism. In *in vitro* tests peripheral blood mononuclear cells (PBMC) of cows not pre-stressed by ketosis *in vivo* responded more sensitively to increased concentrations of BHB as evidenced by both, the proliferative capability and the increased release of nitric oxide in the PBMC supernatant. The results indicate that even slightly diminished immune cell reactions might imply an immunosuppression during periparturient period and confirmed statements that beside NEFA, BHB also elicits effects on immune cells.

For further investigations and in relation to find new possibilities for ketosis therapy, a dipeptidyl peptidase-4 (DPP4) inhibitor (BI 14332) was tested. The inhibition of DPP4 via specific inhibitors is known to result in improved insulin sensitivity and decreased accumulation of hepatic fat in rodents and type II diabetic human patients. With first occurrence of serum BHB concentrations ≥ 1.2 mM BI 14332 was administered daily for 7 days (intravenous, 0.3 mg/kg body weight). The treatment did not alter the main markers of ketosis (BHB, liver lipid content), but NEFA and triglyceride concentrations in blood were decreased after the treatment. Therefore, a positive impact on the pathways of energy metabolism, resulting in a reduced NEB-induced lipolysis may be assumed. Furthermore, the results suggest that the DPP4 inhibition acts also at cellular level. The increase in the lymphocyte $CD4^+/CD8^+$ ratio for treated versus untreated cows was less strong and indicated that the peripartal immune dysregulation of ketotic cows may be positively modulated by BI 14332. A moderate correlation between NEFA and the $CD4^+/CD8^+$ ratio (r = 0.40) clarified how close changes at the biochemical level are connected to immune dysregulation.

Kurzfassung

Während der Transitphase weisen Milchkühe einen erhöhten Energiebedarf auf, wobei die Futteraufnahme gleichzeitig gesenkt ist. Es kommt verstärkt zum Abbau von Körperreserven, insbesondere von Depotfett. Die resultierende negative Energiebilanz (NEB) ist gekennzeichnet durch hohe Konzentrationen an nicht-veresterten Fettsäuren (NEFA) im Blut. Mit steigenden NEFA-Konzentrationen werden diese in der Leber oxidiert (β-Oxidation) und über das dabei entstehende Acetyl-CoA in Ketonkörper umgewandelt (Ketogenese). Ketonkörper werden von der Leber in den Blutkreislauf abgegeben und stellen eine Energiequelle für verschiedene Organe (z.B. das Gehirn) dar. β-Hydroxybutyrat (BHB) gilt als bedeutendster „Ketonkörper" und als Indikator für subklinische (BHB im Blut > 1.2 mM) und klinische Ketosen (BHB im Blut ≥ 3.0 mM). Ziel der vorliegenden Dissertation war die Schaffung einer Tiermodells zur Induktion subklinischer Ketosen bei der Milchkuh, um so unter kontrollierten Bedingungen den ketotischen Stoffwechsel und mögliche Auswirkungen auf die Immunantwort zu untersuchen. Dabei wurden Kühe mit einer Körperkondition (BCS) von mindestens 3.0 für die letzten 6 Wochen der Trächtigkeit mit einer hochenergetischen Futterration versorgt (7.7 MJ NE_L/kg Trockenmasse). *Post partum* erfolgte eine verzögerte Steigerung der Energiezufuhr um die postpartale Lipolyse zusätzlich zu stimulieren. Insgesamt wiesen 80% der Kühe eine Ketose auf (81% subklinisch, 19% klinisch) und zeigten höhere Blutkonzentrationen an BHB, NEFA und ausgewählten Leberenzymen als gesunde, normalkonditionierte Kühe der Kontrollgruppe. Subklinische Kühe gaben zudem mehr Milch (Δ = 4.4 kg/Tag), wobei der Fett/Protein-Quotient der Milch über 1.5 lag, was auf eine übermäßige Lipolyse hinweist. Gravierende Auswirkungen auf immunologische Parameter konnten nicht gezeigt werden, jedoch ließ sich aus den durchgeführten *in vitro*-Untersuchungen ableiten, dass bereits eine subklinische Ketose die Immunzellreaktionen vermindert und so das Risiko von Folgeerkrankungen erhöht sein kann.

Mit dem Ziel neue Therapiemaßnahmen bei Ketose zu entwickeln, wurde erstmalig der Einsatz eines Dipeptidylpeptidase-4 (DPP4) Inhibitors (BI 14332) getestet. Untersuchungen bei Nagern und Diabetes-Typ II-Patienten zeigen, dass durch eine DPP4-Inhibierung die Insulinsensitivität verbessert werden kann, während es in der Leber gleichzeitig zu reduzierten Fetteinlagerungen kommt. In der vorliegenden Dissertation wurden subklinisch ketotische Kühe über 7 Tage (1 x täglich) mit dem DPP4-Inhibitor behandelt (intravenös, 0.3 mg/kg Lebendmasse). Durch die Behandlung konnte der Gehalt an BHB im Blut und der Leberfettgehalt der Kühe nicht beeinflusst werden, jedoch waren die Konzentrationen an NEFA und Triglyceriden mit Behandlungsende reduziert. Die BI 14332-Behandlung lässt einen positiven Einfluss auf den Energiestoffwechsel vermuten, so dass die durch die NEB induzierte Lipolyse leicht reduziert war. Auch Einflüsse auf zellulärer Ebene sind vorstellbar. So weist ein weniger starker Anstieg des $CD4^+/CD8^+$-Quotienten bei T-Lymphozyten auf eine verbesserte Immunregulation im peripartalen Zeitraum hin. Letztlich verdeutlicht der positive Zusammenhang der NEFA-Konzentration im Blut mit dem $CD4^+/CD8^+$-Quotienten (r = 0.40) wie eng biochemische Veränderungen auch mit Dysregulationen des Immunsystem einhergehen und umgekehrt.

Contents

List of Abbreviations

ADA	adenosine deaminase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
a.p.	ante partum
BHB	β-hydroxybutyrate
BCS	body condition score
DPP4	dipeptidyl peptidase-4
DM	dry matter
GIP	gastric inhibitory peptide
GfE	Gesellschaft für Ernährungsphysiologie
GLP-1	glucagon-like peptide-1
HC	higher condition group
Hp	haptoglobin
IL	interleukin
LC	lower condition group
NEB	negative energy balance
NEFA	nonesterified fatty acids
NE_L	net energy for lactation
TG	triglycerides
TNF-α	tumor necrosis factor-α
PBMC	peripheral blood mononuclear cells
p.p.	post partum
PPAR	peroxisome proliferator-activated receptor
RQUICKI	revised quantitative insulin sensitivity check index

List of Tables

Paper III

List of Figures

Paper II

Paper III

1. Introduction

Ketosis is an important clinical or subclinical disease of the high yielding dairy cow, which occurs typically during early lactation. The pathogenesis of bovine ketosis is incompletely understood, but it requires the combination of intense adipose tissue mobilization with increased blood concentrations of free fatty acids and ketone bodies, as well as a high demand of glucose.

1.1 Ketogenesis and ketosis in dairy cows

To meet the requirements for maintenance and milk production, cows like other mammals are able to make use of two sources of nutrients – feed intake and body reserves. During ruminal microbial fermentation short-chain fatty acids are released. Of these, only propionate (one of the most abundant short-chain fatty acid with 15-40% of total released organic acids), valerate and isobutyrate can serve as glucogenic precursors for the net synthesis of glucose (Aschenbach et al., 2011; Larsen and Kristensen, 2009). If the feed intake is insufficient to meet the energy requirements, ruminal production is insufficient to provide glucogenic precursors. An efficient gluconeogenesis is most important in high-yielding dairy cows because it is the major pathway for maintaining an adequate glucose supply for the mammary gland (Reynolds et al., 1988). In case of hypoglycemia, the mobilization of non-esterified fatty acids (NEFA) and glycerol via lipolysis from adipose tissue is induced. NEFA and glycerol are transported to the liver through the bloodstream. β-oxidation takes place in the hepatic mitochondria, where NEFA are metabolized to acetyl-CoA. Hepatic ketogenesis results from a lack of oxaloacetate necessary to condense with acetyl-CoA to enter the citric cycle. Therefore, the excess of acetyl-CoA is converted into the ketone body acetoacetate within three steps. Acetoacetate enters the blood, or is reduced to β-hydroxybutyrate (BHB). The alternative path of acetoacetate is spontaneous decarboxylation to acetone (Berg et al., 2002). Tissues other than the liver (notably brain, skeletal and cardiac muscle) can use ketone bodies, but the accumulation exceeds the rate of peripheral utilization and increased values in blood are detectable. The resulting condition is called ketosis. The ketone bodies acetoacetate and BHB are excreted in milk and urine; acetone is primarily expired by the lungs (e.g. von Soosten et al., 2015).

Furthermore, feeding silage with high contents of butyric acid increases the risk of ketosis. This induced condition is sometimes called nutritional ketosis. Butyrate is a precursor of acetyl-CoA and is therefore ketogenic (Oetzel, 2007). Silages that are chopped too wet or that are low in

water-soluble carbohydrates favor growth of *Clostridium sp.* bacteria which ferment some carbohydrates to butyric acid instead of the desired lactic acid (Tveit et al., 1992).

BHB is more stable in blood than acetoacetate or acetone and is therefore often used to characterize the ketotic status of the dairy cow. Depending on the literature, the most commonly used cut-off point for ketosis is 1.2 mM or 1.4 mM BHB in blood serum. This kind of ketosis is called subclinical ketosis as usually no clinical signs occur. Clinical ketosis normally involves much higher concentrations of BHB, about 3 mM or more (Duffield, 2000; Oetzel, 2007).

Ketosis may be primary or secondary. Primary ketosis results as peripartal energy deficit and is therefore an integral part of the "fat cow syndrome", respectively it is caused by a lack of glucose during the first weeks of lactation. Secondary ketosis is the result of diseases causing the reduction in appetite, like displaced abomasum, metritis or mastitis (Dirksen et al., 2012).

1.1.1 The energy metabolism during early lactation

In dairy cows, the metabolism needs to be adapted to the regular cyclic course related to calving and lactation. In the last part of lactation and the beginning of the dry period, the cow is usually in an anabolic stage and from a few weeks before to about one month after calving in a catabolic state (Holtenius and Holtenius, 1996). Figure 1 refers to the negative energy balance (NEB) and the loss of appetite, which are prerequisites for the onset of ketosis soon after parturition. The Figure describes the relationship of metabolic imbalances and the onset of inflammatory processes with compromised immune competence.

In early lactation, glucose is taken up insulin-independently by the mammary gland. The ratio of growth hormone to insulin is high in blood, which induces mobilization of fatty acids from adipose tissue. Accompanying metabolic adaptations include increased hepatic gluconeogenesis, reduced glucose utilization by peripheral tissues and increased peripheral tissue utilization of NEFA and BHB. The liver re-esterifies NEFA to triglycerides (TG), which can be re-exported as part of very-low-density lipoproteins, but in condition of elevated hepatic NEFA uptake, TG accumulate. This feature contributes to a fatty liver. Hormones, such as adrenalin, noradrenalin and estrogen are positively associated with calving stress and support the increase of NEFA in blood and the synthesis of TG by the liver (Grummer, 1993). During fatty liver, metabolic imbalances become worse because hepatic ketogenesis, oxidative processes and gluconeogenesis from different gluconeogenic precursors are strongly decreased. The pancreatic insulin and

glucagon secretion and the peripheral glucose uptake are inhibited in cows with fatty liver (Bobe et al., 2004). Many of these cows had severe hepatic lipidosis, and insulin sensitivity decreased with increasing severity of ketosis (Ohtsuka et al., 2001). Especially cows selected for high milk yield are of risk to develop insulin resistance, which is also associated with greater body lipid mobilization and a lower body condition score (BCS) nadir (Roche, 2006; Smith and McNamara, 1989). Furthermore, increased resistance of adipose tissue to insulin initiates NEFA mobilization, hence potentially creating a vicious cycle of NEFA mobilization and reduction of dry matter intake (DMI) during the late prepartum period (Grummer et al., 2004).

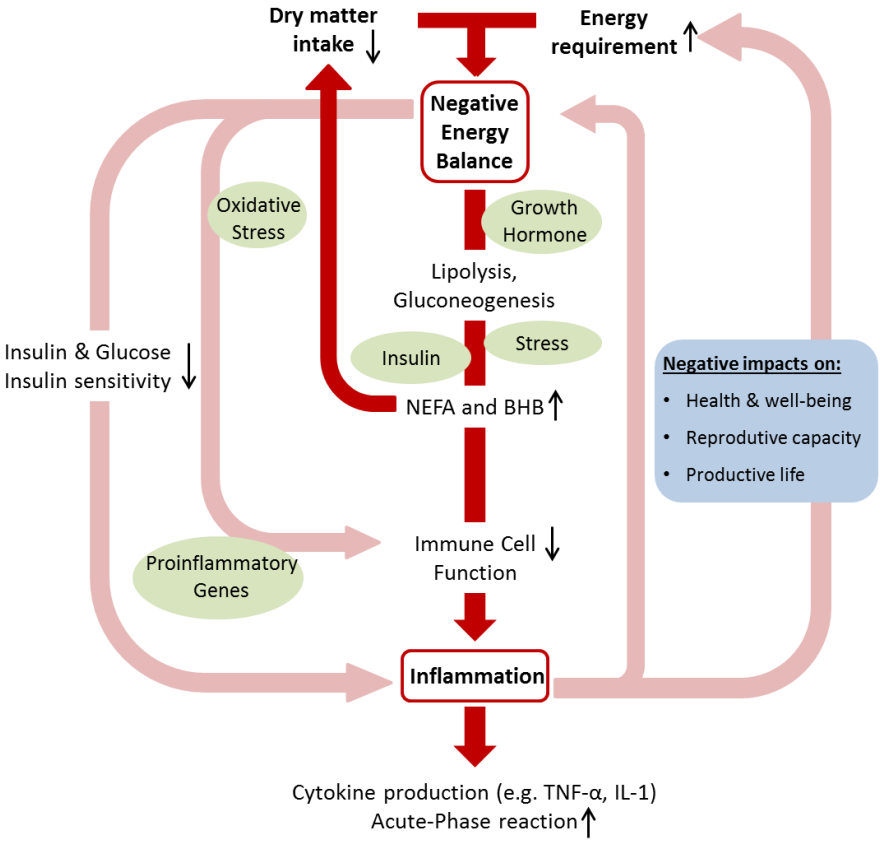

Figure 1. Major interactions between the immune, endocrine and metabolic system in dairy cows during the transition period (adapted from Esposito et al., 2014). Inflammation *post partum*, high cellular metabolism and upregulated immune gene expression increase energy requirements in case of a reduced dry matter intake. NEFA = non-esterified fatty acids, BHB = β-hydroxybutyrate, TNF-α = tumor necrosis factor-α, IL-1 = interleukin 1.

1.1.2 Immune-modulatory consequences of ketosis

According to LeBlanc (2010), ketosis may not have direct effects on the immune system but can indirectly affect the compromised immune response around calving. The metabolic effects of inflammation include adipose tissue mobilization, breakdown of liver glycogen, and liver TG accumulation. More specifically, tumor necrosis factor- α (TNF-α) is associated with hepatic lipidosis, impaired insulin sensitivity, and direct stimulation of lipolysis (Kushibiki et al., 2001; Ohtsuka et al., 2001). Further pro-inflammatory cytokines, such as interleukin (IL)-1 and IL-6, are known to trigger the acute phase reaction. Haptoglobin (Hp), as most important acute phase protein in cattle, is positively associated to NEFA and fatty liver (Saremi et al., 2012; Uchida et al., 1993) and a transiently higher body temperature around calving (Cooke and Bohnert, 2011). The production of reactive oxygen species in early lactating cows, accompanied by increased NEFA concentrations, stimulates excessive lipid peroxide formation, and both the transition to lactation and high body condition are associated with increased plasma markers of lipid peroxidation (Bernabucci et al., 2005). Reactive oxygen species are especially harmful to immune cells and can decrease the ability of the immune system to respond to infections (Spears and Weiss, 2008). Investigations of the lymphocyte function in case of ketosis were mainly focusing on direct effects of BHB and NEFA. In particular, high concentrations of NEFA decrease the proliferative capability of peripheral blood mononuclear cells (PBMC) and the production of interferon-γ, *in vivo* and *in vitro* (Scalia et al., 2006; Ster et al., 2012; Suriyasathaporn et al., 2000). For BHB, concentrations > 1.5 mM are considered critically with regard to the function of bovine myeloid and erythroid progenitor cells and to the oxidative burst activity of neutrophils (Hoeben et al., 1999; Hoeben et al., 1997). In contrast, even much higher BHB concentrations (up to 6.25 mM) did not suppress ConA-stimulated PBMC proliferation (Franklin et al., 1991) *in vitro*.

1.2 Incidence and relevance of subclinical ketosis

The primary risk period for hyperketonemia is commonly reported for the first two months of lactation (Duffield, 2000). More recent work showed that advances in genetics and feeding management have pushed the metabolic challenge closer to calving, namely in the first two weeks after calving (McArt et al., 2012a; Suthar et al., 2013). McArt et al. (2012a) reported an average incidence of subclinical ketosis of 43%, with a peak incidence at day 5 *post partum* (*p.p.*,

29%). In Western Europe the herd average of ketosis is 39%, with highest values in France (53%) and lowest in the United Kingdom and Italy (both 31%; (Berge and Vertenten, 2014). To briefly summarize, the early incidence of subclinical ketosis during the early lactation was found to affect 40 to 60% of cows in herds undergoing repeated testing (Asl et al., 2011; Duffield et al., 1998) and is much higher than the 2 to 15% incidence found with clinical ketosis (Duffield, 2000).

Cows with ketosis are at an increased risk of developing *p.p.* diseases, such as lameness, displaced abomasum, gastrointestinal disorders or metrits (Berge and Vertenten, 2014; Ospina et al., 2010a; Suthar et al., 2013). Each 0.1 mM increase in BHB concentrations in blood above 1.2 mM increases the risk of displaced abomasum by 1.1 and the risk for a herd removal by 1.4 (McArt et al., 2012b). Furthermore, subclinically ketotic cows have decreased milk yields with a high milk fat content and low milk protein in early lactation (Ospina et al., 2010b), as well as an impaired reproductive performance 50 to 100 days later, attributed to a delayed commencement of ovulation (Butler, 2003; Walsh et al., 2007). The negative impacts of subclinical ketosis result in an estimated economic loss of US $104 per case (McArt et al., 2014).

1.3 Prevention

Recommendations for prevention are focused on the nutritional management of the dry and the transition cow. Body condition should start to be managed in late lactation, when cows might become too fat. A BCS of 3.25 at calving is desirable to allow for adequate body fat reserves to support all energy requirements for maintenance, fetal growth and lactation while minimizing tissue energy mobilization and fatty acid infiltration to the liver (Ingvartsen and Moyes, 2013). Next to this, the avoidance of ketogenic feedstuffs and increased frequency of concentrate feeding are suggested as preventive measures (Gustafsson et al., 1995; Tveit et al., 1992). In addition to appropriate nutrition, feed additives might be useful to avoid ketosis. Well studied additives than reduce the risk of ketosis, are niacin (supplementation prior to calving), propylene glycol (prophylactical administration) or monensin (Duffield et al., 1998; Morey et al., 2011; Niehoff et al., 2009; Nielsen and Ingvartsen, 2004).

1.4 Therapeutic measures

1.4.1 General aspects

Treatment of ketosis aims at reestablishing normoglycemia and reducing serum ketone body concentrations. Administration (via intramuscular injection) of dextrose solution and glucocorticoids, like dexamethasone and isoflupredone acetate, also in combination with glucose as repeated measures, are common therapeutic options. According to Gordon et al. (2013), the most effective treatment is 300 mL of 100% propylene glycol orally once daily for 5 days. Cases of ketosis occurring during the first two weeks after calving are described as very refractory to therapy. Therefore, a long-acting insulin preparation may be beneficial, as insulin suppresses both adipose mobilization and ketogenesis (Gordon et al., 2013; Sakai et al., 1993). Nevertheless, the authors conclude, that there is still a lack of well-designed clinical trials for the treatment of ketosis.

1.4.2 Dipeptidyl peptidase-4 and new therapeutic concepts

Dipeptidyl peptidase-4 (DPP4), also known as CD 26, was discovered first in 1966 (Hopsu-Havu and Glenner, 1966). It is an intrinsic membrane-spanning glycoprotein and a serine exopeptidase, which prefers substrates with an amino-terminal proline or alanine at position 2. Dipeptidyl peptidase-4 plays a major role in glucose metabolism and is responsible for the degradation of the incretin hormones gastric inhibitory peptide (GIP) and glucagon-like peptide-1 (GLP-1; Drucker and Nauck, 2006). Both peptides reveal a highly conserved alanine at position 2.
Further action of DPP4 is amplified by the multitude of bioactive substrates which act as biochemical messengers in many tissues, including the immune and the neuroendocrine system.

1.4.2.1 The incretin metabolism and the enzymatic activity of DPP4

The term “incretin effect” was originally used to describe the fact that an oral glucose load produces a greater insulin response than an isoglycemic intravenous glucose infusion. The difference between the two kinds of glucose administration was attributed to the incretin hormones GIP and GLP-1. The corresponding G-protein-coupled receptors are expressed on pancreatic α- and β-cells and different peripheral tissues, such as central nervous system, gastrointestinal tract, heart and lungs. Its activation increases cyclic adenosine monophosphate

(cAMP) levels and insulin secretion in a glucose dependent manner. A sustained incretin binding to the receptor increases insulin biosynthesis via activation of protein kinase A. In addition, GLP-1 suppresses glucagon release from α-cells, which reduces the hepatic glucose production (Kazafeos, 2011). Nauck and colleges demonstrated a reduced incretin effect during type II diabetes and showed a preserved action of GLP-1 and a diminished GIP responsivity to glucose in clinical models of type II diabetes (Nauck, 1998; Nauck et al., 1993).

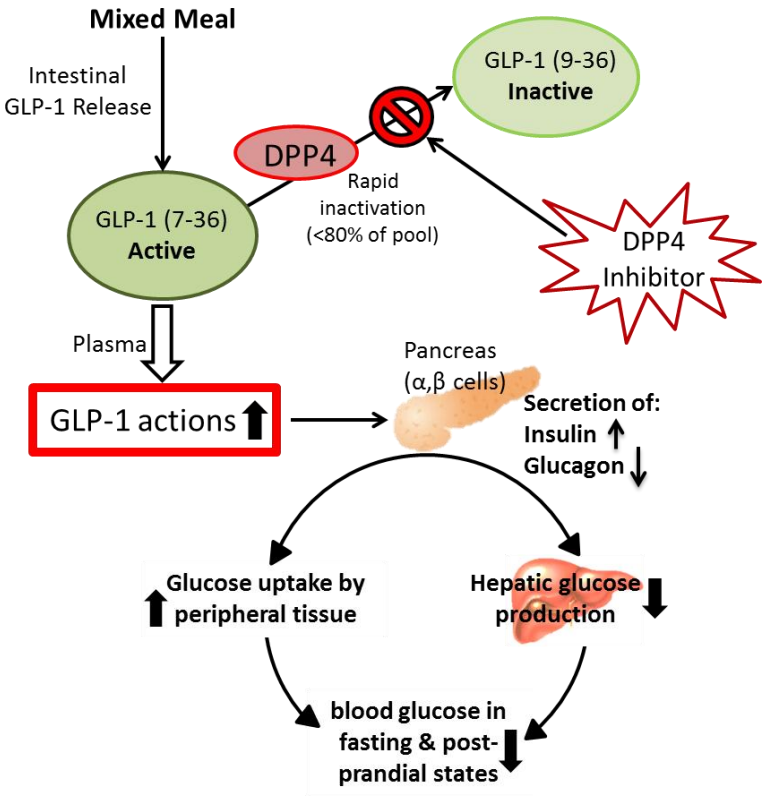

Figure 2. Secretion and metabolism of glucagon-like peptide-1 (GLP-1; adapted from Cefalu, 2010). After food ingestion, GLP-1 is released by the small intestine in its active form (7-36) in plasma, which is rapidly degraded to the inactive form (9-36) by the enzyme dipeptidyl peptidas-4 (DPP4). Incretin therapy can increase available GLP-1 activity by the use of selective DPP4 inhibitors. GLP-1 acts on the pancreatic α- and β-cells to increase insulin release and to suppress glucagon release, than decreasing hepatic glucose production.

Owing to this important issue, type II diabetes therapy aims to a continuous concentration of active GLP-1, circulating in blood in form of GLP-1 (7-36). It is released from the small intestine. Mentlein et al. (1993) were the first who identified DPP4 as the enzyme which is responsible for the enzymatic degeneration of GLP-1 *in vitro*. Within minutes of eating, 80 – 90

% of total plasma GLP-1 is inactive (= GLP-1 (9-36); Deacon et al., 1995). Therefore, incretin based therapies show great promise for the treatment of type II diabetes (Juillerat-Jeanneret, 2014; White, 2008). Figure 2 shows the mechanism of active GLP-1 in consideration of DPP4 inhibition, like it is well studied in rodents and humans.

Relling and Reynolds (2007) were the first who detected GLP-1 and GIP in plasma of cows, with significantly increased concentrations after calving, while insulin and glucose concentrations were lower *p.p.* than *ante partum* (*a.p.*). Similar to monogastrics, dietary energy particularly from unsaturated fatty acids has been shown to enhance the secretion of GLP-1 in steers (Taylor-Edwards et al., 2010). In dairy cows, the concentration of GLP-1 was unaffected by abomasal infusion of glucose (Larsen et al., 2010), but was increased after infusion of soybean oil and casein (Relling and Reynolds, 2008), respectively by feeding a diet with added unsaturated fatty acids (Bradford et al., 2008). The lipid-induced elevation in circulating GLP-1 is believed to play a role in the short-term control of feed intake in cattle (Bradford et al., 2008; Relling and Reynolds, 2008), but the wide range of tissues expressing the GLP-1 receptor (multitude of gut segments, pancreas, spleen and kidney) suggest that GLP-1 may have multiple physiological functions beyond the control of feed intake (Pezeshki et al., 2012). Expression of bovine DPP4 shows a pattern of expression similar to that in humans, with highest values in jejunum and ileum (Connor et al., 2010). Studies show that the DPP4 expression and circulating GLP-1 concentrations in blood are strongly regulated by the stage of lactation. Plasma concentrations of GLP-1 increase after calving (Relling and Reynolds, 2007). The DPP4 mRNA expression is lowest in prepubertal heifers, greatest in nonlactating cows, and intermediate among lactating cows (Connor et al., 2010).

1.4.2.2 DPP4 and its role in immune function

Next to GLP-1, there is a multitude of substrates for DPP4, e.g. various chemokines, neuropeptide Y, growth hormone releasing factor, insulin-like growth factor and prolactin (Schuppan et al., 2010). The cell surface expression of DPP4 is upregulated in activated human lymphocytes and correlates with the production of interferon-γ (Salgado et al., 2000).

In 1993, DPP4 was identified as binding protein for adenosine deaminase (ADA). The ADA catalyzes the irreversible deamination of adenosine to inosine and is part of the cellular and humoral immunity. It is expressed in all tissues and mainly on T-lymphocytes. The model of

Morimoto and Schlossman (1998) describes a DPP4-ADA-adenosine-interaction in which DPP4 modulates the concentration of extracellular adenosine, which provides negative signals to T-cells (De Meester et al., 1999; Morimoto and Schlossman, 1998). Moreover, Yu et al. (2011) showed that DPP4 is able to enhance lymphocyte proliferation of humans *in vitro*, even independently of enzyme activity and ADA-binding. Fischer 344 CD26-deficiet rats have markedly reduced numbers of $CD4^+$ T-cells and a decreased T-cell recruitment compared to wild-type F344 (Kruschinski et al., 2005). In CD26 (-/-) mice, the cytokine response to pokeweed mitogen is altered with reduced concentrations of IL-2 and IL-4, as well as delayed production of interferon-γ (Yan et al., 2003). In brief, DPP4 contributes to the regulation, maturation and migration of $CD4^+$ T-cells, natural killer cells, cytokine production, T-cell dependent antigen production and immunoglobulin isotype switching of B-cells (Gorrell et al., 2001; Yan et al., 2003).

1.4.3 Use of DPP4 inhibitors and general health claims

Incretin-based therapies include both GLP-1 receptor agonists (administered subcutaneously) and DPP4 inhibitors (administered orally). There are of great scientific interest to treat type II diabetes in human patients. The first DPP4 inhibitor was developed and tested in the late 1990s in animal models. According to the literature, isoleucine thiazolidide, vildagliptin, NVP-DPP728 and valine-pyrrolidide were used to inhibit DPP4, mainly in rodents. These agents improved glucose tolerance, induced greater levels of GLP-1(7-36), reduced body weight gain, potentiated the plasma insulin response to intra-gastric glucose and increased hepatic and peripheral insulin sensitivity (Flatt et al., 2008).

In clinical development the inhibitors have received the class name of “gliptins” or “incretin enhancers”. The first DPP4 inhibitor for clinical use to treat type II diabetes was sitagliptin, which was launched in Mexico in 2006 and in the United States and Germany in 2007 (Flatt et al., 2008). Today, vildagliptin, saxagliptin and alogliptin are further DPP4 inhibitors but are still under development. A multitude of studies demonstrates positive, anti-diabetic effects with enhanced postprandial and fasting glycemic control, as well as a possible improvement and preservation of the β-cell function and mass (Neumiller, 2009). The named inhibitors provide a selective and nearly complete inhibition of DPP4 for >12 h (Flatt et al., 2008).

Because type II diabetes is also known as a low grade systemic inflammatory disease, especially in patients with metabolic syndrome and/or atherosclerosis, studies focused more and more on

the anti-inflammatory effects of DPP4 inhibitors. These results are contradicting. Due to the inhibition of DPP4, the production of proinflammatory cytokines (e.g. IL-1 β, IL-6, IL-8, TNF-α) is reduced, *in vitro* and *in vivo* (Rizzo et al., 2012; Ta et al., 2011). In contrast, Anz et al. (2014) found no influence of sitaglipitin, saxagliptin and vildagliptin on the immune response in terms of cytokine production (IL-6, IL-10, IL-12, interferon-γ), co-stimulation (e.g. molecules of major histocompatibility complex class II) and T-cell proliferation or migration.

2. Scope of the thesis

Taking the background information into consideration, DPP4 is a key enzyme in intermediary metabolism by regulating important glycemic pathways. At the same time DPP4 shows immune modulatory characteristics, especially at a cellular level. The ketotic metabolic status of high yielding dairy cows during early lactation exhibits parallels with the metabolic situation in human patients with type II diabetes and fatty liver. Therefore, DPP4 inhibitors might counteract typical ketotic processes in the dairy cow. In the present research and in cooperation with Boehringer Ingelheim, a DPP4 inhibitor (BI 14332) was tested for its effects on bovine ketosis. The experimental trail was conducted to induce an artificial ketotic status in Holstein Friesian cows for treating them subsequently with the named DPP4 inhibitor.

- Overfeeding in the dry period and a reduced feed intake *p.p.* result in a distinct NEB and an excessively stimulated lipomobilisation. With the onset of lactation, cows will enter a ketotic metabolic status, characterized by BHB concentrations in blood serum ≥1.2 mM (**Paper I**).
- The ketotic metabolism, in particular BHB as most important component, causes immune-modulatory alterations (**Paper II**).
- The treatment of bovine ketosis by BI 14332 adjusts elevated variables of the fatty liver syndrome and ketosis to the physiological range. In consequence hepatic lipidosis is reduced (**Paper III**).
- The inhibition of DPP4 via BI 14332 protects against inflammatory processes and influences the immune response (**Paper III**).

To investigate the effects of DPP4 inhibition via BI 14332 during ketosis, first the induction of a ketotic status was necessary, realized by a feeding trial with 30 primi- and multiparous cows. The experimental period started six weeks before the expected parturition until the 56th day in milk. With first occurrence of serum BHB ≥ 1.2 mM, BI 14332 was administered (intravenously, daily, for 7 days).

3. Paper I (Published in Journal of Dairy Research, 2014, 81:257-266)

Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: An animal model to investigate subclinical ketosis

Kirsten Schulz[1], Jana Frahm[1*], Ulrich Meyer[1], Susanne Kersten[1], Dania Reiche[2], Jürgen Rehage[3] and Sven Dänicke[1]

[1]Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, D-38116 Braunschweig, Germany
[2]Boehringer Ingelheim Vetmedica, D-55216 Ingelheim am Rhein, Germany
[3]Clinic for Cattle, University of Veterinary Medicine, Bischofsholer Damm 15, D-30173, Germany

[*] For correspondence; e-mail: Jana.Frahm@fli.bund.de

Abstract

Subclinical ketosis is a metabolic disorder which often goes undiagnosed and leads to constricted performance and an impairment of the general condition. In the current study subclinical ketosis was characterized by a β-hydroxybutyrate (BHB) concentration of > 1.2 mmol/l in blood serum. To generate this metabolic situation, an animal model was created. The model, based on group-specific interaction of dietary energy supply and body condition, is appropriate for testing medical effectiveness to treat this kind of ketosis and its concomitants. During the trial, 18 dairy cows (primiparous and pluriparous) were assigned, according to their body condition score (BCS) 6 weeks before expected parturition, to a normal [6.78 MJ net energy for lactation (NEL)/kg dry matter; 20% concentrate] and a high-energy feeding group (7.71 MJ NEL/kg dry matter; 60% concentrate). Therefore cows with the highest BCS were allocated to the high-energy group to enhance the contrast to the control group. Statistical analysis was done using the MIXED procedure of SAS. Effects were declared significant when P-values were ≤ 0.05. Owing to the higher energy concentration and dry matter intake, the energy intake and balance was significantly higher in the high-energy feeding group, with strong effects on lipid metabolism and health in blood and liver post partum. Within the first 2 weeks after calving, 8 out of 9 cows (89

%) of the high-energy group had BHB values indicative of subclinical ketosis. These cows also had significantly higher values of non-esterified fatty acids (NEFA), aspartate transaminase (AST) and glutamate dehydrogenase (GLDH) post partum, as well as a raised total lipid content of the liver. RQUICKI, a calculated parameter which is based on serum concentrations of glucose, insulin and NEFA to assess the insulin sensitivity, was not affected by treatment. Therefore, RQUICKI does not seem to be the right parameter for diagnosing decreased insulin sensitivity in cows affected by subclinical ketosis. The milk fat and the fat:protein ratio of the high-energy group was also higher, even though there was no decrease in milk yield for cows with subclinical BHB values.

Keywords: Animal model, dairy cow, body condition score, subclinical ketosis, hepatic lipidosis, energy balance.

Introduction

It is well established that in early lactation, the available metabolisable energy (ME) from dry matter intake (DMI) is not sufficient to satisfy the energy requirements of the dairy cow. The required energy for milk (E_L) and maintenance (E_M) cannot be covered only by feed intake alone (Bauman & Currie, 1980). Owing to early lactation energy balance, the dairy cow mobilises energy from body mass (mainly fat reserves), resulting in increased lipolysis. Differences from 8-56 kg of body fat mobilisation were observed in Dutch and Holstein Frisian cows during the first 8 weeks after calving (Tamminga et al., 1997). Such a strong fat mobilization induces an imbalance of the fat- and carbohydrate metabolism with a typical increase of ketone bodies and the possibility for its manifestation as subclinical or clinical ketosis. According to the literature, studies using BHB to define a subclinical ketosis, report ranges of values from 1.0 mmol/L to 1.4 mmol/l as threshold (Duffield, 2000). Dirksen et al. (2012) summarised the literature and defined a level of < 1.2 mmol BHB in blood serum as physiological, characterized by a balanced energy supply. So ketogenesis during early lactation, produced in a range of < 1.2 mmol/l BHB is a considerable metabolic pathway to compensate the insufficient intake of glucose precursors, while increasing concentrations up to 3 mmol/l are defined as subclinical ketosis (Dirksen et al., 2012; Duffield, 2000). Recently, an average incidence of subclinical ketosis of 43% was reported, with a peak incidence 5 d after calving (McArt et al., 2012).

In addition, ketosis is associated with hepatic lipidosis. The extent of the negative energy balance (NEB) and the body condition during dry period determine the potential development of a fatty liver, which results as an increased hepatic uptake of non-esterified fatty acids (NEFA) from blood. The NEFAs are mobilized from adipose tissue, in an amount greater than needed, so that the excess is transported to the liver (Bobe et al., 2004), inducing a fatty liver with an impairment of the general condition in dairy cattle. In particular the body condition score (BCS) is still an object of research for the prevention of excessive negative metabolic changes in dairy cows, as it is a parameter which shows good correlation with increased risk of fatty liver or ketosis (Bernabucci et al., 2005; Bewley & Schutz, 2008; Roche et al., 2013).

Current publications describe the changes in blood profile in regard to ketosis and hepatic lipidosis over a defined period around calving (Asl et al., 2011; Gonzalez et al., 2011; Stengarde et al., 2011). Next to this, in Europe, a monensin slow-release bolus was recently permitted as a ketosis prophylactic measure which hints at the practical relevance of this disease (Day, 2013). The current literature and the continuing interest in metabolic changes during early lactation, underline the usefulness of the present work.

The object of the study was to create an animal model which induces BHB values indicative for subclinical ketosis (BHB in blood > 1.2 mmol/l) due to a heightened lipomobilisation, and to monitor how this physiological condition affects bovine metabolism. We hypothesize that the combination of overfeeding in the dry period and a reduced feed intake post partum will result in a NEB that will cause ketosis. With the model the relationship between blood profile and metabolic diseases can be elucidated, with the possibility of showing pharmacological effects to medicate subclinical ketosis and the fatty liver syndrome. The continued productivity of high-yielding dairy cows and the still existing clinical picture of ketosis, especially with regard to pluriparous cows during transition period, account for the importance of prophylaxis and therapy.

Materials and methods

Experimental design

The experiment was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI) in Braunschweig, Germany. In the study 18 pregnant and healthy German Holstein cows, 10 pluriparous and 8 primiparous, were selected according to BCS (Edmonson et al., 1989), using a 5-point scale. Nine cows (5 pluriparous, 4 primiparous)

with a BCS of 2.61 ± 0.09 were selected to a normal condition group (NC), acting as a control group, and 9 cows (5 pluriparous, 4 primiparous) with a BCS of 3.19 ± 0.09 were selected to the experimental group, called higher condition group (HC). To enhance the contrast between control- and experimental group, the cows with the highest BCS were allocated to the experimental group. The BCS at the time of classification between these two groups was statistically different ($P < 0.01$) and was then established weekly. The experimental period started 6 weeks before expected parturition and continued until the 56th day after calving.

Table 1. Ingredients and chemical composition of concentrate and total mixed ration of the pre-partum diet

	Concentrate	TMR[†]	
Ingredients, %			
Wheat	41.0		
Dried sugar beet pulp	30.5		
Rapeseed meal	20.0		
Soybean meal	6.5		
Vitamin/mineral premix[§]	2.0		
		HC[‡]	**NC**[‡]
Dry matter (DM), g/kg	877	489	384
Nutrients [g/kg DM]			
Crude ash	58	55	51
Crude protein	197	140	117
Ether extract	27	33	35
Crude fibre	101	163	201
Acid detergent fibre (ADF)	136	199	231
Neutral detergent fibre (NDF)	279	394	429
Energy[¶], MJ NEL/kg DM	8.6	7.7	6.8

[†]Total mixed ration on DM basis (75% corn silage, 25% grass silage on DM basis)
[§]High condition (HC) cows were fed a concentrate proportion of 60% in the diet; Normal condition (NC) cows were fed a concentrate proportion of 20% in the diet
[‡]Per kg of mineral feed: 10g Ca, 60g P, 120g Na, 60g Mg, 800,000 IU vitamin A, 100,000 IU vitamin D_3, 2500mg vitamin E, 4000mg Mn, 6000 mg Zn, 1250mg Cu, 100mg I, 35mg Co, 50mg Se
[¶]Calculation based on nutrient digestibilities masured with wethers (GfE, 1991) and values from feed tables (DLG, 1997)

Prepartum, the cows of NC group were fed with an energetically adequate ration, based on the recommendations of the German Society of Nutrition Physiology (GfE, 2001), of 80% roughage (75% corn silage and 25% grass silage based on DM content) and 20% concentrate, whereas the cows of HC group where fed a ration, consisting of 40% roughage and 60% concentrate which led to an energetic oversupply (Table 1). After calving, all animals were initially fed with a standardized TMR for lactation, consisting of 30% concentrate. The ingredients and chemical composition for the lactation TMR are given in Table 2. Immediately after calving, the

concentrate proportion was raised stepwise, applied by a computerized feeding station (Insentec, B.V., 1274 Marknesse, The Netherlands), from 30% to 50% within the first 2 or 3 weeks for NC or HC group, respectively. The initialized increase occurred more slowly in the HC group (Figure 1), with the additional aim to stimulate postpartal lipolysis. Cows were fed ad libitum and had free access to water.

Table 2. Ingredients and chemical composition of concentrate and total mixed ration during lactation period

	Concentrate	TMR[†]
Ingredients (%)		
Wheat	41.0	
Dried sugar beet pulp	30.3	
Rapeseed meal	20.0	
Soybean meal	6.5	
Vitamin/mineral premix[‡]	2.0	
Calcium carbonate	0.2	
Dry matter [g/kg]	875	393
Nutrients [g/kg DM]		
Crude ash	62	56
Crude protein	202	122
Ether extract	28	32
Crude fibre	72	194
Acid detergent fibre (ADF)	96	222
Neutral detergent fibre (NDF)	222	431
Energy[§] [MJ NEL/kg DM]	8.7	7.0

[†]Total mixed ration on DM basis (70% roughage (75% corn silage, 25% grass silage) + 30% concentrate)

[‡]Per kg of mineral feed: 170g Ca, 50g P, 120g Na, 45g Mg, 800,000 IU vitamin A, 100,000 IU vitamin D_3, 4000mg vitamin E, 4000mg Mn, 6000mg Zn, 1300mg Cu, 120mg I, 35mg Co, 40mg Se

[§]Calculation based on nutrient digestibilities masured with wethers (GfE, 1991) and values from feed tables (DLG, 1997)

The classification of a cow as healthy, subclinical ketotic or clinic ketotic was based on the concentration of BHB in blood serum. Therefore, BHB values between 1.2 – 2.5 mmol/l were characterized as a subclinical metabolic status, lower values meant a healthy animal, whereas higher values indicated clinical ketosis.

Sample Preparation and Measurement

The daily individual feed intake was recorded for the whole experimental time (computerized feeding station: Type RIC, Insentec, B.V., 1274 Marknesse, The Netherlands). Representative concentrate samples were taken once, grass and maize silage twice a week, while TMR samples

were collected daily and pooled monthly. Feedstuffs were analyzed for dry matter (DM), crude protein (CP), crude ash (CA), ether extract (EE), crude fibre (CF), neutral detergent fibre (NDF) and acid detergent fibre (ADF), according to the guidelines of the Association of German Agricultural Analytic and Research Institute (VDLUFA, 1993).

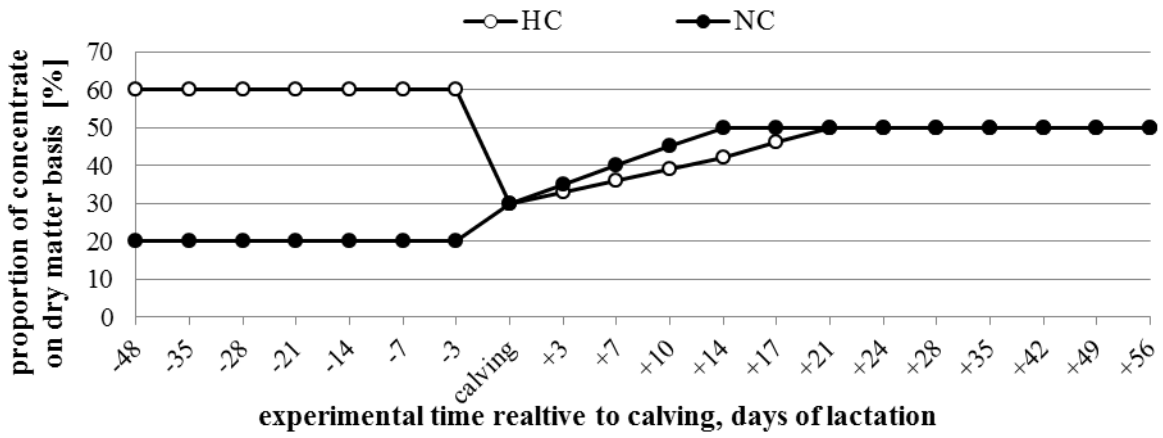

Fig. 1. Concentrate proportion of the diet (%) of high (HC) and normal (NC) condition groups during experiment.

Milking took place twice a day at 05.30 and 15.30. Milk yield was recorded using automatic milk counters (Lemmer Fullwood GmbH, 53797 Lohmar, Germany) and body weight was always determined when leaving the milking parlor. Milk samples were taken twice a week and stored at 4°C for analyzing fat, protein, lactose and urea concentration, using infrared milk analyzer (Milkoscan FT 6000 in combination with a Fossomatic 5000; Foss Electric, 3400 Hillerød, Denmark).

At day -48 (control sample; 48 ± 7.19 days antepartum), -14, -7, -3, 1, 3, 7, 10, 14, 17, 21, 24, 28, 35, 42 and 56 (relative to calving) blood samples for clinical-chemical parameters were taken from the vena jugularis. Immediately after centrifugation (Heraeus Varifuge® 3.0R, 2000g, 15°C; 15 minutes) serum concentrations of BHB, NEFA, glucose and triglycerides (TG) were measured using an automatic analyzing system, based on a photometric measurement (Eurolyser, Type VET CCA, 5020 Salzburg, Austria). Serum concentration of total protein, albumin, cholesterol, urea, aspartate-aminotransferase (AST), γ-glutamyl transferase (γ-GT) and glutamate dehydrogenase (GLDH) were measured the same way. The concentration of insulin was analyzed by the endocrinology laboratory of the Cattle Clinic (University of Veterinary Medicine, 30173 Hanover, Germany) using radioimmunoassay (RIA). Samples were stored at -80°C interim.

Liver samples were taken on day -14, 7, 21, 35 and 56 (relative to calving). Therefore approximately 200 mg of tissue were removed from bovine liver by minimally invasive, transcutaneous cutting biopsy needle technique under local anesthesia. The acquired liver tissue was analyzed for the content of total lipid (TL). The gravimetrical method used, based on the publication by Starke et al. (2010). For the determination in milligrams per gram of fresh liver weight, the TL was extracted from homogenized tissue samples with hexane:isopropanol (mixing ratio 3:2, continual agitation, 20°C, 24h).

Calculations

The net energy lactation (NEL) of concentrates was calculated by using the nutrient digestibility from studies with wethers according to GfE (1991), whereas the data for corn- and grass silage are based on feed tables (DLG, 1997). According to formulas published by GfE (2001), the requirement for maintenance (E_M), milk production (E_L) and the energy balance were calculated as follows:

Maintenance requirement: E_M [MJ NEL/d] = 0.293 × body weight [kg]$^{0.75}$

Energy content of milk: [MJ/kg] = 0.95 + 0.38 × Milk fat [%] + 0.21 × Milk protein [%]

Requirement for milk production:
E_L [MJ NEL/d] = Energy content of milk [MJ NEL/kg] + 0.07 × Milk yield [kg/d]

4% Fat-corrected milk (FCM) was calculated based on the equation of Gaines (1928):
FCM [kg/d] = ((milk fat [%] × 0.15) + 0.4) × milk yield [kg/d]

Calculation of the energy balance as follows:
Energy balance [MJ NEL/d] = energy intake [MJ NEL/d] - (E_M [MJ NEL/d] + E_L [MJ NEL/d])

Before calving, the energy balance was calculated by subtracting the requirement of maintenance and for pregnancy (GfE 2001) from daily net energy intake. During the last 6 weeks of gestation 13 MJ NEL/d are required in addition, with an increase to 18 MJ NEL/d for the last 3 weeks.
In particular to the insulin sensitivity, the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) was calculated by Holtenius and Holtenius (2007):

$$RQUICKI = \frac{1}{\log Insulin[\mu U/ml] + \log Glucose\ [mg/dl] + \log NEFA\ [mmol/l]}$$

Statistical Analysis

For the statistical analysis, the whole trial period became classified into three time periods. Period 1 describes the time prepartum and summarized the days -48, -14, -7 and -3. Period 2 centralizes the first 2 weeks post partum (including days 1, 3, 7, 10 and 14), while in Period 3 the time points from the lactation week 3 (day 17, 21, 24, 28, 35, 42 and 56) are summarized. According to this classification all parameters of the performance and the clinical chemistry were evaluated.

All statistics were performed by using the MIXED procedure of SAS (Software package, Version 9.1, SAS Institute, 2004). Effects were declared significant when P-values were ≤ 0.05 after Tukey test. Each parameter was analyzed by a compound symmetry covariance structure. For clinical-chemical parameters the model contained feeding group and period, including the interaction between the factors. The performance was evaluated similarly, but in consideration of the parity as additional fixed factor. All results are represented at least square means (LSmeans) and standard error of the mean (SEM).

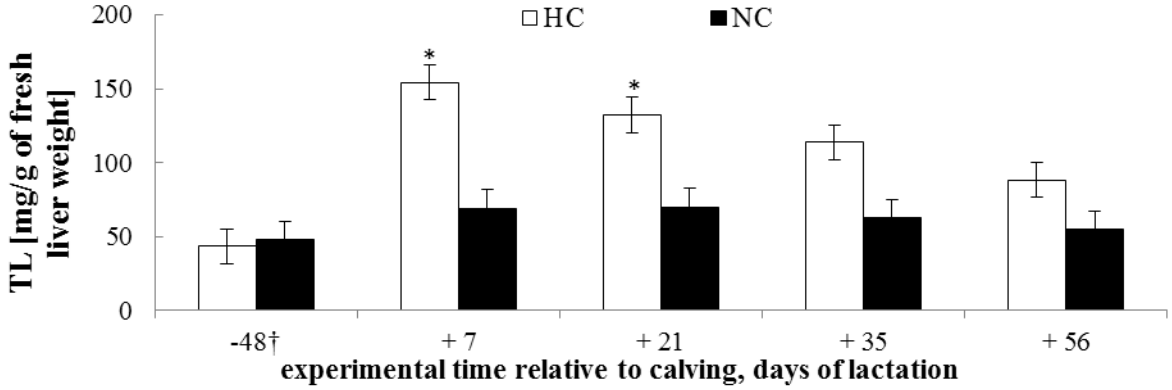

Fig. 2. Total lipid (TL) content of the liver of high (HC) and normal (NC) condition group at sampling points during experiment (†6 weeks before expected parturition). Values are LSMeans ± SEM; *P ≤ 0.01.

Results

Over the entire trial, six out of nine cows of the high condition (HC) group developed a subclinical form of ketosis (BHB in blood serum > 1.2 mmol/l), two cows had BHB values indicative for clinical ketosis (BHB > 2.5 mmol/l) and one cow remained healthy. The two cows with clinical ketosis had the highest BHB values with 4.39 mmol/l and 2.92 mmol/l on day 7 and

10 after calving, respectively. In the normal condition (NC) group eight out of nine animals stayed healthy. The diseased cow had the same indicative serum concentrations like the subclinical ketotic cows in HC group. All 13 parameters of the clinical chemistry, including RQUICKI, are shown for both feeding groups in Table 3. Insulin and urea levels are significantly higher in HC group in Period 1 ($P < 0.0001$). Serum concentrations of BHB and NEFA differ significantly among the groups in Period 2, as does AST. In the 3rd period, the urea level is significantly higher and the GLDH level significantly lower in NC vs. HC cows. The TL content of the liver is also shown in Table 3, with high significant intergroup differences in both periods post partum ($P < 0.01$). A graphical presentation for the evaluated days of experiment is given in Figure 2. There is a significant difference between both groups on day +7 (154.06 mg/g in HC vs. 69.23 mg/g in NC) and day +21 (132.33 mg/g in HC vs. 69.83 mg/g in NC). Detailed evaluations of BHB and NEFA for the single time points are shown in Figure 3, with a significant difference after calving.

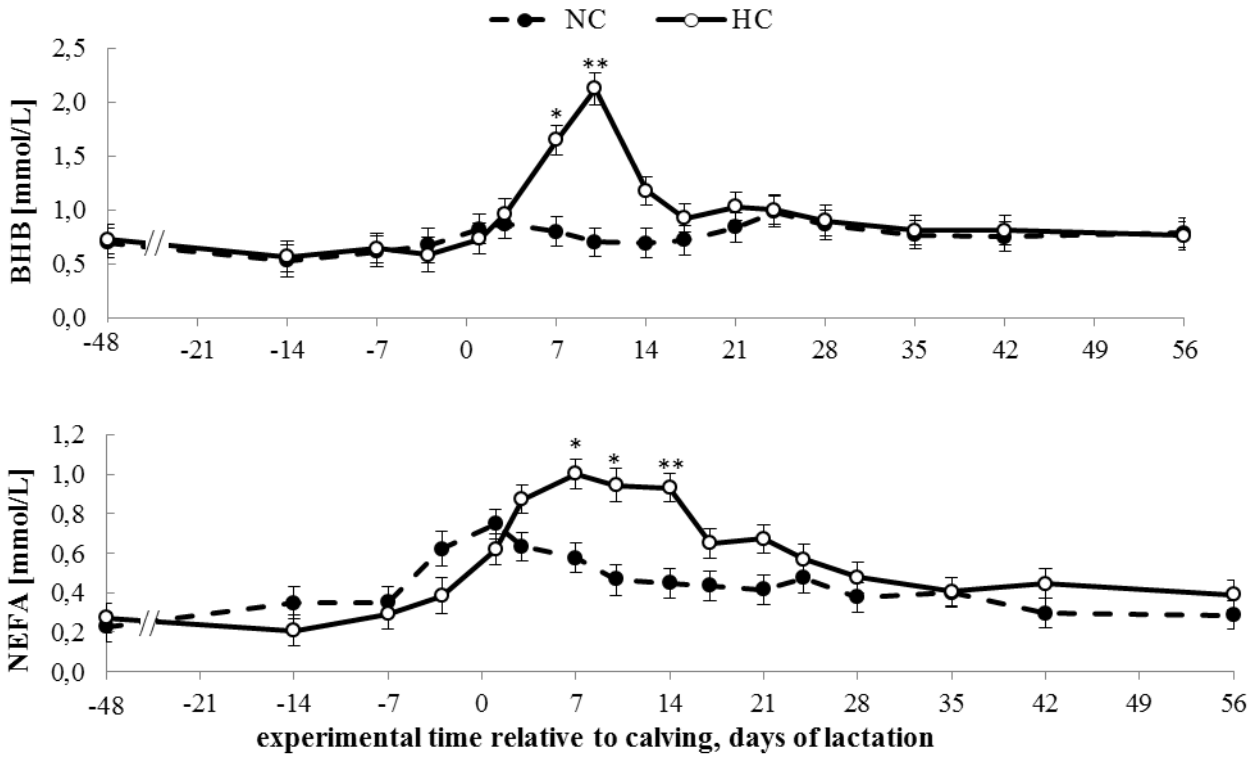

Fig. 3. Values of beta-hydroxy butyrate (BHB) and non-esterified fatty acids (NEFA) in blood serum of high (HC) and normal (NC) condition group at sampling points during experiment. Values are LSMeans ± SEM; *$P < 0.05$; **$P < 0.01$.

Changes of BCS in the two groups are demonstrated in Figure 4. At the time of classification (6 weeks before expected parturition), the difference in BCS between HC and NC was significant ($P < 0.01$). During the further course of the experiment, the BCS showed significant differences with regard to the group ($P < 0.0001$) and the lactation week ($P = 0.0052$), even though the interaction of group and lactation week was not significantly affected ($P = 0.820$; data not shown). The first marked increase (although the increase was found to be not significant) took place 4 weeks post partum for both groups (Figure 4).

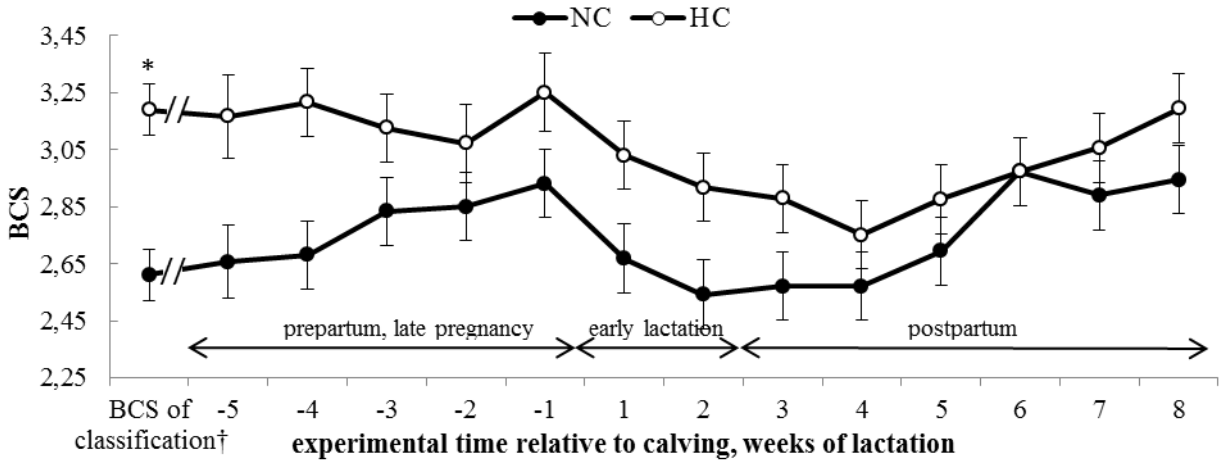

Fig. 4. Comparison of the body condition score (BCS) of high (HC) and normal (NC) condition groups during the experiment (†6 weeks before expected parturition). LSMeans ± SEM; *P < 0.01.

The results for the performance parameters are given in Table 4. Based on the individual feed intake prepartum, there is a high significant difference ($P < 0.01$) between groups for DMI, net energy intake and energy balance in Period 1. Figure 5 shows the energy balance for every single week of the experimental time with a significant difference between HC and NC 3 weeks before calving ($P = 0.0046$). In addition, the NC cows overcome their negative energy balance post partum faster than the HC animals, namely in week 7 of lactation.

Table 3. Blood serum parameters of the clinical chemistry and the total lipid content of the liver of high (HC) and normal (NC) condition group within Period 1 (6 weeks before expected parturition till calving), Period 2 (first 2 weeks postpartum) and Period 3 (2^{nd} week – 8^{th} week postpartum) (LSMeans ± SEM).

	Period 1			**Period 2**			**Period 3**		
	Treatment[†]			Treatment[†]			Treatment[†]		
	HC	NC	*P*-value	HC	NC	*P*-value	HC	NC	*P*-value
BHB, mmol/l[‡]	0.64 ± 0.09	0.63 ± 0.09	1.000	1.30 ± 0.08	0.78 ± 0.08	0.0002	0.89 ± 0.08	0.81 ± 0.08	0.981
NEFA, mmol/l	0.28 ± 0.05	0.37 ± 0.05	0.812	0.88 ± 0.04	0.58 ± 0.04	< 0.0001	0.52 ± 0.04	0.38 ± 0.04	0.132
Glucose, mg/dl	68.61 ± 2.91	66.82 ± 3.04	0.998	67.92 ± 2.60	64.12 ± 2.53	0.900	62.97 ± 2.25	63.07 ± 2.24	1.000
Triglyceride, mg/dL	21.07 ± 0.77	21.55 ± 0.79	0.998	12.41 ± 0.69	10.43 ± 0.67	0.308	11.28 ± 0.59	11.05 ± 0.58	1.000
Albumin, g/L	32.72 ± 0.80	30.99 ± 0.82	0.659	34.69 ± 0.76	32.11 ± 0.75	0.153	34.33 ± 0.71	34.18 ± 0.71	1.000
Total protein, g/L	66.17 ± 1.73	63.99 ± 1.75	0.950	71.22 ± 1.64	68.08 ± 1.61	0.749	72.23 ± 1.54	73.02 ± 1.53	0.999
Cholesterol, mg/dL	92.29 ± 7.55	92.14 ± 7.65	1.000	78.20 ± 7.20	82.82 ± 7,12	0.998	142.89 ± 6.83	129.34 ± 6.81	0.724
Urea, mg/dL	22.53 ± 0.92	14.88 ± 0.94	< 0.0001	18.11 ± 0.82	15.53 ± 0.80	0.215	15.57 ± 0.69	18.72 ± 0.69	0.0181
γ-GT, U/L	17.04 ± 3.22	16.24 ± 3.28	1.000	20.20 ± 3.04	19.99 ± 2.99	1.000	31.28 ± 2.85	22.31 ± 2.83	0.226
AST, U/L	54.78 ± 5.21	49.40 ± 5.30	0.979	97.11 ± 4.92	73.01 ± 4.85	0.0075	81.21 ± 4.57	65.82 ± 4.56	0.165
GLDH, U/L	6.63 ± 2.52	7.04 ± 2.57	1.000	11.17 ± 2.43	9.39 ± 2.37	0.995	26.73 ± 2.18	15.20 ± 2.17	0.0030
Insulin, mU/mL	30.32 ± 2.01	16.74 ± 2.06	< 0.0001	7.02 ± 1.87	10.43 ± 1.83	0.888	12.97 ± 1.70	14.56 ± 1.70	0.999
RQUICKI[¶]	0.38 ± 0.01	0.41 ± 0.01	0.172	0.42 ± 0.01	0.42 ± 0.01	1.000	0.41 ± 0.01	0.42 ± 0.01	0.897
TL in liver, mg/g	43.26 ±12.02	48.13 ± 12.75	0.999	154.06 ± 12.02	69.23 ± 12.75	0.0001	110.45 ± 8.97	62.04 ± 9.52	0.0058

[†]Treatments: High condition (HC) cows were fed a concentrate proportion of 60% during Period 1, after calving the proportion raised from 30 to 42% (Period 2) till 50% (Period 3). Normal condition (NC) cows were fed a concentrate proportion of 20% during Period 1, which was raised from 30 to 50% in Period 2 and stayed at 50% during Period 3.

[‡]BHB, β-hydroxybutyrate; NEFA, non-esterified fatty acids; γ-GT, γ-glutamyltransferase; AST, aspartate aminotransferase; GLDH, glutamate dehydrogenase; TL, total lipid

[¶]Revised quick insulin sensitivity index, calculated by Holtenius and Holtenius (2007)

Table 4. Performance, milk composition and energetic variables of high (HC) and normal (NC) condition group within Period 1 (6 weeks before expected parturition till calving), Period 2 (first 2 weeks postpartum) and Period 3 (2nd week – 8th week postpartum) (LSMeans ± SEM).

	Period 1			Period 2			Period 3		
	Treatment†			Treatment†			Treatment†		
	HC	NC	*P*-value	HC	NC	*P*-value	HC	NC	*P*-value
DMI, kg/d	15.8 ± 0.6	12.5 ± 0.6	0.0019	12.0 ± 0.71	13.7 ± 0.7	0.533	18.1 ± 0.6	18.7 ± 0.6	0.960
Net energy intake, MJ/d	124.1 ± 4.2	84.2 ± 4.1	0.0000	83.9 ± 5.2	95.8 ± 5.2	0.585	126.1 ± 4.1	131.1 ± 4.1	0.974
Live weight, kg	708 ± 15	679 ± 14	0.773	586 ± 15	562 ± 15	0.872	589 ± 14	595 ± 14	0.999
Energy balance, MJ NEL/d	61.9 ± 7.3	26.2 ± 6.7	0.0141	-6.1 ± 7.8	17.3 ± 7.8	0.305	-22.1 ± 6.3	-0.2 ± 6.3	0.172
Body condition score	3.18 ± 0.10	2.79 ± 0.09	0.0656	2.94 ± 0.10	2.59 ± 0.10	0.194	2.92 ± 0.09	2.77 ± 0.09	0.882
Milk yield, kg/d				27.6 ± 1.2	26.2 ± 1.2	0.849	34.0 ± 1.1	29.6 ± 1.1	0.0316
Milk fat, %				6.04 ± 0.31	4.33 ± 0.31	0.0010	4.33 ± 0.24	4.03 ± 0.24	0.816
Milk fat, kg/d				1.68 ± 0.12	1.16 ± 0.12	0.0216	1.55 ± 0.10	1.24 ± 0.10	0.146
FCM‡, kg/d				36.7 ± 1.9	29.0 ± 1.9	0.0234	35.4 ± 1.8	29.7 ± 1.8	0.108
Milk protein, %				3.43 ± 0.08	3.51 ± 0.08	0.882	3.23 ± 0.05	3.29 ± 0.05	0.826
Milk protein, kg/d				1.07 ± 0.10	1.10 ± 0.10	0.994	1.16 ± 0.08	1.06 ± 0.08	0.807
Milk lactose, %				4.76 ± 0.11	4.93 ± 0.11	0.655	5.00 ± 0.08	5.08 ± 0.08	0.896
Milk lactose, kg/d				1.35 ± 0.08	1.72 ± 0.06	0.918	1.28 ± 0.08	1.52 ± 0.06	0.137
Fat:protein ratio in milk				1.76 ± 0.10	1.25 ± 0.10	0.0018	1.35 ± 0.07	1.23 ± 0.07	0.691

†Treatments: High condition (HC) cows were fed a concentrate proportion of 60% during Period 1, after calving the proportion raised from 30 to 42% (Period 2) till 50% (Period 3). Normal condition (NC) cows were fed a concentrate proportion of 20% during Period 1, which raised from 30 to 50% in Period 2 and stayed at 50% during Period 3.

‡4% fat-corrected milk

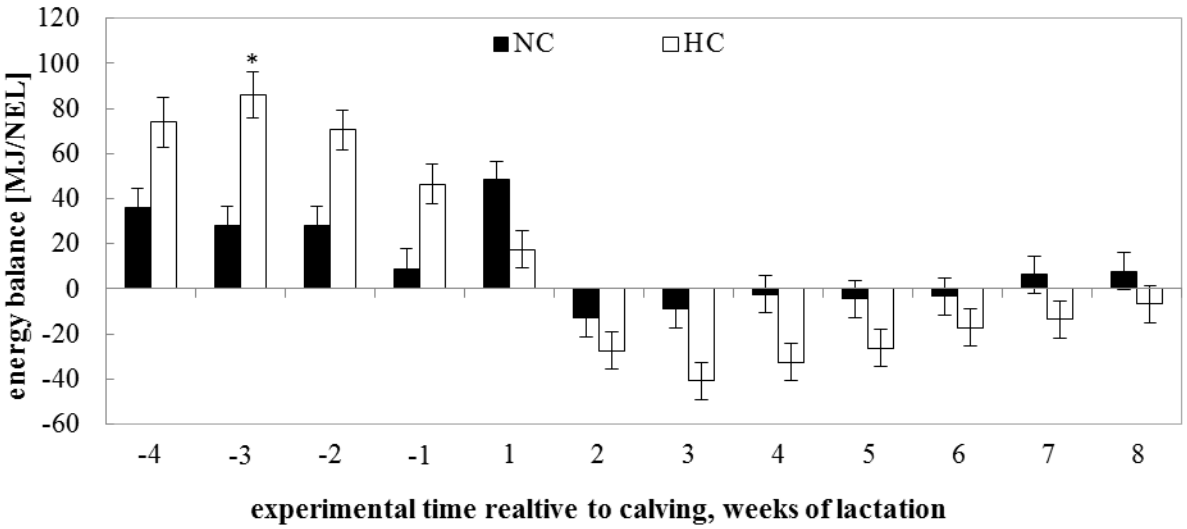

Fig. 5. Energy balance of high (HC) and normal (NC) condition groups during the experiment. Values are LSMeans ± SEM; *P ≤ 0.01.

A high statistical relevance is also shown in the 2nd period in regard to milk fat, absolute as well as relative (Table 4). Due to the higher milk fat content in HC group, also the parameters FCM and FPR (fat:protein ratio of the milk) clarify a significantly higher level compared to the NC feeding group. In Period 3 the milk yield differs significantly between the two groups. Thereby the average milk production of the HC group is 4.35 kg per day higher than the production within the NC group. There are no more differences observed in Period 3.

Discussion

Our animal model to investigate subclinical ketosis was based on a factorial design with three important factors: The BSC 6 weeks before expected parturition and the subsequent classification to high and normal condition, the group-individual DM and energy intake prepartum, as well as the initialized increase in energy input till the week 3 after calving. Bobe et al. (2004) reviewed that studies that investigate the effects of BCS, prepartal and postpartal diet in a factorial design are warranted. Due to the model, the changed physiological situation of HC cows could be compared to the healthy NC cows.

Bewley and Schutz (2008) reviewed that fat cows tend to lose more body condition during early lactation than thin cows. As the present study based on a group-specific feeding management, these data are difficult to compare. Due to the accelerate proportion of concentrate for the NC cows during the first 2 weeks after calving, the differences in BCS post partum between NC and HC become less pronounced. Nevertheless, BCS at calving represents an important risk factor for

subsequent development of subclinical ketosis during lactation (Duffield, 2000). In the present study 6 out of 9 cows of the HC group developed subclinical ketosis. These 6 cows had an average BCS of 3.50 one week before calving. In the NC group 8 out of 9 cows stayed healthy and had an average BCS of 2.90 one week before calving, which was significantly different to the BCS of HC cows with subclinical ketosis at that time ($P = 0.016$; data not shown). This difference in BCS endorsed the success of our animal model, as the artificial provoked subclinical ketosis is a result of a higher BCS combined with experimental feeding management ante- and post partum.

Owing to the feeding management in the animal model, DMI was higher in the HC group prepartum and higher in the NC group post partum. It was not clear whether or not the energy balance for the time prepartum could be calculated according to the formulas published by GfE (2001). The high proportion of concentrate in HC group during dry period came along with 41% wheat, which is highly degradable in the rumen, and a low crude fibre content (16.3%). So the authors cannot exclude the possible development of an at least mild acidosis. In this situation a reduced digestion of the ingredients is possible (Owens et al., 1998). Despite this, the energy balance was significantly higher in HC cows within the experimental dry period. Additionally, NC cows were able to compensate their NEB faster (Figure 5). This was partly due to the faster increase of energy for NC cows post partum and partly to the lower milk yield in Period 3 (Table 4). These results are comparable to other studies (Holcomb et al., 2001; Vickers et al., 2013). In addition, the various nutrient and energy intake prepartum was also reflected by the significant different serum blood concentrations of urea and insulin in Period 1 (Table 3), caused by the higher concentration of crude protein and energy in HC feedingstuff.

Regarding the milk parameters, significant differences in our results were typical for subclinical ketosis and confirm other findings (Miettinen & Setälä, 1993; Duffield, 2000; LeBlanc, 2010). According to the literature, the milk yield is significantly decreased in almost every case of ketosis, even subclinical ketosis (Duffield, 2000). For the present study this did not apply. In the HC group, where 89% had BHB values indicative for clinical or subclinical ketosis, the milk yield was significantly higher during Period 3 ($\Delta = 4.35$ kg/d). The milk yield was also higher in Period 2 ($\Delta = 1.4$ kg/d), even if this difference was not statistically relevant. A few studies with similar results were found. The comparison of healthy and subclinically ketotic cows show continuously higher milk yields until the 6th week of lactation in the group of subclinical cows (Asl et al., 2011). In comparison of cows with lower (≤ 3.25) and higher BCS (> 3.25), the

average milk yield is numerically higher for the fatter ones (Busato et al., 2002). Quite in line with this, Domecq et al. (1997) found, in a study with 779 cows, that a one-point increase (Edmonson-scale) between dry-off and parturition is associated with 546 kg more milk in the first 120 days of lactation. Based on our data and consistent with these findings, cows with a higher BCS at calving tended to yield more milk. We suggest that our HC cows, which underwent a slower energy adaption during the first 3 weeks post partum, used the mobilized body mass as an energy source for milk production, even if the same cows had BHB values indicative of clinical or subclinical ketosis. That a fat body condition is related to a higher milk yield is also described by Heuer et al. (1999). They equate the higher milk production with a FPR > 1.5, which coincides with high lipolysis and an elevated risk for ketosis (Heuer et al., 1999; Buttchereit et al., 2010). Consistent with those studies, we found a FPR of 1.76 in HC group during Period 2 which was significantly different from the FPR of NC cows during Period 2 (Table 4).

According to the serum parameters, bovine subclinical ketosis in combination with high lipomobilisation can be diagnosed if the following values are on par: BHB > 1.2 mmol/l, glucose < 2.5 mmol/l, TG < 0.08 mmol/l and NEFA > 0.4 mmol/l (Oetzel, 2004; Gonzalez et al., 2011; Dirksen et al., 2012). This combination applied to eight out of nine cows from the HC group, in which all eight cows had BHB values indicative for clinical or subclinical ketosis during the ongoing trial.

Our results show that the ketotic metabolic status strongly affected liver metabolism, reflected by the difference of the TL content pre- and post partum (Table 3 & Figure 2). Normally the TL content of the liver alone allows no adequate inference to classify a fatty liver. Nevertheless a TL content of 3 - 6% is considered as physiological and should be in this range for the time prepartum and from the 8th week after calving (Fürll, 1989). Ametaj et al. (2005) established cows with a TL content more than 10% as cows with fatty liver. In the present paper, within the HC group, a content of 15.4% and 11% TL were measured during Period 2 and 3. Depending on the available literature, such a concentration of TL can be classified as mild fatty infiltration, which is almost physiological (Đokovic´ et al., 2012). Due to the infiltration of fat, lesions in hepatic tissues appear and cause increased blood levels of specific enzymes (Bobe et al., 2004). Gonzalez et al. (2011) describe an AST level of higher than 100 U/L as indicative for hepatic lesions. In our case, seven out of nine cows from the HC group (77.8%) showed AST concentrations higher than 100 U/L during early lactation. These seven cows also included the two clinically ketotic animals (BHB > 2.5 mmol/l; AST maximum of 219.44 U/L) and not the

one who stayed healthy (AST maximum of 81.83 U/L). The seven cows together offered an average TL content of 17% and 15% for the days 7 and 21, relative to calving (data not shown). Next to AST, GLDH is useful for analyzing longer acting influences, so that a variation of the activity occurs about 3 to 5 weeks after the impacts. This fact is verified by the high level of GLDH, exceeding the physiological level of 10 U/L (Dirksen et al., 2012) and the significant difference between the feeding groups in Period 3 (Table 3). In summary, even a liver lipid content of about 15% can be classified as mild to moderate fatty liver if further parameters like AST show increased levels, far beyond their physiological range. However, it has to be supposed that the fatty degeneration was reversible, because the TL values and serum concentrations of AST, BHB and NEFA decreased and normalized towards the end of the trial.

An insulin resistance is present, when higher than normal insulin concentrations are needed to achieve normal metabolic responses and has already been demonstrated in case of hepatic lipidosis of dairy cattle (Oikawa & Oetzel, 2006). Holtenius and Holtenius (2007) consider, that different kinds of glucose tolerance tests for clinical investigations are too time-consuming and not suitable for cows. They assess insulin sensitivity on the basis of RQUICKI, and found that this calculated parameter might be useful to identify a disturbed insulin function in cows. In our study, post partum RQUICKI was lowest on day +1 (data not shown) for both groups. However, there is no significant difference between groups to any point of time (Table 3) and consequently no relationship between RQUICKI and subclinical ketosis. These results are contrary to Stengarde et al. (2011), who describe RQUICKI as a more sensitive parameter for metabolic imbalances than separate evaluation of glucose, insulin and NEFA. However, the respective serum values in the present study were substantially higher. For example the serum NEFA and BHB levels in HC group during Period 2 are nearly twice as high compared to Stengarde et al. (2011). This induced a pathological metabolic situation and confirms the statement, that RQUICKI has a low discrimination power in diagnosing decreased insulin sensitivity in cows affected by a metabolic disease (Kerestes et al., 2009).

In conclusion, we give an example of how the physiological situation of subclinical ketosis affects the whole bovine metabolism. Therefore, the prerequisite was the development of an animal model that induces exactly such a metabolic situation. The described animal model based on a combination of three different influencing factors: First, the overfeeding in the dry period; second the decelerated energy supply with concentrate in the first 3 weeks of lactation and at last, the grouping of cows with a higher BCS 6 weeks before expected parturition to the experimental

group to enhance the contrast compared to the animals of the control group. We confirm that the combination of the named factors cause the success of our investigation. We summarize, that the cows of experimental group developed a subclinical ketosis in the time of early lactation and provide the opportunity to investigate metabolic interrelations of subclinical ketosis, as well as an appropriate model for testing medical effectiveness to treat ketosis and fatty liver syndrome.

The authors would like to thank the co-workers of the Institute of Animal Nutrition of the Friedrich-Loeffler-Institute (FLI) in Braunschweig Germany in performing experiment and analyses. Furthermore we gratefully acknowledge Boehringer Ingelheim for its cooperation and the financial support (Boehringer Ingelheim Vetmedica, D-55216 Ingelheim am Rhein, Germany).

References

Ametaj BN, Bradford BJ, Bobe G, Nafikov RA, Lu Y, Young JW & Beitz DC 2005 Strong relationships between mediators of the acute phase response and fatty liver in dairy cows. Canadian Journal of Animal Science 85 165-175

Asl AN, Nazifi S, Ghasrodashti AR & Olyaee A 2011 Prevalence of subclinical ketosis in dairy cattle in the Southwestern Iran and detection of cutoff point for NEFA and glucose concentrations for diagnosis of subclinical ketosis. Preventive veterinary medicine 100 38-43

Bauman DE & Currie WB 1980 Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63 1514-1529

Bernabucci U, Ronchi B, Lacetera N & Nardone A 2005 Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. Journal of dairy science 88 2017-2026

Bewley JM & Schutz MM 2008 Review: an interdisciplinary review of body condition scoring for dairy cattle. The Professional Animal Scientist 24 507-529

Bobe G, Young JW & Beitz DC 2004 Invited review: pathology, etiology, prevention, and treatment of fatty liver in dairy cows. Journal of Dairy Science 87 3105-3124

Busato A, Faissle D, Kupfer U & Blum JW 2002 Body condition scores in dairy cows: associations with metabolic and endocrine changes in healthy dairy cows. Journal of veterinary medicine. A, Physiology, pathology, clinical medicine 49 455-460

Buttchereit N, Stamer E, Junge W & Thaller G 2010 Evaluation of five lactation curve models fitted for fat:protein ratio of milk and daily energy balance. Journal of Dairy Science 93 1702-1712

Day J 2013 Elanco launches ketosis bolus in Europe. [Online. Access Date 22/10/13]. Available:http://www.kcanimalhealth.com/index.php/news/media-coverage/elanco-launches-ketosis-bolus-in-europe/

Dirksen G, Gründer H & Stöber M 2012 Die klinische Untersuchung des Rindes [The clinical investigation of bovine animals]. Stuttgart: Enke

DLG (German Agricultural Society) 1997 DLG Futterwerttabellen Wiederkäuer [Feed value tables for ruminants] 7th edition. Frankfurt am Main: DLG-Verlag

Đokovic´ R, Šamanc H, Petrovic´ MD, Ilic´ Z & Kurc´ubic´ V 2012 Relationship among blood metabolites and lipid content in the liver in transitional dairy cows. Biotechnology in Animal Husbandry 28 705-714

Domecq JJ, Skidmore AL, Lloyd JW & Kaneene JB 1997 Relationship Between Body Condition Scores and Milk Yield in a Large Dairy Herd of High Yielding Holstein Cows. Journal of Dairy Science 80 101-112

Duffield T 2000 Subclinical ketosis in lactating dairy cattle. The Veterinary clinics of North America. Food Animal Practice 16 231-253

Edmonson AJ, Lean IJ, Weaver LD, Farver T & Webster G 1989 A body condition scoring chart for Holstein dairy cows. Journal of Dairy Science 72 68-78

Fürll M 1989 Vorkommen, Ätiologie, Pathogenese, Diagnostik und medikamentelle Beeinflussung von Leberschäden beim Rind. [Incidence, etiology, pathogenesis, diagnosis and

medical influence of liver damage in cattle.]. Vet. med. Habilitation University Leipzig, Germany

Gaines WL 1928 The energy basis of measuring milk yield in dairy cows. Illinois Agricultural Experiment Station Bulletin 308 401-438

GfE (Society of Nutrition Physiology) 1991 Leitlinien für die Bestimmung der Verdaulichkeit von Rohnährstoffen an Wiederkäuern [Guidelines for determining the digestibility of crude nutrients in ruminants]. Journal of Animal Physiology and Animal Nutrition 65 229-234

GfE (Society of Nutrition Physiology) 2001 Empfehlungen zur Energie- und Nährstoffaufnahme der Milchkühe aud Aufzuchtrinder [Recommendations of energy and nutrient supply for dairy cows and breeding cattle]. Frankfurt am Main: DLG Verlag

Gonzalez FD, Muino R, Pereira V, Campos R & Benedito JL 2011 Relationship among blood indicators of lipomobilization and hepatic function during early lactation in high-yielding dairy cows. Journal of Veterinary Science 12 251-255

Heuer C, Schukken YH & Dobbelaar P 1999 Postpartum Body Condition Score and Results from the First Test Day Milk as Predictors of Disease, Fertility, Yield, and Culling in Commercial Dairy Herds. Journal of Dairy Science 82 295-304

Holcomb CS, Van Horn HH, Head HH, Hall MB & Wilcox CJ 2001 Effects of prepartum dry matter intake and forage percentage on postpartum performance of lactating dairy cows. Journal of Dairy Science 84 2051-2058

Holtenius P & Holtenius K 2007 A model to estimate insulin sensitivity in dairy cows. Acta veterinaria Scandinavica 49 29

Kerestes M, Faigl V, Kulcsar M, Balogh O, Foldi J, Febel H, Chilliard Y & Huszenicza G 2009 Periparturient insulin secretion and whole-body insulin responsiveness in dairy cows showing various forms of ketone pattern with or without puerperal metritis. Domestic Animal Endocrinology 37 250-261

LeBlanc S 2010 Monitoring metabolic health of dairy cattle in the transition period. The Journal of reproduction and development 56 (Suppl.) S29-35

McArt JA, Nydam DV & Oetzel GR 2012 Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of Dairy Science 95 5056-5066

Miettinen PVA & Setälä JJ 1993 Relationships between subclinical ketosis, milk production and fertility in Finnish dairy cattle. Preventive Veterinary Medicine 17 1-8

Oetzel GR 2004 Monitoring and testing dairy herds for metabolic disease. The Veterinary clinics of North America. Food Animal Practice 20 651-674

Oikawa S & Oetzel GR 2006 Decreased insulin response in dairy cows following a four-day fast to induce hepatic lipidosis. Journal of Dairy Science 89 2999-3005

Owens FN, Secrist DS, Hill WJ & Gill DR 1998 Acidosis in cattle: a review. Journal of Animal Science 76 275-286

Roche JR, Kay JK, Friggens NC, Loor JJ & Berry DP 2013 Assessing and managing body condition score for the prevention of metabolic disease in dairy cows. Veterinary Clinics of North America: Food Animal Practice 29 323-336

SAS Institute 2004. SAS/STAT 9.1 User's guide. SAS Inst. Inc., Cary, North Carolina.

Starke A, Haudum A, Busche R, Beyerbach M, Danicke S & Rehage J 2010 Technical note: Analysis of total lipid and triacylglycerol content in small liver biopsy samples in cattle. Journal of Animal Science 88 2741-2750

Stengarde L, Holtenius K, Emanuelson U, Hultgren J, Niskanen R & Traven M 2011 Blood parameters in Swedish dairy herds with high or low incidence of displaced abomasum or ketosis. The Veterinary Journal 190 124-130

Tamminga S, Luteijn PA & Meijer RGM 1997 Changes in composition and energy content of liveweight loss in dairy cows with time after parturition. Livestock Production Science 52 31-38

VDLUFA (Association of German Agricultural Analysis and Research Centers) 1993 Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch). Vol III. Die chemische Untersuchung von Futtermitteln. Darmstadt, Germany: VDLUFA-Verlag

Vickers LA, Weary DM, Veira DM & von Keyserlingk MA 2013 Feeding a higher forage diet prepartum decreases incidences of subclinical ketosis in transition dairy cows. Journal of Animal Science 91 886-894

4. Paper II (Published in Archives of Animal Nutrition, 2015, 69:113-127)

Effects of elevated parameters of subclinical ketosis on the immune system of dairy cows: *in vivo* and *in vitro* results

Kirsten Schulz[a], Jana Frahm[a*], Susanne Kersten[a], Ulrich Meyer[a], Dania Reiche[b], Helga Sauerwein[c], Sven Dänicke[a]

[a]Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig, Germany; [b]Boehringer Ingelheim Vetmedica Ingelheim am Rhein, Germany; [c]Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, Bonn, Germany

[*]Corresponding author. Email: jana.frahm@fli.bund.de

Abstract

Using an established model in which subclinical ketosis is induced, the response of differential blood counts and levels of various haematological variables, including the inflammatory marker haptoglobin (Hp), were tested over the last 6 weeks of parturition until the 56th day post-partum in cows with lower or higher body condition scores (LBC and HBC, respectively; n = 9/group). Animals in the HBC group evidenced subclinical ketosis whereas LBC animals were metabolically healthy. For *in vitro* examination with β-hydroxybutyrate (BHB) as further stimulus, peripheral blood mononuclear cells (PBMC) counts of cows with and without subclinical ketosis (n = 5/group) were observed. Counts of leucocytes, granulocytes and lymphocytes (LY) peaked at day 1 post-partum in HBC cows, with a more marked increase in heifers. In subclinical ketosis LY count increased again, with significantly higher values in the HBC group. The red blood cell (RBC) profile was affected by parity (counts were higher in heifers). Hp showed a positive linear correlation with BHB and non-esterified fatty acids (NEFA; R^2 = 0.41). PBMC from cows that were not pre-stressed with subclinical ketosis were more sensitive to increasing levels of BHB *in vitro*, as evidenced by both their higher proliferative capability and increased release of nitric oxide (NO). In summary, cows with subclinical ketosis showed a heightened immune response compared with metabolically healthy individuals, based on increased LY counts, increasing stimulative properties of PBMC and a relationship between Hp and typically increased values of BHB and NEFA. Concentrations of BHB *in vivo* during

subclinical ketosis did not alter the proliferative capability of bovine PBMC *in vitro*, which was first significantly decreased at a dosage of 5 mM BHB.

Keywords: dairy cows; subclinical ketosis; haematology; heifers; ketosis; peripheral blood mononuclear cells; β-hydroxybutyrate

Introduction

Dairy cows undergo distinct metabolic stress during the transition from pregnancy to lactation and are particularly susceptible not only for metabolic diseases but also for infections. The proportion of dairy cows undergoing a transient period of subclinical ketosis in the first month after calving has recently been estimated at 50% (Esposito et al. 2014).

This condition is characterized by increased lipomobilisation and thus rising concentrations of non-esterified fatty acids (NEFA) in the circulation. During the typical negative energy balance in the first weeks of lactation, the complete oxidation of NEFA is compromised resulting in increasing generation of ketone bodies (e.g. of ß-hydroxybutyrate [BHB], used as marker for ketosis) and also in lipid accumulation in the liver. Haptoglobin (Hp), one of the most important acute-phase proteins in cattle, is positively correlated to NEFA and increased liver lipid contents (Uchida et al. 1993). Around calving, the circulating concentrations of Hp exceed the pre-partal levels by a factor of about 4.5 (Hachenberg et al. 2007) and reach values up to 5 mg/ml in the first week after calving (Saremi et al. 2012). Similar to Hp, nitric oxide (NO) is also part of the inflammatory reaction in the dairy cow (Trevisi et al. 2013). These are generated by phagocytes (monocytes, macrophages and neutrophils), and released after enzymatic activation via cytokines. In human studies, increasing NEFA concentrations augment the production of reactive nitrogen species which are derived from NO (Valko et al. 2007). In bovine peripheral blood mononuclear cells (PBMC), NO production is increased after parturition (Lessard et al. 2004) and during mastitis (Bouchard et al. 1999).

Investigations on the lymphocyte function in ketosis have mainly focused on the direct effects of BHB and NEFA. Lacetera et al. (2004) indicated that NEFA reduced mitogen-induced DNA synthesis and the secretion of interferon-gamma (IFN-γ) and immunoglobulin M (IgM) from lymphocytes of dairy heifers in vitro. Albeit at lower doses, NEFA were also shown to inhibit proliferation and IFN-γ mRNA expression of PBMC (Renner et al. 2013). Concentrations of BHB ≤1.5 mM in vivo stimulated the ability of PBMC in response to a mitogen (Dänicke et al.

2012), while older studies found a depressing effect on bovine PBMC proliferation (Targowski and Klucinski 1983) and the phagocytic activity of polymorphonuclear neutrophil leucocytes (Klucinski et al. 1988). Moreover, even much higher concentrations (up to 6.25 mM) did not suppress stimulated PBMC proliferation in vitro (Franklin et al. 1991).

These findings relate well to the common observation that cows which seem to be most stressed by nutritional and environmental factors, as judged by excessive loss of body condition, are the most likely to become ill. Therefore, the purpose of the present study was to investigate the effects of elevated parameters of bovine ketosis on the immune system. Using a recently established model in which subclinical ketosis is induced, the responses of differential blood counts and various haematological variables, including the inflammatory marker Hp, were tested. The functionality of PBMC was assessed during the time around calving, comparing proliferative capability between cows with and without subclinical ketosis. In addition, PBMC were stimulated in vitro with increasing concentrations of BHB, the metabolite most likely to elicit the effects of ketosis in immune cells.

Materials and methods

Animals, trials and treatment

The experiments were carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI) in Braunschweig (Germany). The animals were housed in free-stall barns and milked twice daily. Two trials were performed for the present study. Experiments and procedures were conducted according to the Animal Welfare Act with the approval of the Lower Saxony State Office for Consumer Protection and Food Safety in Oldenburg (Germany).

Trial 1

The factorial design is described in detail elsewhere (Schulz et al. 2014). In brief, 18 pregnant and healthy German Holstein cows (10 pluriparous and 8 primiparous) were assigned to 2 feeding groups (n = 9/group) according to their body condition score (BCS; 5-point scale; Edmonson et al. 1989). Cows with higher BCS (HBC; BCS 3.19 ± 0.09) were fed a ration including 60% concentrate (7.7 MJ NEL/kg dry matter [DM]), which led to energy oversupply. Cows with lower BCS (LBC; BCS 2.61 ± 0.09) were fed an energy-adequate ration with 20% concentrate (6.8 MJ NEL/kg DM). The feed was aimed to be offered for ad libitum intake. After

calving, the concentrate proportion was increased stepwise, regulated by a computerised feeding station, from 30 to 50% within the first 2 or 3 weeks for the LBC and HBC groups, respectively. Initial increase occurred more slowly in the HBC group, with the additional aim of stimulating post-partum lipolysis. The animals in the HBC group evidenced subclinical ketosis, LBC animals being metabolically healthy. Concentrations of BHB between 1.2 and 2.5 mM in blood serum were characterised as subclinical metabolic status; lower values indicated a healthy animal whereas higher values, combined with clinical signs, indicated clinical ketosis.

At days 0 (control sample; 48 ± 7.19 days pre-partum) and -14, -7, -3, 1, 3, 7, 10, 14, 17, 21, 24, 28, 35, 42 and 56 (relative to calving), blood samples were taken from the jugular vein. Haematology was performed in EDTA whole blood using an automatic analyzer (Celltac α MEK-6450, Nihon Kohden, Qinlab Diagnostik, Weichs, Germany). Fifteen variables were measured, including leucocytes (WBC), erythrocytes (RBC) and thrombocytes. Clinical chemistry data for serum were reported previously (Schulz et al. 2014). The determination of Hp was done using ELISA as described by Hiss et al. (2004). The limit of detection was 0.07 mg/ml.

Functional tests of PBMC were performed on days -14, +7, +21 and +56. Unless otherwise stated, all chemicals were purchased from Biochrom AG (Berlin, Germany). Cells were separated from heparinised blood by gradient centrifugation using a Ficoll separation solution (L 6115), and diluted with PBS (L 1835) at a ratio of 1:1. After centrifugation (550 g, 30 min, 20°C) the interphase was harvested and washed with PBS (330 g, 8 min, 20°C). The pellet was resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (F 1295), supplemented with 5% FBS (S 0615), 1 M HEPES buffer (L 1613), 2 mM L-glutamin (K 0282), 5 mM β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany, M 7522), penicillin (100 U/ml) and streptomycin (A 2212, 0.1 mg/ml). Samples were frozen and stored at -80°C until cell proliferation assays were performed. Concanavalin A (ConA, 2.5 µg/ml final, Sigma-Aldrich, Steinheim, Germany, C 5275) was used as mitogen to stimulate T lymphocytes. Cultures were assayed in quintuplicate and seeded onto 96-well plates (1 · 105 cells/well) with a final volume of 200 µl/well. Plates were incubated for 69.5 h (37°C, 5% CO2). After centrifugation (200 g, 5 min, 20°C), 100 µl of the supernatant were removed and Alamar Blue (AB) was added (1:10 final). The fluorescence of the AB reduction product resorufin was measured after 2.5 h at 540 and 590 nm (Tecan infinite M200, Groedig, Austria).

Trial 2

Trial 2, an in vitro dose-response study with BHB, was integrated into another experiment. This experiment followed the same factorial design and feeding management as described for Trial 1. On day 7 post-partum, PBMC were obtained from the blood of 10 different pluriparous cows (5 each LBC and HBC). The BCS was significantly different between the groups six weeks before expected calving (HBC, 4.05 ± 0.17; LBC, 2.80 ± 0.17). The HBC cows had serum BHB values indicative of subclinical ketosis (BHB ≥ 1.2 mM). LBC cows apparently stayed healthy (BHB < 1.2 mM).

For in vitro investigations PBMC were prepared following the same procedure as for ex vivo. β-hydroxybutyrate (Sigma-Aldrich, Steinheim, Germany, 1668985G) was diluted in RPMI-1640 medium to obtain a stock solution (80 mM), so that dose response studies were performed with 20, 10, 5, 2.5, 1.25 and 0.625 mM as well as a medium control (0 mM). The metabolic activity of the cells was determined by AB again, also with and without ConA as mitogen stimulus.

To assess the release of NO by macrophages in the cell culture supernatant of bovine PBMC, its production was determined as the accumulated level of nitrite. A dose-response examination with 20, 10, 5, 2.5, 1.25, 0.625 and 0 mM was assayed in quintuplicates, seeded onto 96-well plates (1 · 105 cells/well). Lipopolysaccharide (LPS, purified from Escherichia coli 0111:B4, Sigma-Aldrich, L 2630 129K4025, Steinheim, Germany) was used as positive control at a dilution of 1 µg/ml. Plates were incubated for 24 h (37°C, 5 % CO2) and then centrifuged (200 g, 5 min, 20°C). For NO determination, a colorimetric reaction with Griess reagent [0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride, 1 % sulfanilamide and 5 % H_3PO_4] was used. The cell culture supernatant (100 µl) and 100 µl of Griess reagent were mixed and incubated in the dark for 5 min at room temperature. All measurements were done in duplicate. Absorbance was measured at 540 nm using a microplate reader (Tecan infinite M200, 5082 Groedig, Austria). Nitrite concentration in the samples was determined from a sodium nitrite standard curve (0.25 – 50 µM). In regard to concentrations below the limit of detection, a constant NO release value of 0.125 µM was assumed.

Calculation and statistics

The results of the *ex vivo* and *in vitro* examination of PBMC were expressed as stimulation index (SI), which is defined as the ratio between fluorescence in the AB assay of ConA-stimulated and -non-stimulated PBMC:

(1) $$SI = \frac{Fluorescence\ of\ ConA\ stimulated\ PBMC}{Fluorescence\ of\ nonstimulated\ PBMC}$$

The dose-response curves were fitted to the following nonlinear regression equation (Mercer et al. 1987):

(2) $$SI = \frac{R_0 \cdot {K_{05}}^b + R_{max} \cdot Con^b}{{K_{05}}^b + Con^b}$$

where R_0 is the intercept on ordinate (SI at 0 µM), R_{max} is the asymptotic SI when *Con* converges to infinity, *Con* is the BHB concentration [mM], K_{05} is the SI at $0.5 \cdot (R_{max} + R_0)$ and *b* is the apparent kinetic order. In regard to the mean SI of the separate curves for HBC and LBC animals, R_0 and R_{max} were defined, respectively.

To investigate changes in haematological variables and Hp over the peri-partal period, the experiment was classified into three periods: (1) days 42, 14, 7 and 3 pre-partum; (2) days 1, 3, 7, 10 and 14 post-partum; and (3) days 17, 21, 24, 28, 35, 42 and 56 post-partum. Data for the measured variables were analysed as repeated measures by the Mixed procedure of SAS (SAS Institute 2004) and are presented as least square means (LSmeans) and standard error (SE) of the mean. Effects are considered significant at p-values ≤0.05 using Tukey´s test; a trend was noted when $0.05 < p < 0.10$. These parameters were analysed using a compound symmetry covariance structure. The model included feeding group (HBC or LBC), time point (day/period) and parity (heifer or pluriparous cow) as fixed factors, including their interactions. Because of the repeated measurements during the experiment, individual animal effects were considered using the Repeated procedure. Regression analysis was carried out using Statistica 10 for the WindowsTM operating system (StatSoft 2011).

Results

Haematology

The results for haematological variables in the different experimental periods are shown in Table 1. There was a significant triple interaction (Group x Time x Parity) for WBC, GR, lymphocytes

(LY), RBC, haemoglobin (HGB) and haematocrit (HCT). The concentrations of these variables during the experiment are plotted in Figure 1 and 2.

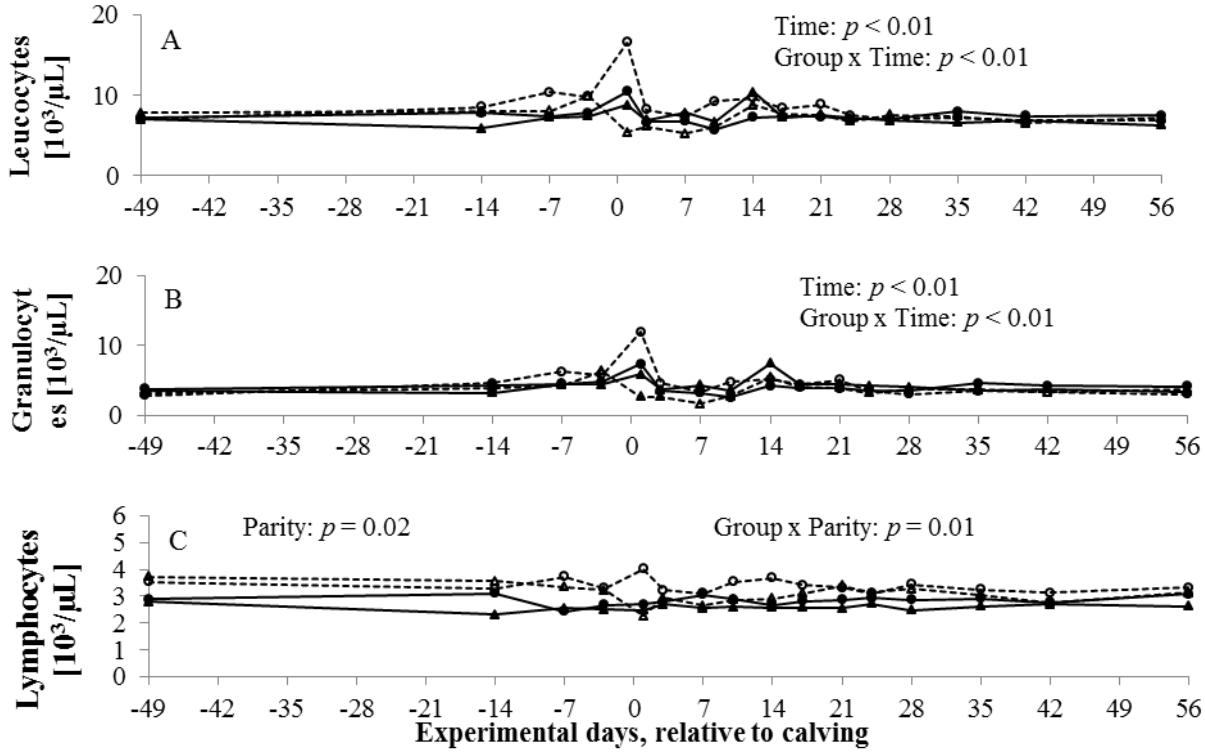

Figure 1. Concentrations of leucocytes (A), granulocytes (B) and lymphocytes (C) in cows of a higher (HBC) and lower condition (LBC). HBC cows suffered from subclinical ketosis (β-hydroxybutyrate ≥1.2 mM in blood) during the first two weeks post-partum, while LBC cows stayed metabolically healthy. Comparison between primiparous and pluriparous cows (LSmeans). The statistical analysis included the Group (HBC versus LBC), Parity (cow versus heifer), Day of experiment and interactions. For all parameters, the triple interaction Group x Day x Parity was significant ($p < 0.05$). Fix factors and interactions not included in the graph are not significant ($p > 0.05$). Note: HBC cows (●), HBC heifers (○), LBC cows (▲) and LBC heifers (Δ).

Almost all variables changed with time. The concentrations of WBC and GR showed similar patterns and peaked at day +1 for the HBC group, with a significant increase in heifers (Δ day -3 to day +1: 6.77 · 103 µl-1). In the LBC group, peak values were reached on day +14. Regardless of parity, both variables were increased on day +1 in HBC versus LBC cows.

The LY concentration in pluriparous animals was lower than in heifers (p = 0.015). The concentration decreased to nadir at day +1 in LBC heifers while HBC heifers reached peak values on that day (Δ = 1.75 · 103 µl-1, p = 0.016). During Period 2, HBC cows showed higher LY concentrations (p < 0.05) than LBC cows.

For both groups, HGB and HCT decreased significantly from Periods 1 and 2 to 3, and showed significant inter-group differences for Periods 2 (HGB and HCT) and Period 3 (HGB only). Comparable effects were found for the absolute concentration of RBC and mean corpuscular haemoglobin concentration (MCHC). Therefore, MCHC was affected by time, group and interaction, with significantly higher values for the HBC group during Period 2. Mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), like the variable thrombocytes, changed with time whereas group and interaction were insignificant. The RBC profile was affected by parity: RBC, MCV, MCH and platelet distribution width (PDW) were higher in heifers than in pluriparous cows whereas HGB and HCT were lower in heifers than in cows (Table 1 and Figures 1 and 2).

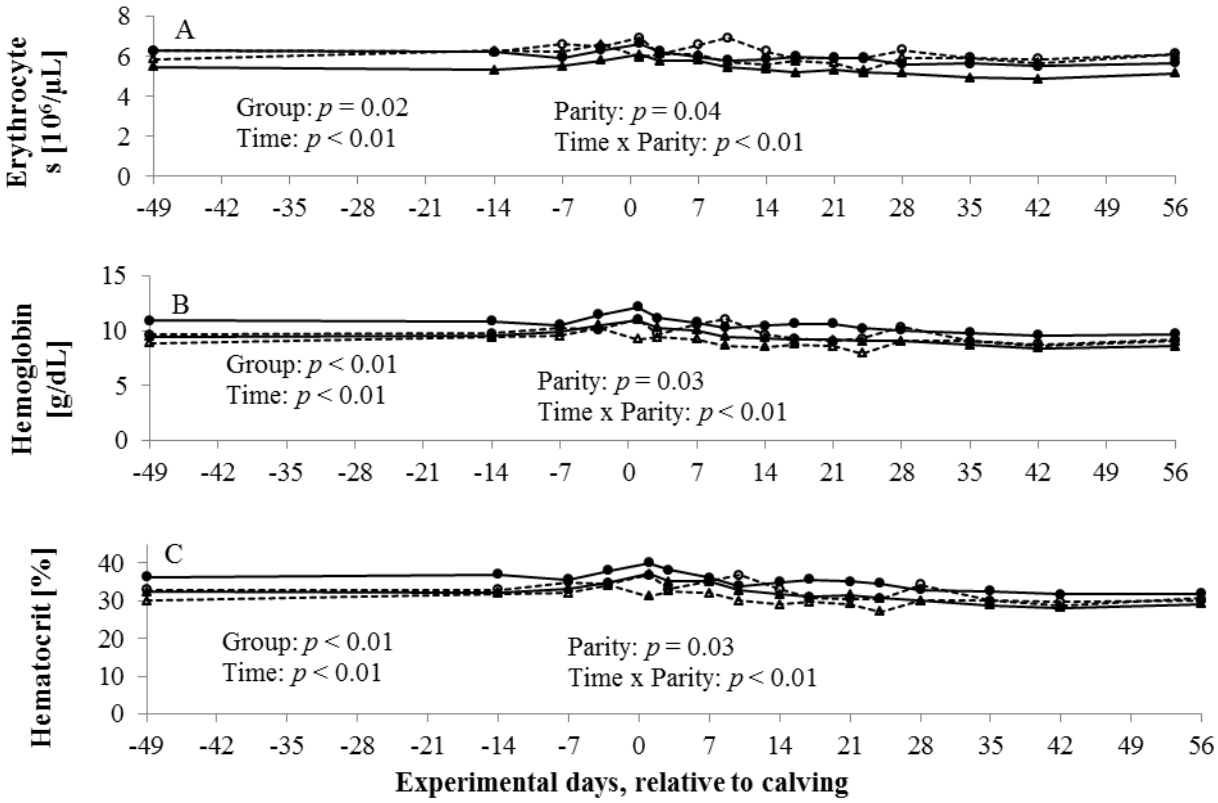

Figure 2. Concentrations of erythrocytes (A), haemoglobin (B) and haematocrit (C) in cows of a higher (HBC) and lower body condition (LBC). HBC cows suffered from subclinical ketosis (β-hydroxybutyrate ≥1.2 mM in blood) during the first two weeks post-partum, while LBC cows stayed metabolically healthy. Comparison between primiparous and pluriparous cows (LSmeans). The statistical analysis included the Group (HBC versus LBC), Parity (cow versus heifer), Day of experiment and interactions. For all parameters, the triple interaction Group x Day x Parity was significant ($p < 0.05$). Fix factors and interactions not included in the graph are not significant ($p > 0.05$). Note: HBC cows (●), HBC heifers (○), LBC cows (▲) and LBC heifers (Δ).

Haptoglobin

Time-dependent alterations in serum Hp concentrations within the experimental periods are shown in Table 1. For both feeding groups, the increase from Period 1 to Period 2, as well as the decrease from Period 2 to Period 3, was significant. Hp concentrations were affected neither by group nor parity, but there was a significant Period x Parity interaction. For heifers and cows, both the increase from Period 1 to Period 2 and the decrease from Period 2 to Period 3 were significant, with greater differences for heifers. In Period 2, heifers had numerically greater Hp concentrations than pluriparous cows (Δ = 0.56 mg/ml), albeit the level of significance was not reached. Haptoglobin concentrations peaked on day 3 post-partum, with 3.08 and 2.52 mg/ml for HBC and LBC, respectively. In the HBC group the increase from days 1 to 3 was significant. Nadir concentrations were observed six weeks before expected calving, with values ≤0.07 mg/ml in both groups.

A moderate correlation was noted for serum Hp with BHB and NEFA values across all periods. Goodness of fit (R^2) was 0.27 for BHB ($p < 0.001$) and 0.40 for NEFA ($p < 0.001$) for all cows, irrespective of group. A combined multiple regression of Hp versus BHB and NEFA increased R^2 to 0.41 ($p < 0.001$) and is shown in Figure 3. The correlation effect became stronger when only the data of the HBC group were taken into account. For HBC cows alone, R^2 was 0.52 ($p < 0.001$) for BHB and 0.60 for NEFA ($p < 0.001$), and the multiple regression of Hp versus BHB and NEFA increased R^2 to 0.63 ($p < 0.001$).

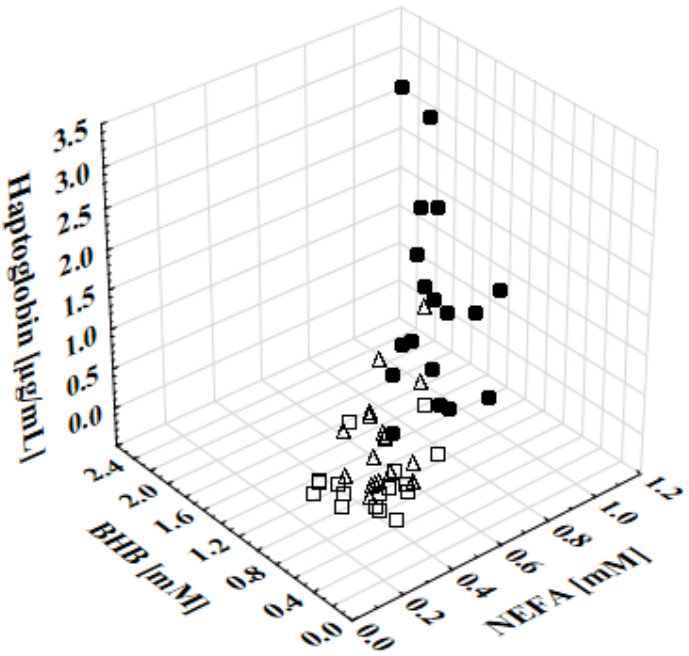

Figure 3. Combined linear multitude regression (3D scatter-plot). Relationship between serum concentrations of haptoglobin (z-axis), β-hydroxybutyrate (BHB; y-axis) and non-esterified fatty acid (NEFA; x-axis) of dairy cows (n = 18) across periods: z = -0.5588 + 1.9155x + 0.3401y (R^2 = 0.41; $p < 0.01$). Note: Period 1 (day -49 until calving □), Period 2 (first two weeks post-partum ●) and Period 3 (week 3-8 post-partum Δ).

Table 1. Haematological variables (whole blood analysis), including serum haptoglobin concentration, compared between cows of a higher condition (HBC) with β-hydroxybutyrate concentrations in blood serum indicative for subclinical ketosis (1.3 ± 0.08 mM during Period 2) and cows of a lower condition (LBC), which remained metabolically healthy (LSmeans ± SE).

	LBC group (n = 9)			HBC group (n = 9)			Probability[‡]				
Variable	Period 1[†]	Period 2[¶]	Period 3[+]	Period 1	Period 2	Period 3	Group	Period	Parity	Group x Period	Period x Parity
Leucocytes [10^3/µl]	7.72 ± 0.46	7.22 ± 0.41	7.11 ± 0.38	8.22 ± 0.45	8.45 ± 0.42	7.48 ± 0.38	0.163	0.038	0.209	0.257	0.095
Lymphocytes [10^3/µl]	3.03 ± 0.15	2.66 ± 0.14	2.86 ± 0.14	3.16 ± 0.15	3.14 ± 0.14	3.09 ± 0.14	0.174	0.003	0.015	0.003	0.002
Monocytes [10^3/µl]	0.20 ± 0.03	0.18 ± 0.02	0.18 ± 0.02	0.19 ± 0.03	0.20 ± 0.02	0.22 ± 0.02	0.378	0.816	0.360	0.579	0.058
Granulocytes [10^3/µl]	4.20 ± 0.40	3.98 ± 0.33	3.82 ± 0.30	4.54 ± 0.39	4.72 ± 0.35	3.86 ± 0.30	0.304	0.087	0.920	0.431	0.271
Erythrocytes [10^6/µl]	5.87 ± 0.14	5.79 ± 0.13	5.44 ± 0.13	6.30 ± 0.14	6.31 ± 0.13	5.87 ± 0.13	0.024	<0.001	0.039	0.617	0.306
Haemoglobin [g/dl]	9.63 ± 0.21	9.51 ± 0.19	8.80 ± 0.19	10.43 ± 0.20	10.64 ± 0.19	9.65 ± 0.18	0.002	<0.001	0.019	0.273	0.237
Hematocrit [%]	32.45 ± 0.66	32.62 ± 0.61	29.61 ± 0.58	35.25 ± 0.65	35.74 ± 0.62	32.10 ± 0.59	0.003	<0.001	0.018	0.622	0.209
MCV[$] [fl]	55.68 ± 1.10	56.59 ± 1.09	54.79 ± 1.09	56.07 ± 1.10	56.82 ± 1.09	54.93 ± 1.09	0.887	<0.001	<0.001	0.684	0.783
MCH[§] [pg]	16.53 ± 0.34	16.49 ± 0.34	16.29 ± 0.34	16.60 ± 0.34	16.92 ± 0.34	16.48 ± 0.34	0.635	<0.001	<0.001	0.084	0.760
MCHC[‖] [g/dl]	29.68 ± 0.15	29.14 ± 0.12	29.71 ± 0.11	29.57 ± 0.15	29.77 ± 0.13	30.08 ± 0.11	0.018	<0.001	0.552	0.017	0.828
RDW[♦] [%]	15.06 ± 0.27	15.10 ± 0.26	14.42 ± 0.25	15.10 ± 0.27	15.42 ± 0.26	14.89 ± 0.25	0.439	<0.001	0.697	0.103	<0.001
Thrombocytes [10^3/µl]	447.6 ± 25.1	479.7 ± 21.8	539.4 ± 20.5	452.9 ± 24.8	471.4 ± 22.5	516.0 ± 20.6	0.740	<0.001	0.470	0.670	0.249
PCT[#] [%]	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.156	<0.001	0.727	0.193	0.428
PDW[◊] [%]	17.44 ± 0.23	17.18 ± 0.20	16.81 ± 0.19	17.98 ± 0.23	17.87 ± 0.21	17.52 ± 0.19	0.013	0.002	0.006	0.872	0.527
Haptoglobin [mg/ml]	0.26 ± 0.21	1.42 ± 0.17	0.38 ± 0.15	0.09 ± 0.20	1.61 ± 0.18	0.34 ± 0.16	0.959	<0.001	0.927	0.575	0.009

Note: [†]Period 1, 6 weeks before expected parturition until calving; [¶]Period 2, first 2 weeks post-partum; [+]Period 3, week 3–8 post-partum; [$]MCV: mean corpuscular volume, [§]MCH: mean corpuscular haemoglobin, [‖]MCHC: mean corpuscular haemoglobin concentration, [♦]RDW: red blood cell distribution width, [#]PCT: Relative volume of thrombocytes, [◊]RDW: Platelet distribution width; [‡]Triple interaction of Group x Period x Parity was statistically significant for the parameters leucocytes, lymphocytes, granulocytes, erythrocytes, haemoglobin and haematocrit. In none of the investigated parameters was the interaction Group x Parity significant.

PBMC proliferation ex vivo

The SI of PBMC differed significantly over time, but was insignificant between the groups on the days evaluated (Table 2). In both groups, SI was lowest on day 56 after calving and differed significantly to all other days within the HBC group. SI was affected by parity and tended to be higher for primiparous versus pluriparous cows (6.89 versus 5.97, $p = 0.092$). Seven days after calving, when the HBC cows became subclinically ketotic, the SI of pluriparous cows was higher (7.12 ± 0.75) than that of primiparous cows (6.53 ± 0.84), and that of primi- and pluriparous LBC individuals (heifers: 6.14 ± 1.09; pluriparous cows: 5.52 ± 0.97) even though inter- or intra-group differences were insignificant.

Within the HBC group, the mean SI of days when serum BHB value ≥1.2 mM was compared to that when the same cows had BHB concentrations within the physiological range. At a BHB concentration of ≥1.2 mM (LSmean of 1.6 mM), SI increased to 7.04, while BHB values <1.2 mM (LSmean of 0.76 mM) resulted in lower SI (5.88 SI-units; $p = 0.038$).

Table 2. Functionality of peripheral blood mononuclear cells (PBMC), expressed as stimulation index (SI[†]), between cows of a higher condition (HBC) with β-hydroxybutyrate concentrations in blood serum indicative of subclinical ketosis (1.65 ± 0.15 mM on day +7) and cows of a lower condition (LBC), which stayed metabolically healthy (LSmeans ± SE).

	Experimental day (relative to calving)				Probability[‡]			
	Day -14	Day +7	Day +21	Day +56	Group	Time	Parity	Group x Time
Group LBC (n = 9)	8.22 ± 0.73	5.83 ± 0.73	7.46 ± 0.80	5.43 ± 0.73	0.248	< 0.001	0.092	0.207
Group HBC (n = 9)	6.92 ± 0.56	6.83 ± 0.56	6.78 ± 0.56	4.00 ± 0.60				

Note: [†]SI is defined as the ratio between fluorescence in the Alamar Blue assay of concanavalin A-stimulated and -non-stimulated PBMC; [‡]Interactions of fixed factors (Group, Day, Parity) not listed are not significant.

Dose-response studies in vitro

Dose-response curves (based on SI obtained in the AB assay) were fitted to Equation (2) and used to calculate IC50 values. IC50 values (means ± SD) for BHB on day 7 after calving were 6.93 ± 1.29 and 6.63 ± 2.79 mM for the LBC and HBC groups, respectively ($p = 0.872$). SI decreased significantly starting at a concentration of 5 mM BHB for both groups (Figure 4). Furthermore, there was a highly significant group effect and an interaction of Group x BHB concentration ($p = 0.038$).

For investigation of NO release in the supernatants of LPS-stimulated and -non-stimulated PBMC, 30 % of results were below the limit of detection (0.125 µM); LPS-non-stimulated cells predominated (70 %). Statistical analysis in the form of a graph is shown in Figure 5. Within the LBC group, LPS-stimulated cells without BHB (medium control) and cells incubated with 5 mM BHB released significantly more NO compared with LPS-stimulated cells from the LBC group incubated with 20 mM BHB. There were no significant differences among the groups in regard to the concentration of BHB for different BHB concentrations. LPS significantly increased NO release levels in the PBMC supernatant in both groups. Across all concentrations of BHB, LPS-induced increase in NO was greater in LBC cows (LBC: 0.503 µM versus HBC: 0.339 µM, p = 0.027). Setting the medium control at 100 %, NO release in the presence of BHB remained lower, independent of LPS application. The physiological concentration of 0.625 mM BHB for unstimulated cells from LBC animals proved the exception, being increased by 20% compared with the BHB-less control.

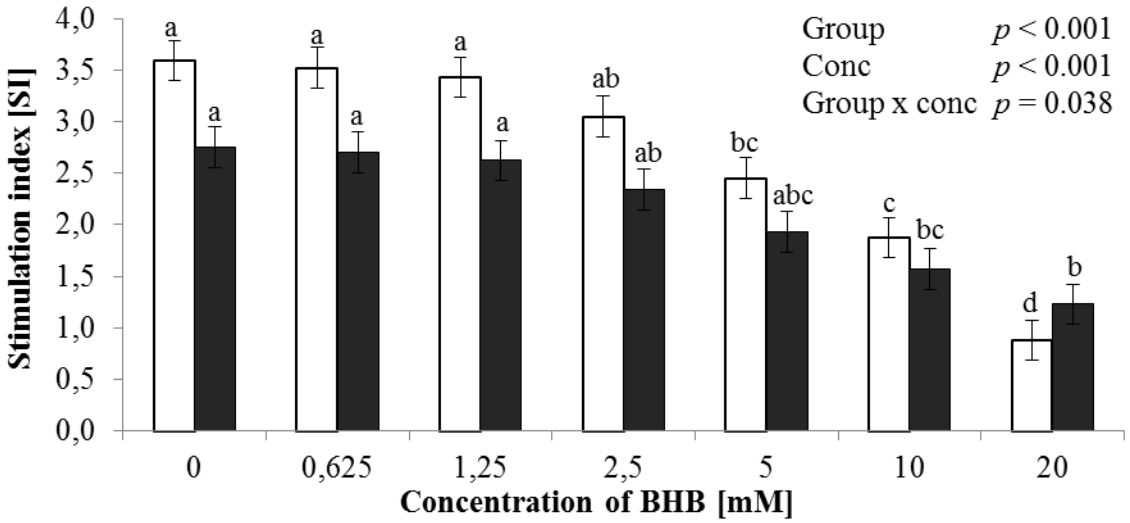

Figure 4. Proliferative capability of bovine peripheral blood mononuclear cells *in vitro*, expressed as stimulation index (LSmeans ± SE).

Notes: Cows with a higher body condition [HBC (■); n = 5] had β-hydroxybutyrate (BHB) concentrations in blood indicative of subclinical ketosis (≥1.2 mM), while cows with a lower body condition [LBC (□); n = 5] stayed apparently healthy (< 1.2 mM BHB). The statistical analysis included Group (HBC and LBC), Concentration (Conc) of BHB and Interactions.

[a - d]: Different letters indicate significant differences within the same group ($p < 0.05$)

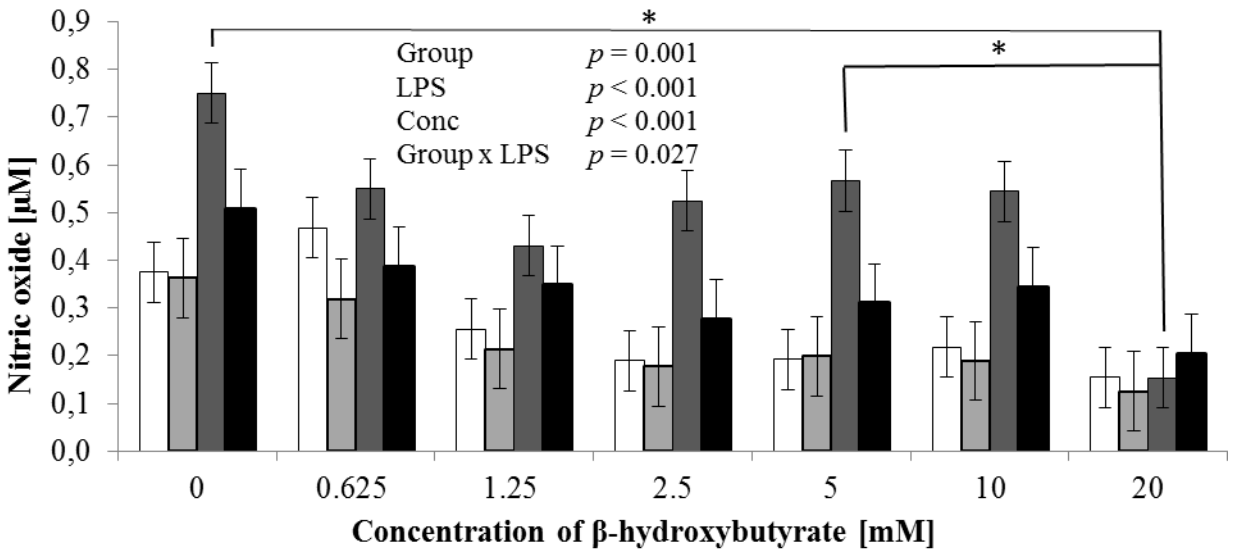

Figure 5. Release of nitric oxide of bovine peripheral blood mononuclear cells *in vitro* (LSmeans ± SE).
Notes: Cows with a higher body condition [HBC; $n = 5$] had β-hydroxybutyrate (BHB) concentrations in blood indicative of subclinical ketosis (≥ 1.2 mM), while cows with a lower body condition [LBC; $n = 5$] stayed apparently healthy (<1.2 mM BHB). Lipopolysaccharide (LPS) was used as both stimulus and positive control. The statistical analysis included Group (HBC and LBC), Concentration (Conc) of BHB and LSP (stimulated or non-stimulated) and Interactions. Interactions not included in the graph are not significant ($p > 0.05$).
LBC without LPS (□), HBC without LPS (■), LBC + LPS (■), HBC + LPS (■)]. * $p \leq 0.05$.

Discussion

It is known that nutrition plays a key role in the immune response and that nutrients and metabolites can influence several aspects of the immune system (Ingvartsen and Moyes 2012). For example, high concentrations of ketone bodies inhibit lymphocyte blastogenesis, oxidative burst and IgM secretion (Nonnecke et al. 1992, Sato et al. 1995) while changes in energy balance influence, for example, the acute-phase response and cytokine production (Moyes et al. 2009). Subclinical ketosis occurs mostly during the first four weeks after calving and therefore peri-partum physiological alterations and a supply of energy with essential nutrients are likewise important for the immune system.

Hematological variables

For almost all elevated parameters, parity has a significant influence. Especially in heifers, concomitant peri-partum metabolic and endocrine changes include modified haematological variables (e.g. WBC and RBC counts, haemoglobin). In part, these modifications are normal, reflecting the specific status of heifers compared with pluriparous cows (Kraft and Duerr 2005, Mallard et al. 1998).

When group-specific differences were evaluated, the varying BCS of LBC and HBC animals during the experiment must be considered (Schulz et al. 2014). It has long been known that enhanced body condition increases the risk of peri-partum complications (Roche et al. 2009). A common cause of dystocia is the disproportion between the birth canal and the size of the foetus, which is particularly important in primiparous cows (Nahkur et al. 2011). An energy oversupply during the dry period may result in higher foetal growth rates. In the present investigation HBC calves appeared to have a higher birth weight but the proportion of male and female calves was imbalanced [HBC: 25% male (51.60 ± 2.6 kg), 75% female (40.5 ± 2.4 kg) versus LBC: 67% male (40.9 ± 4.1 kg), 33% female (42.4 ± 4.5 kg)]. Differences with regard to ease of calving between the groups were not detected but in particular HBC heifers seemed to be more prone to dystocia, reflected by higher concentrations of LY and GR. Haematological variables remained almost unaffected by subclinical ketosis. The small peaks of LY and RBC HBC heifers around day +10, and a significantly higher MCHC starting in Period 2 for the HBC animals, are linked to ketotic status. In human studies, alcohol-related fatty liver results in increased MCHC (Wickramasinghe et al. 1994). Pathophysiological hepatic alterations in HBC cows, characterized by liver lipid contents of about 15% and rising concentrations of aspartate transaminase and glutamate dehydrogenase (Schulz et al. 2014), indicate that hepatic stress may also be related to increasing MCHC.

Serum Hp concentrations

Based on the literature, Hp was expected to differ significant between cows with and without the presence of fatty liver. The present results show that Hp concentrations in ketotic cows were twofold higher compared with those detected by Ametaj et al. (2005) in cows with fatty liver. The significant Parity x Period interaction reflects an influence of parity, resulting in higher Hp concentrations in the primiparous cows. The case of liver lipid content showed the opposite, with values nearly twofold those of pluriparous compared with primiparous cows (data not shown). Although a pro-inflammatory situation is typical for the time around calving (Hachenberg et al. 2007, Saremi et al. 2014), the increase in Hp concentrations seen here in primiparous cows may indicate that peri-partum changes were more stressful for heifers than for pluriparous cows, independent of feeding group. Therefore, increases in Hp concentration in pluriparous HBC cows due to hepatic lipidosis resulted in no significant difference between feeding groups. However, the heightened level of hepatic lipidosis in HBC animals was indirectly related to Hp, as 63 % of Hp variance could be explained by BHB and NEFA concentrations. Recent studies showed similar results and positive correlations, either between Hp and NEFA (Saremi et al. 2012) or Hp and BHB (Fathi et al. 2013).

Functionality of bovine PBMC ex vivo and in vitro

Several studies reported reduced lymphocyte responsiveness around calving (Mallard et al. 1998). For example, Kehrli et al. (1989) demonstrated that lymphocyte blastogenic responses of Holstein heifers increased two weeks pre-partum and also reported a marked impairment of PBMC blastogenesis during the first week post-partum. The present results confirmed this

immunosuppression: the proliferative capability, expressed as SI, of PBMC from cows with no evidence of ketosis was higher on day 14 pre-partum than on day 7 post-partum (ΔSI = - 2.39; Table 2); and they are in accord with observations from Renner et al. (2012). In addition, the present authors expected that the proliferative capacity of lymphocytes would be greater in heifers than in cows (Mehrzad and Zhao 2008) and demonstrated a similar trend between primi- and pluriparous animals (Δ = 0.92, P = 0.092). Interestingly, the SI of pluriparous HBC cows peaked shortly after calving and during subclinical ketosis. This might reflect a better coping capacity in animals that had previously experienced such a metabolic situation, with improved PBMC proliferative ability. Dänicke et al. (2012) described BHB values of up to 1.5 mM in blood serum stimulating bovine PBMC in response to ConA. A similar calculation of the existing data confirmed this observation and showed that time points when BHB values were above the physiological range, proliferative capability was enhanced. In agreement with these results, a marked increase in BHB concentrations up to 1.2 mM did not impair the responsiveness of PBMC to ConA, which was also shown in the in vitro experiments. The present results indicate that the concentrations of BHB occurring in vivo during subclinical ketosis, did not alter the proliferative capability of bovine PBMC in vitro (BHB dosage of 1.25 mM), which was first significantly decreased at a dosage of 5 mM BHB. Earlier studies yielded similar results, and even heightened concentrations of BHB significantly diminished ConA-stimulated PBMC proliferation in vivo (> 6.25 mM) and ex vivo (> 2.0 mM) (Franklin et al. 1991, Sato et al. 1995). PBMC counts in LBC cows were not pre-stressed by subclinical conditions in vivo and were therefore more sensitive during the in vitro investigation, because increasing the concentration of BHB in vitro reduced the difference in SI for cells from LBC versus HBC cows, with analogous results for the release of NO in the PBMC supernatant.

To our knowledge, the release of NO in the PBMC supernatant media, obtained from healthy cows and cows with subclinical ketosis, has not been reported to date. Similar to Hp, NO is shown to increase during inflammatory diseases such as bovine mastitis and various observations indicate a close relationship between tumor necrosis factor-α and NO production in lactating cows (Hirvonen et al. 1999, Kushibiki et al. 2003). In rat macrophages, reduced NO production was linked to a decreased capacity for glucose utilisation in these cells (Alvarez et al. 1996). In ketosis, glucose levels are diminished and PBMC in cows with greater glucose utilisation (LBC group) might produce more NO, accompanied by a higher susceptibility of NO-producing cells, compared with PBMC of cows suffering from a subclinical ketosis (HBC group). Nevertheless, further research, also with respect to the results of in vivo investigations, will be necessary.

Conclusion

The present study showed that almost all parameters were influenced by parity. Therefore, elevated parameters in heifers were closely related to higher peri-partum stress, related to increased impact associated with dystocia. Cows with subclinical ketosis showed a heightened immune response based on increased concentrations of LY, increasing stimulative properties of PBMC and a relationship between Hp and typically increased values of BHB and NEFA. Concentrations up to 1.2 mM BHB had no negative impact according to the proliferative

capability of PBMC, and inhibitory effects were limited to concentrations of 5 mM and higher during in vitro investigation. The PBMC count of cows metabolically healthy was not pre-stressed by elevated BHB concentrations in vivo and was more sensitive during in vitro investigation, with a higher proliferative capability and increased release of NO.

Acknowledgements

The authors would like to thank the co-workers of the Institute of Animal Nutrition (FLI) and Institute of Animal Science (Physiology and Hygiene Unit) in Braunschweig and Bonn for their assistance in performing experiment and analyses. Furthermore, we gratefully acknowledge Boehringer Ingelheim for cooperation (Boehringer Ingelheim Vetmedica, D-55216 Ingelheim am Rhein, Germany).

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Alvarez E, Machado A, Sobrino F, Santa Maria C. 1996. Nitric oxide and superoxide anion production decrease with age in resident and activated rat peritoneal macrophages. Cell Immunol. 169:152-155.

Ametaj BN, Bradford BJ, Bobe G, Nafikov RA, Lu Y, Young JW, Beitz DC. 2005. Strong relationships between mediators of the acute phase response and fatty liver in dairy cows. Can J Anim Sci. 85:165-175.

Bouchard L, Blais S, Desrosiers C, Zhao X, Lacasse P. 1999. Nitric oxide production during endotoxin-induced mastitis in the cow. J Dairy Sci. 82:2574-2581.

Dänicke S, Kowalczyk J, Renner L, Pappritz J, Meyer U, Kramer R, Weber E-M, Döll S, Rehage J, Jahreis G. 2012. Effects of conjugated linoleic acids fed to dairy cows during early gestation on hematological, immunological, and metabolic characteristics of cows and their calves. J Dairy Sci. 95:3938-3953.

Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G. 1989. A body condition scoring chart for Holstein dairy cows. J Dairy Sci. 72:68-78.

Esposito G, Irons PC, Webb EC, Chapwanya A. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim Reprod Sci. 144:60-71.

Fathi E, Hamali H, Kaleibar MT. 2013. Application of acute phase proteins as indicators of ketosis and their relation to energy metabolites in post-calving dairy cows. Int J Recent Sci Res. 4:842 - 845.

Franklin ST, Young JW, Nonnecke BJ. 1991. Effects of ketones, acetate, butyrate, and glucose on bovine lymphocyte proliferation. J Dairy Sci. 74:2507-2514.

Hachenberg S, Weinkauf C, Hiss S, Sauerwein H. 2007. Evaluation of classification modes potentially suitable to identify metabolic stress in healthy dairy cows during the peripartal period. J Anim Sci. 85:1923-1932.

Hirvonen J, Eklund K, Teppo AM, Huszenicza G, Kulcsar M, Saloniemi H, Pyorala S. 1999. Acute phase response in dairy cows with experimentally induced Escherichia coli mastitis. Acta Vet Scand. 40:35-46.

Hiss S, Mielenz M, Bruckmaier RM, Sauerwein H. 2004. Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. J Dairy Sci. 87:3778-3784.

Ingvartsen KL, Moyes K. 2012. Nutrition, immune function and health of dairy cattle. Animal. 7:112-122.

Kehrli ME, Jr., Nonnecke BJ, Roth JA. 1989. Alterations in bovine lymphocyte function during the periparturient period. Am J Vet Res. 50:215-220.

Klucinski W, Degorski A, Miernik-Degorska E, Targowski S, Winnicka A. 1988. Effect of ketone bodies on the phagocytic activity of bovine milk macrophages and polymorphonuclear leukocytes. Zentralbl Veterinarmed A. 35:632-639.

Kraft W, Dürr U. 2005. Klinische Labordiagnostik in der Tiermedizin [Clinical laboratory diagnostics of veterinary medicine] Stuttgart: Schattauer Verlag.

Kushibiki S, Hodate K, Shingu H, Obara Y, Touno E, Shinoda M, Yokomizo Y. 2003. Metabolic and lactational responses during recombinant bovine tumor necrosis factor-α treatment in lactating cows. J Dairy Sci. 86:819-827.

Lacetera N, Scalia D, Franci O, Bernabucci U, Ronchi B, Nardone A. 2004. Short communication: effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J Dairy Sci. 87:1012-1014.

Lessard M, Gagnon N, Godson DL, Petit HV. 2004. Influence of parturition and diets enriched in n-3 or n-6 polyunsaturated fatty acids on immune response of dairy cows during the transition period. J Dairy Sci. 87:2197-2210.

Mallard BA, Dekkers JC, Ireland MJ, Leslie KE, Sharif S, Lacey Vankampen C, Wagter L, Wilkie BN. 1998. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. J Dairy Sci. 81:585-595.

Mehrzad J, Zhao X. 2008. T lymphocyte proliferative capacity and CD4+/CD8+ ratio in primiparous and pluriparous lactating cows. J Dairy Res. 75:457-465.

Mercer LP, Dodds SJ, Smith DL. 1987. New method for formulation of amino acid concentrations and ratios in diets of rats. J Nutr. 117:1936-1944.

Moyes KM, Drackley JK, Salak-Johnson JL, Morin DE, Hope JC, Loor JJ. 2009. Dietary-induced negative energy balance has minimal effects on innate immunity during a Streptococcus uberis mastitis challenge in dairy cows during midlactation. J Dairy Sci. 92:4301-4316.

Nahkur E, Ernits E, Jalakas M, Järv E. 2011. Morphological characteristics of pelves of estonian holstein and estonian native breed cows from the perspective of calving. Anat Histol Embryol. 40:379-388.

Nonnecke BJ, Franklin ST, Young JW. 1992. Effects of ketones, acetate, and glucose on in vitro immunoglobulin secretion by bovine lymphocytes. J Dairy Sci. 75:982-990.

Renner L, Kersten S, Duevel A, Schuberth H-J, Dänicke S. 2013. Effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid, linoleic acid, phytanic acid and the combination of various fatty acids on proliferation and cytokine expression of bovine peripheral blood mononuclear cells. Nutrients. 5:2667-2683.

Renner L, Pappritz J, Kramer R, Kersten S, Jahreis G, Dänicke S. 2012. Fatty acid profile and proliferation of bovine blood mononuclear cells after conjugated linoleic acid supplementation. Lipids Health Dis. 11:63-67.

Roche JR, Friggens NC, Kay JK, Fisher MW, Stafford KJ, Berry DP. 2009. Invited review: body condition score and its association with dairy cow productivity, health, and welfare. J Dairy Sci. 92:5769-5801.

Saremi B, Al-Dawood A, Winand S, Müller U, Pappritz J, von Soosten D, Rehage J, Dänicke S, Häussler S, Mielenz M, Sauerwein H. 2012. Bovine haptoglobin as an adipokine: serum concentrations and tissue expression in dairy cows receiving a conjugated linoleic acids supplement throughout lactation. Vet Immunol Immunopathol. 146:201-211.

Saremi B, Winand S, Friedrichs P, Kinoshita A, Rehage J, Dänicke S, Häussler S, Breves G, Mielenz M, Sauerwein H. 2014. Longitudinal profiling of the tissue-specific expression of genes related with insulin sensitivity in dairy cows during lactation focusing on different fat depots. Plos One. 9:e86211.

SAS Institute. 2004. The SAS/STAT 9.1 User's Guide. Vol. 1-7. In: SAS Institute Cary, NC.

Sato S, Suzuki T, Okada K. 1995. Suppression of mitogenic response of bovine peripheral blood lymphocytes by ketone bodies. J Vet Med Sci. 57:183-185.

Schulz K, Frahm J, Meyer U, Kersten S, Reiche D, Rehage J, Dänicke S. 2014. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis,

energy balance and ketogenesis: an animal model to investigate subclinical ketosis. J Dairy Res. 81:257-266.

StatSoft I. 2011. STATISTICA for Windows Operating System (Version 10.0). In: Tulsa (OH): StatSoft.

Targowski SP, Klucinski W. 1983. Reduction in mitogenic response of bovine lymphocytes by ketone bodies. Am J Vet Res. 44:828-830.

Trevisi E, Bertoni G, Lombardelli R, Minuti A. 2013. Relation of inflammation and liver function with the plasma cortisol response to adrenocorticotropin in early lactating dairy cows. J Dairy Sci. 96:5712-5722.

Uchida E, Katoh N, Takahashi K. 1993. Appearance of haptoglobin in serum from cows at parturition. J Vet Med Sci. 55:893-894.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 39:44-84.

Wickramasinghe SN, Corridan B, Hasan R, Marjot DH. 1994. Correlations between acetaldehyde-modified haemoglobin, carbohydrate-deficient transferrin (CDT) and haematological abnormalities in chronic alcoholism. Alcohol Alcohol. 29:415-423.

5. Paper III (published in PLOS ONE, 2015, 10(8):e0136078)

Effects of inhibiting dipeptidyl peptidase-4 (DPP4) in cows with subclinical ketosis

Kirsten Schulz[1], Jana Frahm[1]*, Susanne Kersten[1], Ulrich Meyer[1], Jürgen Rehage[2], Marion Piechotta[2], Maria Meyerholz[2], Gerhard Breves[3], Dania Reiche[4], Helga Sauerwein[5], Sven Dänicke[1]

[1] Institute of Animal Nutrition, Federal Research Institute for Animal Health, Friedrich-Loeffler-Institute, Braunschweig, Germany

[2] Clinic for Cattle, University of Veterinary Medicine Hannover, Hannover, Germany

[3] Department of Physiology, University of Veterinary Medicine Hannover, Hannover, Germany

[4] Boehringer Ingelheim Vetmedica, Ingelheim am Rhein, Germany

[5] Physiology and Hygiene Unit, Institute of Animal Science, University of Bonn, Bonn, Germany

* Corresponding Author: E-Mail: jana.frahm@fli.bund.de

Abstract

The inhibition of dipeptidyl peptidase-4 (DPP4) via specific inhibitors is known to result in improved glucose tolerance and insulin sensitivity and decreased accumulation of hepatic fat in type II diabetic human patients. The metabolic situation of dairy cows can easily be compared to the status of human diabetes and non-alcoholic fatty liver. For both, insulin sensitivity is reduced, while hepatic fat accumulation increases, characterized by high levels of non-esterified fatty acids (NEFA) and ketone bodies. Therefore, in the present study, a DPP4 inhibitor was employed (BI 14332) for the first time in cows. In a first investigation BI 14332 treatment (intravenous injection at dosages of up to 3 mg/kg body weight) was well tolerated in healthy lactating pluriparous cows (n = 6) with a significant inhibition of DPP4 in plasma and liver. Further testing included primi- and pluriparous lactating cows suffering from subclinical ketosis (β-hydroxybutyrate concentrations in serum > 1.2 mM; n = 12). The intension was to offer effects of DPP4 inhibition during comprehensive lipomobilisation and hepatosteatosis. The cows of subclinical ketosis were evenly allocated to either the treatment group (daily injections, 0.3 mg BI 14332/kg body weight, 7 days) or the control group. Under condition of subclinical ketosis, the impact of DPP4 inhibition via BI 14332 was less, as in particular β-hydroxybutyrate and the

hepatic lipid content remained unaffected, but NEFA and triglyceride concentrations were decreased after treatment. Owing to lower NEFA, the revised quantitative insulin sensitivity check index (surrogate marker for insulin sensitivity) increased. Therefore, a positive influence on energy metabolism might be quite possible. Minor impacts on immune-modulating variables were limited to the lymphocyte $CD4^+/CD8^+$ ratio for which a trend to decreased values in treated versus control animals was noted. In sum, the DPP4 inhibition in cows did not effect glycaemic control like it is shown in humans, but was able to impact hyperlipemia, as NEFA and TG decreased.

Introduction

Dipeptidyl peptidase-4 (DPP4) plays a major role in glucose metabolism and is responsible for the degradation of incretin hormones, such as glucagon-like peptide-1 (GLP-1). Human GLP-1 is released from the small intestine in response to oral glucose [1]. It stimulates insulin secretion via activating specific receptors on the islet β-cells, suppresses glucagon secretion, inhibits gastric emptying and reduces appetite [2]. Furthermore, chronic elevated concentrations of GLP-1 were shown to result in reduced hepatic fat accumulation and significantly lower TG concentrations in rat and mouse model [3]. However, after enzymatic degeneration via DPP4, which occurs within minutes following ingestion, only 10-20% of active GLP-1 remains in blood. Today, DPP4 inhibitors are employed in human medicine to prolong the beneficial incretin effects, in particular to improve insulin sensitivity, with the aim to treat type II diabetes [4].

In high-yielding dairy cows, the metabolic status around calving and the onset of lactation exhibits huge parallels to patients suffering from type II diabetes and non-alcoholic fatty liver diseases, as comprehensive physiological challenges are necessary to coordinate the metabolic alterations. In the transition from late pregnancy and early lactation, decreases in insulin concentration and peripheral insulin responsiveness suppress glucose consumption by peripheral, insulin-dependent tissues (skeletal muscle, adipose tissue) and thus enhance the availability of glucose for the insulin-independent mammary gland [5]. The adaption to the negative energy balance (NEB) is often related to metabolic dysfunctions, such as excessive lipid accumulation in the liver and ketosis [6-8], characterized by increased concentrations of non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHB). Due to the infiltration of fat, lesions in hepatic tissues appear and cause increased blood levels of specific enzymes, such as γ-glutamyl transferase (γ-GT), aspartate transaminase (AST) or glutamate dehydrogenase (GLDH) [9]. Furthermore, a fatty

liver contributes the development of hepatic insulin resistance and influences body´s immune system negatively. In particular, the impacts of tumor necrosis factor-α and acute phase protein reactions are well studied in cows with fatty liver and its role in immune response [10,11].

Little is known about the metabolism of incretins and its interaction with DPP4 in ruminants. In contrast to monogastric species, the cow does not rely on glucose absorption in the small intestine but uses short chain fatty acids from ruminal fermentation for her energy supply with propionate as main substrate for gluconeogenesis. However, increasing dietary energy supply has been shown to enhance the secretion of GLP-1 in steers [12] and abomasal infusion of lipid and casein, but not glucose, increased the GLP-1 concentration in cows [13,14]. The fat-induced elevation in circulating GLP-1 is believed to play a role in the short-term control of feed intake in cattle [14,15], but the wide range of tissues expressing the GLP-1 receptor (gut segments, pancreas, spleen and kidney) suggest that GLP-1 may have multiple physiological functions beyond the control of feed intake [16]. The DPP4 expression and the circulating GLP-1 concentrations in blood depend on stage of lactation. While GLP-1 concentrations increase with onset of lactation, the expression of DPP4 decreases [13,17].

Taking the background information into consideration, DPP4 is a key enzyme in intermediary metabolism by regulating important glycemic pathways. Therefore, it was possible that DPP4 inhibitors could counteract typical ketotic processes in the dairy cow. Within the present research a DPP4 inhibitor (BI 14332) was employed to regulate typically increased parameters of bovine ketosis to the physiological range, respectively to compensate a distinct NEB. Therefore, we first established appropriate dosage of BI 14332 to effectively decrease DPP4 activity in plasma and liver from healthy lactating dairy cows. The second aim was to verify the efficacy of the derived dose and dosing regimen in cows with subclinical ketosis based on evaluation of various endpoints, such as clinical-chemical parameters and immune traits as well as liver lipid concentration.

Materials and Methods

Ethic statement

The experiments were approved by the competent authority, the lower Saxony state office for consumer protection and food safety (LAVES; Trial 1: file no. 33.9-42502-05-11A172, Trial 2:

file no. 33.14-42502-04-11/0444; Oldenburg, Germany). The regulations of the German Animal Welfare Act (TierSchG) in its respective edition were met.

Experimental design

The investigations about the pharmacokinetics and pharmacodynamics (PK/PD) of BI 14332 were performed at the Clinic for Cattle, University of Veterinary Medicine in Hannover, Germany (Trial 1). The experiment aimed in evaluating the effectiveness of DPP4 inhibition in dairy cows with subclinical ketosis was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI) in Braunschweig, Germany (Trial 2).

Trial 1

Six lactating and clinically healthy German Holstein cows (pluriparous) were treated with three different doses, i.e. 0.3, 1.0 and 3.0 mg/kg body weight (BW; injection volume: 0.01 – 0.1 mL/kg) of BI 14332 (n = 2/dosage; i.v.).

To evaluate the concentration of BI 14332 and the DPP4 activity in plasma, samples were collected 24 h before the injection, 0, 0.25, 0.5, 1, 2, 4, 6, 12, 24, and 48 h after the injection. In addition, liver samples were collected according to Starke et al. [18] 24 h before injection and 4, 24 and 48 h thereafter to evaluate the hepatic DPP4 activity.

For an in vitro activity assay, potassium EDTA plasma samples of three healthy Holstein Frisian cows were incubated with 0, 1, 3, 10, 30 and 100 nM BI 14332.

Trial 2

Using an animal model in which subclinical ketosis is induced [19]; the impact of DPP4 inhibition via BI 14332 on metabolic variables and on immune function was investigated. β-hydroxybutyrate concentrations between 1.2 – 2.5 mM in blood serum were defined as a subclinical ketotic status [20].

The chemical compositions of concentrate and total mixed ration are shown in Table 1. For more details regarding feeding management refer to Schulz et al. [19]. In brief, 20 pregnant and healthy German Holstein cows with a mean body condition score (BCS) of 3.16 ± 0.06 [21] were assigned to the experimental group. For the last six weeks of parturition, cows were fed with a high energetic ration (7.7 MJ NEL/kg dry matter). The allocation of cows with a BCS of at least 3.0 and a high energetic energy supply ante partum resulted in a higher condition at the time of

calving (called "higher condition" cows; HC). The aim was to enhance lipomobilisation post partum. Immediately after calving, the energy supply by concentrate feeding was reduced first and raised stepwise (from 30% to 50% for the first three weeks of lactation).

Table 1. Ingredients and chemical composition of concentrate and total mixed ration.

	Ante partum diet[a]		Post partum diet[b]	
	Concentrate	TMR	Concentrate	TMR
Ingredients, %				
Wheat	41.0		41.0	
Dried sugar beet pulp	30.5		30.3	
Rapeseed meal	20.0		20.0	
Soybean meal	6.5		6.5	
Vitamin/mineral premix	2.0[c]		2.0[d]	
Calcium carbonate	-		0.2	
Dry matter (DM), g/kg	877	489	875	393
Nutrients [g/kg DM]				
Crude ash	58	55	62	56
Crude protein	197	140	202	122
Ether extract	27	33	28	32
Crude fibre	101	163	72	194
Acid detergent fibre (ADF)	136	199	96	222
Neutral detergent fibre (NDF)	279	394	222	431
Energy[e], MJ NEL/kg DM	8.6	7.7	8.7	7.0

[a]Total mixed ration (TMR) on DM basis (40% roughage (75% corn silage, 25% grass silage) + 60% concentrate.
[b]Total mixed ration on DM basis (70% roughage (75% corn silage, 25% grass silage) + 30% concentrate.
[c]Per kg of mineral feed: 10g Ca, 60g P, 120g Na, 60g Mg, 800,000 IU vitamin A, 100,000 IU vitamin D_3, 2500mg vitamin E, 4000mg Mn, 6000 mg Zn, 1250mg Cu, 100mg I, 35mg Co, 50mg Se
[d]Per kg of mineral feed: 170g Ca, 50g P, 120g Na, 45g Mg, 800,000 IU vitamin A, 100,000 IU vitamin D_3, 4000mg vitamin E, 4000mg Mn, 6000mg Zn, 1300mg Cu, 120mg I, 35mg Co, 40mg Se
[e]Calculation based on nutrient digestibilities masured with wethers (GfE, 1991) and values from feed tables (DLG, 1997)

Two cows from HC group were excluded from the experiment because of health problems, which were not due to the experimental design. Out of the 18 HC cows, 12 cows developed subclinical ketosis (serum BHB concentration ≥ 1.2 and < 2.5 mM). Six HC cows were treated with BI 14332 (HC-BI) over a period of 7 days (daily i.v. injections, 0.3 mg/kg BW/day) and the remaining six subclinical cows formed the control group (HC-Con) without treatment. Subclinical ketosis was diagnosed on day +3 (1 HC-Con cow), day +7 (5 HC-BI cows and 3 HC-Con cows) or on day +10 (1 HC-BI cow and 2 HC-Con cows), relative to calving. The remaining six HC cows were either affected with clinical ketosis (n = 3, BHB in serum > 2.5 mM) or stayed apparently healthy (BHB < 1.2 mM, n = 3). Blood samples were collected at day "0", i.e. 48 ± 7.2 days before expected calving, and then on day -14, -7, -3, +1, +3, +7, +10, +14, +17, +21,

+24, +28, +35, +42 and +56 (relative to calving) from the V. jugularis; liver biopsies were taken on day -14, +7, +21, +35 and +56.

Sample Preparation and Analysis

Trial 1

The DPP4 activity in plasma and liver was assessed by a semi-quantitative assay with fluorescence detection at the Institute for Clinical Research and Development (Mainz, Germany). The method was validated for the detection of DPP4 activity in human plasma samples [22]. The dose dependently inhibition of DPP4 by increasing BI 14332 in nanomolar concentrations (Fig. 1) showed that DPP4 activity in bovine samples is detectable by the method as well. The fluorescence measured as relative fluorescence units (RFU) is equivalent to the DPP4 activity in the sample. The baseline/pre-dose activity was set to 100% and all other activities measured in blood samples of the individual cows were calculated as the respective percentage of the baseline DPP4 activity.

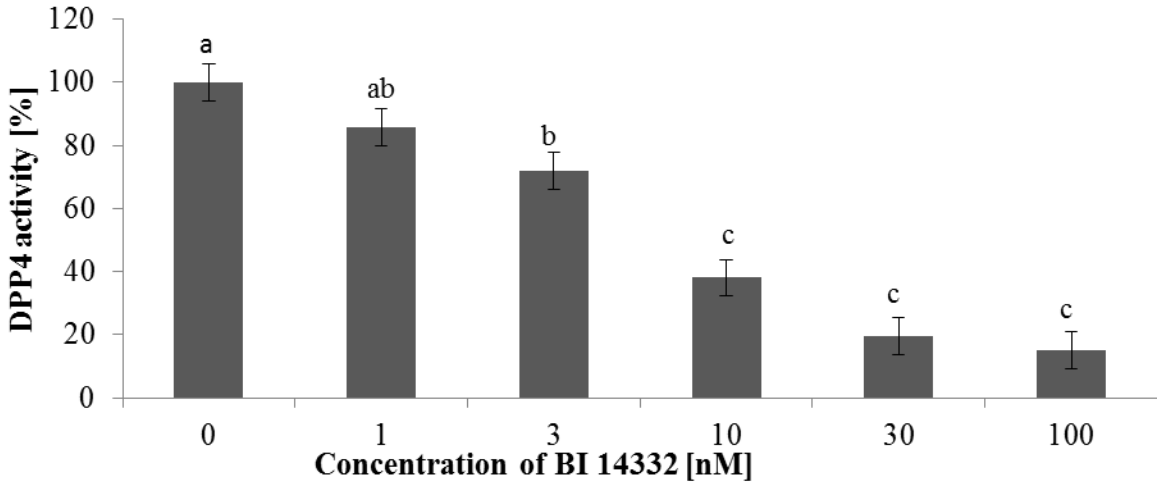

Fig. 1. Dipeptidyl peptidase-4 (DPP4) activity assessed in vitro. Potassium EDTA plasma samples of three healthy dairy cows were incubated with different concentrations of BI 14332 (0, 1, 3, 10, 30 and 100 nM; 469 g/mol). The concentration of BI 14332 was significant ($P = 0.006$). a, b, c: Different letters indicate significant differences between dosages ($P < 0.05$, Tukey test).

Homogenized liver samples (20 mg) were mixed with 450 µL DPP4 tissue buffer (25 mM HEPES, 140 mM NaCl, 80 mM MgCl2 and 11.25 mM Triton x-100). The DPP4 tissue buffer containing liver material was collected in a vial, centrifuged (1780 g, 10 min, 4 °C) and the resulting supernatant was stored at -80 °C until analysis for DPP4 activity.

The concentration of BI 14332 in blood plasma was analyzed by the Pharmacelsus GmbH (Saarbrücken, Germany) via LC-MS/MS (Surveyor MS Plus HPLC system, Thermo Fisher Scientific), connected to a TSQ Quantum Discovery Max (Thermo Fisher Scientific) triple quad mass spectrometer. Data handling was done using the standard software Xcalibur 2.0.7.

Trial 2

Clinical chemistry [BHB, NEFA, triglycerides (TG), glucose, γ-glutamyl transferase (γ-GT), aspartate transaminase (AST), glutamate dehydrogenase (GLDH)] was assessed in serum using photometric methods (Eurolyser, Type VET CCA, Eurolyser Diagnostica GmbH). A radioimmunoassay was used to quantify the serum insulin concentrations (IM3210, Insulin IRMA KIT, Immunotech, Beckman Coulter). This immunoradiometric test was a "sandwich" type assay. The antibodies used (mouse monoclonal) were directed against two different insulin epitopes. The assay was performed according to the manufacturer's instructions. The intra-assay CV was 7.6 %, and the inter assay CV was 10.7 %. The lowest detection limit was 3.95 µU/ml. Haptoglobin (Hp) was measured by ELISA as described elsewhere [23] and the total lipid content in liver samples (~100 mg) was assessed using a gravimetrical method [18].

The daily dry matter intake (DMI) was recorded for the whole experimental time (computerized feeding station: Type RIC, Insentec). Milking took place twice a day at 05.30 and 15.30. Milk yield was recorded using automatic milk counters (Lemmer Fullwood GmbH).

Hematological analyses were performed in EDTA whole blood using an automatic analyzer (Celltac α MEK-6450, Nihon Kohden, Qinlab Diagnostik).

Functional tests (ex vivo) of peripheral blood mononuclear cells (PBMC) were performed in samples from days -14, +7, +10, +14, +21 and +56 (relative to calving) using the Alamar Blue assay (AB). Concanavalin A (ConA, 2.5 µg/mL final, Sigma-Aldrich) was used as mitogen to stimulate T-lymphocytes. Further details were described previously [24].

For the calculation of $CD4^+/CD8^+$ T-cell population and its $CD4^+/CD8^+$ ratio from data generated by flow cytometry, days relative to calving where pooled in accordance to treatment [day "0" and day -14 ("ante-partum"), two days of treatment ("treatment"), after treatment, i.e. day +17 until day +28 post-partum ("2 weeks post treatment") and day +35, +42 and +56 post-partum ("end of trial")]. A detailed description of the measurements is provided by Stelter et al. [25]. Samples were double stained with monoclonal antibodies for $CD4^+$ (mouse anti bovine CD4:FITC) and

$CD8^+$ (mouse anti bovine CD8:RPE) or the corresponding isotype controls (mouse IgG2a negative control: RPE and mouse IgG2b negative control: FITC; all AbD Serotec).

Statistics and Calculations

Trial 1

The pharmacokinetic parameters were performed using non-linear regression via STATISTICA 10 [26]. The time course of plasma concentration of BI 14332, C_p, was expressed by a sum of two exponential functions:

$$C_p = \sum_{i=1}^{n} a_i \, e^{-b_i t}$$

where a_i and b_i are hybrid coefficients and exponential terms, t is time, and n is the number of exponential terms. According to Frey and Löscher (2009) from the data obtained, area under the concentration-time curves from 0 to 24 h (AUC), terminal half-life ($t_{1/2}$), total body clearance from 0 to 24 h (Cl_{24h}), the Volume of distribution (V_d), and the average steady state concentration (C_{ss}) were calculated.

Area under the curve of DPP4 activity in plasma and liver from 0 to 24 h was calculated using the linear trapezoidal rule:

$$AUC = \sum_{n=1}^{N} \frac{C_n + C_{n+1}}{2} (t_{n+1} - t_n)$$

Trial 2

Insulin sensitivity was estimated by the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) [27]:

$$\text{RQUICKI} = \frac{1}{\log \text{Insulin}\,[\mu\text{U/mL}] + \log \text{Glucose}\,[\text{mg/dL}] + \log \text{NEFA}\,[\text{mmol/L}]}$$

The results of the ex vivo examinations of PBMC were expressed as stimulation index (SI), defined as ratio between the fluorescence in the AB assay of ConA stimulated and nonstimulated PBMC:

$$\text{SI} = \frac{\text{Fluorescence of ConA stimulated PBMC}}{\text{Fluorescence of nonstimulated PBMC}}$$

For statistical analyses the SAS software package [28] was used. Evaluation of goodness of fit was carried out using the corrected Akaike information criterion. All parameters evaluated were compared as dependent variable by the MIXED procedure with a compound symmetry covariance structure. Treatment (HC-Con vs. HC-BI) was considered as fixed factor and sampling dates (time) as a repeated effect, and their respective interaction were included into the model. All results are presented as least square means (LSmeans) and standard errors (SE). Effects were declared to be significant when P-values were ≤ 0.05 after Tukey test for post-hoc analysis, whereas a trend was noted when $0.05 < P < 0.10$.

Data evaluation of hematology, proliferative capability (SI) and clinical chemistry based on pooled sampling days ("week of treatment/observation", "1st week after treatment/observation" and "2nd week after treatment/observation"). The day with first occurrence of serum BHB concentration ≥ 1.2 mM (day of classification) was set as covariate. Data evaluation of milk yield and DMI based on weekly mean values. The first week of lactation was set as covariate. For the proliferative capability, the SI of day +7 post partum was set as covariate. The remaining variables (liver lipid content, parameters of glycemic control, phenotyping T-lymphocytes) were analyzed in accordance to the evaluated sampling days, as described above.

Results

Trial 1

Investigations in vitro

The DPP4 activity in EDTA plasma samples decreased significantly starting at a concentration of 3 nM (≙ 1.407 ng/mL) BI 14332 as shown in Fig. 1. At 100 nM (≙ 46.9 ng/mL) the remaining DPP4 activity was 15.1%.

Pharmacokinetics and pharmacodynamics

The single administration of BI 14332 at 0.3, 1.0 or 3.0 mg/kg BW was well tolerated and a clear BI 14332 plasma concentration-dependent inhibition of the DPP4 activity both in plasma and liver was noted (Fig. 2). The PK/PD variables of BI 14332 and DPP4 activity in plasma and liver are represented in Table 2. The AUC regarding BI 14332 in plasma were dose-dependently increase. The $t_{1/2}$ of BI 14332 was highest for the 0.3 mg/kg BW dosage group, with a 10.5 to 23 h range. The V_d and Cl24h were greatest when 3 mg/kg BW was applied. The C_{ss} decreased

dose-dependently, starting with the highest dosage of BI 14332. Plasma DPP4 activity (Fig. 2A) was significantly inhibited by BI 14332 at all dosages with a remaining maximum activity of 14%, which was in line with an inhibitory power of 86% (1 mg/kg BW; 15 min after injection). The single dose of 1 mg/kg also showed the lowest inhibition at 24 h after injection (~ 70%), while inhibition by the other two dose groups were greater and quite similar (81-87%). Forty-eight hours after injection, the cows treated with 0.3 and 3 mg/kg still had an inhibition of DPP4 activity of about 82% versus 74% for the 1 mg/kg dosage group ($P < 0.05$). In liver, the AUC of DPP4 activity was decreased with increasing dosage (Fig. 2B).

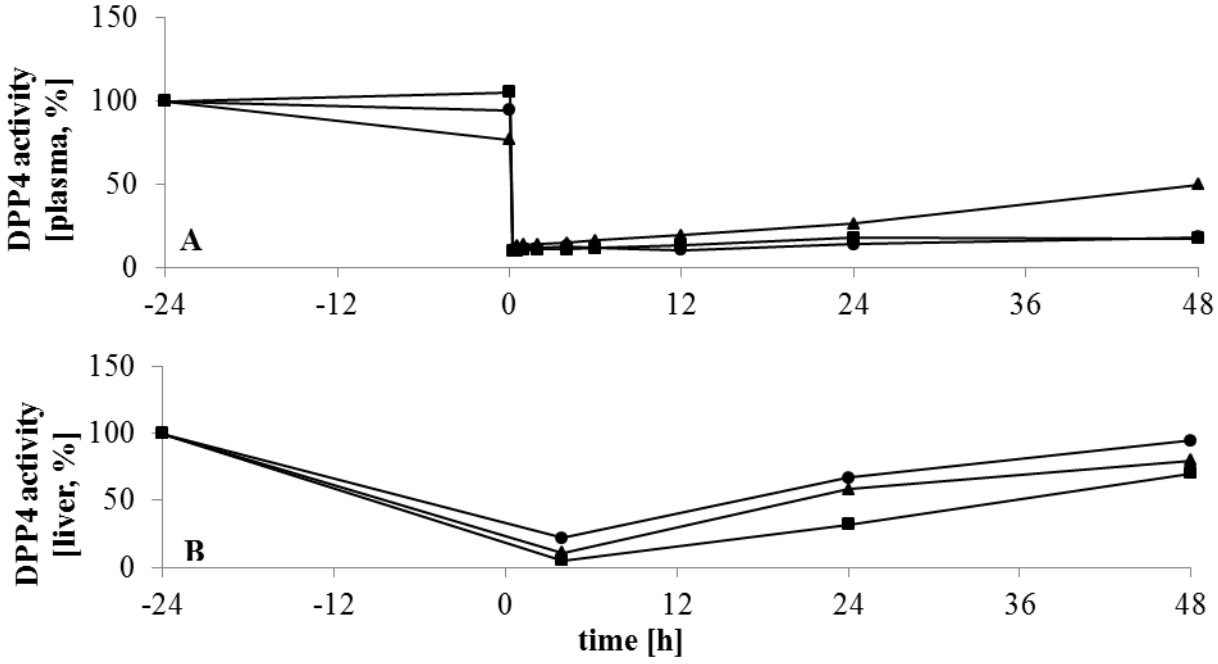

Fig. 2. Inhibition of plasma (A) and liver (B) dipeptidyl peptidase-4 (DPP4) activities after injection of BI 14332. BI 14332 was administered in a single dose of 3 [■], 1 [▲] and 0.3 [●] mg/kg body weight in dairy cows (n = 2/group). Plasma samples (*V. jugularis*) were taken 24 h before and immediately before (time zero "0") injection, as well as 0.25, 0.5, 1, 2, 4, 6, 12, 24 and 48 h post injection. Liver was biopsied 24 h before injection, as well as 4, 24 and 48 h thereafter.

The relationship between the BI 14332 concentration in plasma and the corresponding DPP4 activity in plasma and liver is depicted in Fig. 3, well approximated by a power function. With increasing plasma concentrations of BI 14332 (x-axis), a negative slope for DPP4 activity (y-axes) was observed in liver: $y = 7.72x-0.589$ ($r^2 = 0.72$) and in plasma: $y = 935.31x-0.081$ ($r^2 = 0.76$).

Table 2. Pharmacokinetic parameters of BI 14332 and dipeptidyl peptidase-4 (DPP4) in plasma and liver of six healthy German Holstein cows treated with different dosages of BI 14332 [3, 1 and 0.3 mg/kg body weight (BW); n = 2/dosage group][a].

	Dosage								
	3 mg/kg BW[b]			**1 mg/kg BW**[b]			**0.3 mg/kg BW**[b]		
BI 14332	Cow 1	Cow 2	Mean	Cow 3	Cow 4	Mean	Cow 5	Cow 6	Mean
Plasma									
AUC_{24h} [ng•h/mL]	2076	2784	2430	1769	773	1271	809	783	796
$t_{1/2}$ [min]	198	289	243	116	173	144	630	1386	1008
V_d [L/kg BW]	17.07	30.13	23.60	5.21	12.73	8.97	13.37	20.56	16.97
Cl_{24h} [mL/kg/min]	24.09	17.96	21.02	9.42	21.55	15.49	6.18	6.39	6.28
C_{ss} [ng/mL]	86.49	115.99	101.24	71.94	30.70	51.32	33.69	32.63	33.16
DPP4									
Plasma									
AUC_{24h} [RFU/h]	20491	16560	18525	21034	20135	20584	15729	17914	16822
Δ_{15min} [%]	90	91	91	86	86	86	88	90	89
Δ_{24h} [%]	81	85	83	66	73	70	84	87	85
Liver[c]									
AUC_{24h} [µg•h/mL]	41	74	43	90	45	68	63	112	101
Δ_{4h} [%]	96	94	94	89	89	89	77	79	78
Δ_{24h} [%]	65	71	68	40	44	42	35	31	33

[a]Pharmacokinetic parameters of BI 14332 were evaluated via bi-exponential function (Stat Soft, 2011); AUC for DPP4 activity in plasma and liver was calculated using the trapezoidal rule.

[b]BI 14332 was administrated intravenously (i.v.); plasma samples were taken 24 h before injection, 0, 0.25, 0.5, 1, 2, 4, 6, 12, 24 and 48 h after injection; liver samples were taken 24 h before injection and 4, 24 and 48 h thereafter.

[c]DPP4 activity in liver was normalized to the total protein content of the samples.

Abbreviations: AUC: area under the curve from 0 to 24 h; $t_{1/2}$: terminal half-life; V_d: Volume of distribution; Cl_{24h}: Clearance from 0 to 24 h; C_{ss}: average steady state concentration; $\Delta_{15min}/\Delta_{4h}/\Delta_{24h}$: Inhibitory power of BI 14332 regarding DPP4 activity, calculated as difference before BI 14332 application and the first sample post injection (i.e. 15 min post injection in plasma and 4 h post injection in liver) and 24 h after injection, respectively.

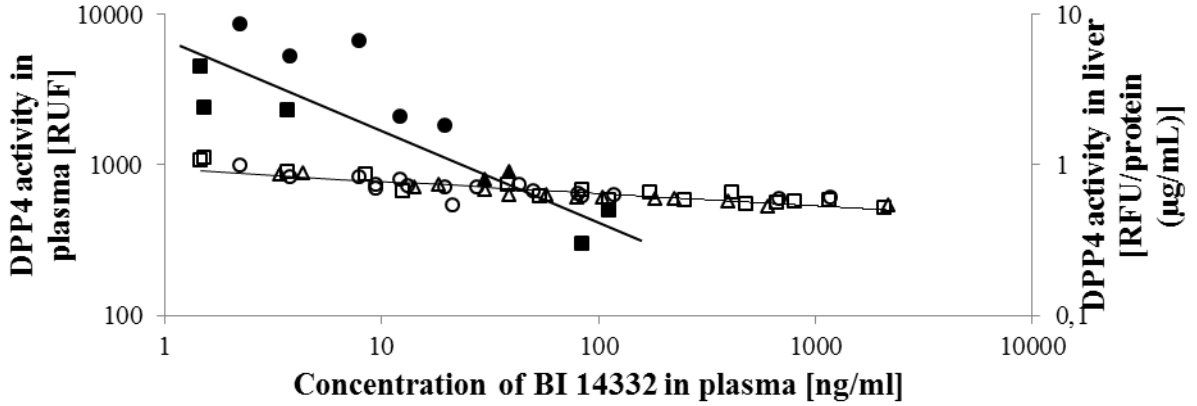

Fig. 3. Inhibition of dipeptidyl peptidase-4 (DPP4) activity in plasma and liver after injection of BI 14332. BI 14332 was administered in a single dose of 3, 1 and 0.3 mg/kg body weight (BW) in dairy cows (n = 2/group). Plasma samples were taken 0.25, 0.5, 1, 2, 4, 6, 12, 24 and 48 h after the injection (*V. jugularis*; 3 mg/kg BW [□]; 1 mg/kg BW [Δ]; 0.3 mg/kg BW [○]). Liver samples were obtained by biopsy 4, 24 and 48 h after the injection (3 mg/kg BW [■]; 1 mg/kg BW [▲]; 0.3 mg/kg BW [●]). BI 14332 (x-axis) was shown to have a strong negative impact on DPP4 activity (y-axes), well approximated by a power function (represented as quasi linear model via log-log transformation) in liver: $y = 7.72x^{-0.589}$ ($r^2 = 0.72$) and plasma: $y = 935.31x^{-0.081}$ ($r^2 = 0.76$).

Trial 2

Clinical chemistry and hepatic lipid content

An overview of the clinical chemical parameters is given in Table 3. A significant group*time interaction was shown for NEFA, TG and GLDH. All three variables changed significantly with time and were additionally influenced by treatment. For TG, this was indicated by a significantly lower concentration in the HC-BI cows during the 1st week after treatment compared to the HC-Con cows. For NEFA, there was a significant decrease in the concentration between the week of treatment to the 1st and the 2nd week post treatment, only within the HC-BI group, while GLDH increased significantly in the HC-Con cows and peaked in the 2nd week post observation. Further time-dependent alterations were detected for glucose, insulin, AST and γ-GT. Glucose and insulin concentrations were significantly greater two weeks post observation than during observation in the HC-Con group. For more detailed evaluation of variables describing glycemic control, Fig. 4 shows RQUICKI and the variables necessary to calculate the index (NEFA, glucose, insulin). The figure reveals significant changes relative to calving and in accordance to treatment. On day +10 post partum, NEFA, glucose and insulin peaked within the HC-BI group,

while RQUICKI decreased to a nadir at that day. For the HC-Con cows there was the opposite effect as substantiated by the significant interaction between group and day for RQUICKI. Starting on day +14 until day +21 post partum, RQUICKI differed markedly between the experimental groups, with higher values for the HC-BI cows.

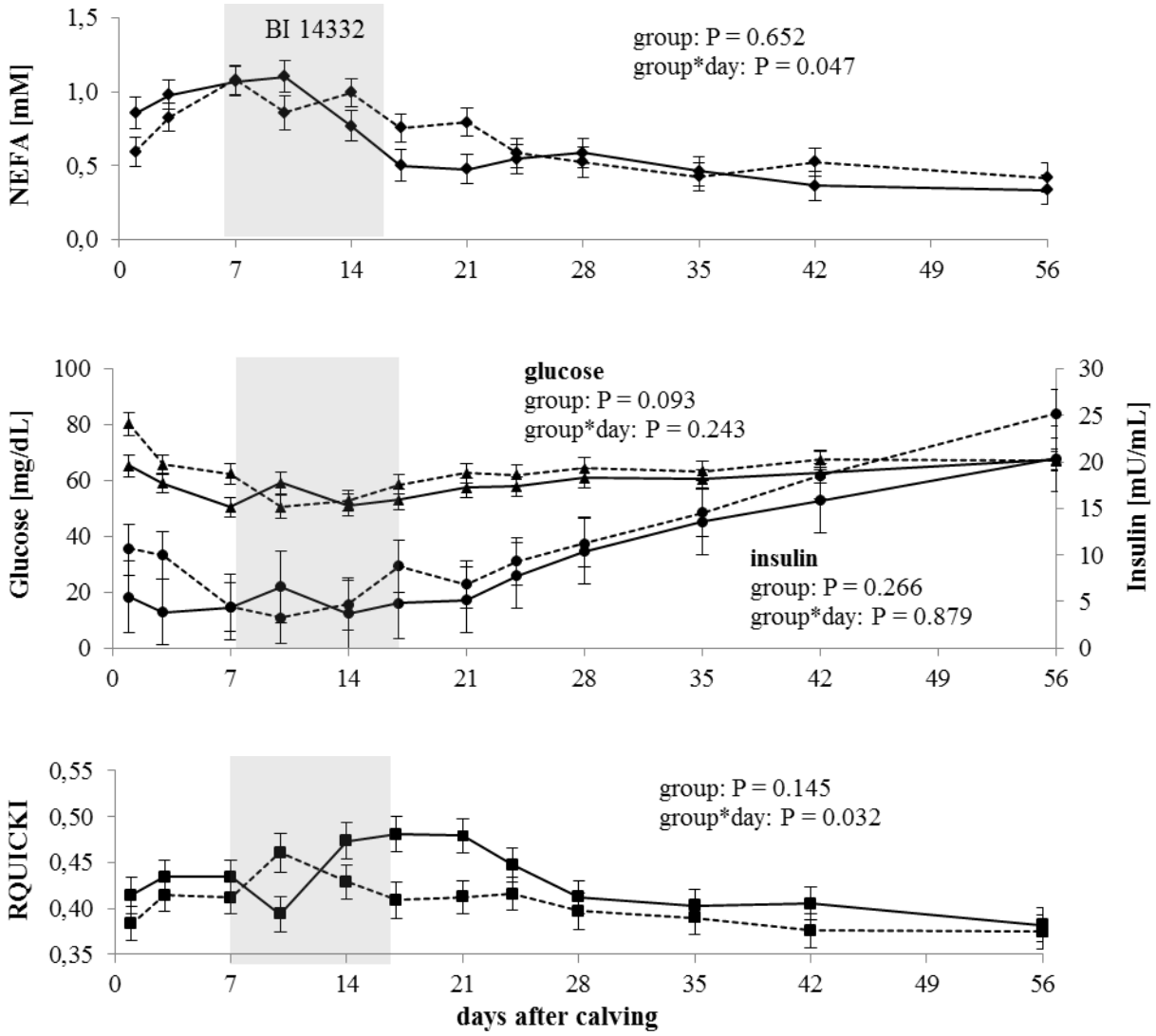

Fig. 4. Concentrations of non-esterified fatty acids (NEFA), glucose and insulin in serum, and insulin sensitivity (RQUICKI) in cows with subclinical ketosis. With the first occurrence of serum β-hydroxybutyrate concentrations ≥ 1.2 mM, cows were treated with BI 14332 [(—) n = 6] or stayed untreated as control [(···) n = 6]. Within the BI 14332 treatment group subclinical ketosis was diagnosed on day +7 (5 cows) and on day +10 (1 cows), relative to calving. Dosage of BI 14332 was 0.3 mg/kg body weight, applied i.v. once a day over a period of 7 days. The statistical analysis included group (BI 14332 treatment vs. control), experimental day (1st day post partum until 56th day post partum), and the interaction ($P < 0.05$, Tukey test). Experimental day differed significantly for all parameters. [NEFA (♦), Glucose (▲), Insulin (●), RQUICKI (■)].

Table 3. Effects of dipeptidyl peptidase-4 (DPP4) inhibition via BI 14332 to blood serum variables of clinical chemistry and insulin sensitivity of cows with subclinical ketosis (LSmeans ± SE).

	HC-BI (n = 6)[a]				HC-Con (n = 6)[a]				Probability[c]		
Parameter	Day of classification[b]	Treatment	1st week after treatment	2nd week after treatment	Day of classification[b]	Observation	1st week after observation	2nd week after observation	group	time	group x time
BHB [mM]	1.63 ± 0.65	1.77 ± 0.38	1.07 ± 0.28	1.56 ± 0.31	1.51 ± 0.23	1.44 ± 0.31	1.15 ± 0.30	1.12 ± 0.32	0.509	0.166	0.520
NEFA [mM]	0.92 ± 0.28	1.09 ± 0.10	0.53 ± 0.07	0.56 ± 0.08	0.94 ± 0.23	0.94 ± 0.08	0.75 ± 0.08	0.55 ± 0.08	0.820	**< 0.001**	**0.026**
Triglyceride [mg/dL]	11.07 ± 2.40	11.20 ± 1.10	9.03 ± 0.74	9.57 ± 0.85	12.34 ± 3.69	11.37 ± 0.87	14.70 ± 0.81	11.02 ± 0.88	**0.014**	0.123	**0.002**
Glucose [mg/dL]	50.68 ± 9.22	55.09 ± 4.49	57.64 ± 3.72	59.86 ± 3.97	63.05 ± 8.52	51.70 ± 3.93	60.84 ± 3.74	62.58 ± 3.89	0.876	**0.018**	0.404
Insulin [mU/mL]	5.20 ± 2.72	4.58 ± 1.79	5.78 ± 1.29	8.63 ± 1.44	6.70 ± 5.62	5.49 ± 1.46	7.17 ± 1.43	11.21 ± 1.48	0.313	**0.001**	0.802
RQUICKI	0.44 ± 0.04	0.43 ± 0.02	0.47 ± 0.01	0.44 ± 0.02	0.41 ± 0.04	0.43 ± 0.02	0.42 ± 0.02	0.39 ± 0.02	0.129	0.127	0.160
Haptoglobin [mg/mL]	1.56 ± 1.63	0.31 ± 0.30	0.13 ± 0.21	0.12 ± 0.23	1.27 ± 1.22	0.76 ± 0.24	0.53 ± 0.23	0.32 ± 0.24	0.168	0.385	0.817
AST [U/I]	108.03 ± 35.3	117.00 ± 10.8	104.86 ± 8.18	86.43 ± 8.92	96.79 ± 21.0	112.65 ± 9.07	105.11 ± 8.68	92.83 ± 9.19	0.941	**0.005**	0.772
γ-GT [U/I]	18.49 ± 3.41	21.67 ± 9.01	31.50 ± 8.15	40.30 ± 8.37	19.95 ± 4.18	22.94 ± 8.44	28.54 ± 8.30	38.85 ± 8.47	0.928	**0.001**	0.868
GLDH [U/I]	8.13 ± 1.56	19.89 ± 7.72	29.75 ± 6.56	27.08 ± 6.88	9.42 ± 2.59	13.59 ± 6.90	20.92 ± 6.67	39.41 ± 6.97	0.918	**0.003**	**0.028**

[a]With first occurrence of serum β-hydroxybutyrate (BHB) concentration ≥ 1.2 mM cows were treated with BI 14332 (HC-BI) or stayed untreated as control group (HC-Con). BI 14332 was applied once a day over a period of 7 days (i.v., 0.3 mg/kg body weight). Subclinical ketosis was diagnosed on day +3 (1 cow), day +7 (8 cows) or on day +10 (3 cows) after calving.

[b]The day of classification (mean ± SD), which was the day with first occurrence of BHB values ≥ 1.2 mM were set as covariate, integrated in the MIXED procedure of SAS [24] with group and time as fixed factors ($P \leq 0.05$; Tukey test).

[c]Significant values are shown in bold.

Abbreviations: NEFA, non-esterified fatty acids; RQUICKI, revised quick insulin sensitivity index; AST, aspartate aminotransferase; γ-GT, γ-glutamyltransferase; GLDH, glutamate dehydrogenase

The BHB concentrations were not different between the groups and the same was true for Hp. The greatest Hp concentrations were detected at the day of classification in both groups; thereafter the concentration decreased continuously. Albeit not reaching the level of significance, the decrease of the Hp concentration seemed to be faster in HC-BI than in the HC-Con cows.
There was also no significant difference for the total liver lipid content between groups (data not shown), even though HC-Con cows had slightly higher lipid contents than the HC-BI cows at all days evaluated post partum (Δ = 13.8 mg/g). For both groups the total hepatic lipid content differed significantly with time and highest contents were detected on day +7 after calving with 161 mg/g (HC-BI) and 175 mg/g (HC-Con).

DMI and milk yield

Statistical evaluation of dry matter intake and milk yield during lactation is shown in Table 4. Data offered a significant time effect for the variables. Milk yield and DMI increased continuously for both subgroups. A group effect, respectively a significant group*time interaction, was not determined for the named parameters.

Table 4. Effects of dipeptidyl peptidase IV (DPP4) inhibition via BI 14332 to dry matter intake (DMI) and milk yield of cows with subclinical ketosis (LSmean ±SE).

	HC-BI (n = 6)[a]				HC-Con (n = 6)[a]				Probability		
Parameter	Co-variate[b]	2nd week of lactation	3rd and 4th week of lactation	5th until 8th week of lactation	Co-variate[b]	2nd week of lactation	3rd and 4th week of lactation	5th until 8th week of lactation	group	time	group x time
DMI [kg/d]	13.3 ± 2.0	13.2 ± 0.7	16.5 ± 0.6	20.4 ± 0.5	12.6 ± 2.6	14.6 ± 0.8	17.4 ± 0.6	20.5 ± 0.5	0.240	**0.001**	0.400
Milk yield [kg/d]	30.7 ± 5.7	31.0 ± 1.7	36.5 ± 1.6	39.1 ± 1.5	30.2 ± 4.5	31.2 ± 1.5	34.5 ± 1.4	37.4 ± 1.4	0.592	**0.001**	0.150

[a]With first occurrence of serum β-hydroxybutyrate (BHB) concentration ≥ 1.2 mM cows were treated with BI 14332 (HC-BI) or stayed untreated as control group (HC-Con). BI 14332 was applied once a day over a period of 7 days (intravenous, 0.3 mg/kg body weight). Subclinical ketosis was diagnosed during 1st and 2nd week of lactation (day +3, day +7 or day +10 after calving).
[b]The first week (mean ± SD) were set as covariate, integrated in the MIXED procedure of SAS [24] with group and time as fixed factors (P ≤0.05; Tukey test).

Hematology and cell function

Variables of the hematology and the SI of PBMC are shown in Table 5. White blood cells (WBC) were significantly influenced by time and time*group. During treatment, leucocytes and granulocytes counts were significantly higher in the HC-BI than in the HC-Con group. The decrease of the named parameters from treatment to the 1st and the 2nd week after treatment was significant in the HC-BI group. The proliferative capability of PBMC in the ex vivo assay did not

change over time. T-cell-phenotyping revealed that the $CD4^+/CD8^+$ ratio of subclinically cows tended to be higher for the HC-Con versus the HC-BI group (P = 0.059, Table 6). For the HC-Con cows the increase from ante partum to treatment period was significant; due to a selective increase of the $CD4^+$ T-cell population (~ 11%) and a slight decrease of the $CD8^+$ T-cell population (~ 10%). In the HC-BI group, both subpopulations decreased ($CD4^+$: ~ 16%, $CD8^+$: ~ 26%) during treatment. Both experimental groups showed significant time effect with highest ratios during observation (HC-Con: 3.28) and two weeks after treatment (HC-BI: 2.60). The individual $CD4^+$ and $CD8^+$ cell populations differed significantly over the time, but neither group nor the group*time interaction were significant.

Discussion

Most of the findings about inhibition of DPP4 are related to type II diabetes in human patients, opening up new perspectives in therapy. The ketotic metabolic status of high yielding dairy cows during early lactation is characterized by some similarities with the metabolic situation of type II diabetes and fatty liver in humans [29].

The first aims of the current investigation were (1) to show that BI 14332 is an effective DPP4 inhibitor and (2) to find the optimal dosage of BI 14332. Therefore, all doses resulted in an inhibition of DPP4 activity in plasma and liver without any indication for adverse side-effects. According to the PK/PD results, we considered the dosage of 0.3 mg/kg BW as most suitable. Albeit the dosage of 3 mg/kg BW yielded the highest AUC of BI 14332, the plasma AUC of DPP4 activity was quite similar in the highest and the lowest dosage group. The terminal t1/2 is the time required to halve the plasma concentration after reaching a steady state equilibrium and has to be known to provide an appropriate length for the dosing interval [30], which was sought to be 24 h. Results confirm dosing decision of 0.3 mg/kg BW, as t1/2 and Cl24h, i.e. the ability to eliminate a drug [31], offered the longest retention period in blood.

The aim of trial 2 was to test whether inhibiting DPP4 may affect blood variables associated with lipid metabolism and glycemic control under catabolic condition. The results showed that the impact of DPP4 inhibition was less in cows of subclinical ketosis, respectively during early lactation. Significant changes and positive impacts were limited to the time after treatment (Table 3; NEFA, TG, GLDH). Lower TG concentrations were also observed by Ben-Shlomo et al. [3].

Table 5. Effects of dipeptidyl peptidase-4 (DPP4) inhibition via BI 14332 to hematological variables and the proliferative capability of PBMC (LSmeans ±SE).

Parameter	HC-BI (n = 6)[1]				HC-Con (n = 6)[1]				Probability		
	Day of Classification[2]	Treatment	1st week after treatment	2nd week after treatment	Day of Classification[2]	Observation	1st week after observation	2nd week after observation	group	time	group x time
SI	6.69 ± 1.29	6.05 ± 0.79	6.26 ± 0.56	3.69 ±1.42	7.27 ±1.62	6.73 ± 0.75	6.49 ± 0.64	6.80 ± 1.37	0.138	0.591	0.453
Leucocytes [10^3/µL]	9.75 ± 2.79	10.17 ± 0.65	7.59 ± 0.47	7.14 ± 0.51	7.35 ± 3.07	7.33 ± 0.52	7.48 ± 0.50	7.05 ± 0.56	0.105	**0.005**	**0.009**
LY [10^3/µL]	3.30 ± 1.07	2.79 ± 0.22	2.86 ± 0.19	2.89 ± 0.20	2.93 ± 0.41	2.95 ± 0.20	2.97 ± 0.19	3.09 ± 0.20	0.549	0.642	0.927
GR [10^3/µL]	5.87 ± 2.01	6.74 ± 0.66	4.01 ± 0.48	3.75 ± 0.52	3.77 ± 2.95	4.00 ± 0.53	3.99 ± 0.51	3.37 ± 0.57	0.106	**0.002**	**0.015**
EO [10^3/µL]	0.40 ± 0.24	0.33 ± 0.12	0.39 ± 0.09	0.29 ± 0.10	0.45 ± 0.26	0.27 ± 0.10	0.34 ± 0.10	0.40 ± 0.11	0.987	0.730	0.426
Erythrocytes [10^6/µL]	5.92 ± 0.42	5.81 ± 0.21	5.69 ± 0.19	5.53 ± 0.20	6.07 ± 0.82	5.90 ± 0.20	5.94 ± 0.19	5.87 ± 0.20	0.407	0.238	0.468
HGB [g/dL]	10.82 ± 0.68	10.23 ± 0.42	9.90 ± 0.37	9.69 ± 0.39	10.52 ± 1.18	10.40 ± 0.39	10.38 ± 0.39	10.31 ± 0.40	0.435	0.331	0.551
HCT [%]	36.30 ± 2.61	34.60 ± 1.36	33.48 ± 1.20	32.27 ± 1.24	35.07 ± 4.09	34.70 ± 1.26	34.59 ± 1.24	34.43 ± 1.30	0.510	0.186	0.347
Thrombocytes [10^3/µL]	514 ± 127	117 ± 653	1029 ± 515	1631 ± 550	453 ± 48	835 ± 561	862 ± 538	909 ± 583	0.933	0.211	0.276

[1]With first occurrence of serum β-hydroxybutyrate ≥ 1.2 mM cows were treated with BI 14332 (HC-BI) or stayed untreated as control (HC-Con). BI 14332 was applied once a day over a period of 7 days (intravenous, 0.3 mg/kg body weight). Subclinical ketosis was diagnosed on day +3 (1 cow), day +7 (8 cows) or on day +10 (3 cows) after calving.
[2]The day of classification (mean ± SD), which was the day with first occurrence of BHB values ≥ 1.2 mM were set as covariate, integrated in the MIXED procedure of SAS [24] with group and time as fixed factors (P ≤0.05; Tukey test). Significant values are shown in bold.
Abbreviations: SI, stimulation index (ratio between the fluorescence in the Alamar Blue assay of concanavalin A-stimulated and unstimulated PBMC); LY, lymphocytes; GR, granulocytes; EO, eosinophile granulocytes; HGB, hemoglobin; HCT, hematocrit

Table 6. Effects of dipeptidyl peptidase IV (DPP4) inhibition via BI 14332 to relative numbers of $CD4^+$ and $CD8^+$ T-lymphocytes (LSmean ±SE).

Parameter	HC-BI (n = 6)[a]				HC-Con (n = 6)[a]				Probability		
	Ante partum	Treatment	2 weeks after treatment	End of trial	Ante partum	Observation	2 weeks after observation	End of trial	group	time	group x time
$CD4^+$ [%]	28.8 ± 2.3^{ab}	24.0 ± 2.9^{a}	33.5 ± 2.0^{b}	34.0 ± 2.37^{b}	28.8 ± 2.1^{a}	2.4 ± 2.7^{ab}	33.2 ± 2.4^{ab}	36.5 ± 2.2^{b}	0.314	**0.001**	0.155
$CD8^+$ [%]	15.6 ± 1.2	11.6 ± 1.6	13.7 ± 1.1	14.4 ± 1.30	12.9 ± 1.2	11.5 ± 1.5	11.7 ± 1.3	13.6 ± 1.2	0.347	**0.045**	0.535
$CD4^+/CD8^+$	1.85 ± 0.25	2.06 ± 0.33	2.60 ± 0.22	2.46 ± 0.27	2.29 ± 0.24^{a}	3.28 ± 0.30^{b}	2.90 ± 0.26^{a}	2.83 ± 0.25^{a}	**0.059**	**0.001**	0.245

[a]With first occurrence of serum β-hydroxybutyrate ≥ 1.2 mM cows were treated with BI 14332 (HC-BI) or stayed untreated as control group (HC-Con). BI 14332 was applied once a day over a period of 7 days (0.3 mg/kg body weight). Subclinical ketosis was diagnosed on day +3 (1 cow), day +7 (8 cows) or on day +10 (3 cows) after calving. Significant values ($P \leq 0.05$) and trends ($P \leq 0.1$) are shown in bold. LSmeans with different superscripts (a-b) within the same group are significantly different.

The researchers show significant reduced levels in DPP4-deficient rats and explain a GLP-1 mechanism in liver which induces a signal for a low energy state. Therefore, protein kinase activities involved in the pathway effect a reduced expression of lipogenesis-related genes. Effects of DMI and milk yield showed that there was no difference between the subgroups (Table 4), which could have explained a lower lipolysis for the HC-BI cows. Therefore, the decrease in NEFA was not caused by a higher DMI or a lower milk yield for those cows compared to the HC-Con cows.

RQUICKI, as surrogate marker to assess insulin sensitivity, includes NEFA in the equation and the lower NEFA concentrations in HC-BI vs. HC-Con rather than the ones of glucose and insulin affected RQUICKI. However, differences between the groups were limited to diverging reaction over time, i.e. the interaction of time and treatment. At day +14 post partum RQUICKI was greater in the BI 14332 treated animals. At this stage, approximately during the middle of the treatment, a steady state plateau is reached and therapeutic efficacy is assumingly complete [32]. In a further study, RQUICKI remained unaffected during subclinical ketosis [19]. We conclude, that RQUICKI may be not sensitive enough to investigate alterations of insulin sensitivity in case of subclinical ketosis, but it is more likely that insulin sensitivity is not, or even very less, influenced by subclinical ketosis and a diminished sensitivity is limited to physiological changes around calving. Nevertheless RQUICKI was sensitive enough to show a transient improvement of insulin sensitivity by BI 14332. Taking into account that DPP4 inhibition was significant (Fig. 2, Table 2) and with respect to investigations made in humans and rodents, one could assume that the retention period of active GLP-1 in the periphery was prolonged. If that is also true in cows, it may explain the positive impacts on lipaemic control. However, the support was not strong enough to affect the entire metabolism.

The fact that effects of DPP4 inhibition were marginal may be linked to the short duration of the treatment period [2]; which was possibly reflected by GLDH. Changes in GLDH became evident only late [19,33] and indicated less hepatic lesions in the second week after BI 14332 treatment. This was not evidenced by a reduced level of hepatic fat in the HC-BI cows. A prolonged treatment period might affect hepatic infiltrations of lipids in a stronger way.

Another aim of trial 2 was to investigate if the inhibition of DPP4 via BI 14332 has immune-modulating effects. The prescribing information of sitagliptin, the first DPP4 inhibitor for clinical use, reports a slight increase in WBC, primarily due to a small increase of neutrophil granulocytes counts [34]. The HC-BI cows had already higher WBC values before the onset of

the treatment, with $\Delta = 2.40 \cdot 103/\mu l$ at the day of classification compared to cows of control group. Therefore, it was questionable, if significant changes of WBC and GR counts were indeed related to BI 14332 or just coincided with time-related alterations around calving. Our investigations regarding the proliferative capability of PBMC showed similar results and immune-modulatory alterations were not evident. Studies employing DPP4 inhibitors showed that T-cell proliferation and cytokine production is inhibited by impaired DNA synthesis [35]. In contrast, Anz et al. [36] showed results, similar to the present. None of the tested DPP4 inhibitors impaired key parameters of the innate and adaptive immune response, which were included in the present study to assess drug safety.

For the current investigation, immune-modulatory effects were limited to differences in $CD4^+/CD8^+$ ratio, which tended to be higher for the HC-Con cows (vs. HC-BI cows), with a significant increase after calving (~ 30%) up to 3.28. A ratio up to 2.5 suggests a physiological situation, while an increased ratio may indicate an immune dysregulation [37,38]. Furthermore it is known that DPP4 is expressed predominantly on T-lymphocytes and most of the T-cells expressing DPP4 belong to the $CD4^+$ population (~ 56%) [39]. The inhibition of DPP4 activity may lower the expression of $CD4^+$ and $CD8^+$ T-cells. This was more pronounced for $CD8^+$ cells and led to a better $CD4^+/CD8^+$ ratio. It suggests an impaired immune defense after calving, when the need for an appropriate defense is highest. In view of the concentrations of the acute-phase protein Hp that is commonly used as marker of inflammation, a beneficial effect of BI 14332 on the immune defense was not supported. Haptoglobin is assumed to be elevated by tissue lesions occurring during birth and by the general proinflammatory situation for the time around calving [40]. It positively correlates with BHB, NEFA and TG [10,24,41]. The minor impact of the DPP4 inhibition regarding these variables may explain for the insignificant differences in Hp concentrations between groups. Nevertheless, in numerical terms alone, within the HC-BI group Hp dropped markedly by about 80% (vs. ~ 40% for HC-Con cows; Table 3) during the treatment. Thus a prolonged treatment period together with a concomitant improvement of hepatic lipid metabolism may also reduce Hp.

Conclusions

The DPP4 activity was determined in plasma and liver samples of dairy cows. The DPP4 inhibitor BI 14332 reduced the enzymatic activity in vivo and showed a fast onset and a long lasting inhibition of DPP4. However, the DPP4 inhibition did not improve the metabolic

disarrangements related to subclinical ketosis. Albeit an improved lipaemic control was observed, as NEFA and TG were decreased after treatment. Unfortunately, the support was not strong enough to affect main markers of ketosis (BHB, hepatic lipid content).

Acknowledgment

The authors would like to thank the co-workers of the Institute of Animal Nutrition (FLI) and Institute of Animal Science (Physiology and Hygiene Unit) in Braunschweig and Bonn in performing experiment and analyses.

Author Contributions

Conceived and designed the experiments: SD UM SK DR JR GB. Performed the experiments: KS JF MP MM HS. Analyzed the data: KS JF DR. Wrote the paper: KS SD HS.

References

1. Deacon CF. Circulation and degradation of GIP and GLP-1. Horm Metab Res 2004; 36:761 765.

2. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 2006; 368: 1696-1705.

3. Ben-Shlomo S, Zvibel I, Shnell M, Shlomai A, Chepurko E, Halpern Z et al. Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase. J Hepatol 2011; 54: 1214-1223.

4. Flatt PR, Bailey CJ, Green BD. Dipeptidyl peptidase IV (DPP IV) and related molecules in type 2 diabetes. Front Biosci 2008; 13: 3648-3660.

5. Bell AW, Bauman DE. Adaptations of glucose metabolism during pregnancy and lactation. J Mammary Gland Biol Neoplasia 1997; 2: 265-278.

6. Hove K. Insulin secretion in lactating cows: responses to glucose infused intravenously in normal, ketonemic, and starved animals. J Dairy Sci 1978; 61: 1407-1413.

7. Ohtsuka H, Koiwa M, Hatsugaya A, Kudo K, Hoshi F, Itoh N et al. Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. J Vet Med Sci 2001; 63: 1021-1025.

8. McArt JA, Nydam DV, Oetzel GR, Overton TR, Ospina PA. Elevated non-esterified fatty acids and beta-hydroxybutyrate and their association with transition dairy cow performance. Vet J 2013; 198: 560-570.

9. Bobe G, Young JW, Beitz DC. Invited review: pathology, etiology, prevention, and treatment of fatty liver in dairy cows. J Dairy Sci 2004; 87: 3105-3124.

10. Saremi B, Al-Dawood A, Winand S, Müller U, Pappritz J, von Soosten D et al. Bovine haptoglobin as an adipokine: serum concentrations and tissue expression in dairy cows receiving a conjugated linoleic acids supplement throughout lactation. Vet Immunol Immunopathol 2012; 146: 201-211.

11. Saremi B, Winand S, Friedrichs P, Kinoshita A, Rehage J, Dänicke S et al. Longitudinal profiling of the tissue-specific expression of genes related with insulin sensitivity in dairy cows during lactation focusing on different fat depots. PLoS One 2014; 9: e86211.

12. Taylor-Edwards CC, Burrin DG, Matthews JC, McLeod KR, Holst JJ, Harmon DL. Expression of mRNA for proglucagon and glucagon-like peptide-2 (GLP-2) receptor in the ruminant gastrointestinal tract and the influence of energy intake. Domest Anim Endocrinol 2010; 39: 181-193.

13. Larsen M, Relling AE, Reynolds CK, Kristensen NB. Effect of abomasal glucose infusion on plasma concentrations of gut peptides in periparturient dairy cows. J Dairy Sci 2010; 93: 5729-5736.

14. Relling AE, Reynolds CK. Abomasal infusion of casein, starch and soybean oil differentially affect plasma concentrations of gut peptides and feed intake in lactating dairy cows. Domest Anim Endocrinol 2008; 35: 35-45.

15. Bradford BJ, Harvatine KJ, Allen MS. Dietary unsaturated fatty acids increase plasma glucagon-like peptide-1 and cholecystokinin and may decrease premeal ghrelin in lactating dairy cows. J Dairy Sci 2008; 91: 1443-1450.

16. Pezeshki A, Muench GP, Chelikani PK. Short communication: expression of peptide YY, proglucagon, neuropeptide Y receptor Y2, and glucagon-like peptide-1 receptor in bovine peripheral tissues. J Dairy Sci 2012; 95: 5089-5094.

17. Connor EE, Baldwin RLt, Capuco AV, Evock-Clover CM, Ellis SE, et al. Characterization of glucagon-like peptide 2 pathway member expression in bovine gastrointestinal tract. J Dairy Sci 2010; 93: 5167-5178.

18. Starke A, Haudum A, Busche R, Beyerbach M, Dänicke S, Rahge J. Technical note: analysis of total lipid and triacylglycerol content in small liver biopsy samples in cattle. J Anim Sci 2010; 88: 2741-2750.

19. Schulz K, Frahm J, Meyer U, Kersten S, Reiche D, Rehage J et al. Effects of prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy balance and ketogenesis: an animal model to investigate subclinical ketosis. J Dairy Res 2014; 81: 257-266.

20. Duffield T. Subclinical ketosis in lactating dairy cattle. Vet Clin North Am Food Anim Pract 2000; 16: 231-253.

21. Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G. A body condition scoring chart for Holstein dairy cows. J Dairy Sci 1989; 72: 68-78.

22. Huttner S, Graefe-Mody EU, Withopf B, Ring A, Dugi KA. Safety, tolerability, pharmacokinetics, and pharmacodynamics of single oral doses of BI 1356, an inhibitor of dipeptidyl peptidase 4, in healthy male volunteers. J Clin Pharmacol 2008; 48: 1171-1178.

23. Hiss S, Mielenz M, Bruckmaier RM, Sauerwein H. Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. J Dairy Sci 2004; 87: 3778-3784.

24. Schulz K, Frahm J, Kersten S, Meyer U, Reiche D, Sauerwein H et al. Effects of elevated parameters of subclinical ketosis on the immune system of dairy cows: in vivo and in vitro results. Arch Anim Nutr 2015; 69: 113-127.

25. Stelter K, Frahm J, Paulsen J, Berk A, Kleinwachter M, Selmar D et al. Effects of oregano on performance and immunmodulating factors in weaned piglets. Arch Anim Nutr 2013; 67: 461-476.

26. StatSoft I. STATISTICA for Windows Operating System (Version 10.0). 2011. Tulsa (OH): StatSoft.

27. Holtenius P, Holtenius K. A model to estimate insulin sensitivity in dairy cows. Acta Vet Scand 2007; 49: 29.

28. SAS Institute. The SAS/STAT 9.1 User's Guide. 2004; Vol. 1-7. SAS Institute Cary, NC.

29. Tilg H, Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab 2008; 19: 371-379.

30. Toutain PL, Bousquet-Melou A. Plasma terminal half-life. J Vet Pharmacol Ther 2004; 27: 427-439.

31. Toutain PL, Bousquet-Melou A. Plasma clearance. J Vet Pharmacol Ther 2004; 27: 415-425.

32. Frey H, Löscher W. Lehrbuch der Pharmakologie und Toxikologie für die Veterinärmedizin [Pharmacology and Toxicology in Veterinary Medicine]. Stuttgart: Enke; 2009.

33. Dirksen G, Gründer H, Stöber M. Die klinische Untersuchung des Rindes [The clinical investigation of bovine animals]. Stuttgart: Enke; 2012.

34. White JR. Dipeptidyl peptidase-IV inhibitors: pharmacological profile and clinical use. Clin Diab 2008; 26: 53-57.

35. Gorrell MD, Gysbers V, McCaughan GW. CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand J Immunol 2001; 54: 249-264.

36. Anz D, Kruger S, Haubner S, Rapp M, Bourquin C, Endres S. The dipeptidylpeptidase-IV inhibitors sitagliptin, vildagliptin and saxagliptin do not impair innate and adaptive immune responses. Diabetes Obes Metab 2014; 16: 569-572.

37. Mehrzad J, Zhao X. T lymphocyte proliferative capacity and CD4+/CD8+ ratio in primiparous and pluriparous lactating cows. J Dairy Res 2008; 75: 457-465.

38. Asai K, Kai K, Rikiishi H, Sugawara S, Maruyama Y, Yamaguchi T et al. Variation in CD4+ T and CD8+ T lymphocyte subpopulations in bovine mammary gland secretions during lactating and non-lactating periods. Vet Immunol Immunopathol 1998; 65: 51-61.

39. Munoz E, Blazquez MV, Madueno JA, Rubio G, Pena J. CD26 induces T-cell proliferation by tyrosine protein phosphorylation. Immunology 1992; 77: 43-50.

40. Hachenberg S, Weinkauf C, Hiss S, Sauerwein H. Evaluation of classification modes potentially suitable to identify metabolic stress in healthy dairy cows during the peripartal period. J Anim Sci 2007; 85: 1923-1932.

41. Fathi E, Hamali H, Kaleibar MT. Application of acute phase proteins as indicators of ketosis and their relation to energy metabolites in post-calving dairy cows. Int J Rec Sci Res 2013; 4: 842 - 845.

6. General discussion and conclusion

A recently published study underlines the relevance of subclinical ketosis in dairy herd management and therefore the motivation in animal science for further research. In brief, the authors highlighted the need to conduct more studies on the associations between subclinical ketosis and the risk of diseases, changes in milk production and reproductive parameters (Raboisson et al., 2014). The present work focused on cows suffering from a subclinical ketosis during early lactation with the aim to determine changes in metabolism, performance and parameters of the immune response (Paper I and II). The animal model will be useful for testing interventions available for diagnosis, prevention and treatment. The inhibition of DPP4 was a new possibility to influence a ketotic metabolism in cows, providing new knowledge of cows during subclinical ketosis and around calving (Paper III).

The animal model of subclinical ketosis based on two relevant components – the classification of groups according to the BCS six weeks before expected calving and the feeding management *a.p.* and *p.p.*. An extended overfeeding period *a.p.* or feed intake restriction or deprivation during early lactation to induce a ketotic state *p.p.* were also described in other studies (Kuhla et al., 2009; Loor et al., 2007; Petzold et al., 2015). The "Utrecht fatty liver model of dairy cows" (Geelen and Wensing, 2006) was aimed to find an adequate model in which hepatic lipidosis and associated problems of production, health and fertility can be studied. Without the classification of cows according to their BCS, the combination of overconditioning *a.p.* and feed restriction *p.p.* used herein induced an increased lipolysis in adipose tissue and TG accumulation in the liver following calving.

In the present model of subclinical ketosis, the BCS six weeks before expected parturition included the assignment of cows with an average BCS of 3.19 to a higher condition group (HC), and those cows with an average BCS of 2.61 to a lower condition group (LC). In brief, body condition scoring has been widely accepted as the most practical method for assessing changes in energy reserves. An optimum early lactation BCS (3.0 to 3.5) and a controlled loss of body condition of 0.5 to 1.0 is associated with optimal peak milk production, health, and reproductive performance (Bewley and Schutz, 2008; Roche et al., 2009). Fat cows tend to lose more body condition during early lactation than thin cows (Lacetera et al., 2005). The same is true for multiparous versus primiparous cows (Domecq et al., 1997). Owing to a positive nonlinear relationship between calving BCS and milk yield, researchers show that maximum milk

production is associated with BCS at calving of 3.25 to 3.5 (Berry et al., 2007), even though cows with a calving BCS of ~3.5 have 2 times higher risk to develop ketosis compared to cows calving at BCS ≤ 3.25 (Gillund et al., 2001), respectively, compared to cows at BCS 2.0 (Rasmussen et al., 1999). The literature reveals the fragile balance between the request for maximum milk yield and the risk of ketosis, respectively, suggesting why dairy cows, especially cows of the Holstein breed, with higher milk production suffer from subclinical ketosis more often. In sum, the intensification of milk production has led to a worsening of the average body condition of the cow, partly as a consequence of antagonistic genetic correlations between milk yield and BCS (Kadarmideen and Wegmann, 2003; Pryce et al., 2011). Therefore, in the present investigation the selection for BCS was equivalent to a genetic selection, accompanied by the fact that indirect relationships to other traits are possible. For example, the BCS at calving affects systemic and hepatic transcriptome indicators of inflammation and nutrient metabolism in grazing dairy cows, whereby cows of a higher calving BCS show an up-regulated hepatic expression of peroxisome proliferator-activated receptor-α-targets (PPARα; one of the key hepatic transcription factors) in the fatty acid oxidation and ketogenesis pathways, along with gluconeogenetic genes (Akbar et al., 2015).

In ruminants, most of the available glucose is derived from hepatic gluconeogenesis and regulation of glucose homeostasis is known to be mediated by insulin and glucagon. During hyperglycemia (blood glucose >79 mg/dl; (Vogel et al., 2011), insulin secretion is increased to maintain plasma glucose concentrations at a physiological level. The present investigation achieved a well-controlled blood glucose concentration and no differences between the experimental groups were shown (Paper I and Paper III). In contrast, the insulin concentration was strongly affected. The higher concentrations of insulin *a.p.* as a response to energy overfeeding (Paper I) might be a result of a higher consumption of corn, followed by increased gluconeogenesis and coupled with stimulated pancreatic insulin secretion. Also the combination with an exacerbated peripheral insulin resistance (defined as either decreased sensitivity or responsiveness of insulin-sensitive tissues to insulin; (Kahn, 1978) might be possible (Ji et al., 2012). In the late pregnancy and early lactation, insulin concentration decreases and the peripheral insulin responsiveness suppresses glucose consumption by peripheral, insulin-dependent tissues (skeletal muscle, adipose tissue). Thus the availability of glucose for the insulin-independent mammary gland is enhanced (Bell, 1995). Nevertheless, severe undernutrition can cause insulin levels to fall and in overweight cows hyperinsulinaemia may be

related to insulin resistance (Wathes et al., 2007). Owing to these observations, differences in insulin sensitivity between the experimental groups seemed to be expectable.

At this point, BI 14332 may play a key role as it should improve insulin sensitivity by inhibiting DPP4. To our knowledge, the employment of a DPP4 inhibitor in dairy cows was tested herein for the first time. The increasing concentrations of GLP-1 and GIP after calving (Faulkner and Martin, 1999; Relling and Reynolds, 2007) are associated with an increase in gut tissue mass with positive impacts on nutrient absorption. A prolonged effectiveness of active GLP-1(7-36) *p.p.* via DPP4 inhibition was expected to reduce hepatic lipidosis, with positive impacts on energy metabolism (Paper III). This assumption was made because chronically elevated concentrations of GLP-1 resulted in reduced hepatic lipid contents and significantly lower TG concentrations in rat and mouse models (Ben-Shlomo et al., 2011). The researchers delineate a GLP-1 signaling pathway in the liver, in which GLP-1 induces a signal for a low energy status via the cAMP/AMP-activated protein kinase (AMPK) pathway, resulting in a reduced expression of lipogenesis-related genes. The AMPK has emerged as a key regulator of skeletal muscle fat metabolism. For example, it regulates the fatty acid transport into the mitochondria, the capacity of the fatty acid oxidation by phosphorylation (Thomson and Winder, 2009) and AMPK mediates the metabolic effects of hormones such as leptin, glucocorticoids or insulin (Lim et al., 2010). Seo et al. (2008) showed that the food intake lowering effect of GLP-1 is caused by reversing the fasting-induced increase in hypothalamic AMPK activity and authors conclude that anorectic effects of GLP-1 are mediated, at least in part, by the hypothalamus. In cattle, protein expression of AMPK has been investigated in the liver during mobilization of body reserves (Kuhla et al., 2009) and was increased in adipose tissue both during early lactation (Locher et al., 2012) and independent from lactation with decreasing DMI (Locher et al., 2015). Furthermore, the bovine hepatic mRNA expression of PPARγ, a lipogenic and adipogenic gene which is down-regulated by activated AMPK, is negatively associated with NEFA, BHB and back fat thickness and positively with the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) (Saremi et al., 2014). Unfortunately, associations regarding pathways of AMPK and GLP-1 or other incretins in cows are presently lacking. In a simplified scheme and according to the own results, AMPK activity may have been increased during early lactation and under a status of fasting-induced energy deficit, which was reflected by an increased lipolysis to compensate. Because BI 14332 caused a significant inhibition of DPP4 (Paper III) it can be assumed, that a high level of active GLP-1(7-36) circulates in the periphery of treated cows. In this case AMPK activity might

remain on a mean level and the NEB-induced lipolysis will be less strong. As a possible point of entry, the lower TG and NEFA concentration in bovine serum and the higher RQUICKI index after BI 14332 treatment are conceivably (Paper III). With respect to the DMI and unlike to human studies, a reduced appetite was not observed with BI 14332 treatment (Paper III). For the present investigation, this fact has to be considered positively, since physiologically decreased DMI around calving was not further strengthened. Selected variables of the clinical chemistry are summarized in Table 1. The table reflects the first and the second week after calving when the risk of ketosis is highest (McArt et al., 2011) and 80% of the HC cows showed a ketotic status.

The variables assessed were only marginally influenced by the BI 14332 treatment. The data set demonstrates that HC cows treated with BI 14332 do not reach the same metabolic status as cows of the NC group, with the exception of TG. Triglyceride concentrations for HC-BI cows were as high as for the NC cows on day +14 *p.p.* and therefore 20% lower than for the HC-Con cows. A similar development was observed for the NEFA concentration, with values lower for HC-BI cows than for HC-Con cows, but higher than those for NC cows. Particularly interesting for the present investigation, RQUICKI showed a significant interaction between group and time within the two evaluated days and beyond the three experimental groups, as well as the highest index value on day +14 for the HC-BI cows, when BI 14332 treatment was almost terminated. By now, RQUICKI is an established surrogate marker for insulin sensitivity, because in lactating cows about 80% of the cellular glucose uptake occurs independently of insulin (mainly by the mammary gland), whereby the suitability of glucose tolerance tests is inhibited (Holtenius and Holtenius, 2007). Paper I showed that there was no difference in RQUICKI between cows with subclinical ketosis and cows that are metabolically healthy and it is questionable if this surrogate marker is useful for assessing insulin sensitivity in cows suffering from a metabolic disorder. In healthy cows RQUICKI is positively associated to insulin and glucose clearance, and negatively to BCS (Balogh et al., 2008). However, in periparturient cows with changes in BHB, RQUICKI remained unaffected by any measured variable during glucose or insulin tolerance test, and did not correlate with BCS or BCS loss (Kerestes et al., 2009). In the same study, cows with long-term hyperketonemia had decreased peripheral insulin sensitivity and glucose responsiveness to insulin, while RQUICKI was increased, and authors indicated RQUICKI as an index with low discrimination power in diagnosing insulin sensitivity in cows affected by metabolic diseases (Kerestes et al., 2009).

Table 1. Variables of clinical chemistry to elicit information about metabolic health of three different experimental groups[†] of primi- and pluriparous cows.

		BHB [mM]	NEFA [mM]	Triglyceride [mg/dL]	Glucose [mg/dL]	Insulin [mU/mL]	RQUICKI	Haptoglobin [mg/mL]	AST [U/I]	γ-GT [U/I]
LC		0.76 ± 0.27	0.58 ± 0.08	10.65 ± 1.06	62.55 ± 3.28	9.01 ± 1.84	0.43 ± 0.01	1.59 ± 0.46	77.97 ± 10.38	16.99 ± 2.03
HC-Con	Day +7	1.31 ± 0.31	1.03 ± 0.10	13.44 ± 1.26	65.15 ± 3.88	5.78 ± 2.00	0.39 ± 0.02	2.70 ± 0.54	94.94 ± 12.29	17.00 ± 2.40
HC-BI		1.43 ± 0.41	1.28 ± 0.13	15.19 ± 1.65	54.84 ± 5.08	4.95 ± 2.62	0.41 ± 0.02	0.96 ± 0.71	94.57 ± 16.09	17.62 ± 3.15
LC		0.65 ± 0.27	0.42 ± 0.08	9.93 ± 1.06	63.71 ± 3.28	12.58 ± 1.69	0.42 ± 0.01	1.10 ± 0.46	65.15 ± 10.38	17.04 ± 2.03
HC-Con	Day +14	1.07 ± 0.42	0.86 ± 0.13	12.86 ± 1.66	58.10 ± 5.19	6.22 ± 2.69	0.41 ± 0.02	0.42 ± 0.71	92.09 ± 16.11	20.18 ± 3.01
HC-BI		1.53 ± 0.42	0.63 ± 0.13	9.41 ± 1.68	56.45 ± 5.20	3.81 ± 2.68	0.48 ± 0.02	0.17 ± 0.72	96.84 ± 16.42	20.74 ± 3.20
Probability	Group	0.136	**< 0.001**	**0.046**	0.309	**0.031**	0.157	0.420	0.205	0.760
	Parity	0.124	0.438	**0.036**	**0.011**	**0.045**	**0.010**	0.576	**0.014**	0.333
	Time	0.762	**0.005**	0.102	0.667	0.593	**0.046**	**0.012**	0.640	0.201
	Group*Time	0.891	0.130	0.288	0.502	0.497	**0.039**	0.186	0.763	0.580

[†]Cows of higher condition group (HC) suffered from subclinical ketosis, defined as BHB concentration in blood serum ≥1.2 mM. Cows of lower condition group (LC, n = 9) stayed apparently healthy. Subclinical ketosis was diagnosed on day +3, +7 or +10 *post partum*. With first occurrence of subclinically BHB concentrations, cows of HC-BI (n = 6) group were treated with BI 14332 (dipeptidyl peptidase 4-inhibitor; intravenous; 0.3 mg/kg body weight, 7-days period), while HC-Con (n = 6) cows remained untreated as control group.

Significant values ($p < 0.05$) are shown in bold.

Abbreviations: BHB. β-hydroxybutyrate; NEFA. Non-esterified fatty acids; RQUICKI. Revised quick insulin sensitivity index; AST. aspartate aminotransferase; γ-GT. γ-glutamyltransferase

The higher index in cows treated with BI 14332 was attributable to the decreased NEFA concentration (Paper III, Table 1), and RQUICKI seems to be useful to investigate differences reflected e.g. by breed (Bossaert et al., 2009), by parity (Singh et al., 2014) or by various stages of lactation (Bjerre-Harpoth et al., 2012). However, RQUICKI may not be sensitive enough to investigate sensitive metabolic alterations, such as short-term changes or metabolic diseases, especially in case of subclinically disorders (Kerestes et al., 2009). RQUICKI may also not be a reliable marker across the whole range of BCS (Jaakson et al. 2013). Furthermore, the digestive physiology of ruminants complicates investigations of fasting blood concentrations to prepare steady state protocols and calculate surrogate insulin sensitivity indices as suggested in humans.

Regarding variables which are closely associated with the immune system, a slight influence of subclinical ketosis was observed (Paper II). Interestingly, PBMC of cows which were not pre-stressed by subclinical ketosis *in vivo* were more sensitive to increasing levels of BHB *in vitro* as evidenced by both their proliferative capability and the increased release of nitric oxide in the PBMC supernatant. As slightly diminished immune cell responsiveness might imply an increased risk for different diseases (e.g. endometritis, mastitis), consequences on the immune system in case of subclinical ketosis are expectable, with negative impacts on health in general, on fertility and performance. First significant effects on the proliferative capability of PBMC were detected at a dosage of 5 mM BHB. Such BHB concentrations can be expected during clinical ketosis and starvation. Therefore, it is evidenced that next to NEFA (Lacetera et al., 2004; Scalia et al., 2006; Ster et al., 2012) even BHB, as most important ketone body, elicits effects of immune cells. However, the inhibition of DPP4 via BI 14332 seemed not to alter the proliferative capability of bovine PBMC (Paper III). Anz and colleges showed similar results in humans and mice and conclude that these findings are confirming the drug safety (Anz et al., 2014). In view of immune-modulated changes, the lymphocyte $CD4^+/CD8^+$ ratio tended to be lower in BI 14332 treated cows at the time of treatment. An appropriate $CD4^+/CD8^+$ ratio is important to sustain T-cell homeostasis and immune responses. It has been proven that most of the T-cells expressing DPP4 belong to the $CD4^+$ population, whereby expression is tightly regulated upon T-cell activation with an increased DPP4 expression for several days (Lee et al., 2013). If this assumption also applies for cattle, the results show that an immune suppression for BI 14332 untreated cows and the subsequent increased susceptibility to diseases may be associated to an imbalance in the T-cell subpopulation, followed by a reduced T-cytotoxic immune response. The correlation observed in Figure 3 between NEFA and the $CD4^+/CD8^+$ ratio point to the relation

between increased hepatic lipidosis and induction of an immune dysregulation. It is known, that an increased hepatic fatty infiltration correlates strong positively with inflammatory markers, such as haptoglobin (Saremi et al., 2012, Paper II), serum amyloid A (Fathi et al., 2013), or various cytokines (Trevisi et al., 2012). As both, NEFA and $CD4^+/CD8^+$ ratio, were influenced by DPP4 inhibition, a relationship seems possible. As shown in Figure 3, the $CD4^+/CD8^+$ ratio (y-axis) increased with increasing NEFA (x-axis) concentrations linearly with a positive slope: y = 1.376x + 1.9336, which suggests a moderate relationship (r = 0.40). The largest scattering with highest $CD4^+/CD8^+$ ratios and NEFA values were shown for the HC-Con cows (blue triangles), but also for the other two groups outliers were present. According to physiological ranges, defined as maximum $CD4^+/CD8^+$ ratio of 2.5 (Mehrzad and Zhao, 2008) and 0.63 mM NEFA in blood (Dirksen et al., 2012), 48.3% of all dots were within this range.

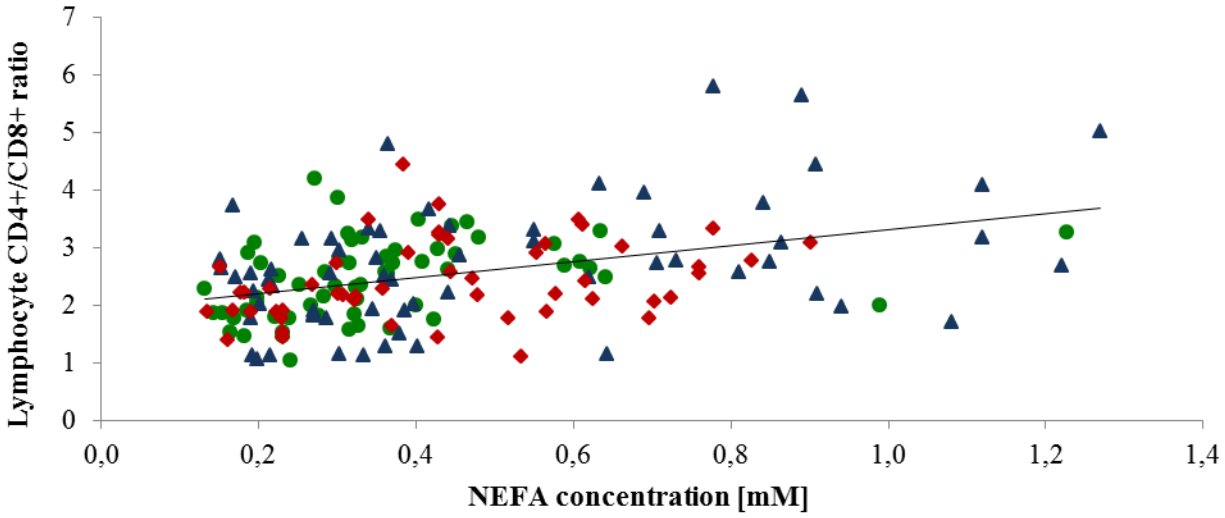

Figure 3. Linear regression between the serum concentrations of NEFA (x-axis) and the lymphocytic $CD4^+/CD8^+$ ratio (y-axis) for the time around calving and during subclinical ketosis. Lower conditioned cows stayed metabolically healthy during the experimental time (LC ●; n = 9). Higher conditioned cows suffered from subclinical ketosis *post partum*, defined as β-hydroxybutyrate concentrations in blood ≥ 1.2 mM, and were either treated with BI 14332 to inhibit dipeptidyl peptidase-4 (HC-BI ♦; n = 6) or stayed untreated as control (HC-Con ▲; n = 6). The $CD4^+/CD8^+$ ratio increased with increasing NEFA concentrations linearly with a positive slope and a moderate correlation (r = 0.40) was observed: y = 1.376x + 1.9336 [Correlation was calculated using Pearson correlation coefficient at $P \leq 0.05$].

Taking older studies and their findings about the lack of association between GIP or GLP-1 and insulin into account, it is doubtful how useful attempt to treat ketotic situation by inhibition of DPP4 are. For the present investigation the GLP-1 concentration in blood could not be measured. Owing to matrix effects the results of the used multispecies assay (Enzyme Linked

Immunosorbent Assay; species reactivity: mammals) were not evaluable. Therefore, the pathways are still unclear and precise statements regarding the interaction of DPP4, GLP-1 and the generated data are lacking, but there is no doubt that further investigations have also to reflect the incretin concentrations to clarify that the incretin activity is prolonged during DPP4 inhibition. Even a more sensitive evaluation of insulin sensitivity (e.g. via hyperinsulinemic euglycemic clamp) may be useful, also to confirm findings made by RQUICKI. An explanation for the minor impact of DPP4 inhibition on variables of energy balance, ketogenesis and/or the immune system may be the duration of treatment period. The BI 14332 was kept rather short compared to studies of humans and rodents (Drucker and Nauck, 2006), where test subjects are usually treated over a period of several weeks. In addition, in humans further proteases such as neutral endopeptidase may inactivate up to half of the GLP-1 entering the circulation (Plamboeck et al., 2005). Human neutral endopeptidase is also known as neprilysin or CD10 and has already been demonstrated in the mammary glands of dairy heifers (Safayi et al., 2012). Therefore, it seems possible, that beside DPP4 other peptidases (i.e. DPP8, DPP9, fibroblast activation protein; Chen and Jiaang, 2011; Lankas et al., 2005) are able to inactivate GLP-1 and/or other incretins in cattle, with potential impacts on energy metabolism.

Conclusion

Under the present conditions (e.g. dosage, duration) a significant inhibition of DPP4 via BI 14332 was shown. Temporarily, the inhibition influenced clinical chemical variables, with positive impacts on energy metabolism. A long-term modeling via BI 14332 was not overserved. In particular, BHB, as indicator for subclinical ketosis, remained unaffected. Also the impact on immune-modulating variables was less, but in a reverse conclusion, no negative influence on general cow health or performance was noticed. Whether treatment impacted physiologically decreased insulin sensitivity could not be finally clarified, as for the present investigation RQUICKI seemed to be too imprecise for reliable statements and further research will be necessary to fill the gap of knowledge. For that and other similar projects, the animal model of subclinical ketosis will be useful. At this, the classification of cows with a lower and higher BCS during dry period, accompanied by a high energetic feeding management *a.p.* and feed restriction *p.p.* for those of higher BCS, caused an increased lipolysis and ketogenesis soon after calving.
Owing to the model of subclinical ketosis, further investigations of the metabolism of transition cows, as well as diagnosis, treatment and prevention of metabolic disorders are possible.

7. Summary

The time between late gestation and early lactation, also called the periparturient period or transition period, is of critical importance for high-yielding dairy cows in terms of health and performance. This period is characterized by numerous physiological challenges and a catabolic state, as the required energy for milk and maintenance cannot be covered only by voluntary feed intake alone. The dairy cow mobilizes energy from body reserves (mainly fat) and passes through a distinct negative energy balance (NEB). During NEB, high concentrations of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) are detectable in blood. Because BHB is the most stable ketone body in blood, it is usually used to define a ketotic status, with 1.2 mM BHB in serum as cut-off point for subclinical ketosis. Concentrations of BHB ≥ 3.0 mM, accompanied by clinical signs, are indicative for clinical ketosis. The first aim of the present dissertation was to induce subclinical ketosis in lactating cows. Owing to a classification of cows with a lower and higher body condition score (BCS; 2.61 ± 0.09 versus 3.19 ± 0.09; $P < 0.05$) 6 weeks before expected calving, accompanied by feeding an energy-dense ration *a.p.* (7.7 MJ NE_L/kg dry matter) and feed restriction *p.p.* for the cows of the higher BCS group, an excessively stimulated lipomobilisation was induced. In sum, 80% of the cows in the higher BCS group had ketosis (81% subclinical and 19% clinical ketosis), with significantly higher serum concentrations of BHB, NEFA and liver-function enzymes (e.g. aspartate transaminase), as well as a higher liver lipid content in the 2nd week *p.p.* (154 mg/g) compared to cows of the lower BCS group, which stayed apparently healthy during the experimental trail. For assessing insulin sensitivity, which is known to decrease around calving, the revised quantitative insulin sensitivity check index (RQUICKI) was used as surrogate marker, but remained unaffected by the ketotic status. Variables about immune function were slightly influenced by subclinical ketosis; expect that in *in vitro* tests peripheral blood mononuclear cells (PBMC) of cows not pre-stressed by subclinical ketosis *in vivo* responded more sensitively to increasing levels of BHB as evidenced by both, the proliferative capability and the increased release of nitric oxide in the PBMC supernatant. The results indicate that even slightly diminished immune cell responsiveness might imply an immunosuppression during the periparturient period. Moreover, the proliferative capability of PBMC decreased with increasing BHB concentrations *in vitro*. First significant effects were shown at 5 mM BHB. The results confirmed statements that beside NEFA, BHB also elicits effects of immune cells.

As the metabolic situation of cows during the periparturient period has some similarities to type II diabetes and non-alcoholic fatty liver in humans, another aim of the dissertation was to investigate how the inhibition of dipeptidyl peptidase-4 (DPP4) affects variables associated with NEB and subclinical ketosis. DPP4 inhibitors are successfully used to treat type II diabetes in humans, as they improve insulin sensitivity and glucose tolerance, while accumulation of hepatic fat is decreased. DPP4 is known to be an incretin hormones (e.g. glucagon-like peptide-1; GLP-1) degrading enzyme. Therefore, beneficial effects of DPP4 inhibition result from the prolonged effectiveness of incretins. Enables by the cooperation with Boehringer Ingelheim we were able to test the DPP4 inhibitor BI 14332. With the first occurrence of serum BHB $\geq$ 1.2 mM concentrations BI 14332 was administered daily for 7 days (intravenous, 0.3 mg/kg body weight). Treatment with BI 14332 did not alter main markers of ketosis (BHB, liver lipid content), but triglycerides and NEFA concentrations in blood were decreased after the treatment, with triglyceride values as low as in metabolically healthy cows. Caused by the decreased NEFA concentrations, even RQUICKI changed, so that improved insulin sensitivity was assumed. Therefore, the prolonged retention period of incretin hormones may impact pathways of energy metabolism positively, resulting in a reduced NEB-induced lipolysis. Unfortunately, RQUICKI as marker for insulin sensitivity seemed to be insufficiently precise within the present experimental design. Considering various hematological and immune-related variables, differences between cows with subclinical ketosis treated with BI 14332 and those who remained untreated were limited to the lymphocyte $CD4^+/CD8^+$ ratio for which a trend to decreased values in the treated animals was noted. The results indicated that the peripartal immune dysregulation in ketotic cows was ameliorated by BI 14332.

In brief, the inhibition of DPP4 had some advantages in the periparturient cow and provided new knowledge in animal science, then stimulating further research to clarify the specific interaction of incretin hormones and DPP4, as well as its inhibition, in the dairy cow. A further point of entry may refer to the positive impacts on insulin sensitivity via DPP4 inhibition. Therefore, more sensitive methods to evaluate and to confirm the statements made by RQUICKI are necessary. For future research projects, the described animal model for subclinical ketosis will be useful in assessing new metabolic pathways, on biochemical and cellular level, and also to develop new approaches related to diagnosis, treatment and prevention.

8. Zusammenfassung

Die Wochen um die Abkalbung stellen für den Stoffwechsel einer Milchkuh eine besondere Herausforderung dar. Während dieser Zeit, die auch als peripartale Phase bezeichnet wird, ist die Futteraufnahme physiologisch gemindert. Gleichzeitig steigt durch die Trächtigkeit, die Ausbildung des Euters und die Milchbildung der Energiebedarf. Die Kuh gerät in eine katabole Stoffwechsellage. Über den Abbau körpereigener Energiereserven (vornehmlich Depotfett) wird das Energiedefizit möglichst ausgeglichen. Wie groß das Energiedefizit und die daraus resultierende negative Energiebilanz (NEB) sind, definiert sich u.a. über die Konzentration an nicht veresterten Fettsäuren (NEFA) im Blut. Bei anhaltend hoher Lipolyse erfolgt in der Leber eine vermehrte Bildung von Ketonkörper, wobei β-Hydroxybutyrat (BHB) als der bedeutendste „Ketonkörper" erachtet wird und als Indikator für subklinische (BHB-Konzentration im Blut > 1.2 mM) und klinische Ketosen (BHB-Konzentration im Blut > 3 mM) gilt. Ziel der vorliegenden Dissertation war die Schaffung eines Tiermodells zur Induktion subklinischer Ketosen bei der Milchkuh, um unter kontrollierbaren Bedingungen den ketotischen Stoffwechsel und mögliche Auswirkungen auf Leistungsparameter und die Immunantwort zu untersuchen. Dabei wurden 20 Kühe (primi- und pluripare Tiere) mit einer Körperkondition (BCS) von mindestens 3.0 für die letzten 6 Wochen der Trächtigkeit mit einer hochenergetischen Futterration versorgt (7.7 MJ NE_L/kg Trockenmasse). *Post partum* erfolgte eine verzögerte Steigerung der Energiezufuhr über die ersten 3 Wochen um die postpartale Lipolyse zusätzlich zu stimulieren. Insgesamt wiesen 80% dieser Kühe eine Ketose auf (81% subklinische, 19% klinische Ketose), gekennzeichnet durch signifikant höhere Blutkonzentrationen an BHB und NEFA, sowie deutlich erhöhte Leberfettwerte (154 mg/g) im Vergleich zur Kontrollgruppe. Die Kontrolltiere (n = 10) hatten zum Zeitpunkt der Einteilung (6 Wochen *a.p.*) einen BCS < 3.0 und wurden im Versuchsverlauf adäquat (entsprechend der Leitlinien der Gesellschaft für Ernährungsphysiologie) gefüttert, so dass 90% der Tiere *p.p.* anhand klinisch-chemischer Variablen als metabolisch gesund eingestuft werden konnten. Die Insulinsensitivität, die um die Abkalbung herum physiologisch abnimmt, wurde anhand eines Ersatzmarkers, dem sogenannten RQUICKI (revised quantitative insulin sensitivity check index), bestimmt. Hierbei konnten keine Unterschiede zwischen Kühen mit und ohne subklinischer Ketose festgestellt werden. Im Hinblick auf die Immunantwort zeigten die durchgeführten *in vitro* Untersuchungen, dass die mononukleären Zellen des peripheren Blutes (PBMC) der metabolisch gesunden Kühe sensibler auf ansteigende BHB-Konzentrationen

reagierten. Dies konnte durch die Proliferationsfähigkeit der Zellen und die Stickoxid-Freisetzung im PBMC-Überstand festgestellt werden. Die Ergebnisse lassen rückschließen, dass bereits leicht verminderte Immunzellreaktionen eine Immunsuppression während der peripartalen Phase implizieren, so dass das Risiko für Folgeerkrankungen, besonders für Infektionserkrankungen, steigt. Des Weiteren sank die Proliferationsfähigkeit der PBMC bei BHB-Konzentrationen in Blut ab 5 mM (*in vitro*). Hier bestätigt sich, dass neben NEFA auch BHB Immunzellreaktionen beeinflusst.

Da die Stoffwechsellage peripartaler Kühe Ähnlichkeiten mit dem Krankheitsbild bei Typ 2 Diabetes und (nicht alkoholbedingter) Fettleber aufweist, bestand ein weiteres Ziel dieser Arbeit darin, zu untersuchen wie sich die Inhibierung der Dipeptidylpeptidase-4 (DPP4) auf ausgewählte Parameter der NEB und subklinischen Ketose auswirkt. Dipeptidylpeptidase-4 ist ein Enzym im Inkretinstoffwechsel. Das Inkretinhormon Glucagon-like-peptide 1 (GLP-1) wird mit der Nahrungsaufnahme aus dem Dünndarm freigesetzt. Im Pankreas bewirkt es eine Stimulation der Insulinproduktion bei gleichzeitiger Reduzierung der Glucagonbildung. Innerhalb weniger Minuten nach der Nahrungsaufnahme wird GLP-1 durch DPP4 in eine inaktive Form überführt. DPP4-Inhibitoren bewirken, dass aktives GLP-1 länger wirken kann, was bei Mensch und Nager zu einer Verbesserung der Insulinsensitivität und Glucosetoleranz führt, während Fetteinlagerungen in der Leber reduziert sind. Im vorliegenden Versuch wurden subklinisch ketotische Kühe mit dem DPP4-Inhibitor BI 14332 behandelt (Boehringer Ingelheim, tägliche intravenöse Injektion über 7 Tage, Dosis: 0.3 mg/kg Lebendmasse). Anhand der Ergebnisse konnte festgestellt werden, dass die Hauptmerkmale einer ketotischen Stoffwechsellage (BHB, Leberfettgehalt) unbeeinflusst von der DPP4-Hemmung via BI 14332 blieben. Allerdings waren NEFA- und Triglyceridkonzentrationen im Blut mit Behandlungsabschluss reduziert und sanken im Fall der Triglyceride auf ein ähnliches Niveau wie bei metabolisch gesunden Kühen zum gleichen Zeitpunkt. Aufgrund der reduzierten NEFA-Konzentration im Blut war RQUICKI erhöht, weshalb eine Verbesserung der Insulinsensitivität angekommen werden könnte. Allerdings zeigten die durchgeführten Untersuchungen auch, dass RQUICKI möglicherweise nicht ausreichend sensibel war um im vorliegenden Fall konkrete Aussagen über die Insulinsensitivität zu treffen. Hinsichtlich ihres Immunsystems wiesen BI 14332 behandelte Tiere einen tendenziell kleineren $CD4^+/CD8^+$ Quotienten auf, verglichen mit den unbehandelten Tieren der Kontrollgruppe. Bei den Kontrolltieren stieg der Quotient im Beobachtungszeitraum auf > 3.0 an. Im selben Zeitraum verblieb der Quotient bei behandelten Tiere auf einem niedrigeren Niveau

und stieg *p.p.* nicht an. Diese Ergebnisse deuten an, dass eine mögliche Immundysregulation bei Kühen mit subklinischer Ketose durch die DPP4-Inhibierung positiv beeinflusst wurde.

Zusammenfassend bleibt zu sagen, dass durch die Hemmung der DPP4 wenige, aber positive Effekte in der peripartalen Phase gezeigt werden konnten. Weiterführende Studien könnten hier anknüpfen und den noch wenig erforschten Zusammenhang zwischen GLP-1 und DPP4, sowie deren Inhibierung, bei der Milchkuh genauer untersuchen. Des Weiteren muss die Insulinsensitivität stärker berücksichtigt und mögliche positive Einflüsse mit sensibleren Methoden festgestellt werden, auch um die in dieser Dissertation gezeigten Ergebnisse mittels RQUICKI bestätigen zu können. Für solche und ähnliche Forschungsvorhaben stellt das Tiermodell zur Induktion subklinischer Ketosen eine adäquate Voraussetzung dar und bietet im Rahmen der Tiergesundheit neue Forschungsmöglichkeiten mit Bezug auf Diagnose, Prävention und Therapie von Milchkühen im peripartalen Zeitraum.

9. List of references

Akbar, H., Grala, T.M., Vailati Riboni, M., Cardoso, F.C., Verkerk, G., McGowan, J., Macdonald, K., Webster, J., Schutz, K., Meier, S., Matthews, L., Roche, J.R., Loor, J.J., 2015. Body condition score at calving affects systemic and hepatic transcriptome indicators of inflammation and nutrient metabolism in grazing dairy cows. J Dairy Sci 98, 1019-1032.

Anz, D., Kruger, S., Haubner, S., Rapp, M., Bourquin, C., Endres, S., 2014. The dipeptidylpeptidase-IV inhibitors sitagliptin, vildagliptin and saxagliptin do not impair innate and adaptive immune responses. Diabetes Obes Metab 16, 569-572.

Aschenbach, J.R., Penner, G.B., Stumpff, F., Gabel, G., 2011. Ruminant Nutrition Symposium: Role of fermentation acid absorption in the regulation of ruminal pH. J Anim Sci 89, 1092-1107.

Asl, A.N., Nazifi, S., Ghasrodashti, A.R., Olyaee, A., 2011. Prevalence of subclinical ketosis in dairy cattle in the Southwestern Iran and detection of cutoff point for NEFA and glucose concentrations for diagnosis of subclinical ketosis. Prev Vet Med 100, 38-43.

Balogh, O., Szepes, O., Kovacs, K., Kulcsar, M., Reiczigel, J., Alcazar, J., Keresztes, M., Febel, H., Bartyik, J., Fekete, S.G., 2008. Interrelationships of growth hormone AluI polymorphism, insulin resistance, milk production and reproductive performance in Holstein-Friesian cows. Vet Med (Praha) 53, 604-616.

Bell, A.W., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci 73, 2804-2819.

Ben-Shlomo, S., Zvibel, I., Shnell, M., Shlomai, A., Chepurko, E., Halpern, Z., Barzilai, N., Oren, R., Fishman, S., 2011. Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase. J Hepatol 54, 1214-1223.

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Biochemistry, Fifth Edition. W.H. Freeman.

Berge, A.C., Vertenten, G., 2014. A field study to determine the prevalence, dairy herd management systems, and fresh cow clinical conditions associated with ketosis in western european dairy herds. J Dairy Sci 97, 2145-2154.

Bernabucci, U., Ronchi, B., Lacetera, N., Nardone, A., 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J Dairy Sci 88, 2017-2026.

Berry, D.P., Buckley, F., Dillon, P., 2007. Body condition score and live-weight effects on milk production in Irish Holstein-Friesian dairy cows. Animal 1, 1351-1359.

Bewley, J.M., Schutz, M.M., 2008. Review: an interdisciplinary review of body condition scoring for dairy cattle. Prof Anim Sci 24, 507-529.

Bjerre-Harpoth, V., Friggens, N.C., Thorup, V.M., Larsen, T., Damgaard, B.M., Ingvartsen, K.L., Moyes, K.M., 2012. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation. J Dairy Sci 95, 2362-2380.

Bobe, G., Young, J.W., Beitz, D.C., 2004. Invited review: pathology, etiology, prevention, and treatment of fatty liver in dairy cows. J Dairy Sci 87, 3105-3124.

Bossaert, P., Leroy, J.L., De Campeneere, S., De Vliegher, S., Opsomer, G., 2009. Differences in the glucose-induced insulin response and the peripheral insulin responsiveness between neonatal calves of the Belgian Blue, Holstein-Friesian, and East Flemish breeds. J Dairy Sci 92, 4404-4411.

Bradford, B.J., Harvatine, K.J., Allen, M.S., 2008. Dietary unsaturated fatty acids increase plasma glucagon-like peptide-1 and cholecystokinin and may decrease premeal ghrelin in lactating dairy cows. J Dairy Sci 91, 1443-1450.

Butler, W., 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest Prod Sci 83, 211-218.

Cefalu, W.T., 2010. The physiologic role of incretin hormones: clinical applications. J Am Osteopath Assoc 110, S8-S14.

Chen, S.J., Jiaang, W.T., 2011. Current advances and therapeutic potential of agents targeting dipeptidyl peptidases-IV, -II, 8/9 and fibroblast activation protein. Curr Top Med Chem 11, 1447-1463.

Connor, E.E., Baldwin, R.L., Capuco, A.V., Evock-Clover, C.M., Ellis, S.E., Sciabica, K.S., 2010. Characterization of glucagon-like peptide 2 pathway member expression in bovine gastrointestinal tract. J Dairy Sci 93, 5167-5178.

Cooke, R.F., Bohnert, D.W., 2011. Technical note: bovine acute-phase response after corticotrophin-release hormone challenge. J Anim Sci 89, 252-257.

De Meester, I., Korom, S., Van Damme, J., Scharpe, S., 1999. CD26, let it cut or cut it down. Immunol Today 20, 367-375.

Deacon, C.F., Nauck, M.A., Toft-Nielsen, M., Pridal, L., Willms, B., Holst, J.J., 1995. Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH2-terminus in type II diabetic patients and in healthy subjects. Diabetes 44, 1126-1131.

Dirksen, G., Gründer, H., Stöber, M., 2012. Die klinische Untersuchung des Rindes. Enke, Stuttgart.

Domecq, J.J., Skidmore, A.L., Lloyd, J.W., Kaneene, J.B., 1997. Relationship between body condition scores and milk yield in a large dairy herd of high yielding Holstein cows. J Dairy Sci 80, 101-112.

Drucker, D.J., Nauck, M.A., 2006. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 368, 1696-1705.

Duffield, T., 2000. Subclinical ketosis in lactating dairy cattle. Vet Clin North Am Food Anim Pract 16, 231-253.

Duffield, T.F., Sandals, D., Leslie, K.E., Lissemore, K., McBride, B.W., Lumsden, J.H., Dick, P., Bagg, R., 1998. Efficacy of monensin for the prevention of subclinical ketosis in lactating dairy cows. J Dairy Sci 81, 2866-2873.

Esposito, G., Irons, P.C., Webb, E.C., Chapwanya, A., 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Anim Reprod Sci 144, 60-71.

Fathi, E., Hamali, H., Kaleibar, M.T., 2013. Application of acute phase proteins as indicators of ketosis and their relation to energy metabolites in post-calving dairy cows. Int J Rec Sci Res 4, 842 - 845.

Faulkner, A., Martin, P.A., 1999. Insulin secretion and intestinal peptides during lactation in sheep. J Dairy Res 66, 45-52.

Flatt, P.R., Bailey, C.J., Green, B.D., 2008. Dipeptidyl peptidase IV (DPP IV) and related molecules in type 2 diabetes. Front Biosci 13, 3648-3660.

Franklin, S.T., Young, J.W., Nonnecke, B.J., 1991. Effects of ketones, acetate, butyrate, and glucose on bovine lymphocyte proliferation. J Dairy Sci 74, 2507-2514.

Geelen, M.J., Wensing, T., 2006. Studies on hepatic lipidosis and coinciding health and fertility problems of high-producing dairy cows using the "Utrecht fatty liver model of dairy cows". A review. Vet Q 28, 90-104.

Gillund, P., Reksen, O., Grohn, Y.T., Karlberg, K., 2001. Body condition related to ketosis and reproductive performance in Norwegian dairy cows. J Dairy Sci 84, 1390-1396.

Gordon, J.L., Leblanc, S.J., Duffield, T.F., 2013. Ketosis treatment in lactating dairy cattle. Vet Clin North Am Food Anim Pract 29, 433-445.

Gorrell, M.D., Gysbers, V., McCaughan, G.W., 2001. CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand J Immunol 54, 249-264.

Grummer, R.R., 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. J Dairy Sci 76, 3882-3896.

Grummer, R.R., Mashek, D.G., Hayirli, A., 2004. Dry matter intake and energy balance in the transition period. Vet Clin North Am Food Anim Pract 20, 447-470.

Gustafsson, A., Andersson, L., Emanuelson, U., 1995. Influence of feeding management, concentrate intake and energy intake on the risk of hyperketonaemia in Swedish dairy herds. Prev Vet Med 22, 237-248.

Hoeben, D., Burvenich, C., Massart-Leen, A.M., Lenjou, M., Nijs, G., Van Bockstaele, D., Beckers, J.F., 1999. In vitro effect of ketone bodies, glucocorticosteroids and bovine pregnancy-associated glycoprotein on cultures of bone marrow progenitor cells of cows and calves. Vet Immunol Immunopathol 68, 229-240.

Hoeben, D., Heyneman, R., Burvenich, C., 1997. Elevated levels of beta-hydroxybutyric acid in periparturient cows and in vitro effect on respiratory burst activity of bovine neutrophils. Vet Immunol Immunopathol 58, 165-170.

Holtenius, P., Holtenius, K., 1996. New aspects of ketone bodies in energy metabolism of dairy cows: a review. Zentralbl Veterinarmed A 43, 579-587.

Holtenius, P., Holtenius, K., 2007. A model to estimate insulin sensitivity in dairy cows. Acta Vet Scand 49, 29.

Hopsu-Havu, V.K., Glenner, G.G., 1966. A new dipeptide naphthylamidase hydrolyzing glycyl-prolyl-beta-naphthylamide. Histochemie 7, 197-201.

Ingvartsen, K.L., Moyes, K., 2013. Nutrition, immune function and health of dairy cattle. Animal 7 Suppl 1, 112-122.

Jaakson, H., Ling, K., Samarütel, J., Ilves, A., Kaart, T., Kärt, O., Ots, M., 2013. Blood glucose and insulin response during the glucose tolerance test in relation to dairy cow body condition and milk yield. Vet Med Zoot (Lithuania) 62.

Ji, P., Osorio, J.S., Drackley, J.K., Loor, J.J., 2012. Overfeeding a moderate energy diet prepartum does not impair bovine subcutaneous adipose tissue insulin signal transduction and induces marked changes in peripartal gene network expression. J Dairy Sci 95, 4333-4351.

Juillerat-Jeanneret, L., 2014. Dipeptidyl peptidase IV and its inhibitors: therapeutics for type 2 diabetes and what else? J Med Chem 57, 2197-2212.

Kadarmideen, H.N., Wegmann, S., 2003. Genetic parameters for body condition score and its relationship with type and production traits in Swiss Holsteins. J Dairy Sci 86, 3685-3693.

Kahn, C.R., 1978. Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. Metabolism 27, 1893-1902.

Kazafeos, K., 2011. Incretin effect: GLP-1, GIP, DPP4. Diabetes Res Clin Prac 93 Suppl 1, S32-36.

Kerestes, M., Faigl, V., Kulcsar, M., Balogh, O., Foldi, J., Febel, H., Chilliard, Y., Huszenicza, G., 2009. Periparturient insulin secretion and whole-body insulin responsiveness in dairy cows showing various forms of ketone pattern with or without puerperal metritis. Domest Anim Endocrinol 37, 250-261.

Kruschinski, C., Skripuletz, T., Bedoui, S., Tschernig, T., Pabst, R., Nassenstein, C., Braun, A., von Horsten, S., 2005. CD26 (dipeptidyl-peptidase IV)-dependent recruitment of T cells in a rat asthma model. Clin Exp Immunol 139, 17-24.

Kuhla, B., Albrecht, D., Kuhla, S., Metges, C.C., 2009. Proteome analysis of fatty liver in feed-deprived dairy cows reveals interaction of fuel sensing, calcium, fatty acid, and glycogen metabolism. Physiol Genomics 37, 88-98.

Kushibiki, S., Hodate, K., Shingu, H., Ueda, Y., Shinoda, M., Mori, Y., Itoh, T., Yokomizo, Y., 2001. Insulin resistance induced in dairy steers by tumor necrosis factor alpha is partially reversed by 2,4-thiazolidinedione. Domest Anim Endocrinol 21, 25-37.

Lacetera, N., Scalia, D., Bernabucci, U., Ronchi, B., Pirazzi, D., Nardone, A., 2005. Lymphocyte functions in overconditioned cows around parturition. J Dairy Sci 88, 2010-2016.

Lacetera, N., Scalia, D., Franci, O., Bernabucci, U., Ronchi, B., Nardone, A., 2004. Short communication: effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J Dairy Sci 87, 1012-1014.

Lankas, G.R., Leiting, B., Roy, R.S., Eiermann, G.J., Beconi, M.G., Biftu, T., Chan, C.C., Edmondson, S., Feeney, W.P., He, H., Ippolito, D.E., Kim, D. et al., 2005. Dipeptidyl peptidase IV inhibition for the treatment of type 2 diabetes: potential importance of selectivity over dipeptidyl peptidases 8 and 9. Diabetes 54, 2988-2994.

Larsen, M., Kristensen, N.B., 2009. Effect of abomasal glucose infusion on splanchnic amino acid metabolism in periparturient dairy cows. J Dairy Sci 92, 3306-3318.

Larsen, M., Relling, A.E., Reynolds, C.K., Kristensen, N.B., 2010. Effect of abomasal glucose infusion on plasma concentrations of gut peptides in periparturient dairy cows. J Dairy Sci 93, 5729-5736.

LeBlanc, S., 2010. Monitoring metabolic health of dairy cattle in the transition period. J Reprod Dev 56 Suppl, S29-35.

Lee, S.A., Kim, Y.R., Yang, E.J., Kwon, E.J., Kim, S.H., Kang, S.H., Park, D.B., Oh, B.C., Kim, J., Heo, S.T., Koh, G., Lee, D.H., 2013. CD26/DPP4 levels in peripheral blood and T cells in patients with type 2 diabetes mellitus. J Clin Endocrinol Meta 98, 2553-2561.

Lim, C.T., Kola, B., Korbonits, M., 2010. AMPK as a mediator of hormonal signalling. J Mol Endocrinol 44, 87-97.

Locher, L.F., Rehage, J., Khraim, N., Meyer, U., Dänicke, S., Hansen, K., Huber, K., 2012. Lipolysis in early lactation is associated with an increase in phosphorylation of adenosine monophosphate-activated protein kinase (AMPK)alpha1 in adipose tissue of dairy cows. J Dairy Sci 95, 2497-2504.

Locher, L., Häussler, S., Laubenthal, L., Singh, S.P., Winkler, J., Kinoshita, A., Kenéz, Á., Rehage, J., Huber, K., Sauerwein, H., Dänicke, S., 2015. Effect of increasing body condition on key regulators of fat metabolism in subcutaneous adipose tissue depot and circulation of nonlactating dairy cows. J Dairy Sci 98, 1057-1068.

Loor, J.J., Everts, R.E., Bionaz, M., Dann, H.M., Morin, D.E., Oliveira, R., Rodriguez-Zas, S.L., Drackley, J.K., Lewin, H.A., 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiol Genomics 32, 105-116.

McArt, J.A., Nydam, D.V., Oetzel, G.R., 2012a. Epidemiology of subclinical ketosis in early lactation dairy cattle. J Dairy Sci 95, 5056-5066.

McArt, J.A., Nydam, D.V., Oetzel, G.R., 2012b. A field trial on the effect of propylene glycol on displaced abomasum, removal from herd, and reproduction in fresh cows diagnosed with subclinical ketosis. J Dairy Sci 95, 2505-2512.

McArt, J.A., Nydam, D.V., Oetzel, G.R., Guard, C.L., 2014. An economic analysis of hyperketonemia testing and propylene glycol treatment strategies in early lactation dairy cattle. Prev Vet Med, 170-179.

McArt, J.A., Nydam, D.V., Ospina, P.A., Oetzel, G.R., 2011. A field trial on the effect of propylene glycol on milk yield and resolution of ketosis in fresh cows diagnosed with subclinical ketosis. J Dairy Sci 94, 6011-6020.

Mehrzad, J., Zhao, X., 2008. T lymphocyte proliferative capacity and CD4+/CD8+ ratio in primiparous and pluriparous lactating cows. J Dairy Res 75, 457-465.

Mentlein, R., Gallwitz, B., Schmidt, W.E., 1993. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem, 829-835.

Morey, S.D., Mamedova, L.K., Anderson, D.E., Armendariz, C.K., Titgemeyer, E.C., Bradford, B.J., 2011. Effects of encapsulated niacin on metabolism and production of periparturient dairy cows. J Dairy Sci 94, 5090-5104.

Morimoto, C., Schlossman, S.F., 1998. The structure and function of CD26 in the T-cell immune response. Immunol Rev 161, 55-70.

Nauck, M.A., 1998. Glucagon-like peptide 1 (GLP-1): a potent gut hormone with a possible therapeutic perspective. Acta Diabetol 35, 117-129.

Nauck, M.A., Heimesaat, M.M., Orskov, C., Holst, J.J., Ebert, R., Creutzfeldt, W., 1993. Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. J Clin Invest 91, 301-307.

Neumiller, J.J., 2009. Differential chemistry (structure), mechanism of action, and pharmacology of GLP-1 receptor agonists and DPP-4 inhibitors. J Am Pharm Assoc (2003) 49 Suppl 1, S16-29.

Niehoff, I.D., Hüther, L., Lebzien, P., 2009. Niacin for dairy cattle: a review. Br J Nutr 101, 5-19.

Nielsen, N.I., Ingvartsen, K.L., 2004. Propylene glycol for dairy cows: A review of the metabolism of propylene glycol and its effects on physiological parameters, feed intake, milk production and risk of ketosis. Anim Feed Sci Technol 115, 191-213.

Oetzel, G.R., 2007. Herd-level ketosis–diagnosis and risk factors, In: Proceedings of the 40th annual conference of bovine practitioners, Vancouver, Canada.

Ohtsuka, H., Koiwa, M., Hatsugaya, A., Kudo, K., Hoshi, F., Itoh, N., Yokota, H., Okada, H., Kawamura, S., 2001. Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. J Vet Med Sci 63, 1021-1025.

Ospina, P.A., Nydam, D.V., Stokol, T., Overton, T.R., 2010a. Association between the proportion of sampled transition cows with increased nonesterified fatty acids and beta-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. J Dairy Sci 93, 3595-3601.

Ospina, P.A., Nydam, D.V., Stokol, T., Overton, T.R., 2010b. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. J Dairy Sci 93, 1596-1603.

Petzold, M., Meyer, U., Kersten, S., Spilke, J., Breves, G., Dänicke, S., 2015. Impacts of CLA and dietary concentrate proportion on blood metabolite concentration and proliferation of peripheral blood mononuclear cells of periparturient dairy cows. Animal 9, 481-489.

Pezeshki, A., Muench, G.P., Chelikani, P.K., 2012. Short communication: expression of peptide YY, proglucagon, neuropeptide Y receptor Y2, and glucagon-like peptide-1 receptor in bovine peripheral tissues. J Dairy Sci 95, 5089-5094.

Plamboeck, A., Holst, J.J., Carr, R.D., Deacon, C.F., 2005. Neutral endopeptidase 24.11 and dipeptidyl peptidase IV are both mediators of the degradation of glucagon-like peptide 1 in the anaesthetised pig. Diabetologia 48, 1882-1890.

Pryce, J.E., Gredler, B., Bolormaa, S., Bowman, P.J., Egger-Danner, C., Fuerst, C., Emmerling, R., Solkner, J., Goddard, M.E., Hayes, B.J., 2011. Short communication: genomic selection using a multi-breed, across-country reference population. J Dairy Sci 94, 2625-2630.

Raboisson, D., Mounie, M., Maigne, E., 2014. Diseases, reproductive performance, and changes in milk production associated with subclinical ketosis in dairy cows: a meta-analysis and review. J Dairy Sci 97, 7547-7563.

Rasmussen, L.K., Nielsen, B.L., Pryce, J.E., Mottram, T.T., Veerkamp, R.F., 1999. Risk factors associated with the incidence of ketosis in dairy cows. Ani Sci 68, 379-386.

Relling, A.E., Reynolds, C.K., 2007. Plasma concentrations of gut peptides in dairy cattle increase after calving. J Dairy Sci 90, 325-330.

Relling, A.E., Reynolds, C.K., 2008. Abomasal infusion of casein, starch and soybean oil differentially affect plasma concentrations of gut peptides and feed intake in lactating dairy cows. Domest Anim Endocrinol 35, 35-45.

Reynolds, C.K., Huntington, G.B., Tyrrell, H.F., Reynolds, P.J., 1988. Net portal-drained visceral and hepatic metabolism of glucose, L-lactate, and nitrogenous compounds in lactating holstein cows. J Dairy Sci 71, 1803-1812.

Rizzo, M.R., Barbieri, M., Marfella, R., Paolisso, G., 2012. Reduction of oxidative stress and inflammation by blunting daily acute glucose fluctuations in patients with type 2 diabetes: role of dipeptidyl peptidase-IV inhibition. Diabetes care 35, 2076-2082.

Roche, J.F., 2006. The effect of nutritional management of the dairy cow on reproductive efficiency. Anim Reprod Sci 96, 282-296.

Roche, J.R., Friggens, N.C., Kay, J.K., Fisher, M.W., Stafford, K.J., Berry, D.P., 2009. Invited review: body condition score and its association with dairy cow productivity, health, and welfare. J Dairy Sci 92, 5769-5801.

Safayi, S., Korn, N., Bertram, A., Akers, R.M., Capuco, A.V., Pratt, S.L., Ellis, S., 2012. Myoepithelial cell differentiation markers in prepubertal bovine mammary gland: Effect of ovariectomy. J Dairy Sci 95, 2965-2976.

Sakai, T., Hayakawa, T., Hamakawa, M., Ogura, K., Kubo, S., 1993. Therapeutic effects of simultaneous use of glucose and insulin in ketotic dairy cows. J Dairy Sci 76, 109-114.

Salgado, F.J., Vela, E., Martin, M., Franco, R., Nogueira, M., Cordero, O.J., 2000. Mechanisms of CD26/dipeptidyl peptidase IV cytokine-dependent regulation on human activated lymphocytes. Cytokine 12, 1136-1141.

Saremi, B., Al-Dawood, A., Winand, S., Muller, U., Pappritz, J., von Soosten, D., Rehage, J., Dänicke, S., Häussler, S., Mielenz, M., Sauerwein, H., 2012. Bovine haptoglobin as an adipokine: serum concentrations and tissue expression in dairy cows receiving a conjugated linoleic acids supplement throughout lactation. Vet Immunol Immunopathol 146, 201-211.

Saremi, B., Winand, S., Friedrichs, P., Kinoshita, A., Rehage, J., Dänicke, S., Häussler, S., Breves, G., Mielenz, M., Sauerwein, H., 2014. Longitudinal profiling of the tissue-specific expression of genes related with insulin sensitivity in dairy cows during lactation focusing on different fat depots. Plos One 9, e86211.

Scalia, D., Lacetera, N., Bernabucci, U., Demeyere, K., Duchateau, L., Burvenich, C., 2006. In vitro effects of nonesterified fatty acids on bovine neutrophils oxidative burst and viability. J Dairy Sci 89, 147-154.

Schuppan, D., Gorrell, M.D., Klein, T., Mark, M., Afdhal, N.H., 2010. The challenge of developing novel pharmacological therapies for non-alcoholic steatohepatitis. Liver Int 30, 795-808.

Seo, S., Ju, S., Chung, H., Lee, D., Park, S., 2008. Acute effects of glucagon-like peptide-1 on hypothalamic neuropeptide and AMP activated kinase expression in fasted rats. Endocr J 55, 867-874.

Singh, S.P., Häussler, S., Heinz, J.F., Saremi, B., Mielenz, B., Rehage, J., Dänicke, S., Mielenz, M., Sauerwein, H., 2014. Supplementation with conjugated linoleic acids extends the adiponectin deficit during early lactation in dairy cows. Gen Comp Endocrinol 198, 13-21.

Smith, D.J., McNamara, J.P., 1989. Lipolytic response of bovine adipose tissue to alpha and beta adrenergic agents 30 days pre- and 120 days postpartum. Gen Pharmacol 20, 369-374.

Spears, J.W., Weiss, W.P., 2008. Role of antioxidants and trace elements in health and immunity of transition dairy cows. Vet J 176, 70-76.

Ster, C., Loiselle, M.C., Lacasse, P., 2012. Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. J Dairy Sci 95, 708-717.

Suriyasathaporn, W., Heuer, C., Noordhuizen-Stassen, E.N., Schukken, Y.H., 2000. Hyperketonemia and the impairment of udder defense: a review. Vet Res 31, 397-412.

Suthar, V.S., Canelas-Raposo, J., Deniz, A., Heuwieser, W., 2013. Prevalence of subclinical ketosis and relationships with postpartum diseases in European dairy cows. J Dairy Sci 96, 2925-2938.

Ta, N.N., Schuyler, C.A., Li, Y., Lopes-Virella, M.F., Huang, Y., 2011. DPP-4 (CD26) inhibitor alogliptin inhibits atherosclerosis in diabetic apolipoprotein E-deficient mice. J Cardiovasc Pharmacol 58, 157-166.

Taylor-Edwards, C.C., Burrin, D.G., Matthews, J.C., McLeod, K.R., Holst, J.J., Harmon, D.L., 2010. Expression of mRNA for proglucagon and glucagon-like peptide-2 (GLP-2) receptor in the ruminant gastrointestinal tract and the influence of energy intake. Domest Anim Endocrinol 39, 181-193.

Thomson, D.M., Winder, W.W., 2009. AMP-activated protein kinase control of fat metabolism in skeletal muscle. Acta Physiol (Oxf) 196, 147-154.

Trevisi, E., Amadori, M., Cogrossi, S., Razzuoli, E., Bertoni, G., 2012. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Res Vet Sci 93, 695-704.

Tveit, B., Lingaas, F., Svendsen, M., Sjaastad, O.V., 1992. Etiology of acetonemia in Norwegian cattle. 1. Effect of ketogenic silage, season, energy level, and genetic factors. J Dairy Sci 75, 2421-2432.

Uchida, E., Katoh, N., Takahashi, K., 1993. Appearance of haptoglobin in serum from cows at parturition. J Vet Med Sci 55, 893-894.

Vogel, K.D., Claus, J.R., Grandin, T., Oetzel, G.R., Schaefer, D.M., 2011. Effect of water and feed withdrawal and health status on blood and serum components, body weight loss, and meat and carcass characteristics of Holstein slaughter cows. J Anim Sci 89, 538-548.

von Soosten D., Leppert J., Boeker P., Meyer U., Frahm J., Dänicke S., 2015. Indicator substances in breath samples of early lactation dairy cows for detection of subclinical ketosis. Proceedings of the Society of Nutrition Physiology 24, 99.

Walsh, R.B., Walton, J.S., Kelton, D.F., LeBlanc, S.J., Leslie, K.E., Duffield, T.F., 2007. The effect of subclinical ketosis in early lactation on reproductive performance of postpartum dairy cows. J Dairy Sci 90, 2788-2796.

Wathes, D.C., Fenwick, M., Cheng, Z., Bourne, N., Llewellyn, S., Morris, D.G., Kenny, D., Murphy, J., Fitzpatrick, R., 2007. Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. Theriogenology 68, Supplement 1, S232-S241.

White, J.R., 2008. Dipeptidyl peptidase-IV inhibitors: pharmacological profile and clinical use. Clin Diabetes 26, 53-57.

Yan, S., Marguet, D., Dobers, J., Reutter, W., Fan, H., 2003. Deficiency of CD26 results in a change of cytokine and immunoglobulin secretion after stimulation by pokeweed mitogen. Eur J Immunol 33, 1519-1527.

Yu, D.M., Slaitini, L., Gysbers, V., Riekhoff, A.G., Kahne, T., Knott, H.M., De Meester, I., Abbott, C.A., McCaughan, G.W., Gorrell, M.D., 2011. Soluble CD26 / dipeptidyl peptidase IV enhances human lymphocyte proliferation in vitro independent of dipeptidyl peptidase enzyme activity and adenosine deaminase binding. Scand J Immunol 73, 102-111.

Danksagung

An dieser Stelle danke ich allen, die mich auf ganz vielfältige Weise unterstützt haben – sowohl bei der Durchführung des Versuchs, als auch beim Erstellen dieser Arbeit.

Ich danke Herrn Prof. Dr. Dr. Sven Dänicke für die Möglichkeit am Institut für Tierernährung zu promovieren. Danke auch für die Unterstützung bei der Durchführung des Versuchs und den vielen hilfreichen Diskussionen im Rahmen dieser Arbeit.
Auch Frau Dr. Dania Reiche, sowie der Boehringer Ingelheim Vetmedica GmbH, sei für die fachliche und finanzielle Unterstützung beim Anfertigen dieser Arbeit gedankt.

Mein besonderer Dank gilt Frau Prof. Dr. Dr. Helga Sauerwein. Ohne Sie und Ihr Team wäre ich nie auf die Idee gekommen zu promovieren. Ich danke Ihnen für die fortwährende Unterstützung aus der Ferne, dafür dass Sie für mich immer erreichbar waren und mir mit Optimismus zur Seite standen. Auch möchte ich mich recht herzlich bei Frau PD Dr. Sarah Egert für die Übernahme der Zweitbetreuung bedanken.

Darüber hinaus danke ich allen Mitarbeitern am Institut für Tierernährung in Braunschweig für die Unterstützung im Rahmen dieser Arbeit. Ein besonderer Dank geht dabei an die Abteilung Immuno-Nutrition, allen voran an Frau Dr. Jana Frahm, Nicola Mickenautsch und Susanne Bühler.
Liebe Jana, ich weiß, du hattest es nicht immer leicht mit mir. Ich danke dir, dass du mir immer ruhig und warmherzig zur Seite standest und natürlich für all das, was du mir im Labor beigebracht hast. Es hat mir sehr viel Spaß gemacht!
Liebe Nicola, auch dir sei von ganzem Herzen gedankt. Generationen von Doktoranden wären völlig kopflos ohne dich! Du bist eine wahnsinnige Hilfe - im Stall, im Labor und überhaupt. Danke, dass immer Verlass auf dich ist!
Liebe Susanne, ich bin wahnsinnig froh dich zu kennen. Die Zeit, die du dir immer genommen hast, obwohl du sie nicht hattest, um mich zu unterstützen und dich in meine Probleme hineinzudenken war unheimlich wertvoll und hat viel zum Gelingen dieser Arbeit beigetragen. Du warst menschlich und fachlich eine riesige Unterstützung. Ich drück dir beide Daumen - Du bist wunderbar und packst das!

Auf keinen Fall zu vergessen sei meine liebe Bürokollegin Annerose. Mit deiner Gelassenheit und deinem Frohsinn hast du mich mehr als 3 Jahre ertragen - Danke dafür! An dieser Stelle danke ich auch Heinz und Dirk für die vielen lustigen Momente im Tierhaus.

Auch danke ich Herrn Dr. Meyer und Herrn Dr. Berk. Ersteren dafür, dass er mir bei Fragen rund um die Wiederkäuerernährung immer eine sehr große Hilfe war, aber auch für seinen Verdienst als der wohl zuverlässigste „Kaffeedienst-Partner“. Zweiteren besonders für Fachsimpeleien über Laufschuhe, Trainingszeiten und die optimale Wettkampfvorbereitung☺. Vielen lieben Dank dafür.

Ich danke allen lieben Kollegen und „Leidensgenossen“ am Institut für Tierernährung, die mich während meiner Doktorandenzeit begleitet haben. Ganz besonders Katrin, Caroline, Stefanie, Lara und Jonas für die Freundschaft, die auch außerhalb des Instituts besteht.

Von ganzen Herzen danke ich meiner Familie, besonders meiner Mama und Oma Lisa, für ihre Liebe und für die Gewissheit, dass sie immer an meiner Seite sind.

Last but not least danke ich „meinen“ Jungs. Rossi, Michi und Pierre – Ihr habt Braunschweig zu meinem Zuhause gemacht!