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Nutritional strategies and their influence on the expression of nutrient sensing G protein-coupled receptors in adipose tissue of dairy cows

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Nutritional strategies and their influence on the expression of nutrient sensing G protein-coupled receptors in adipose tissue of dairy cows

Adipose tissue (AT) plays a central role in the regulation of metabolism at the onset of lactation as a major contributor of energy through the mobilization of nonesterified fatty acids. This time period is especially crucial, because the feed intake does not increase to the same extent as the energy requirements for milk synthesis. To improve the metabolic situation of dairy cows during this time, different nutritional interventions can be applied. A pivotal role in the coordination of metabolic processes is taken by nutrient sensing G protein-coupled receptors (GPCR), which can modulate, for example adipocyte metabolism as a consequence of the extracellular availability of nutrients. The main objective of this thesis was to study the effect of conjugated linoleic acids (CLA), nicotinic acid (NA) and dietary energy density on the mRNA expression of different GPCR relevant for nutrient sensing, namely the free fatty acid receptors (*FFAR*) *1*, *2*, and, *3* and hydroxycarboxylic acid receptor (*HCAR*) *2*, in bovine AT. Additionally, this thesis investigated the difference in the mRNA expression of the aforementioned receptors between AT from subcutaneous (SC) and visceral (VC) locations. Moreover, their mRNA expression from late pregnancy up to the entire following lactation cycle with special emphasis on the transition period have been studied. For these purposes, separate feeding trials were conducted. In the CLA-1 trial, the mRNA abundance of *FFAR 1* and *2* and *HCAR2* was measured in AT biopsies (SC AT from tail head) from multiparous cows (n=21) taken at d -21, 21, 105, 196, and 252 relative to calving. In the CLA-2 trial, the mRNA abundance of *FFAR 1*, *2* and *3* and *HCAR2* was measured in AT samples from 3 SC depots (tail head, sternum, and withers) and from 3 VC depots (retroperitoneal [RP], mesenteric, and omental) from primiparous cows slaughtered at d 1 (n=5), 42 (n=10) and 105 (n=10) relative to calving. In both trials, the animals were allocated either to a group receiving CLA or to a group receiving a control fat supplement for the period of d 1 to 182 (CLA-1 trial) or d 1 to 105 (CLA-2 trial) of lactation. In the NA trial, 20 multiparous cows were divided into a group fed a diet supplemented with NA and a group fed a diet without supplemented NA. Half of each group was fed either a high-concentrate (HC, n=10) diet with a 60:40 concentrate-to-roughage ratio or a low-concentrate (LC, n=10) diet with a 30:70 concentrate-to-roughage ratio on a dry matter basis from d 1 to d 21 relative to calving. The mRNA abundance of *FFAR 1*, *2* and *3* and *HCAR2* was measured in biopsies of SC AT (tail head) and RP AT obtained at d -21, 1 and 21 relative to calving. In all trials, the mRNA abundance of the target genes was measured by quantitative PCR. The effects of CLA on the investigated AT were limited to the mRNA abundance of *FFAR1* in the omental and RP AT of the primiparous animals (CLA-2 trial), whereby in both tissues the mRNA abundance of *FFAR1* was higher in the CLA-treated animals than in the control animals at d 105 relative to calving and in RP AT additionally at d 42 relative to calving. The supplementation of NA to the animals' diet showed no influence on the herein investigated receptors' mRNA expression in SC and RP AT. However, the mRNA abundance of *FFAR2* was altered due to the different energy densities of the diet fed the first three weeks after calving. At d 21 after calving, the *FFAR2* mRNA abundance was 2.5-fold higher in the RP AT of the LC cows than in the HC cows. The expression of *FFAR3* mRNA was inversely related to *FFAR2* mRNA, with a lower *FFAR3* mRNA abundance at d -21 than at d 1 relative to calving. The mRNA abundance of *HCAR2* in AT was not influenced either by the different applied nutritional strategies or by the progress of the transition period. In summary, the effects of the different nutritional strategies on the expression of the nutrient sensing receptors investigated herein were rare or, in the case of NA, absent. The results of the present thesis provide to the basic understanding of the regulation of *FFAR 1*, *2* and *3* and *HCAR2* in different bovine AT at the level of transcription.

Einfluss verschiedener Fütterungsstrategien auf die Expression von *nutrient sensing* G-Protein-gekoppelten Rezeptoren im Fettgewebe der Milchkuh

Zu Beginn der Laktation, nimmt das Fettgewebe (AT) eine zentrale Rolle in der Stoffwechselregulation mit der Energiebereitstellung durch die Mobilisierung von nicht-veresterten Fettsäuren ein. Dieser Zeitraum ist besonders kritisch, da die Futteraufnahme nicht im gleichen Maß ansteigt wie der Energiebedarf für die Synthese der Milch. Zur Verbesserung der Stoffwechselsituation in dieser Zeit können verschiedene Fütterungsstrategien angewendet werden. Eine entscheidende Bedeutung in der Koordination von Stoffwechselprozessen spielen *nutrient sensing* G-Protein-gekoppelte Rezeptoren (GPCR), welche imstande sind z.B. den Fettzellstoffwechsel der extrazellulären Verfügbarkeit von Nährstoffen anzupassen. Das Hauptziel dieser Arbeit war die Untersuchung des Einflusses einer Supplementation von konjugierten Linolsäuren (CLA), einer Supplementation von Nikotinsäure (NA) und von Futterrationen mit unterschiedlicher Energiedichte auf die mRNA-Expression verschiedener *nutrient sensing* GPCR, namentlich der freie Fettsäuren-bindenden Rezeptoren (*FFAR*) *1*, *-2* und *-3*, sowie des Hydroxycarbonsäuren-bindenden Rezeptors (*HCAR*) 2, im bovinen AT. Ebenfalls, wurden die Unterschiede zwischen subkutan (SC) *versus* viszeral (VC) lokalisiertem AT in der mRNA-Expression der oben genannten Rezeptoren untersucht. Es wurde auch ihre mRNA-Expression von der späten Trächtigkeit bis zur folgen Laktation mit besonderem Augenmerk auf die Transitphase untersucht. Zu diesem Zweck erfolgten separate Fütterungsversuche. Im CLA 1-Versuch wurde die mRNA-Menge des *FFAR1*, *-2* und des *HCAR2* im SC AT (vom Schwanzansatz) pluriparer Tiere (n=21), welches an d 21 *ante partum* (a.p.), sowie an d 21, 105, 196, und 252 *post partum* (p.p.) entnommen wurden, gemessen. Zusätzlich wurde im CLA 2-Versuch die mRNA-Menge des *FFAR1*, *-2*, *-3* und des *HCAR2* in Proben aus 3 SC Fettdepots (vom Schwanzansatz, vom Brustbein, vom Widerrist) und 3 VC Fettdepots (retroperitoneal [RP], mesenterial und omental) von primiparen Kühen gemessen, welche bei der Schlachtung an d 1 (n=5), d 42 (n=10) und d 105 (n=10) p.p. gewonnen wurden. In beiden Versuchen wurden die Tiere jeweils aufgeteilt in eine Gruppe, die ein CLA-Supplement oder eine Gruppe, die ein Kontrollsupplement von d 1 bis d 182 p.p. (CLA 1-Versuch) oder von d 1 bis d 105 p.p. (CLA 2-Versuch) erhielt. Im NA-Versuch wurden 20 pluripare Kühe aufgeteilt in eine Gruppe mit (n=10) und eine Gruppe ohne (n=10) NA-Supplementation des Futters. Je die Hälfte der Tiere einer Gruppe erhielt entweder eine Futterration mit einem hohen Kraftfutteranteil von 60 % und einem niedrigen Raufutteranteil von 40% (HC; n=10) oder mit einem niedrigen Kraftfutteranteil von 30 % und einem hohen Raufutteranteil von 70% (LC; n=10). Die Fütterung der verschiedenen Rationen bzw. die NA-Supplementation des Futters erfolgte von d 1 bis d 21 p.p. Die mRNA-Menge von *FFAR1*, *-2*, *-3*, and *HCAR2* wurde in Bioptaten aus dem SC AT (vom Schwanzansatz) und RP AT, welche an d 21 a.p., d 1 p.p. und d 21 p.p. entnommen wurden, quantifiziert. In allen Versuchen wurde die mRNA-Menge der Zielgene mittels quantitativer PCR gemessen. Ein Einfluss der CLA-Supplementation des Futters zeigte sich in Unterschieden in der *FFAR1* mRNA-Menge im omentalen und RP AT der primiparen Tiere, wobei die mRNA-Menge des *FFAR1* in beiden AT höher an d 105 p.p. und im RP AT zusätzlich an d 42 p.p. in der CLA-Gruppe im Vergleich zur Kontrollgruppe war. Die NA-Supplementation des Futters zeigte keinen Einfluss auf die mRNA-Expression der hier untersuchten Rezeptoren im SC und RP AT der Tiere. Allerdings zeigten die Futterrationen mit unterschiedlicher Energiedichte einen Einfluss auf die *FFAR2* mRNA-Expression. An d 21 p.p. war die *FFAR2* mRNA-Menge um ein 2,5-faches höher im RP AT der Kühe mit der LC-Futterration im Vergleich zu den Kühen mit der HC-Futterration. Die mRNA-Expression von *FFAR3* verhielt sich umgekehrt proportional zu der von *FFAR2*; mit einer niedrigeren *FFAR3* mRNA-Menge an d 21 a.p. im Vergleich zu d 1 p.p. Die mRNA-Menge von *HCAR2* in den untersuchten AT wurde weder von den verschiedenen Fütterungsstrategien beeinflusst, noch zeigte diese Veränderung im Verlauf der Transitphase. Zusammenfassend lässt sich sagen, dass die mRNA-Expression der hier untersuchten *nutrient sensing* GPCR durch die unterschiedlichen Fütterungsstrategien kaum beeinflusst wurde, wobei die Supplementation von NA keinerlei Einfluss zeigte. Die Ergebnisse dieser Arbeit tragen zum grundlegenden Verständnis der Regulation des *FFAR1*, *-2* und *-3*, sowie des *HCAR2* in verschiedenen bovinen AT auf der Ebene der Transkription bei.

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List of abbreviations

ADIPOQ	adiponectin
ADP	adenosine diphosphate
AMP	adenosine monophosphate
a.p.	antepartum (ante partum)
AT	adipose tissue
ATP	adenosine triphosphate
BAT	brown adipose tissue
BHBA	β-hydroxybutyrate (BHB in Manuscript 1)
*c*9,*t*11	*cis*-9,*trans*-11
cAMP	cyclic adenosine monophosphate
CLA	conjugated linoleic acids
DIM	day(s) in milk
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
EB	energy balance
FFA	free fatty acids
FFAR	free fatty acid receptor (FFA in Manuscript 1)
FFAR2L	free fatty acid receptor 2-like
GLP	glucagon-like peptide
GPCR	G protein-coupled receptor
GSIS	glucose-stimulated insulin secretion
HC	high-concentrate
HCAR	hydroxycarboxylic acid receptor (HCA in Manuscript 1)
HDL	high-density lipoprotein
IL-6	interleukin-6
LA	linoleic acid
LC	low-concentrate
LDL	low-density lipoprotein
MFD	milk fat depression
NA	nicotinic acid
NAD	nicotinamide adenine dinucleotide

NADP	nicotinamide adenine dinucleotide phosphate
NAM	nicotinamide
NEB	negative energy balance
NEFA	nonesterified fatty acids
NFC	nonfiber carbohydrates
PG	prostaglandin
p.p.	postpartum (post partum)
PPAR	peroxisome proliferator-activated receptor
RP	retroperitoneal
SC	subcutaneous
SCFA	short-chain fatty acids
SNP	single nucleotide polymorphism
SREBP	sterol response element-binding protein
SUCNR	succinate receptor
SVF	stromal vascular fraction
*t*10,*c*12	*trans*-10,*cis*-12
TAG	triacylglycerides
TNF-α	tumor necrosis factor-α
UCP-1	uncoupled protein-1
VC	visceral
VLDL	very-low-density lipoprotein
WAT	white adipose tissue

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1 Introduction

The genetic selection during the last few decades has succeeded in producing dairy cattle breeds with substantially increased milk yields, which has not remained without consequences for animal health and welfare. The high demands on the animals' metabolism in adapting to the requirements of lactation are often related to metabolic disorders, reduced fertility, lameness and various infectious diseases in the periparturient period. Overcoming these restrictions in the effectiveness of milk production implies challenges for dairy farming. To minimize the incidence of disease in the periparturient period, different management and nutritional strategies are used in practice, e.g. providing nutritional supplements or increasing the energy content in the diet. Adipose tissue contributes crucially to lactation through the mobilization of energy, therefore the physiological processes and changes in this organ are of particular importance at the onset of lactation, a period characterized by a comprehensive negative energy balance. As the negative energy status offers a special approach in studying certain G protein-coupled receptors that are involved in nutrient sensing, some insights into their expression patterns in different bovine adipose tissues during energy deficit can be provided. Identification and a better understanding of possible adjustments of these modulatory factors involved in nutrient sensing in the different adipose tissue depots could help to improve strategic concepts for nutrition management of dairy cattle in the critical time around parturition and thus contribute to animal welfare.

1.1 Adipose tissues

Based on diverse biochemical and functional characteristics, adipose tissue (AT) can be classified into two distinct types: white AT (WAT) and brown AT (BAT). White AT is the predominant and most abundant type of AT in adult, nonhibernating mammals (Ahima, 2006; Marra and Bertolani, 2009) and forms the focus of this thesis. Brown AT differs from WAT mainly by its multilocular adipocytes and its higher number of mitochondria expressing the tissue-specific uncoupling protein-1 (*UCP-1*). Mediated by UCP-1, which allows BAT a respiratory oxidation of fatty acids uncoupled from the production of adenosine triphosphate (ATP), BAT is able to produce local heat also known as nonshivering thermogenesis (Cannon and Nedergaard, 2004). Brown AT was considered to exist only in a limited number of mammalian species and in specific physiological conditions such as in neonates and infants (Gesta et al., 2007). However, there is evidence that functional BAT also occurs in adult humans and that brown adipocytes can be found in WAT depots as so-

called “brown-in-white” or “beige” adipocytes (Cypess et al., 2009; Cinti, 2012; Beranger et al., 2013) and in WAT depots of adult fattening cattle where the mRNA expression of the brown adipocytes-specific *UCP-1* has been demonstrated (Asano et al., 2013).

1.1.1 Structure and function of white adipose tissue

The highly vascularized WAT is a loose connective tissue (Wronska and Kmiec, 2012) that contains mainly unilocular adipocytes sized 20 to 200 µm, with the size of the adipocyte depending on its functional and developmental stage (Cinit, 2001; Frühbeck, 2008). The characteristic spherical shape of adipocytes can be attributed to one single large lipid droplet within the adipocyte, which is surrounded by a thin layer of cytoplasm. The lipid droplet displaces the nucleus and other cell organelles, such as the mitochondria, to the margin of the adipocyte where they form a thickening in the cytoplasmic rim surrounding the lipid vacuole; this gives adipocytes their characteristic signet ring morphology (Frühbeck, 2008). Besides adipocytes, which account for about one third of all cell types in WAT, WAT contains cells of the stromal vascular fraction (SVF; Marra and Bertolani, 2009). The SVF includes, for example, multipotent stem cells, macrophages and other infiltrating immune cells, fibroblasts, preadipocytes, endothelial cells of blood and lymphatic vessels, sympathetic nerve fibers and various other cell types (Hauner, 2005; Ahima, 2006; Wronska and Kmiec, 2012). Chemically, human WAT is mainly composed of triacylglycerides (TAG, 80 to 85 % of tissue weight), and the remaining part consists of water, proteins and minerals (Shen et al., 2003; Trayhurn et al., 2006).

Its chemical composition reflects well the major function of WAT as a store of energy in the form of TAG. In times of excess energy intake, nonesterified fatty acids (NEFA) are synthesized and accumulated in WAT as TAG; in periods when energy expenditure exceeds the energy intake, those NEFA are released (Bing and Trayhurn, 2009). The two major metabolic processes responsible for the accumulation and breakdown of TAG in WAT are lipogenesis and lipolysis, respectively (Boschmann, 2001).

Other than the described role as a sink and source for energy, WAT was discovered as an endocrine organ in the last few decades. In the early 1990s, it was described that the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is synthesized and released by adipocytes (Hotamisligil et al., 1993). Shortly thereafter, the secretory role of WAT was affirmed with the identification of the hormone leptin and its corresponding *OB* gene in 1994 (Zhang et al., 1994). Today, leptin is well known as a key hormone in the regulation of food

intake as a signal of satiety, of energy expenditure, in the regulation of the innate immune system, and the modulation of glucose and fat metabolism (Blüher and Mantzoros, 2009). Such factors, which are mainly expressed in WAT in a regulated manner, are collectively termed adipokines (adipose tissue cytokines), whereby the adipocytes as well as the cells of the SVF participate in secretion (Marra and Bertolani, 2009; Wronska and Kmiec, 2012). The adipokines enable WAT to cross-talk with other organs including, for example, the brain, liver and skeletal muscle. During the last few years an array of adipokines have been identified (Bing and Trayhurn, 2009), and the most important ones are summarized in Figure 1.

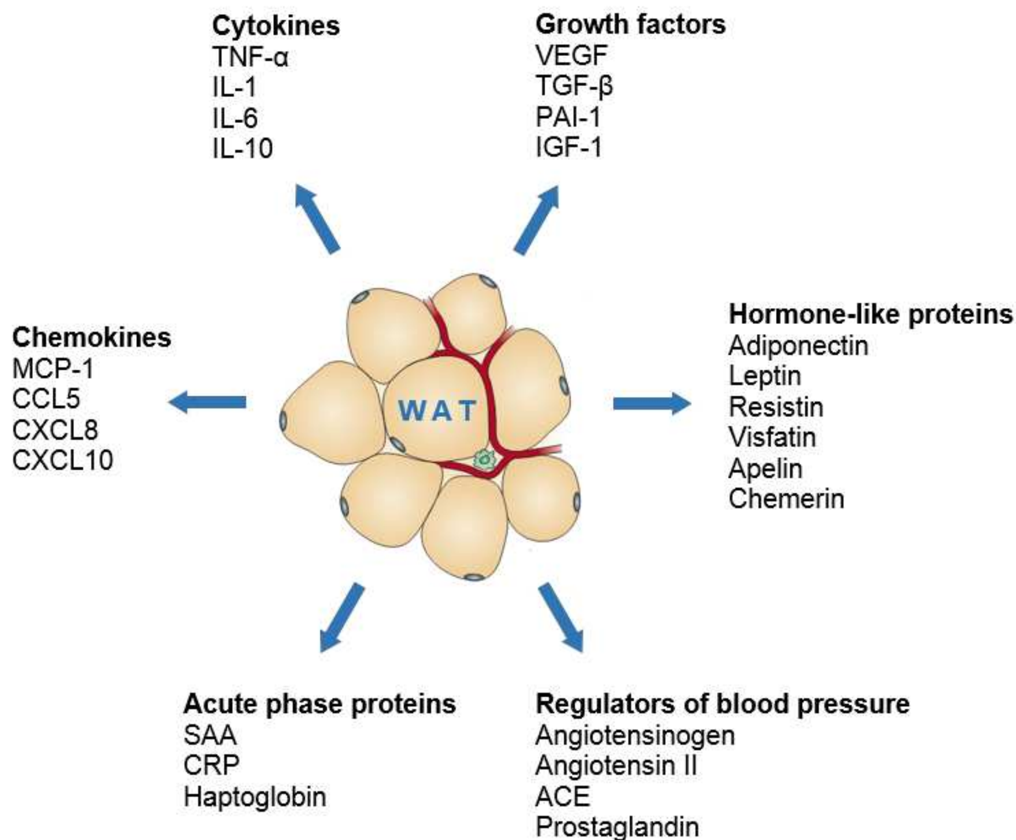

Figure 1. Schematic overview of some adipose tissue-secreted factors. WAT secretes a multiplicity of factors that can act autocrine or paracrine at local level or they can signal in an endocrine manner to modulate appetite, nutrient metabolism, insulin sensitivity, inflammation and adipose tissue development (modified from Trayhurn et al. (2006), Lago et al. (2007) and Jagannathachary and Kamaraj (2010)). ACE: angiotensin-converting enzyme; CCL: CC-chemokine ligand; CRP: c-reactive protein; CXCL: CXC-chemokine ligand; IGF-1: insulin-like growth factor-1; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; PAI-1: plasminogen activator inhibitor-1; SAA: serum amyloid A; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor; WAT: white adipose tissue.

Due to the expression and secretion of adipokines, WAT exerts important influences on numerous metabolic and physiological processes such as appetite regulation, lipid metabolism, insulin sensitivity, adipocyte proliferation and differentiation, angiogenesis, immune responses, reproduction, blood coagulation and blood pressure regulation (Wronska and Kmiec, 2012). Their participation in a wide range of systemic processes brought adipokines into the focus of life science, for example as the link between obesity and insulin resistance as well as other pathological conditions (Antuna-Puente et al., 2008). It is worth mentioning that mammalian AT also functions as a thermal insulator and mechanical cushion (Shen et al., 2003).

1.1.2 Subcutaneous versus visceral adipose tissue

Considering the anatomical location, WAT can be distinguished into subcutaneous (SC), visceral (VC) and intraorgan AT (Boschmann, 2001). Table 1 shows the corresponding major depots in humans with a description of their location. As the SC and VC are the major AT depots in the human body (Ahima, 2006), this chapter aims to compare the anatomical and physiological differences between these two depots.

Table 1. White adipose tissue depots in humans classified according to their anatomic location (modified from Boschmann (2001), Wronska and Kmiec (2012) and Fitzgibbons and Czech (2014))

Depot	Location description
Subcutaneous adipose tissues	
abdominal	In the hypodermis back and anterior of the abdomen
gluteo-femoral	In the hypodermis of the gluteo-femoral region
mammary	Fat pad in the hypodermis of the mammary
Visceral adipose tissues	
omental	Superficial surround the intestines
mesenteric	Deeply buried around the intestines
retroperitoneal	Near the kidneys, dorsal side of the abdominal cavity
epicardial	Adjacent to the myocardium and encased by the visceral pericardium
paracardial	Surround the parietal pericardium
Intraorgan adipose tissues	
intrahepatic	Fat depots in the liver
intra- and intermuscular	Fat depots between the muscle fibers and between muscle groups

The SC AT is located under the skin in the hypodermis, where it forms a fat layer. The VC AT can be found around the organs in the abdominal cavity and mediastinum (Wronska and Kmiec, 2012). In humans, the SC AT depot is the largest one, accounting for about 80 % of the total body fat, but when the TAG storage capacity of SC AT is exceeded or impaired, fat accumulation in the VC depots is elevated. Furthermore, the amount of VC AT in the human body depends on gender and age (Wajchenberg et al., 2002). In contrast, the fat distribution in cattle, measured by separation and weighing of single carcass tissues, seems to be different: The intermuscular fat depots represent at least a two-fold greater proportion of the total carcass fat than SC AT (Gibb et al., 1992; Mahgoub, 1995). Compared to beef breeds, dairy cattle breeds deposit relatively more fat in the VC than in the SC AT depots (Wright and Russel, 1984). However, the weight of individual cattle body fat depots is likely to vary depending on growth, stage of lactation and feeding (Williams, 1978; Butler-Hogg et al., 1985; von Soosten et al., 2011; Drackley et al., 2014). Beside their sizes, the depots also differ in their blood drainage: The SC depots and the VC retroperitoneal (RP) AT drain into the systemic circulation via the inferior *Vena cava*, while omental and mesenteric AT drain blood directly to the liver through the portal vein. This has important consequences for the effects of mediators released from the SC and RP AT; they will exert systemic rather than hepatic effects. However, the omental and mesenteric adipocytes can provide free fatty acids (FFA) and adipokines directly to the liver by the portal drainage (He et al., 2008; Ibrahim, 2010). Based on this link, the evident "portal vein theory" asserts that a high rate of lipolysis in these two VC AT leads to increased hepatic fat accumulation and liver insulin resistance (Kabir et al., 2005; He et al., 2008). Generally, VC AT is more vascularized and innervated than SC AT (Ibrahim, 2010).

Compared to VC AT, the SC AT contains a higher portion of small adipocytes, which are more insulin-sensitive and have a higher affinity for FFA and TAG accumulation than large adipocytes. Thus, the adipocytes from VC depots are more insulin-resistant than those from SC AT (Ibrahim, 2010). For cows it has been reported that the area of adipocytes in the RP depot is consistently larger than the area of adipocytes in the SC depot independent of the nature of the forage fed (Faulconnier et al., 2007), and surprisingly also compared to adipocytes from omental and mesenteric depots (Pike and Roberts, 1984; Akter et al., 2011).

Lipolysis in adipocytes is regulated to a great extent by catecholamines (e.g. adrenaline and noradrenaline) through the lipolytic β-adrenergic receptors and the anti-lipolytic α_2-adrenergic receptor (Langin, 2006). With regard to the molecular differences between VC and SC AT, VC AT seems to have a higher catecholamine-induced lipolytic rate due to a

higher density of β_3-adrenergic receptors than SC AT (Ibrahim, 2010). Additionally, VC adipocytes are less sensitive to α_2-adrenergic receptor-dependent inhibition of lipolysis (Ibrahim, 2010). There is evidence that RP AT from dairy cattle also has a higher lipolytic activity than AT from the SC depots due to higher expression of hormone-sensitive lipase and a higher NEFA release in response to a catecholamineric stimulus in RP AT *ex vivo* (Locher et al., 2011; Kenéz et al., 2013). In addition, compared to other fat depots, mainly RP AT is mobilized during early lactation in dairy cows (von Soosten et al., 2011) and lactation-induced effects on adipocyte size, with smaller adipocytes in mid lactation compared to 1 d after calving, are limited to RP AT (Akter et al., 2011). The apoptotic activity identified by staining apoptosis-specific deoxyribonucleic acid (DNA) fragments was lowest in RP AT when compared other VC and SC fat depots from heifers (Häussler et al., 2013).

The adipocytes from SC fat depots of nonobese humans secrete more leptin and adiponectin (ADIPOQ) than those from VC fat depots (Wajchenberg et al., 2002; Wronska and Kmiec, 2012). A higher concentration of some inflammatory cytokines such as interleukin-6 (IL-6), TNF-α and C-reactive protein in the circulation is associated with abdominal obesity (Ibrahim, 2010). The release of IL-6 and prostaglandin (PG) E_2 from VC AT explants was greater than that from SC AT explants (Fain et al., 2004). In humans, the properties of the SC and VC AT depots as described before result in increased metabolic and cardiovascular risks associated with excessive accumulation of VC AT. However, the depot-specific characteristics of AT from ruminants, in particular from dairy cattle, need further investigation.

1.1.3 Physiological adaptations during the transition period

In humans, the relatively small additional costs of lactation can be sufficiently covered by the diet, by large SC body fat depositions when dietary intake is restricted, and to a certain extent by metabolic adaptations. In many other mammalian species, in particular species domesticated for dairy production, lactation is a process requiring a very high energy allocation (Prentice and Prentice, 1988). In this context, the term “transition period” comprises the period from late pregnancy to early lactation in dairy cattle, defined as the time from 3 weeks before to 3 weeks after calving (Grummer, 1995). With the onset of lactation, the energy requirements of dairy cattle for milk production increase considerably while the energy intake by voluntary feed intake does not, thus result in a negative energy balance (NEB). The main organ sites where adaptations to NEB occur are AT, liver, muscle,

gut and the mammary gland; their metabolic and endocrine regulation and coordination are key components of NEB compensation (Drackley, 1999; Herdt, 2000). In particular, the AT, as described in a previous section, represents the energy storage of the body and thus provides energy for maintenance, lactogenesis and galactopoesis by releasing NEFA during the NEB in early lactation. Given that the sensitivity to lipolytic signals (e.g. epinephrine and norepinephrine) is enhanced, lipolysis is the dominating pathway in adipocytes during the NEB occurring with the onset of lactation, whereas lipid deposition by lipogenesis is considerably shut down during this time. Likewise the absorption and the *de novo* synthesis of fatty acids are downregulated in adipocytes. The consequence of the increased lipolysis is the release of fatty acids from AT. Those fatty acids are released as NEFA from AT into the circulation and represent the major energy source for cattle during the transition period (McNamara and Hillers, 1986; Ingvartsen, 2006). Another major consequence of the high nutrient demand for milk production in the transition period is an increased state of insulin resistance in AT and muscle, which reduces peripheral glucose uptake and thus facilitates the flow of nutrients away from maternal stores to the mammary gland (Bell and Bauman, 1997; Ingvartsen, 2006). The glucose uptake of the mammary gland seems to be independent from insulin, as in lactating cows as well as in lactating nonruminants, the mammary transport of glucose is largely facilitated by the insulin-independent glucose transporter (GLUT) 1 and not by the insulin-responsive GLUT4 (Bell and Bauman, 1997). The interaction of several hormones creates an environment that favors the mobilization of fatty acids from adipocytes and reduces the glucose uptake during the NEB, e.g. the low insulin concentrations in the periparturient period, the reduced insulin-like growth factor-I and elevated growth hormone concentrations in the circulation (Esposito et al., 2014).

Figure 2 illustrates the relationship between AT lipid metabolism and the lipid metabolism of the liver as well as the mammary gland, since these two organs, as mentioned before, are also important for the metabolic compensation of the NEB. Some major factors that promote either lipogenesis or lipolysis are also listed in Figure 2.

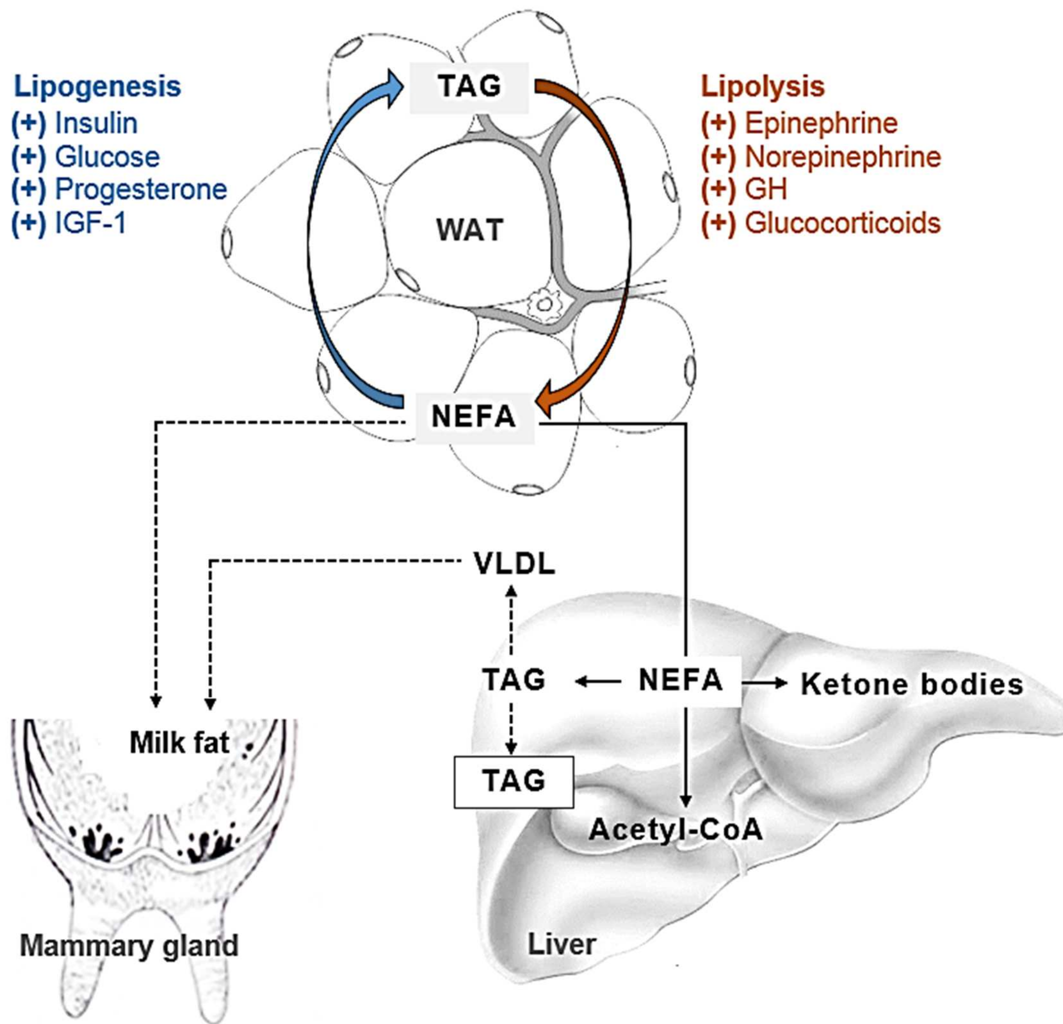

Figure 2. Schematic overview of lipid metabolism in adipose tissue, liver and mammary gland (modified from Drackley (1999) and Esposito et al. (2014)). The factors promoting (+) either lipogenesis or lipolysis are listed under the corresponding pathway. Dashed lines indicate processes that occur at low rates or only during certain physiological states e.g. the negative energy balance at the onset of lactation. GH: growth hormone; IGF-1: insulin-like growth factor-1; NEFA: nonesterified fatty acids; TAG: triacylglycerides; VLDL: very-low-density lipoprotein; WAT: white adipose tissue.

The excessive lipid mobilization from AT is linked with greater incidence of health problems during the transition period. The extremely high rates of NEFA release from AT increase accordingly the NEFA uptake by the liver and its TAG content (Figure 2). If the NEFA cannot be completely metabolized via the Krebs cycle because of a low accessibility of oxaloacetate, the NEFA are converted to ketone bodies (acetoacetic acid, acetone and β-hydroxybutyrate [BHBA]) (Ingvartsen, 2006). The elevated plasma levels of ketone bodies are associated with ketosis and an excessive lipid and TAG accumulation in the liver characterizing the fatty liver syndrome (Drackley, 1999; Ingvartsen, 2006; Esposito et al., 2014). Several studies indicate that a subclinical course of both diseases can be a relevant

problem for up to 50 % of dairy cattle in early lactation (Ingvartsen, 2006; Esposito et al., 2014).

In addition, the expression and secretion of several adipokines, AT-derived messenger molecules, are altered in adaptation to the catabolic state of adipocytes. The plasma leptin concentrations decrease in late pregnancy and early lactation (Leury et al., 2003; Hachenberg et al., 2007). The same applies to ADIPOQ, an adipokine with insulin-sensitizing, food intake-stimulating and energy expenditure-decreasing activity (Kadowaki et al., 2008), which shows decreased concentrations with the onset of lactation (Giesy et al., 2012; Singh et al., 2014a). The decline of the mRNA expression of both adipokines in SC AT from late pregnancy to early lactation has also been confirmed (Koltes and Spurlock, 2012; Saremi et al., 2014).

1.1.4 Nutrient sensing in adipose tissue

The term “nutrient sensing” describes the ability of cells to assess the current availability or changes in the availability of intra- and extracellular nutrients or energy metabolism intermediates, and to transduce this sensory information into adjustment of the cells’ metabolism (Obici and Rossetti, 2003; Lindsley and Rutter, 2004; Blad et al., 2012). Some authors prefer instead the term “metabolic sensing” (Blad et al., 2012; Prentki et al., 2013). The plasma levels of energy metabolites and metabolic intermediates vary depending on the nutrient composition of the food intake (Blad et al., 2012), thus it may not be possible to make a clear delineation between the terms “nutrient” and “metabolic” sensing.

Cells have a variety of mechanisms to sense nutrients and their metabolites resulting in an adjustment of their metabolism. One key cellular energy sensor is the adenosine monophosphate (AMP)-activated protein kinase (AMPK), which senses the nutrient availability indirectly over the cellular energy status and consequently regulates glucose metabolism, fatty acid metabolism, transcription and protein synthesis through multiple cellular mechanisms. Another well-studied nutrient sensing pathway is that involving the mammalian target of rapamycin protein kinase, which senses the availability of amino acids and the cellular energy status and regulates accordingly the cell growth (Lindsley and Rutter, 2004). In mammalian AT, the activation of the hexosamine biosynthesis pathway is suggested as a signal of cellular satiety resulting in a downregulation of the insulin-dependent glucose uptake as well as glycogen synthesis and induces the expression of leptin (Obici and Rossetti, 2003; Lindsley and Rutter, 2004). The nutrient sensing G protein-coupled receptors (GPCR) take a pivotal role in the coordination of metabolic processes,

because of their ability to modulate the cells' metabolism according to nutrient availability. Figure 3 shows a model of the regulation of adipocyte function through GPCR and lists major GPCR involved in nutrient sensing in AT. Some nutrient sensing GPCR are highly expressed by adipocytes and can be therapeutic targets to regulate lipolysis in dyslipidemic or obese individuals (Blad et al., 2012).

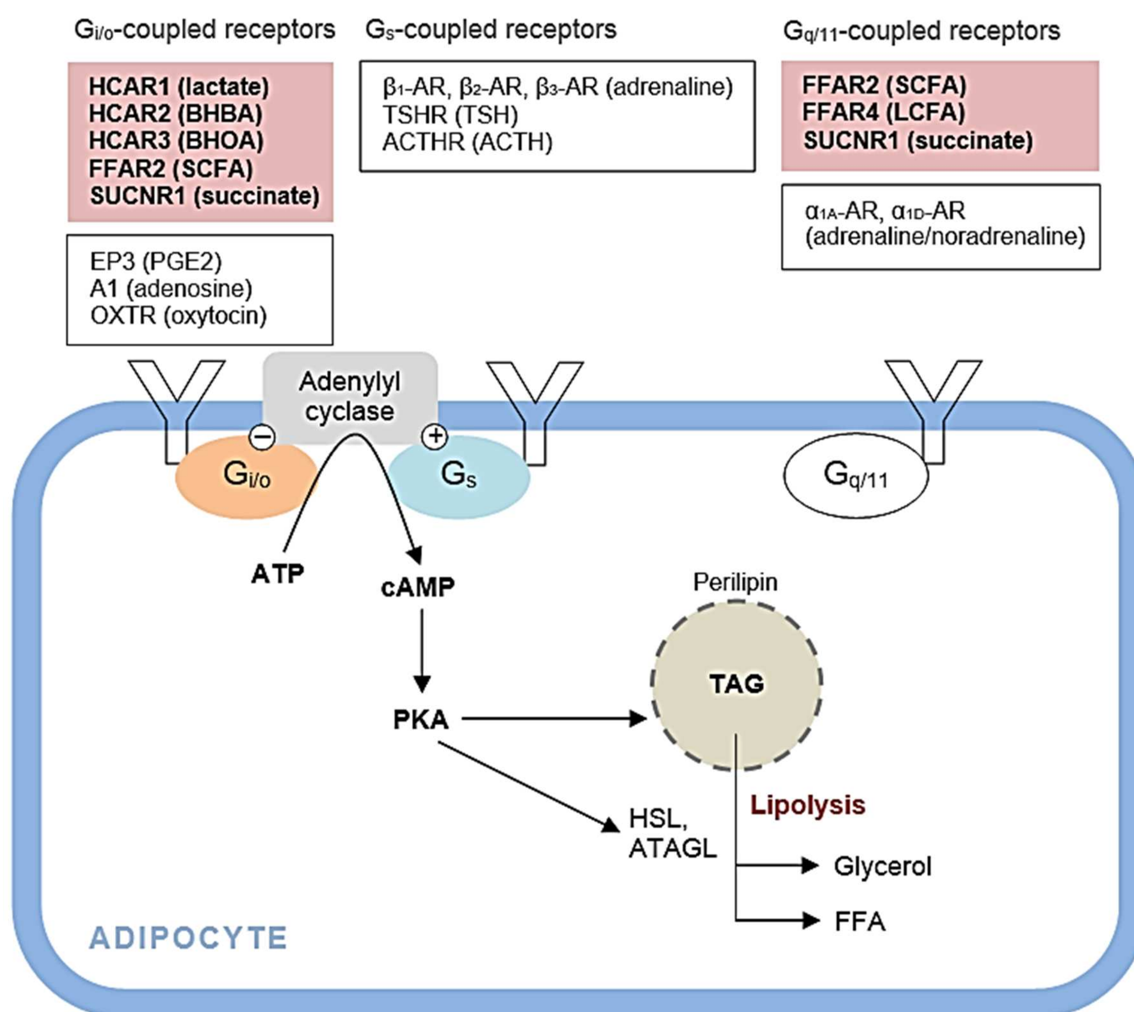

Figure 3. Simplified model of the regulation of lipolysis in adipocytes through G protein-coupled receptors (modified from Blad et al. (2012)). The receptors are classified based on their coupled G proteins. The nutrient sensing GPCR are listed in the red boxes with their corresponding ligands in parentheses. A1: adenosine receptor A1; ACTH: adrenocorticotropic hormone; ACTHR: ACTH receptor; AR: adrenergic receptor; ATAGL: adipose TAG lipase; BHBA: β-hydroxybutyrate; BHOA: β-hydroxyoctanoate; EP3: prostaglandin E receptor 3; FFA: free fatty acids; FFAR: free fatty acid receptor; HCAR: hydroxycarboxylic acid receptor; HSL: hormone sensitive lipase; LCFA: long-chain fatty acids; OXTR: oxytocin receptor; PGE2: prostaglandin E2; SCFA: short-chain fatty acids; SUCNR1: succinate receptor 1; TAG: triacylglycerides; TSH: thyroid-stimulating hormone; TSHR: TSH receptor.

The next chapter aims to provide a detailed description of nutrient sensing through GPCR and, in particular, of their involvement in the regulation of the lipolytic response of adipocytes to the availability of extracellular nutrients or energy metabolism intermediates. It also aims to describe the signaling pathways displayed in Figure 3 in more detail.

1.2 G protein-coupled receptors involved in nutrient sensing

The term “G protein-coupled receptor” refers to a group of receptors whose amino acid sequence contains seven transmembrane helices and an interaction of these heterotrimeric receptors with their corresponding ligands empowers them for signal transduction by binding and activating intracellular G proteins (Karnik et al., 2003). Four main G proteins can be distinguished based on the signaling pathways they induce: G_s activates adenylyl cyclase; $G_{i/o}$ inhibits adenylyl cyclase; $G_{q/11}$ activates phospholipase C; and $G_{12}/_{13}$ activates adenylyl cyclase 7, a specific adenylyl cyclase isoform (Hamm, 1998; Jiang et al., 2008). Figure 4 gives examples of nutrient sensing GPCR as classified by the nature of their corresponding ligands.

Regulation of lipolysis in adipocytes through GPCR, as displayed in Figure 3 (previous chapter), takes part through different signaling pathways. Activation of G_s-coupled receptors leads to an increased formation of cyclic AMP (cAMP) through activation of adenylyl cyclase. Elevated levels of cAMP activate protein kinase A, which stimulates the hormone-sensitive lipase and the adipose TAG lipase to hydrolyze the TAG into FFA and glycerol. The activity of G_s-coupled receptors is counteracted by an activation of $G_{i/o}$-coupled receptors by inhibiting adenylyl cyclase activity. Like G_s-coupled receptors, $G_{q/11}$-coupled receptors also stimulate lipolysis (Blad et al., 2012) through molecular mechanisms that involve calmodulin, phospholipase C and protein kinase C (Carmen and Víctor, 2006; Chaves et al., 2011). Table 2 gives an overview of the herein considered GPCR involved in nutrient and metabolic sensing, their major actions and their ligands.

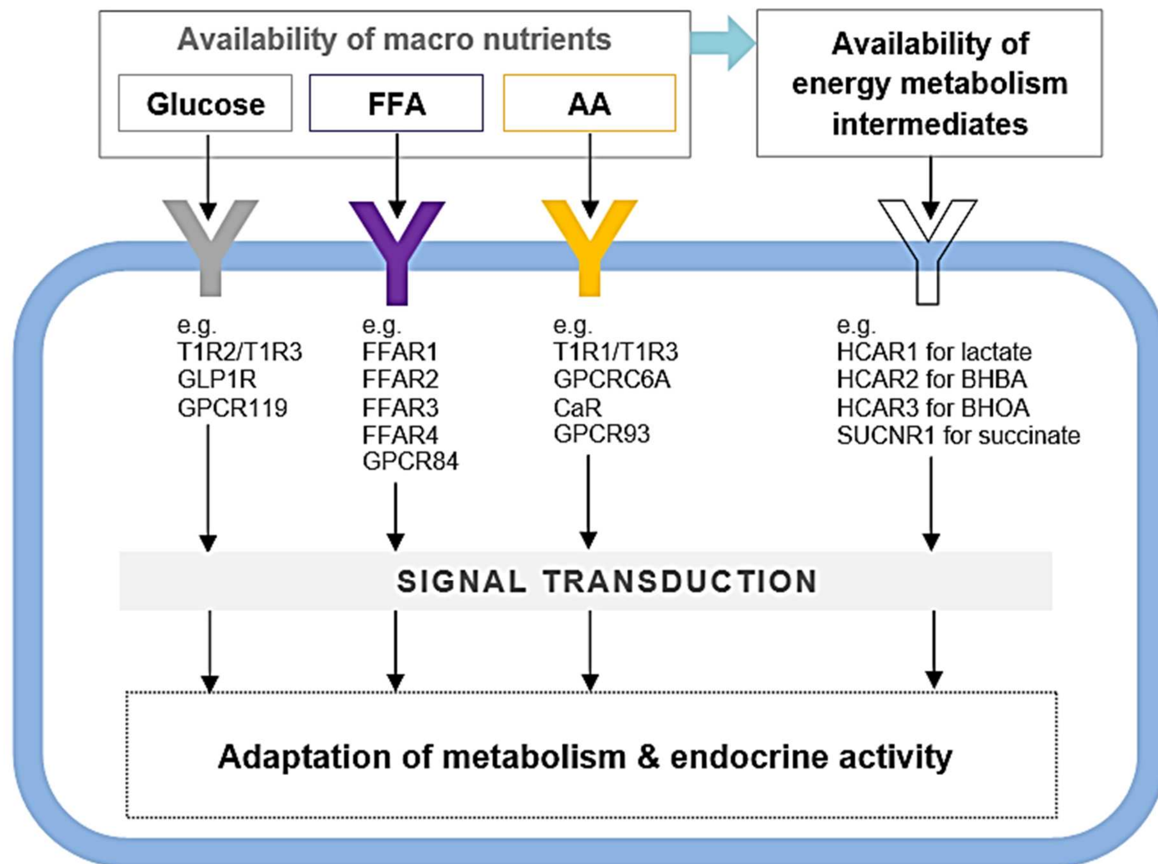

Figure 4. Schematic overview of nutrient signaling by G protein-coupled receptors in a mammalian cell (modified from Wellendorph et al. (2009), Blad et al. (2012) and Prentki et al. (2013)). The receptors are classified based on sensitivity to the breakdown products from the macro nutrients proteins, lipids, and carbohydrates or to energy metabolism intermediates. AA: amino acids; BHBA: β-hydroxybutyrate; BHOA: β-hydroxyoctanoate; CaR: calcium-sensing receptor; FFA: free fatty acids; FFAR: free fatty acid receptors; GLP1R: Glucagon-like peptide 1 receptor; GPCR: G protein-coupled receptor; HCAR: hydroxycarboxylic acid receptor; SUCNR1: succinate receptor 1; T1R: taste 1 receptor.

Table 2. Selected nutrient sensing G protein-coupled receptors, their major actions and ligands (modified from Stoddart et al. (2008) and Offermanns et al. (2011))

Name	GPCR	Signal	Major action	Ligands
FFAR1	40	$G_{q/11}$ and $G_{i/o}$	Pancreatic glucose-stimulated insulin secretion	MCFA, LCFA (C6-C22)
FFAR2	43	$G_{i/o}$ and $G_{q/11}$	Inhibition of lipolysis	SCFA (C1-C6)
FFAR3	41	$G_{i/o}$	Anti-inflammatory action	SCFA (C1-C6)
HCAR2	109A	$G_{i/o}$	Inhibition of lipolysis	Niacin, BHBA

BHBA: β-hydroxybutyrate; LCFA: long-chain fatty acids; MCFA: medium-chain fatty acids; SCFA: short-chain fatty acids.

The free fatty acid receptors (FFAR) 1, FFAR2 and FFAR3 belong to the group of nutrient sensing GPCR because of their ability to transduce extracellular concentrations of specific FFA and to initiate adaptive responses such as the secretion of hormones (Covington et al., 2006; Hirasawa et al., 2008). The hydroxycarboxylic acid receptor (HCAR) 2 is activated by BHBA as the endogenous ligand and suppresses lipolysis during starvation. In addition, HCAR2 is also an important target for nicotinic acid (NA) at pharmacological doses and for other antilipolytic drugs (Ahmed et al., 2009; Offermanns et al., 2011). Specific phenolic acids (e.g. *trans*-cinnamic acid) are also able to activate HCAR2 and thus suppress lipolysis in adipocytes, but with a lower binding potency than NA does (Ren et al., 2009).

1.2.1 Free fatty acid receptor 1

In 2003, three working groups deorphanized FFAR1 (previously known as GPCR40) independently and identified a range of medium- and long-chain saturated and unsaturated fatty acids as ligands for this receptor (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003). Another receptor for medium- and long-chain fatty acids is FFAR4 (previously known as GPCR120), which has an amino acid sequence identity with FFAR1 of 10 % (Ichimura et al., 2014). Free fatty acid receptor 1 mainly couples to the $G_{q/11}$ protein, but there is evidence that the receptor additionally couples weakly to the $G_{i/o}$ protein (Stoddart et al., 2008). Based on the measurements of the FFAR1-mediated calcium mobilization, saturated fatty acids show different potencies to activate FFAR1 depending on their chain length. In contrast, across the unsaturated fatty acids the potency is probably not associated with their carbon chain length or their degree of saturation (Briscoe et al., 2003). The *FFAR1* gene is mainly expressed in the pancreatic islets of humans and likewise in rodents, in particular in the insulin-producing β-cells. In addition, *FFAR1* mRNA is present in several other tissues or cells, such as the brain, liver, heart, skeletal muscle, immune cells, predominantly in monocytes, in enteroendocrine cells and in breast cancer cells (Brown et al., 2005; Stoddart et al., 2008).

In normal β-cell function, FFAR1 seems to play an important role with its ability to augment glucose-stimulated insulin secretion (GSIS) in response to short-term elevated FFA in the circulation. This relationship has been studied in FFAR1 knockout mice models in which the pancreatic islets from FFAR1 knockout mice showed a reduced capacity to augment GSIS on exposure to fatty acids (Steneberg et al., 2005; Latour et al., 2007). Despite the augmentation of GSIS by short-term elevation of circulating FFA, their long-term elevation has been shown to impair insulin secretion. The available studies investigating whether the

effect of long-term elevated NEFA in the circulation is mediated by FFAR1 show contrasting results (Steneberg et al., 2005; Latour et al., 2007; Kebede et al., 2008; Lan et al., 2008). When fasting NEFA are high it seems that a cross-talk between PPAR-γ and FFAR1 signaling regulates the insulin secretion in humans (Wagner et al., 2014). In addition, it has been suggested that the underlying mechanism of FFAR1-mediated insulin secretion from β-cells comprises an activation of the cAMP-dependent protein kinase and a reduction of voltage-gated potassium current leading to an increase in calcium ions and insulin secretion (Feng et al., 2006). Due to its mediating role in insulin secretion and as a possible link between obesity and type 2 diabetes, FFAR1 seems to be an interesting target in the treatment of type 2 diabetes (Briscoe et al., 2006; Bing and Trayhurn, 2009; Feng et al., 2012). The development of a promising selective partial agonist of FFA1, TAK-875, was stopped due to its liver toxicity although it is not known whether the effect was candidate- or mechanism-specific (Ichimura et al., 2014; Srivastava et al., 2014). Knockout mice models also indicate that FFAR1 plays a role in glucagon release from α-cells (Flodgren et al., 2007; Lan et al., 2008) and in secretion of glucagon-like peptide (GLP)-1, glucose-dependent insulinotropic polypeptide and cholecystokinin from murine enteroendocrine cells (Edfalk et al., 2008; Liou et al., 2011). In addition, there is evidence of an involvement of FFAR1 in cell proliferation, breast cancer progression (Yonezawa et al., 2004; Hardy et al., 2005) and decreased hepatic lipid accumulation accompanied with decreased expressions of lipogenesis-related proteins in mice fed a high fat diet (Ou et al., 2014).

In 2008, Yonezawa et al. identified the FFAR1 ortholog in cattle; the amino acid sequence of the bovine FFAR1 is 84 % and 82 %, identical to the human FFAR1 and mouse FFAR1, respectively. Furthermore, *FFAR1* mRNA was detected in bovine mammary epithelial cells as well as in the bovine mammary gland at different stages of lactation. The same study provided evidence that linoleic and oleic acid are involved in cellular signaling in bovine mammary epithelial cells through activation of FFAR1 (Yonezawa et al., 2008). Another working group showed the presence of an FFAR1 ortholog in bovine neutrophils, suggesting that the oleic acid-induced release of granules from neutrophils is mediated by FFAR1 (Hidalgo et al., 2011). Additionally, it seems that FFAR1 might also be involved in adhesion, chemotaxis, granule release and intracellular responses in bovine neutrophils induced by linoleic acid (Mena et al., 2013). For deeper insights into the role of FFAR1 in bovine AT, further research is required.

1.2.2 Free fatty acid receptor 2 and 3

At the same time as FFAR1, FFAR2 and FFAR3 (previously known as GPCR43 and GPCR41, respectively) were deorphanized as targets for short-chain fatty acids (SCFA). A third member of this SCFA binding receptor family, GPCR42, results most likely from a recent tandem gene duplication event of *FFAR3* as both receptor sequences differ in only six amino acids. Whether the gene is an inactive pseudogene or a functional gene in some species or species populations is still unknown (Brown et al., 2003; Liaw and Connolly, 2009). Table 3 lists the potency rank order of saturated fatty acids at FFAR2 and FFAR3 from different studies.

Table 3. Potency rank order of saturated fatty acids with different carbon chain lengths in activating the free fatty acid receptors 2 or 3

Receptor	Species	Potency rank order	Reference
FFAR2	Human	C2 = C3 = C4 > C5 > C6 = C1	Brown et al. (2003)
	Human	C3 > C2 = C4 > C6 > C5 > C1	Le Poul et al. (2003)
	Human/ mouse	C3 > C2 = C4 > C1	Nilsson et al. (2003)
	Human	C3 = C2 > C1 = C5	Hudson et al. (2012)
	Bovine	C6 > C5 > C4 = C7 > C3 = C8 > C2 = C9	Hudson et al. (2012)
FFAR3	Human	C3 = C5 = C4 > C2 > C1	Brown et al. (2003)
	Human	C3 > C4 > C5 > C6 > C2	Le Poul et al. (2003)
	Human	C2 > C4 = C6 = C3 > C7 = C5	Hudson et al. (2012)
	Bovine	C2 > C4 > C7 = C3 = C6 = C5	Hudson et al. (2012)

C1: formate; C2: acetate; C3: propionate; C4: butyrate; C5: valerate; C6: caporate; C7: heptanoate; C8: caprylate; C9: pelargonate; FFAR: free fatty acid receptor.

For the human FFAR2, the rank order of potency was consistent between the different studies, with the highest binding potencies of acetate and propionate followed by butyrate and then valerate and formate (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). In contrast, the bovine FFAR2 responds with a completely different rank order of potency to the SCFA compared to the human ortholog of FFAR2, showing the highest affinity to caproate followed by valerate and then butyrate and heptaonate. This could be related to the fact that ruminants are exposed to higher SCFA concentrations due to fermentation of dietary fiber in the rumen and might indicate that FFAR2 has different functions in cattle as compared to humans (Hudson et al., 2012). The predominant SCFA produced by rumen

fermentation of dietary fiber are acetate, propionate and butyrate. Compared to human blood from the portal vein with total concentrations ranging from 0.16 to 0.36 mM of these three SCFA, sheep blood from this vessel showed concentrations of 1.60 mM (Bergman, 1990). The activation potencies of SCFA at the human FFAR3 were in a range similar to those at human FFAR2, with high affinity of the receptor to propionate, valerate and butyrate followed by acetate, and then caproate (Brown et al., 2003; Le Poul et al., 2003). The bovine and human FFAR3 showed comparable affinities to the different SCFA (Hudson et al., 2012).

FFAR2 is mainly expressed in immune cells e.g. neutrophils, monocytes, peripheral blood mononuclear cells, and lymphocytes. In addition, *FFAR2* mRNA was detected in skeletal muscle and heart (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003), in a breast cancer cell line (Yonezawa et al., 2004), and in murine WAT from different depots (Xiong et al., 2004; Hong et al., 2005). In humans, the highest expression of *FFAR3* occurs in WAT, but the receptors' mRNA has also been detected in various other tissues, e.g. pancreas, spleen, lung, brain, bone marrow, and intestine. There is evidence that *FFAR3* expression increases during adipocyte differentiation (Brown et al., 2003). In cattle, the expression of both receptors has been detected in various tissues, including the liver, heart, spleen, skeletal muscle, kidney, brain, adrenal gland, lung, pancreas, intestine, colon, rumen, and omasum (Wang et al., 2009). Both receptors' mRNA is expressed in SC AT of dairy cows, whereas *FFAR3* mRNA abundance seems to be upregulated from antepartum (a.p.) to postpartum (p.p.; Lemor et al., 2009). The expression of both receptors' mRNA also occurs in bovine preadipocytes isolated from an SC fat depot around the sternum and increases during a 13 d differentiation period (Hosseini et al., 2012). In another ruminant species, the goat, the mRNA encoding for *FFAR3* was upregulated in SC AT, but not in RP AT, in response to propionate infusion *in vivo* (Mielenz et al., 2008).

Some studies demonstrated stimulatory effects of SCFA on leukocyte chemotaxis, whereas the recruitment of leukocytes over SCFA is mediated by activation of FFAR2 (Le Poul et al., 2003; Maslowski et al., 2009; Sina et al., 2009; Vinolo et al., 2011). The propionate-induced granule release from bovine neutrophils is mediated by FFAR2 (Carretta et al., 2013). Additionally, an activation of FFAR2 by acetate or propionate stimulates *in vitro* adipocyte differentiation and development, increases the intracellular lipid content and inhibits lipolysis (Hong et al., 2005). The receptors' ability to inhibit lipolysis is strongly supported by results from FFAR2 knockout mouse models (Ge et al., 2008). An *in vitro* study showed that SCFA stimulate GLP-1 secretion via the FFAR2 (Tolhurst et al., 2012).

Moreover, FFAR2 activation by SCFA has been shown to suppress insulin signaling in murine AT, leading to inhibition of fat accumulation in this tissue and instead lipids and glucose were primarily utilized in muscle tissue (Kimura et al., 2014). In nonruminants SCFA are produced by gut microbial fermentation, thus the FFAR2 links the metabolic activity of the gut microbiota with the host body energy homoeostasis (Kimura et al., 2013). Therefore, the FFAR2 might act as a sensor for excessive dietary energy and thus control energy balance through the insulin pathway in AT (Kimura et al., 2014).

Activation of FFAR3 in adipocytes stimulates the production and secretion of leptin (Xiong et al., 2004). Furthermore, FFAR3-deficient mice showed increased intestinal transit rates and thus a reduced intestinal absorption, which might indicate that the receptor is involved in the secretion of gut motility-influencing hormones, such as peptide YY and GLP-1, from enteroendocrine cells (Samuel et al., 2008). Studies on 3T3-L1 adipocytes showed that both propionate and valerate increase insulin-stimulated glucose uptake and also that FFAR3 activation plays a role in the enhanced insulin responsiveness by these two SCFA (Han et al., 2014). Propionate administration increases body the energy expenditure and heart rate of adult wild-type mice; in FFAR3 knockout mice this regulation of the sympathetic nervous system activity by propionate was abolished, suggesting that FFAR3 functions as an energy sensor in the nervous system (Kimura et al., 2011). In another study, lean mass and energy expenditure were reduced in male FFAR3 knockout mice, indicating that SCFA may raise energy expenditure by activating FFAR3 (Bellahcene et al., 2013).

Both receptors transduce signals by coupling to $G_{i/o}$ proteins and FFAR2, but not FFAR3, is able to couple efficiently to $G_{q/11}$ (Brown et al., 2003; Le Poul et al., 2003). In 2009, Wang et al. identified and characterized the genes encoding *FFAR2* and *FFAR3* in cattle, with amino acid sequences being more than 75 % identical to the human orthologues of both receptors. This working group also provided evidence of a coupling of these two bovine receptors to $G_{i/o}$ proteins (Wang et al., 2009).

Recently, Zhang et al. identified the *FFAR2*-like (*FFAR2L*) gene in ten mammalian species (including pig, cat, elephant, and giant panda). Functional studies with the porcine FFAR2L showed structural similarity with FFAR2 and similar, but not identical, properties to FFAR2; like FFAR2, FFAR2L is also able to bind to SCFA. The expression of this novel receptor in pigs was mainly restricted to the gastrointestinal tract where *FFAR2* and *FFAR2L* seem to be co-expressed. However, in humans and cattle, the chromosomal region predicted to encode *FFA2L* was identified as being truncated, indicating that this gene might be a pseudogene without biological function in these species (Zhang et al., 2014).

1.2.3 Hydroxycarboxylic acid receptor 2

The HCAR2, also referred to as GPCR109A, HM74a in human, PUMA-G in mouse or niacin receptor 1, belongs to the receptor family of hydroxycarboxylic acid receptors (Offermanns et al., 2011). Two other receptors for hydroxylcarboxylic acids are HCAR1 and HCAR3, also referred to as GPCR81 and GPCR109B, respectively (Offermanns et al., 2011). Pharmacological doses of NA have been used for decades as antilipolytic drugs for the treatment of dyslipidemic states in humans, but only in 2003 was the HCAR2 discovered as a target for NA mediating its antilipolytic effects in adipocytes (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003). In addition, the ketone body BHBA was shown to be an endogenous ligand for HCAR2 (Taggart et al., 2005). Ketone bodies are mainly produced by the liver depending on the delivery of FFA from adipocytes during severe deficiency in energy intake (Gille et al., 2008). The antilipolytic effects exerted by activation of HCAR2 by BHBA functions as a negative feedback mechanism to adjust an optimal lipolytic activity to the requirements of prolonged starvation (Gille et al., 2008). Additionally, some SCFA, e.g. valerate, caporate, capylate, and butyrate, were shown to activate the human HCAR2, but it seems unlikely that physiological concentrations of these SCFA reach sufficient levels to activate the receptor in humans (Taggart et al., 2005). The highest expression of human and murine *HCAR2* was found in WAT and BAT (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003), and apart from AT, *HCAR2* is also expressed in various immune cells, e.g. neutrophils, macrophages and dendritic cells, but not in B and T lymphocytes, human eosinophils, or immature bone marrow cells (Schaub et al., 2001; Kostylina et al., 2008; Tang et al., 2008). In addition, the receptor's mRNA is also expressed in epidermal Langerhans cells, in epithelial cells of the retinal pigment, of the ileum and of the colon, as well as in primary human keratinocytes, in human brain and in a human epidermoid carcinoma cell line (Maciejewski-Lenoir et al., 2006; Zhou et al., 2007; Miller and Dulay, 2008; Tang et al., 2008; Martin et al., 2009; Thangaraju et al., 2009; Cresci et al., 2010). There is no functional peroxisome proliferator-activated receptor (PPAR)-response element in the promoter region of the *HCAR2* gene, but the expression of *HCAR2* seems to be increased by activation of PPAR-γ (Jeninga et al., 2009). The expression of *HCAR2* is also up-regulated by certain cytokines, e.g. interferon-γ and TNF-α (Schaub et al., 2001).

Beside the antilipolytic effect of pharmacological doses of NA mediated through HCAR2 as mentioned before, BHBA is also able to inhibit lipolysis in adipocytes (Taggart et al., 2005). Suppression of lipolysis by HCAR2 activation leads to a decreased release of FFA from adipocytes and supply to the liver. This in return reduces the liver synthesis of TAG, of very-

low-density lipoprotein (VLDL) and of low-density lipoprotein (LDL) cholesterol levels (Carlson, 1963), all accounting for the antidyslipidemic properties of HCAR2 activation. Otherwise, hepatic overexpression of HCAR2 in mice unexpectedly reduced plasma concentrations of high-density lipoprotein (HDL) cholesterol in plasma, evidently due to an HCAR2-dependent reduction in cAMP production, leading to reduced efflux of cholesterol to apolipoprotein A-I *in vivo* (Li et al., 2010). However, in isolated murine macrophages, NA treatment enhanced the efflux of cholesterol to apolipoprotein A-I-containing HDL particles in an HCAR2-dependent manner (Lukasova et al., 2011). The role of HCAR2 activation in the reported increase of circulating HDL cholesterol concentrations in dyslipidemic individuals in response to the pharmacological doses of NA is not clear, just as the mechanism of how NA increases HDL cholesterol has not been clarified yet (Gille et al., 2008). The effect of NA in adipocytes mediated through HCAR2 activation may not provide a full explanation for all alterations in plasma lipid concentrations, which might also involve direct effects of NA on immune cells, e.g. macrophages (Kamanna and Kashyap, 2008; Lukasova et al., 2011). In addition, there is evidence that HCAR2 plays a physiological role in immune cells, e.g. macrophages and neutrophils. The receptor is recognized as being involved in the recruitment of neutrophils (Chen et al., 2014). Moreover, HCAR2 seems to be involved in the regulation of prostanoid release (e.g. PGE_2, PGD_2) from human macrophages (Gaidarov et al., 2013).

The stimulation of the secretion of ADIPOQ from adipocytes by NA is mediated through HCAR2 *in vivo* (Plaisance et al., 2009), which has also been proven for differentiated bovine preadipocytes (Kopp et al., 2014). Another ligand of HCAR2, *trans*-cinnamic acid, is also able to stimulate ADIPOQ secretion in 3T3-L1 adipocytes apparently via activation of HCAR2 (Kopp et al., 2014).

Furthermore, HCAR2 mediates an undesirable side effect of NA or HCAR2-targeting drugs, the so-called cutaneous flushing reaction, a result of cutaneous vasodilatation associated with tingling and burning of the affected skin areas (Benyó et al., 2005; Maciejewski-Lenoir et al., 2006; Gille et al., 2008; Offermanns et al., 2011). The increase in dermal blood flow proceeds in two phases. The first is induced by HCAR2 activation in epidermal Langerhans cells and results in the formation of PGD_2 and PGE_2, two vasodilatory prostanoids. The second phase is induced by HCAR2 activation in keratinocytes, which increases PGE_2 release (Benyó et al., 2005; Cheng et al., 2006; Gille et al., 2008; Hanson et al., 2010).

Due to the fact that the receptor is also expressed in intestinal epithelial cells and one of its ligands, butyrate, is present in millimolar concentrations in the lumen, it has been suggested

that HCAR2 mediates the suppressive function of butyrate in the development of colon cancer (Thangaraju et al., 2009). The actions of HCAR2 are induced by receptors coupling to $G_{i/o}$ proteins (Taggart et al., 2005; Gaidarov et al., 2013). It seems that the signaling via HCAR2 is dependent on the cell type. In adipocytes, HCAR2 exerts its activity through a $G_{\alpha i}$ subunit that mediates the inhibition of adenylyl cyclase and thus the reduction of intracellular cAMP concentrations. In macrophages, HCAR2 exerts signaling via $G_{\beta\gamma}$ subunits augmenting intracellular cAMP concentrations when additional G_s stimuli are present (Gaidarov et al., 2013).

The bovine sequence of HCAR2 shares 83 % homology with human HCAR2 and it has been suggested that the sequence encodes for a receptor that is able to bind NA (Titgemeyer et al., 2011). Protein and mRNA of the receptor have been detected in bovine *M. longissimus*, one VC AT (RP AT), 2 SC AT depots (back fat and tail head fat) and different regions of the brain. Surprisingly, the highest expression was observed in bovine liver: Immunofluorescence performed with a primary anti-HCAR2 and fluorescent-labeled secondary antibody showed that the protein is also expressed in parenchymal cells and not exclusively by immune cells (Titgemeyer et al., 2011). When compared to other species, e.g. man and mouse (Tunaru et al., 2003; Wise et al., 2003), the high expression of *HCAR2* in bovine liver seems unusual (Titgemeyer et al., 2011). Additionally, BHBA and butyrate inhibit lipolysis in bovine adipocytes (Metz et al., 1974). The HCAR2-mediated pathway also seems to be functional in bovine AT and activated by both NA and BHBA *in vitro* (Kenéz et al., 2014). Furthermore, the *HCAR2* mRNA expression is downregulated from late pregnancy to 3 weeks after calving, indicating the relevance of an HCAR2-mediated regulation of lipolysis during the transition period (Lemor et al., 2009).

1.3 Nutritional supplements and other strategies in dairy cattle feeding

Passing through the transition period without production diseases increases the profitability of the high-yielding dairy cow, therefore the management of nutrition of the animals has received enormous interest during the last few decades (Drackley, 1999; Ingvartsen, 2006). To overcome the critical transition period, different nutritional strategies can be applied to improve the animals' health by supporting the metabolic adaptations that dairy cows undergo during this period. Established possible interventions are the use of fatty acids (e.g. conjugated linoleic acids [CLA], eicosapentaenoic acid, docosahexanoic acid), the use of methyl donors (e.g. choline, methionine) in the transition diet, and the manipulation of the

energy density of the diet (Esposito et al., 2014). The nutritional strategies considered in this thesis are the use of CLA and nicotinic acid (NA), and the modulation of energy density.
Concentrates containing nonfiber carbohydrates (NFC; starch provided by grains) can be used to increase the energy density of the diet by increasing the digestibility and the passage rate of the diet as well as the ruminal production of propionate (Rabelo et al., 2001; Drackley et al., 2005; Esposito et al., 2014). Fatty acids can also be used to increase the energy density (Drackley et al., 2005). The additive feeding of CLA as well as the modulation of energy density targets amelioration of the NEB during the transition period, whereby CLA reduces the output of energy via milk by reducing mammary synthesis of fatty acids (Loor and Herbein, 1998), and a higher dietary energy density might increase the energy intake.
The antilipolytic properties of NA have been well studied in humans and for decades NA has been used as a potent lipid-modifying drug (Gille et al., 2008). Based on its antilipolytic properties, some studies tested NA as a feed additive for high-yielding dairy cattle to prevent fatty liver and ketosis due to a limitation of excessive lipid mobilization (Waterman and Schultz, 1972; Fronk and Schultz, 1979). A reduced mobilization of FA from AT in response to NA might contribute to a reduced risk of metabolic disorders among dairy cattle in the transition period (Yuan et al., 2012).

1.3.1 Conjugated linoleic acids

Supplementing CLA in the diet of dairy cows enhances their content in milk and thereby contributes to the nutritive value of milk and milk products (Griinari and Baumann, 1999; Dhiman et al., 2000). In addition, CLA have been shown to limit the extent of the comprehensive physiological NEB by decreasing milk fat secretion and in turn decreasing the caloric demand of milk production in early-lactating dairy cows, whereby particularly the *trans*-10,*cis*-12 (*t*10,*c*12) CLA isomer is known to induce milk fat depression (Bauman et al., 2000).

1.3.1.1 Structure and origins

The term "conjugated linoleic acids" refers to a mixture of positional and geometric isomers of octadecadienoic acid with a conjugated double-bond system. The different CLA isomers result from the position of the double bond pair (e.g. 8-10, 9-11, and 10-12) separated by a single bond and from the four possible configurations (*cis-trans*, *trans-cis*, *cis-cis*, or *trans-trans*) of the double bond (Bauman et al., 2000). Naturally, CLA are widespread in plant and

animal tissues, whereas the highest amounts occur in food products (milk and meat) derived from ruminant animals in the range of 2–7 mg per g of total fat (Chin et al., 1992). The predominant CLA in products derived from ruminants is the *cis*-9,*trans*-11 (*c*9,*t*11) isomer (Chin et al., 1992; Bauman et al., 2000). Beside the *c*9,*t*11 isomer, the *trans*-10,*cis*-12 (*t*10,*c*12) isomer has also been shown to possess biological activities (Banni, 2002). Commercially available CLA supplements mainly contain a 1:1 mixture of these two CLA isomers (Banni, 2002). Usually the commercially available CLA is synthetically prepared from linoleic acid (LA) from, for example, safflower oil by alkaline isomerization (Koba and Yanagita, 2014). Figure 5 shows the structure of LA and the two main isomers, *c*9,*t*11 and *t*10,*c*12 CLA, deriving from LA.

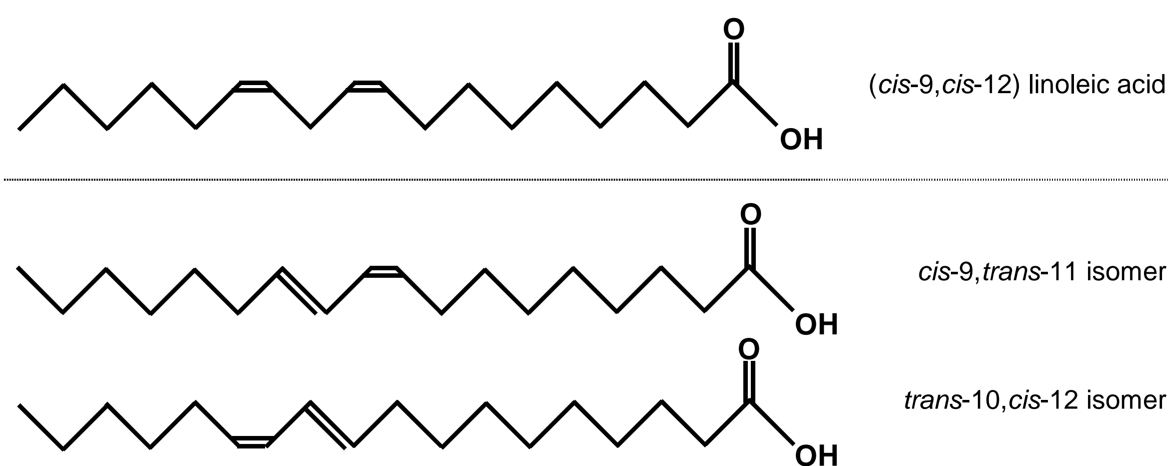

Figure 5. Chemical structures of linoleic acid and its two main derivatives (modified from Tsuzuki et al. (2006))

The CLA in ruminants' milk and meat arise from the production at two sites: One source is the biohydrogenation of LA into stearic acid by rumen bacteria (such as *Butyrivibrio fibrisolvens*) leading to CLA as an intermediate product, and the other source is the CLA synthesis by animal tissues from *trans*-11 octadecenoic acid (Bauman et al., 2000). Dairy cows' dietary intake of unsaturated fatty acids, such as LA, ranges from 300 g/d (2 % of dry matter intake [DMI]) without fat supplements to 700 g/d (5 % of DMI) with fat supplemented to their diet (Santos, 2011). Figure 6 displays the endogenous synthesis of *c*9,*t*11 CLA at these two sites, whereat it is the predominant isomer, representing at least 80 % of the total CLA content in milk and meat from cattle (Bauman et al., 2000). In contrast, the *t*10,*c*12 CLA represents less than 2 % of the total milk CLA (Piperova et al., 2000).

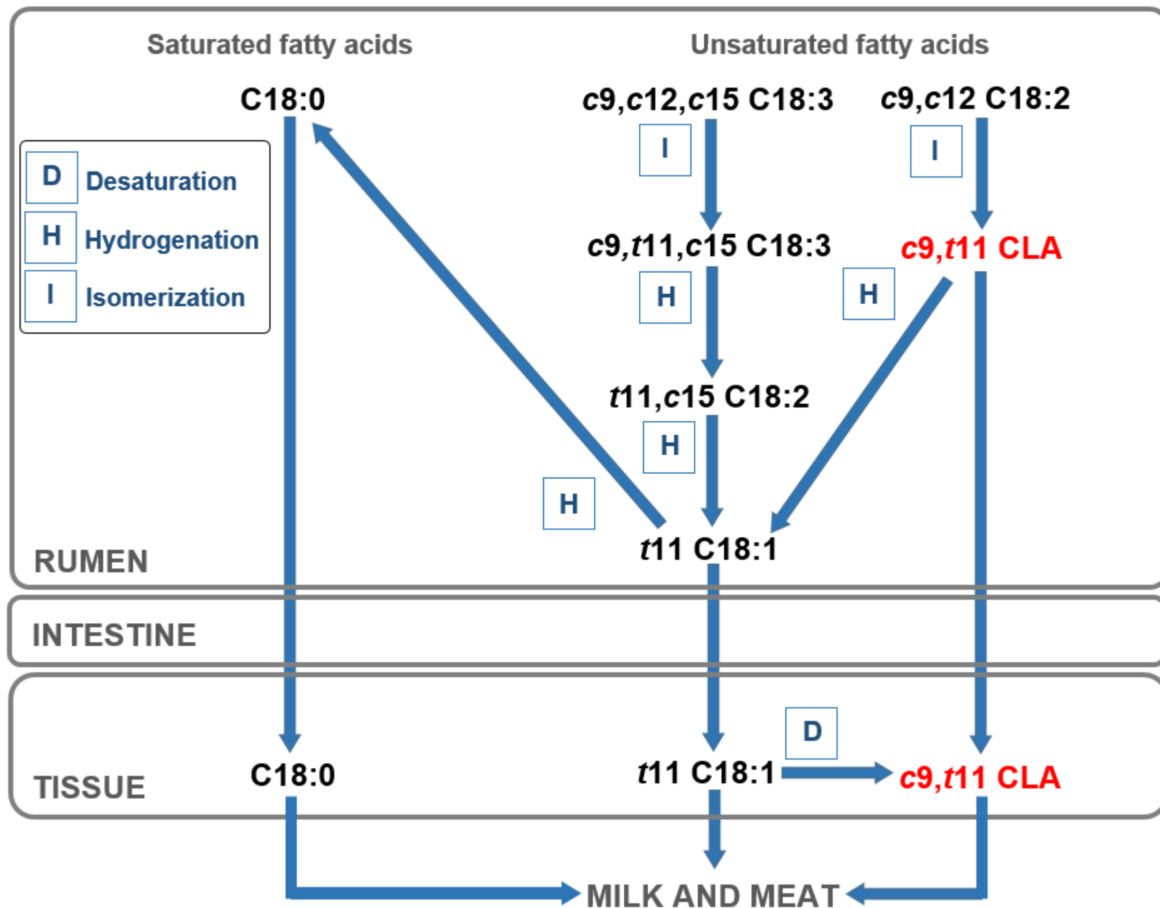

Figure 6. Predominant pathways of C18 fatty acids including the rumen and tissue biosynthesis of *cis*-9,*trans*-11 conjugated linoleic acid (modified from Griinari and Baumann (1999))

When TAG, the major form of dietary lipids, enter the rumen, the ester linkages are hydrolyzed through catalysis by microbial lipases (Bauman et al., 2000; Bauman et al., 2011). In the rumen, the *cis*-double bonds of the dietary unsaturated fatty acids are isomerized to form conjugated *cis/trans* double bond systems. Further hydrogenation of the *cis*-double bonds leads to the formation of *trans*-11 octadecenoic acid, also known as vaccenic acid, which is a common intermediate in biohydrogenation. As the hydrogenation of vaccenic acid limits the rate of complete biohydrogenation of unsaturated C18 fatty acids, it accumulates in the rumen. Portions of *c*9,*t*11 CLA and vaccenic acid formed in the rumen escape outright biohydrogenation and are absorbed in the intestine (Griinari and Baumann, 1999). Also, the conversion of vaccenic acid to *c*9,*t*11 CLA catalyzed by Δ^9-desaturase in the tissues accounts for the large portion of CLA in milk and meat. The activity of Δ^9-desaturase appears to be highest in the mammary gland in lactating ruminants, whereas in growing animals AT shows the highest activity of this enzyme (Griinari and Baumann, 1999; Griinari et al., 2000).

It is worth mentioning that several factors, including breed, age, the bacterial rumen population, and diet of the animal, influence the CLA content in milk and meat, wherein dietary factors are of major importance (Bauman et al., 2000; Dhiman et al., 2005). Diets rich in lipid substrates for ruminal synthesis of *c*9,*t*11 CLA (e.g. diets supplemented with plant oils containing high amounts of LA) and diets increasing the rumen population of bacteria involved in the biohydrogenation of unsaturated fatty acids (e.g. diets with a high forage-to-concentrate ratio) elevate the content of CLA in milk fat (Griinari and Baumann, 1999).

1.3.1.2 Effects of conjugated linoleic acids in nonruminants

In various animal and cell culture studies, numerous physiological functions and putative beneficial health effects such as anticarcinogenic, antiobese, and antihypertensive effects, as well as the prevention of atherosclerosis and enhancement of immune function exerted by CLA, have been identified (Oleszczuk et al., 2012; Koba and Yanagita, 2014). The *c*9,*t*11 CLA isomer and the *t*10,*c*12 CLA isomer exert different effects, whereby some effects are exerted solely by one of these isomers and other effects are exerted by both isomers together (Oleszczuk et al., 2012; Koba and Yanagita, 2014).

For instance, *in vitro* studies showed that the anticarcinogenic activity seems to be independent of the CLA isomer used and studies in rats indicate a dose-dependent anticarcinogenic effect of CLA in the range of 0 to 1.5 % of body weight (Koba and Yanagita, 2014). There is also evidence that the growth inhibitory effect on different human cancer cell lines is mediated by activation of PPAR-γ, which induces apoptosis and inhibits the proliferation of different human cancer cell lines (Koba and Yanagita, 2014). However, the mechanisms of the anticarcinogenic properties of CLA remain largely unclear and their potential beneficial effects on different cancer types are ambiguous. The potential anticarcinogenic effects of CLA seem to be dependent on the site of the cancer; from *in vitro* studies and rodent models there is evidence that CLA have potential beneficial effects on colorectal, breast and prostate cancer (McCrorie et al., 2011).

The effects of CLA on body composition as well as their hypolipidemic effects have been attributed to the *t*10,*c*12 isomer rather than to the *c*9,*t*11 isomer (Koba and Yanagita, 2014). Rodent, but not human studies, showed a CLA-induced reduction in energy intake. Nevertheless, the reducing effect of CLA on body fat mass seems to be not solely dependent on energy intake as several studies have reported reduced body fat mass even without changes in the energy intake. Also, the CLA-induced suppression of animal and human

preadipocyte differentiation, the inhibition of lipogenesis and the stimulation of lipolysis may account for the potential of CLA for reducing body fat mass. In WAT from rodents, a mixture of both isomers or the *t*10,*c*12 isomer alone induced the *UCP-2* transcription, which probably enhances the respiratory oxidation of fatty acids (Kennedy et al., 2010). Results from human intervention studies for investigating the CLA effect on body composition in normal weight, overweight and obese subjects are inconsistent. The majority of these studies have shown no effect on body weight and just some reported reduced body fat mass following CLA supplementation. However, the promising evidence of the CLA effect on body composition from animal studies could not be reproduced in human intervention studies (McCrorie et al., 2011).

Numerous animal model studies reported anti-atherosclerotic effects of a mixture of both CLA isomers or the *t*10,*c*12 isomer due to the reduction of circulating total cholesterol, TAG, LDL cholesterol and blood pressure as well as an elevation of HDL cholesterol, whereas human studies did not provide clear evidence of the CLA effect on the risk of cardiovascular disease (Dilzer and Park, 2012). In some human clinical trials, a mixture of both CLA isomers or the *t*10,*c*12 isomer caused unfavorable changes in serum lipids with regard to cardiovascular-related risks, enhanced lipid peroxidation and increased the plasma concentrations of C-reactive protein, an inflammatory marker associated with cardiovascular disease (Oleszczuk et al., 2012). Additionally, CLA binds to FFAR1 with high affinity and one cannot exclude a potential contribution of FFAR1 to β-cell dysfunction through long-term application of CLA (Schmidt et al., 2011).

It has also been suggested that feeding of CLA normalizes impaired glucose tolerance in obese, diabetic rats. Since CLA are able to activate PPAR-γ, the antidiabetic effect of CLA could be mediated by PPAR-γ activation resulting in increased plasma ADIPOQ concentrations and thus amelioration of hyperinsulinemia (Houseknecht et al., 1998), whereas the *t*10,*c*12 CLA seems to be the isomer influencing the glucose tolerance (Koba and Yanagita, 2014). However, studies in humans reported inconsistent results: The majority of studies reported no effects of CLA supplementation on blood glucose and plasma insulin levels or reported impairing effects by both CLA isomers on glucose metabolism (Risérus et al., 2002; Risérus et al., 2004; Dilzer and Park, 2012). Inasmuch as some human and rodent studies indicate that CLA might decrease insulin sensitivity, induce hepatic lipodystrophy and exert pro-atherosclerotic effects, the safety of CLA supplementation for human nutrition is still questionable (Oleszczuk et al., 2012).

1.3.1.3 Effects of conjugated linoleic acids in dairy cows

The *t*10,*c*12 CLA inhibits mammary milk fat synthesis in a dose-dependent manner (Bauman et al., 2000; de Veth et al., 2004) at various stages of lactation (Hutchinson et al., 2011), whereas milk yield, milk lactose and milk protein are generally not affected (Bauman et al., 2008). A key regulator in the milk fat depression (MFD) induced by *t*10,*c*12 CLA is the sterol response element-binding protein (SREBP)-1 as many lipogenic enzymes have SREBP response elements in their promoter region (Bauman et al., 2008). It has been shown that expression of *SREBP-1*, a gene highly expressed in bovine mammary tissue, is decreased during *t*10,*c*12 treatment and thus might downregulate the expression of lipogenic enzymes (Harvatine and Bauman, 2006). In CLA supplementation-induced MFD all fatty acids in milk are reduced, but most of the studies observed a relatively greater reduction in *de novo* synthesized fatty acids (Peterson et al., 2003; Hötger et al., 2013). Therefore it is likely that the *t*10,*c*12 CLA isomer predominantly inhibits *de novo* fatty acid synthesis in the mammary gland (Peterson et al., 2003; Hötger et al., 2013).

Beside the *t*10,*c*12 CLA, two other isomers that are produced as intermediates in rumen biohydrogenation (Bauman et al., 2011), *t*9,*c*11 and *c*10,*t*12 CLA, have also been identified as suppressing milk fat synthesis (Saebø et al., 2005; Perfield et al., 2007). The secretion of milk fat is energetically the most expensive process, as the fat energy content represents 40 to 50 % of the total energy concentration of milk (Bauman and Currie, 1980; Santos, 2011). The ability of CLA to reduce the energy output via milk could therefore be a nutritional strategy for ameliorating the NEB in early lactation (Bauman et al., 2000). However, the findings from some studies on the effects of CLA supplementation on DMI and NEB are contradictory. In some studies a slightly inhibitory effect of CLA supplementation on DMI has been reported (Bauman et al., 2000; Moallem et al., 2010; Pappritz et al., 2011b; von Soosten et al., 2011; Hötger et al., 2013), results from one study indicate an increasing effect of CLA on DMI (Shingfield et al., 2004) and some studies reported no effect of CLA treatment on DMI (Perfield et al., 2002; Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2005). The same applies for the calculated energy balance, whereas a reduction (Shingfield et al., 2004) as well as an aggravation (Pappritz et al., 2011b; Hötger et al., 2013) or no changes in p.p. NEB (Bernal-Santos et al., 2003; Selberg et al., 2004; Castañeda-Gutiérrez et al., 2005; Moallem et al., 2010) in response to CLA supplementation have been reported. The inconsistent results concerning the DMI and the calculated energy balance might be due to the different design of the studies, e.g. the dosage of *t*9,*c*11 CLA and *t*10,*c*12 CLA, the preparation (i.e. rumen protected *versus* non-rumen protected) and form of

application of the CLA supplement, the beginning and duration of the CLA supplementation or the dietary status and the stage of lactation of the investigated cows. The supplementation of CLA may thus not necessarily result in an improvement of energy balance in dairy cows (Hötger et al., 2013). Table 4 provides a summary of the biological responses to CLA supplements in dairy cattle and the applied dosages and preparation forms of CLA used in the different studies. As shown in Table 4, most studies indicate that CLA supplementation has no effect on the concentrations of plasma metabolites such as glucose, NEFA and BHBA or metabolic hormones including insulin, insulin growth factor (IGF)-1, and leptin, whereby most of the studies investigated the effect of supplements containing only the *t*10,*c*12 isomer or a 1:1 mixture of the *t*10,*c*12 and *c*9,*t*11 CLA isomers.

There is evidence that CLA might affect metabolic processes such as endogenous glucose production, a process that ensures the supply of glucose for mammary milk production in ruminants. The measurement of endogenous glucose production by infusion of uniformly labeled ^{13}C glucose and the measurement of enrichment of this tracer by gas chromatography-mass spectrometry revealed lower endogenous glucose production rates in the third week of lactation, whereby the hepatic gene expression of two key enzymes involved in gluconeogenesis also tended to be lower in CLA-treated animals (Hötger et al., 2013).

The aforementioned reducing effect of CLA on body fat content observed in studies with rodents could not be conclusively confirmed for lactating cows. On the one hand, CLA tended to slightly decrease DMI (von Soosten et al., 2012) and a decreased size of adipocytes in VC and SC AT in CLA-treated animals during early lactation has been observed, which prefigures rather lipolytic and/or antilipogenic effects of CLA on AT (Akter et al., 2011).

It was also shown that the decline of serum concentrations of ADIPOQ around parturition is extended in early lactation in response to CLA supplementation (Singh et al., 2014b). On the other hand, body fat mobilization was reduced in animals fed a CLA supplemented diet, suggesting improved utilization of the metabolizable energy (von Soosten et al., 2012), and an increasing effect of t10,c12 CLA-induced MFD on the gene expression of lipogenic genes in AT has been observed (Harvatine et al., 2009). It is conceivable that the CLA effect on bovine AT metabolism observed in some studies is mediated through alterations in nutrient sensing and signaling (de Veth et al., 2009).

Table 4. Summary of the most frequently observed biological responses to conjugated linoleic acids in dairy cows (modified from Bauman et al. (2011))

Process	Effect(s)
Milk	
Protein and lactose	Output unchanged[1, 2, 3, 4]
Fat	Output decreased up to 50 %[1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17]
Fatty acid profile	All FA reduced, but relatively greater reduction in *de novo* synthesized FA[1, 3, 6, 7, 8, 9, 10, 11, 16]
Whole-animal metabolism	
Feed intake	Slight reduction in DMI[1, 10, 12, 13, 17]
Ketogenesis	Plasma BHBA unchanged[3, 5, 11, 12, 17]
Liver lipids	TAG content unchanged[4, 5]
Plasma hormones	
IGF-1	IGF-1 plasma concentration unchanged[1, 11]
Leptin	Leptin plasma concentration unchanged[2]
ADIPOQ	ADIPOQ plasma concentrations decreased[18]
Glucose homeostasis	
Glucose set point	Plasma glucose unchanged[1, 2, 3, 4, 5, 6, 11, 12]
Basal insulin	Plasma insulin unchanged[1, 2, 4, 6, 11, 17]
Stimulated	Glucose response to insulin unchanged[2, 6]
Lipolysis	
Basal	Plasma NEFA unchanged[2, 3, 5, 6, 12, 13, 17]
Stimulated	Response to β-adrenergic stimulation unchanged[6]
Tissue-specific metabolism	
Mammary gland	
Lipid synthesis enzymes	Decreased mRNA abundance of enzymes involved in FA uptake, synthesis, transport, desaturation, and esterification[7, 8, 13]
Transcription factors	Decreased expression and activation of *SREBP1* and *S14*[7, 8, 14], and *PPAR-γ2*[19]
Adipose tissue	
Lipid synthesis enzymes	Increased mRNA expression of transcription factors (*SREBP1* and *S14*) and of enzymes involved in FA synthesis, uptake, and desaturation[9]
Adipocyte size	Decreased adipocyte sizes in subcutaneous and visceral adipose tissue[20]
Liver	
Fatty acid metabolism enzymes	Unchanged expression of key enzymes involved in lipogenesis, ketogenesis, and β-oxidation[16]

Table 4. Continued.

	References	Dosage of *t*10,*c*12 and *c*9,*t*11 isomers and application form of the CLA
1	Bauman et al. (2000)	Abomasal infusion of 10 g/d *t*10,*c*12 or *c*9,*t*11 CLA
2	Baumgard et al. (2002)	Abomasal infusion of 13.6 g/d *t*10,*c*12 CLA
3	Perfield et al. (2002)	126 g/d RPP (10 % *t*10,*c*12 and 9 % *c*9,*t*11 CLA)
4	Castañeda-Gutiérrez et al. (2005)	295 g/d RPP (6 % *t*10,*c*12 and 6 % *c*9,*t*11 CLA)
5	Bernal-Santos et al. (2003)	126 g/d RPP (10 % *t*10,*c*12 and 9 % *c*9,*t*11 CLA)
6	de Veth et al. (2006)	115 g/d RPP (10 % *t*10,*c*12 and 10 % *c*9,*t*11 CLA)
7	Harvatine and Bauman (2006)	Jugular vein infusion of 10 g/d *t*10,*c*12 CLA
8	Gervais et al. (2009)	Jugular vein infusion of 10 g/d *t*10,*c*12 CLA
9	Harvatine et al. (2009)	Abomasal infusion of 7.5 g/d *t*10,*c*12 CLA
10	Moallem et al. (2010)	RPP containing 4.7 g/d *t*10,*c*12 and 4.7 g/d *c*9,*t*11 CLA
11	Hutchinson et al. (2011)	RPP containing 7 g/d *t*10,*c*12 and 7 g/d *c*9,*t*11 CLA
12	Pappritz et al. (2011b)	100 g/d RPP (12 % *t*10,*c*12 and 12 % *c*9,*t*11 CLA)
13	von Soosten et al. (2011)	100 g/d RPP (12 % *t*10,*c*12 and 12 % *c*9,*t*11 CLA)
14	Han et al. (2012)	200 g/d RPP (37 % *t*10,*c*12 and 38 % *c*9,*t*11 Ca-CLA)
15	Hutchinson et al. (2012)	RPP containing (5 g/d *t*10,*c*12 and 5 g/d *c*9,*t*11 CLA)
16	Schlegel et al. (2012)	RPP containing 4 g/d *t*10,*c*12 and 4 g/d *c*9,*t*11 CLA
17	Hötger et al. (2013)	50 g/d RPP (9.3 % *t*10,*c*12 and 10.3 % *c*9,*t*11 CLA)
18	Singh et al. (2014b)	100 g/d RPP (12 % *t*10,*c*12 and 12 % *c*9,*t*11 CLA)
19	Saremi et al. (2014)	100 g/d RPP (12 % *t*10,*c*12 and 12 % *c*9,*t*11 CLA)
20	Akter et al. (2011)	100 g/d RPP (12 % t10,c12 and 12 % c9,t11 CLA)

ADIPOQ: adiponectin; BHBA: β-hydroxybutyrate; CLA: conjugated linoleic acids; DMI: dry matter intake; FA: fatty acid; IGF-1: insulin growth factor-1; MFD: milk fat depression; NEFA: nonesterified fatty acid; PPAR-γ2: peroxisome proliferator-activated receptor γ-2; RPP: rumen-protected preparation; S14: thyroid hormone-responsive spot 14; SREBP: sterol regulatory element-binding protein; TAG: triacylglycerides.

1.3.2 Niacin

The effect of supplementing niacin is not as well studied as the effect of supplementing CLA in dairy cows' diets; however, numerous studies about the effects of applied pharmacological doses of NA in humans and rodents have been published.

1.3.2.1 Structure and sources

Niacin (also known as vitamin B_3) belongs to the group of water-soluble B vitamins and is a generic descriptor for NA (pyridine 3-carboxylic acid) and derivatives exhibiting qualitatively the biological activity of nicotinamide (NAM, pyridine 3-carboxylic acid amide) (Bates, 1998; Carlson, 2005; Combs, 2012). Niacin is defined as a group of dietary precursors to nicotinamide adenine dinucleotide (NAD), other than tryptophan (Penberthy and Kirkland, 2012). As well as NA and NAM, a third form of niacin, NAM riboside, has been discovered (Bieganowski and Brenner, 2004). Causing confusion in the use of the generic and specific terms, in some countries, e.g. North America the term "niacin" refers exclusively to NA (Bates, 1998; Combs, 2012). In this thesis the term "niacin" refers generically to nicotinic acid (NA) and nicotinamide (NAM), whereas the termes "nicotinic acid" and "nicotinamide" refer specifically to each.

Figure 7 shows the chemical structures of the niacin vitamers NA, NAM and NAM riboside. Niacin occurs in foods either as NA, as NAM or as the pyridine nucleotide coenzymes NAD and nicotinamide adenine dinucleotide phosphate (NADP). Another source for nearly all species, including mammals, is the endogenous synthesis of the vitamin from the amino acid L-tryptophan. In ruminants, an important additional source is the production of niacin by rumen microbes (Bates, 1998; Niehoff et al., 2009).

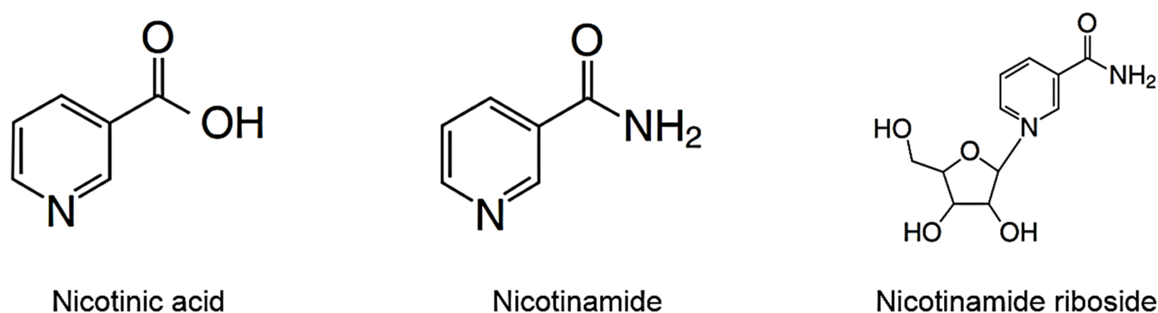

Figure 7: Chemical structures of the niacin vitamers nicotinic acid, nicotinamide and nicotinamide riboside (modified from Penberthy and Kirkland (2012))

The estimated daily requirement of approximately 289 mg/d of niacin for a 650 kg lactating cow with a DMI of 22 kg/d and a milk yield of 35 kg/d seems to be covered by their estimated ruminal synthesis of 1804 mg/d regardless of the absorption rates in the rumen (NRC, 2001). Additionally, the diet seems to influence the ruminal synthesis of niacin, since an effect of the content of nonfiber carbohydrates in the diet as well as an effect of the forage content in the diet on ruminal niacin (both vitamers NA and NAM) synthesis have been reported (Schwab et al., 2006). However, it is not clear which portion of the synthesized niacin is absorbed and which portion is degraded in the rumen (Niehoff et al., 2009) as most NA is absorbed from the duodenum, converted to NAD and afterwards hydrolyzed to NAM, which is the main transport form of niacin in the blood (Henderson and Gross, 1979).

1.3.2.2 Physiological functions and pharmacological actions in nonruminants

As mentioned before, both vitamers of niacin, NA and NAM, are precursors of NAD and NADP, which are ubiquitous coenzymes in numerous redox reactions in the cell (Bates, 1998; Combs, 2012). The coenzyme NAD is used more by redox enzymes for catabolic reactions, e.g. oxidation of substrates in the Krebs cycle), whereas its phosphorylated derivate NADP is used more for anabolic reactions, e.g. as a reducing agent for the synthesis of lipids and steroids (Rongvaux et al., 2003). The coenzyme NAD is able to readily accept and donate electrons in reactions catalyzed by dehydrogenases, leading in particular to the generation of ATP in the mitochondrial electron transport chain (Rongvaux et al., 2003). Adenosine diphosphate (ADP)-ribose transferring enzymes use NAD as a substrate for poly-ADP-ribosylation to repair DNA (Menissier de Murcia et al., 2003) and for mono-ADP-ribosylation with a regulatory function in immune response and GPCR signaling (Corda and Di Girolamo, 2003). Furthermore, NAD is involved in the synthesis of cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate, both of which increase intracellular calcium concentrations (Lee, 2001). The redox state of NAD and NADP regulates the DNA binding activity of transcription factors and thus regulates the expression of genes (Lin and Guarente, 2003). It has also been proposed that an increased flux through the NAD salvage pathway regulates the deacetylase activity of the NAD-dependent silent information regulator 2p and thus the extension of the life span in yeast (Anderson et al., 2002).
The binding to certain P2Y receptors enables NAD to act as a neurotransmitter. For example, in colonic cells, the binding of NAD to P2Y1 inhibits colonic muscle contraction (Penberthy and Kirkland, 2012).

Pellagra is a systemic disease caused by dietary niacin deficiency and is characterized by dermatitis, diarrhea and dementia leading to death if the diet is not improved (Penberthy and Kirkland, 2012). Pellagra occurs mainly in developing nations where corn and its products are the major food source or where the diet is poor in niacin and/or tryptophan (Hegyi et al., 2004). In developed countries, sporadic cases of pellagra occur among chronic alcoholics or people dependent on drugs (e.g. certain tuberculosis medicine or chemotherapeutics) and patients in states of malabsorption (Hegyi et al., 2004; Penberthy and Kirkland, 2012).

Although NA and NAM are equally effective forms of the vitamin, it is exclusively NA that is a powerful pharmacological agent inhibiting lipolysis in AT (Bates, 1998; Combs, 2012). Thus, for decades pharmacological doses of NA have been used in the treatment of dyslipidemia and for the prevention of cardiovascular diseases in humans. At doses higher than 1.5 g/d NA decreases TAG, VLDL, LDL cholesterol, and total cholesterol plasma concentrations while increasing HDL cholesterol plasma concentrations in dyslipidemic individuals (Gille et al., 2008). Due to these changes in plasma lipid concentrations, NA decreases the risk of cardiovascular diseases (Carlson, 2005). A six-month treatment of impaired glucose tolerance with extended-release NA resulted in increased HDL cholesterol and ADIPOQ concentrations, and decreased the fasting concentration of TAG in the circulation (Linke et al., 2009). In addition, the treatment with extended-release NA altered the mRNA abundance of genes in isolated adipocytes from patients' abdominal SC AT. For example, the expression of mRNA of *ADIPOQ* and *PPAR-γ* increased and for hormone sensitive lipase, fatty acid synthase and *HCAR3* decreased compared to the baseline and the patients within the control group (Linke et al., 2009).

As described in a previous section, some plasma lipid altering effects of NA can be explained through activation of HCAR2 in adipocytes leading to an inhibition of lipolysis (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003), but there is also evidence of lipid altering effects of NA independent from activation of HCAR2 (Lauring et al., 2012). It is unlikely that HCAR3, a receptor with at least 1000-fold lower affinity for NA than HCAR2, mediates the pharmacological effects of NA; however, some synthetic agonists (e.g. acifran) are able to activate both receptors with equal affinity (Offermanns et al., 2011). The vitamer NAM is not able to activate either HCAR2 or HCAR3 (Gille et al., 2008).

In human subjects, a major rebound increase of NEFA blood concentrations when NA blood concentrations fall has been observed, because NA acts relatively temporary on lipolysis (Offermanns, 2006). Increased circulating NEFA levels have been shown to decrease insulin sensitivity, which might be a contraindication for the use of NA in type 2 diabetic patients

(Gille et al., 2008). The administration of pharmacological doses of NA can also cause side effects. One side effect mediated through the activation of HCAR2 is NA-induced flushing and is described in a previous section. The treatment of dyslipidemia with extended-release NA is associated with decreased insulin sensitivity, whereas the mechanisms responsible for the insulin resistance caused by extended-release niacin are not known (Fraterrigo et al., 2012). Other possible side effects might be gastrointestional problems when NA is orally administered, chemical hepatitis due to the application of NA in an extended-release form (Dalton and Berry, 1992) and hyperuricemia (Gershon and Fox, 1974). Whether these side effects involve the activation of HCAR2 is currently not known (Gille et al., 2008).

1.3.2.3 Effects of niacin in dairy cows

The results from some studies indicate that niacin supplementation of the diet for dairy cows increases milk yield (Drackley et al., 1998; Lohölter et al., 2013), possibly due to a reduction of subclinical ketosis (Fronk and Schultz, 1979). As mentioned before, NA is also able to induce a flushing reaction primarily characterized by cutaneous vasodilatation through activation of HCAR2 leading to an increased formation of vasodilatory PG. Additionally, the possible alleviation of heat stress in dairy cows in response to NA has been investigated, due to the ability of NA to induce vasodilatation and thus possibly decrease body and skin temperature during thermal stress (Di Costanzo et al., 1997; Zimbelman et al., 2010). Table 5 provides a summary of the most frequently observed biological responses to niacin supplements in dairy cattle and the applied dosages and vitamers in the different studies.
Niacin increases protozoal numbers in the rumen fluid mainly by increasing *Entodinium ssp.* (Erickson et al., 1990; Doreau and Ottou, 1996), a protozoa species that regulates the rumen environment by consuming starch and thus might increase the bacterial population and microbial protein synthesis in the rumen (Erickson et al., 1991). However, the bacterial population in the rumen seems to be unaffected by niacin supplementation (Doreau and Ottou, 1996). The effect of niacin on the ruminal protozoa population could also alter the production of SCFA in the rumen since the presence of some protozoa might increase ruminal butyrate production (Ranilla et al., 2007). As summarized in Table 5, in two studies essentially the proportion of butyric acid in the total rumen SCFA concentrations increased in response to niacin, whereas the total concentration of SCFA and the propionic acid proportions remained unchanged (Erickson et al., 1990; Christensen et al., 1996).

Table 5. Summary of the most frequently observed biological responses to niacin, mainly to the vitamer nicotinic acid, in dairy cows* (modified from Niehoff et al. (2009))

Process	Effect(s)
Milk	
Yield	Increase[1, 2, 3, 4, 5, 6]
Protein yield	Slight increase of output[4, 5, 7, 8]
Fat yield	Slight decrease of output[6, 9]
Whole-animal metabolism during moderate to severe heat stress	
Temperature	Decreased skin temperature[10] and decreased body temperature[11]
Heat loss	Increased evaporative heat loss[11]
Ruminal protozoa population and SCFA	
Protozoa	Increase of protozoa, especially *Entodinium spp.,* in rumen fluid[12, 13]
Total SCFA	Unchanged concentration of total SCFA in the rumen[12, 14, 15, 16]
Acetic acid	Slight reduction of acetic acid portion[15]
Propionic acid	Portion of propionic acid unchanged[12, 14, 15, 16]
Butyric acid	Increase of the portion of butyric acid[12, 15]
Blood metabolites and insulin	
NEFA	Unchanged plasma concentrations[4, 5, 8, 17]
BHBA	Decreased plasma concentrations[1, 8, 18]
Glucose	Plasma concentrations unchanged[4, 5, 7, 8, 9, 16, 17]
Insulin	Plasma concentrations unchanged[7, 17]
Tissue-specific metabolism	
Adipose tissue	
HSL	Unchanged protein expression of HSL[17]
HCAR2	Unchanged mRNA and protein expression of *HCAR2*[19]
Liver	
TAG content	Unchanged content of TAG[9]
HCAR2	Unchanged mRNA and protein expression of *HCAR2*[19]

Table 5. Continued.

	References	Dosage of the applied niacin vitamers in the supplement
1	Fronk and Schultz (1979)	12 g/d NA as treatment on ketosis
2	Kung et al. (1980)	6 g/d niacin (no information on the vitamer used)
3	Muller et al. (1986)	6 g/d NA
4	Cervantes et al. (1996)	12 g/d nicotinamide
5	Drackley et al. (1998)	6 g/d NA
6	Lohölter et al. (2013)	24 g/d of supplement containing at least 99.5 % of NA
7	Horner et al. (1986)	7 g/d niacin as niacin-corn premix
8	Erickson et al. (1992)	12 g/d NA
9	Yuan et al. (2012)	12 g/d rumen-protected supplement containing 65 % NA
10	Di Costanzo et al. (1997)	12 g/d to 36 g/d NA
11	Zimbelman et al. (2010)	12 g/d rumen-protected supplement containing 65 % NA
12	Erickson et al. (1990)	12 g/d supplement containing either nicotinamide or NA
13	Doreau and Ottou (1996)	6 g/d NA via the ruminal cannula
14	Campbell et al. (1994)	12 g/d NA
15	Christensen et al. (1996)	12 g/d NA
16	Madison-Anderson et al. (1997)	12 g/d NA
17	Locher et al. (2011)	24 g/d non-protected supplement (≥99.5 % NA)
18	Morey et al. (2011)	24 g/d encapsulated supplement (40 % bioavailable NA)
19	Titgemeyer et al. (2011)	Abomasal infusion of 16 g/d of NA (≥99.5 %) in steers

*One study included in the table has been performed on steers (reference 19).
BHBA: β-hydroxybutyrate; FA: fatty acids; HCAR2: hydroxycarboxylic acid receptor 2; HSL: hormone sensitive lipase; NA: nicotinic acid; NEFA: nonesterified fatty acids; SCFA: short-chain fatty acids; TAG: triacylglycerides.

In most of the studies investigating the effect of niacin on several blood parameters, niacin supplementation did not alter the blood concentrations of NEFA and glucose (Table 5). The rebound of NEFA blood concentrations after a period of NA application has also been observed in dairy cows, starting 2 to 3 h after abomasal infusion of NA (Pires and Grummer, 2007). A few studies reported a decreasing effect of NA supplementation to the diet on BHBA blood levels. The decrease of BHBA concentrations in response to NA was accompanied by a decrease in NEFA concentrations in the blood of primiparous cows in one study (Morey et al., 2011) and in another study in the blood of ketotic cows at the onset of lactation (Fronk and Schultz, 1979).

However, feeding trials investigating the effect of niacin supplementation to the diet of dairy cows did not provide consistent results due to the different design of the studies, e.g. the vitamer and dosage of the niacin supplement, the beginning and duration of the supplementation or the composition of the diet and the stage of lactation of the investigated

cows (Niehoff et al., 2009). It has been suggested that the response to NA supplementation might be greater in early rather than later stages of lactation (Jaster and Ward, 1990). Additionally, it is currently unknown how much absorption in the rumen or abomasum contributes to the total absorption of niacin (Niehoff et al., 2009).

1.3.3 Effects of modulating the dietary energy density in transition dairy cows

With the onset of lactation, the energy requirements of dairy cattle for milk production and maintenance increase considerably while the energy intake by voluntary feed intake does not increase at the same rate and thus dairy cows undergo a period of NEB (Drackley, 1999; Herdt, 2000). The formulation of diets that minimize this NEB, in particular in the first weeks p.p., not only improves milk yield, but also reduces the mobilization of body reserves and the risk of postparturient health problems (Drackley, 1999). One possible strategy to meet the energy required for milk production and thus reduce the extent of NEB is adaptation of the dietary energy density (Weiss and Pinos-Rodríguez, 2009).

The energy density of cows’ diet depends on its composition as well as its digestibility, including the ability of the cow to absorb the nutrients contained (Santos, 2011). Depending on the level of feed intake and retention time in the rumen, the diet’s energy density is a dynamic value in ruminants; e.g. an increased feed intake results in shorter rumen retention times and thus decreases the overall digestibility of feed (Santos, 2011). Therefore the energy density of the diet can be changed by changing its caloric density or by changing the digestibility of the diet through alteration of its components. For example, sources rich in fatty acids can be used to increase the caloric density of the diet and NFC can be used to increase the digestibility and passage rate of the diet fed (Rabelo et al., 2001; Drackley et al., 2005; Esposito et al., 2014). A usual diet of ruminants contains less than 3 % of fatty acids arising from forage, grains, or seeds that are rich in LA or linolenic acids. Standard concentrate feed contains a large amount of NFC, such as starch, provided by grains (e.g. corn, barley, sorghum, and canola), but also other nutrients such as fatty acids, protein, and neutral detergent fiber provided by whole or processed oilseeds (Santos, 2011). In addition, concentrates are formulated to provide the animals with minerals and vitamins, which are not abundant in forage. As forage is a more economical source of dietary energy and nutrients, the diet of dairy cows should contain the maximum level of forage and also ensure a sufficient supply of energy and nutrients (Miller, 1979). Some benefits of an increased energy density by increasing the concentrate portion containing NFC have been reported and the additional energy might help the dairy cow to overcome the NEB at the onset of lactation,

especially when the voluntary feed intake is reduced (Rabelo et al., 2003). However, forage is an important source of crude fiber and cows' diets should contain more than 18 % crude fiber of the DMI to ensure the stimulation of rumination and salivary secretion to buffer the effect of SCFA produced in the rumen (GfE, 2001; Santos, 2011). Thus, a certain amount of dietary fiber is required to avoid digestive disturbances such as ruminal acidosis, diarrhea, and displaced abomasum (Santos, 2011).

Due to the limited capacity of the rumen and the increased energy requirements at the beginning of lactation, the energy density of the diet is an important criterion for the formulation of the ration. This chapter describes the biological responses to changes of dietary energy density mainly through alteration of the forage-to-concentrate ratio in the pre-fresh and post-fresh transition diet of dairy cows.

1.3.3.1 Effects of modulating the energy density in the pre-fresh diet

Three weeks before calving, dairy cows experience a transient decrease in DMI by up to 30 % and the energy requirements to support the gravid uterus growth increase by about 20 % during this time. Therefore, increasing the energy density of the pre-fresh transition diet may help to maintain energy intake and to rectify the imbalance between energy requirements and dietary energy intake p.p. (Ingvartsen and Andersen, 2000; Huang et al., 2014). At least in multiparous cows, an increased energy density of the diets fed resulted in an increase of energy balance in the late a.p. period until the day of calving (Hayirli et al., 2002; Rabelo et al., 2003). Likewise, the magnitude of increase in energy intake energy due to higher energy density during the a.p. period is higher for multiparous than for primiparous animals (Rabelo et al., 2003). As reported in several studies, the more balanced the energy status is in animals fed a diet with higher energy density, the higher plasma glucose (Minor et al., 1998; Rabelo et al., 2005) and insulin concentrations that are reached (Holcomb et al., 2001; Rabelo et al., 2005). Less NEB also results in lower NEFA concentrations (Minor et al., 1998; Vandehaar et al., 1999; Holcomb et al., 2001; Doepel et al., 2002; Rabelo et al., 2005). In particular, the lower plasma NEFA concentrations indicate less mobilization of AT in animals fed a diet with increased energy density (Rabelo et al., 2005). The effects of the energy density on the hepatic content of TAG are not equivocally reported: In some studies no differences between animals fed a diet with high energy density *versus* animals fed a diet with lower energy density of liver TAG were reported (Minor et al., 1998; Rabelo et al., 2005). Some studies reported reduced liver TAG after calving in response to an increased energy density of the a.p. diet (Vandehaar et al., 1999; Doepel et al., 2002). However, the

energy density of the diet fed during the first 3 weeks after calving seems to have the biggest impact on the metabolic status p.p., whereas the a.p. diet's energy density has only minor effects on the p.p. metabolic status (Vandehaar et al., 1999; Dann et al., 2005; Rabelo et al., 2005).

On the other hand, excessive overfeeding of cows during the last month of gestation has negative effects on the performance in the following lactation. Overconditioning of dairy cows (150 % *versus* 100 % energy consumption of the calculated energy requirements) with the diet fed from 3 weeks before the anticipated calving date up to parturition resulted in decreased milk yield, milk lactose content and DMI in the first 4 weeks p.p., whereas the average EB remained unaffected in this period (Huang et al., 2014). In contrast, in another study cows fed a diet containing 150 % of the required energy had greater milk yield and a greater milk fat percentage, but also a lower DMI and milk lactose during the first 3 weeks of lactation than cows fed diets containing 100 % or 80 % of the required energy (Janovick and Drackley, 2010). Additionally, in overfed cows a greater energy deficit in the first weeks p.p. has been observed compared to cows limited to 100 % of energy requirements in the dry period, whereas the effects diminished as lactation proceeded (Dann et al., 2006; Janovick and Drackley, 2010). An explanation for the more severe NEB could be the sharp decrease of DMI shortly before parturition in the overconditioned animals (Dann et al., 2006; Janovick and Drackley, 2010). At the onset of lactation, the incidence of hyperketonemia is greater in cows fed a diet exceeding energy requirements than in cows fed a controlled-energy diet during the dry period (Mann et al., 2015). When cows consume more than 150 % of the calculated energy requirements provided by a diet with high energy density in the pre-fresh period the reproductive performance also declines, as reflected by a longer time interval between parturition and conception (Cardoso et al., 2013).

Beside the effect of different forage:concentrate ratios, an increased energy density of the a.p. diet through supplementation with plant oil (1.6 % of DMI as calcium salts of soybean oil *versus* no fatty acid supplement) decreased the DMI 3 weeks before calving (Karimian et al., 2015). Controversially, an increased diet energy density for the last 4 weeks prior to calving by supplementation with 6.5 % fatty acids compared to 3.3 % fatty acids based on DMI to cows' diet had no effect on a.p. DMI, body weight, glucose and NEFA concentrations (Afzalzadeh et al., 2010).

In summary, several studies have investigated the potential benefits (e.g. improved metabolic status p.p., reduced incidence of subclinical ketosis and improved reproductive performance) of consuming an energy-controlled diet that meets and does not exceed the

energy requirements during the 3 weeks before calving (Drackley, 1999; Dann et al., 2005; Janovick et al., 2011; Mann et al., 2015). However, feeding trials investigating the effect of the energy density of the pre-fresh diet on dairy cows provide inconsistent results due to different total energy consumption relative to their calculated energy requirements. Additionally, studies differ in the chosen strategy to enhance the energy density, e.g. an increased NFC portion in the diet or the supplementation of fatty acids to the diet. Further research is needed to clarify the discrepancy between the different studies.

1.3.3.2 Effects of modulating the energy density in the post-fresh diet

In common practice, cows are usually fed a diet containing smaller forage portions after calving for 3 to 4 weeks (Rabelo et al., 2003). During the first 3 weeks of lactation, a higher DMI, energy intake and milk yield have been observed when feeding a complete diet containing a higher portion of concentrate than a diet containing a lower portion of concentrate with separate feeding of concentrate and forage (Ingvartsen et al., 2001). An increased dietary energy density by increasing the portion of NFC (47 % *versus* 41 % NFC based on DM) immediately after calving also resulted in a higher energy intake and an increased rate of milk production compared to cows fed a diet with lower energy density (Rabelo et al., 2003). When changing over the diet of all cows to a diet high in energy density after 3 weeks of lactation, the differences in milk production and energy intake between the high and low energy density group disappeared (Rabelo et al., 2003). In addition, animals fed a diet with higher energy density had lower ruminal pH values and higher ruminal propionate concentrations in the third week of lactation (Rabelo et al., 2003). The animals fed with the diet higher in energy density had higher circulating glucose and insulin concentrations during the first 3 weeks of lactation, maybe due to the higher ruminal concentrations of propionate, a glucogenic precursor and a potent insulin secretagogue, that were observed, as suggested by Rabelo et al. (2005). With regard to hepatic lipid metabolism, the cows fed a diet with higher energy density for the first 3 weeks of lactation had lower hepatic TAG and lower blood BHBA concentrations than the cows fed a diet lower in energy density, whereas the blood concentrations of NEFA did not differ between the two groups (Rabelo et al., 2005).

On the other hand, an increased energy density of the p.p. diet by supplementation with soybean oil (1.7 % of calcium salts of soybean oil based on DM in the first 3 weeks of lactation *versus* no supplementation of fatty acids) decreased the DMI and the net energy intake and thus deteriorated the NEB in the animals receiving a diet supplemented with

soybean oil (Karimian et al., 2015). Moreover, the animals fed a diet supplemented with soybean oil 3 weeks after calving had higher plasma TAG concentrations, likely as a result of higher hepatic TAG production due to an increased absorption of fatty acids, compared to the animals fed a diet supplemented without fatty acids during this time (Karimian et al., 2015).

Discrepancies between studies may result from differences in the energy level of the diet, the digestibility of the dietary components and differences in the strategies chosen for increasing the energy density (Karimian et al., 2015). The effects of different dietary energy sources have been described for dairy cows (van Knegsel et al., 2005; van Knegsel et al., 2007b), in particular the effect of lipogenic (diet with a high fiber and/or fat content) *versus* glucogenic (diet with a high starch/NFC content) diets. Lipogenic nutrients originate from the microbial fermentation of fiber in the rumen mainly to acetate and butyrate, or from fatty acids (van Knegsel et al., 2007b). In contrast, glucogenic nutrients originate from starch that has escaped rumen degradation or gluconeogenesis and from propionate, as a major precursor for gluconeogenesis (van Knegsel et al., 2007b).

Several studies reported an improved EB of dairy cows in early lactation when fed a more glucogenic ration compared with an isocaloric, more lipogenic ration, as reflected by a higher calculated EB, lower plasma BHBA and NEFA concentrations as well as lower hepatic TAG content (van Knegsel et al., 2007b; van Knegsel et al., 2014; Chen et al., 2015). A possible reason for the improved EB in the transition period in response to a more glucogenic diet could be the reduced milk fat content and thus a reduced energy output via milk (van Knegsel et al., 2007a; van Knegsel et al., 2014). The contrasting results of studies increasing the energy density through higher portions of NFC in the ration (Rabelo et al., 2003; Rabelo et al., 2005) and those supplementing fatty acids (Karimian et al., 2015) are likely due to the fate (glucogenic or lipogenic) of the nutrients contributing to the dietary energy.

2 Objectives

As mentioned in the introduction, less is known about the regulation of the aforementioned genes expressing the receptors involved in nutrient and metabolic sensing in the AT of dairy cattle. In particular, the AT of dairy cattle at the onset of lactation can offer special insights into the expressional regulation of these receptors in response to a state of NEB, in which AT is mobilized to adapt to the high energy demands of milk production. However, AT from different sites, e.g. SC and VC, may respond differently to the metabolic and endocrine changes related to the onset of lactation. Thus, this thesis aimed:

1) to study the expression of selected GPCR involved in nutrient sensing (*FFAR 1*, *2* and *3* and *HCAR2*) in the AT of dairy cattle from late pregnancy up to the entire following lactation cycle (with special emphasis on the transition period), and
2) to compare the expression of these genes in AT from different SC and VC locations.

In modern dairy cattle husbandry, the welfare of the animals is of special interest and to minimize the incidence of diseases in the transition period due to metabolic stress, different nutritional strategies are used in practice, e.g. feeding of nutritional supplements or increasing the energy density of the post-fresh diet. This thesis also aimed to investigate whether there is an effect of

3) supplementing 100 g/d of rumen-protected CLA (1:1 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12) to the animals' diet from d 1 up to d 182 p.p., or
4) supplementing 24 g/d of non-rumen-protected NA to animals' diet from d 1 to d 21 p.p., or
5) feeding with diets with different energy density (diet with a 60:40 concentrate:roughage ratio *versus* diet with a 30:70 concentrate:roughage ratio on a DM basis) from d 1 to d 21 p.p., on the expression of *FFAR1*, *2* and *3* and *HCAR2* in SC and VC AT of dairy cattle.

Adipose tissue, as the major energy store, plays a central role in the regulation of metabolism at the onset of lactation. Therefore, understanding the expressional regulation of the GPCR investigated herein that are involved in nutrient and metabolic sensing under dynamic physiological changes is highly relevant.

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Energy and metabolic sensing G protein–coupled receptors during lactation-induced changes in energy balance

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Abstract

The free fatty acid receptor FFA1, FFA2, FFA3 and hydroxy-carboxylic acid receptor $(HCA)_2$ are G protein-coupled receptors, acting as energy and metabolic sensors. Herein, we characterized the tissue-specific *mRNA* abundance of genes encoding for these receptors at different stages of lactation. In addition, potential effects of supplementation with or without conjugated linoleic acids (CLA) were tested. Tissues from pluriparous cows (subcutaneous adipose tissue [SAT] and liver) and from primiparous cows (3 SAT locations, 3 visceral adipose tissues, liver, mammary gland and skeletal muscle) were used from 2 separate trials. In primiparous cows, the *mRNA* abundance of all receptors (FFA3 was not detectable by the applied protocol in muscle and udder) was lowest in muscle ($P < 0.05$). With exception of FFA1, gene expression of the investigated receptors was higher in adipose tissue than in the non-adipose tissue. Expression of FFA1 in liver ($P < 0.03$), of FFA2 in SAT ($P < 0.01$) and HCA_2 in SAT ($P < 0.01$) from pluriparous cows changed during the observation period (days -21 to 252 relative to parturition). The correlation between *mRNA* abundance of HCA_2 and peroxisome proliferator-activated receptor gamma (PPARG) and likewise PPARG2 ($P < 0.01$) in SAT indicates a link between HCA_2 and PPARG. Differences in receptor *mRNA* abundance between the CLA-fed and the control animals were scarce and limited to HCA_2 and FFA1 in 1 and 2 time points, respectively (less hepatic HCA_2 *mRNA* in CLA-fed pluriparous cows and greater FFA1 *mRNA* abundance in 2 visceral adipose depots in CLA-treated primiparous cows). In view of metabolic changes occurring during the different phases of lactation, in particular, the altered concentrations of nonesterified fatty acids and β-hydroxybutyrate acting as receptor ligands, the longitudinal tissue specific characterization provided herein allows a first insight into the regulation of these receptors at gene expression level.

Keywords: cow, lactation, transition period, free fatty acid receptor, hydroxy-carboxylic acid receptor, conjugated linoleic acid

Introduction

Lactation, as a highly successful reproductive strategy of mammalian species, requires partitioning of nutrients toward the mammary gland. Almost all mammals undergo a period of negative energy balance at the onset of lactation [1]; in dairy cows selected for high milk yields, the extent of the negative energy balance is particularly pronounced and may last for several weeks [2]. To meet the energy requirements for milk production, body reserves, mainly fat, need to be mobilized because the voluntary feed intake does not increase as fast as does milk production. In early lactating cows, lipolysis ensures the release of nonesterified fatty acids (NEFA) from adipose tissue (AT), which can then be used by other organs [3]. In this context, we herein considered particularly G protein–coupled receptors (GPR) whose activation by nutrients or is related to the secretion of hormones that are involved in metabolic regulation including lipolysis. A group of free fatty acid receptors, including FFA1, FFA2, and FFA3 previously known as GPR40, GPR43, and GPR41, respectively, are receptors for free fatty acids (FFAs) and enable FFAs to act as signal molecules [4]. Indeed these receptors share structural homology, but they differ concerning their agonists and physiological effects.Figure 1summarizes the different actions of the ligands via their corresponding receptors FFA1/2/3 and hydroxy-carboxylic acid receptor (HCA_2). FFA1 is a target for medium and long-chain fatty acids, whereby saturated and unsaturated FFAs are able to activate this receptor [5,6]. Activation of FFA1 amplifies glucose-stimulated insulin secretion (GSIS) and FFA1 is most abundant in insulin-producing pancreaticb-cells and is also expressed in other tissues. In contrast, FFA2 and 3 are both activated by short chain fatty acids with unequal affinities for ligands with different carbon chain lengths [4]. In adipocytes, activation of FFA2 has an inhibitory effect on lipolysis [7] and FFA3 activation stimulates leptin secretion [8]. Recently, Wang et al [9] identified the genes encoding for FFA2/3 on the bovine genome. The HCA_2 (previously termed GPR109A) is activated by niacin and b-hydroxybutyrate (BHB) was identified as an endogenous ligand of HCA_2. Besides its function as metabolic sensor suppressing lipolysis during starvation, HCA_2 is also an important target for a group of antilipolytic drugs and particularly expressed in adipocytes [10,11]. Studies in cell culture and rodents are indicating that conjugated linoleic acids (CLA) act as lipolytic agent [12]. However, the CLA effects in lactating cows are not entirely clarified yet [13]. Among others CLA is an agonist for FFA1, whereby both CLA isomers *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 were found to activate FFA1 [14].

We hypothesized that the FFAs receptor and HCA_2 are involved in the regulation of lipolysis and energy partitioning in dairy cows and that the *mRNA* expression of these receptors may

change depending on the metabolic changes occurring during the lactation cycle in a tissue-specific manner. Using biopsies from liver and subcutaneous fat of pluriparous cows, we aimed to characterize the longitudinal changes from 3 week antepartum until the 36th week of lactation; for assessing the target receptor *mRNA* in additional tissues, in particular visceral fat depots, samples were obtained from primiparous cows that were slaughtered on days 1, 42, or 105 of lactation. In view of CLA being able to activate FFA1, we also aimed to test whether dietary supplementation with CLA might affect the *mRNA* expression of FFA1 but also of FFA2, FFA3, and HCA_2 in a lactation state-specific manner.

Materials and Methods

Animals, sampling and treatments

Two trials were conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany, according to the European Community regulations concerning the protection of experimental animals. Authorization for both experiments was given by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany (File Numbers 33.14.42502-04-071/07 and 33.11.42502-04-071/07). In both trials, cows were fed a partial mixed ration for ad libitum consumption consisting of 37 % concentrate and 63 % silage based on dry matter content during the treatment period. In addition, 3.5 kg concentrate in trial 1 and 4 kg concentrate in trial 2 based on dry matter content were provided by computerized concentrate feeding stations (RIC; Insentec, B.V., Marknesse, Netherlands) in pelleted form. Supplemental, rumen-protected CLA (Lutrell pure; BASF SE, Ludwigshafen, Germany) were added to the pelleted concentrate provided by the concentrate station, containing 12 % each of the *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomer. Besides CLA, the supplement contained stearic, palmitic, and oleic acids. The concentrate fed to the control group contained a rumen-protected fat supplement (Silafat; BASF SE) in which CLA were substituted by stearic acid. Both supplements were fed at a level of 100 g/d during treatment periods. Water was available ad libitum. In both trials, cows were fed according to the recommendations of the German Society of Nutrition Physiology [15]. The cows were housed in group pens according to their feeding group. More details about trial 1 are published by Pappritz et al [16] and about trial 2 by von Soosten et al [17].

Trial 1

Pluriparous German Holstein cows (n = 21) were divided into a CLA (n = 11) and a control group (n = 10) at the day of calving. The respective supplements were fed from day 1 until day 182 postpartum (p.p.). From both feeding groups, biopsies were collected from subcutaneous AT (SAT; tail head fat) and from liver at days -21, 21, 105, 196, and 252 relative to calving.

Trial 2

From 25 primiparous German Holstein cows, 5 animals were slaughtered at day 1 p.p. and the remaining animals were allocated randomly to either CLA or control fat supplement starting from day 1 p.p. Five cows per group were slaughtered at days 42 and 105 p.p. Samples were collected from 3 visceral adipose tissues (VAT; omental, mesenterial, and retroperitoneal), 3 SAT (tail head, withers, and sternum), liver, Musculus semitendinosus, and mammary gland parenchyma after slaughter. The tissue samples from both trials were snap-frozen in liquid nitrogen and stored at -80°C until further processed for RNA extraction.

Determination of body condition score and back fat thickness, blood collection, analysis of NEFA and BHB

Body condition score and back fat thickness recordings, and blood sampling were assembled on the same days as the biopsies. As described earlier [16], body condition score of each animal was estimated using a 5-point system [18], using the average of two scores from different persons as assigned value. Ultrasonic measuring point of back fat thickness from each cow was at a line between the upper range of *tuber coxae* and *tuber ischiadicum* [19]. NEFA and BHB were measured in heparin plasma using an automatic analyzer (Cobas Mira Plus System from Roche Diagnostica Ltd, Basel, Switzerland) with the respective test kits (NEFA: HR(2) R1+R2 Set, WAKO Chemicals GmbH, Neuss, Germany; BHB: RANBUT, RB 1008, Randox Laboratories GmbH, Wülfrath, Germany).

Relative quantification of mRNA

The preparation of the samples including RNA extraction and complementary DNA (cDNA) synthesis are described in detail by Saremi et al [20]. The sequences of the primers used and their required conditions for quantitative PCR are provided in Table 1. All polymerase chain reaction products were confirmed by sequencing. Relative quantification of the target genes

using efficiency corrected data was performed with standard curves diluted from cDNA except in case of FFA3 in trial 2, where a dilution series based on purified amplicon was used. Efficiency corrected data were used for analysis. The reaction was performed in an Mx3000P (Stratagene, Amsterdam, the Netherlands and Agilent, Santa Clara, CA) with a total volume of 10 µL consisting of 2 µL cDNA (diluted 1:4) as template, 1 µL primer mix, 2 µL water, and 5 µL SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Nümbrecht, Germany).

Reference gene stability and data analysis in trial 1

To determine the most stably expressed genes for subsequent data normalization, a set of seven genes was tested separately for each tissue and their stability was evaluated with geNorm [21]. As final reference genes (RG), low density lipoprotein receptor–related protein 10 (LRP10), glyceraldehyde-phosphate-dehydrogenase (GAPDH), and RNA polymerase II (POLR2A) for SAT were used; for liver, eukaryotic translation initiation factor 3, subunit K (EIF3K), LRP10, and POLR2A appeared as the most stable genes [20].

Reference gene stability and data analysis in trial 2

The estimation of the RG was performed with qBASE^{plus} 2.0 (Biogazelle, Ghent, Belgium). Dependent of tissue or tissue combinations, a set of RG with the highest stability in target tissue(s) was selected for normalization. All subsequent calculations and data quality controls were done based on this software [22]. The details about the final RG in trial 2 are provided by Saremi et al [23]. Data are presented as ratios of the *mRNA* abundance of the genes of interest and the geometric mean of the corresponding RG.

Statistical analyses

The statistical analyses were performed with the software package SPSS 20.0 (SPSS Inc, Chicago, IL). Statistical significance was set at $P \leq 0.05$. In trial 1, we used Mann-Whitney *U* test for comparing the control vs the CLA group and Wilcoxon signed-rank test followed by Bonferroni correction to examine time-related changes. For correlation studies, Spearman rank correlation coefficient was calculated. In trial 2, the general linear model, with fixed effects for treatment, date, and the respective interaction were applied. In case of nonnormal distribution and inhomogeneous variances, nonparametric tests (Kruskal-Wallis or Mann-Whitney *U*) were used.

Results

Trial 1

The *mRNA* expression results from trial 1 are summarized in Figure 2. All significant correlation coefficients ($P < 0.01$) from the correlation studies in trial 1 are listed in Table 2. For all 3 genes measured in trial 1, the expression profiles between SAT and liver differed. The *mRNA* abundance of FFA1 in SAT showed no time-related changes during the lactation cycle. In contrast, a time effect with higher FFA1 *mRNA* abundance on 21 d before calving in comparison with 21 d after calving ($P < 0.03$) was observed in liver. The FFA2 *mRNA* abundance in SAT was higher at day 21 in comparison with day 105 after calving ($P < 0.01$). In liver, the *mRNA* abundance of FFA2 remained unchanged during lactation. The *mRNA* abundance of FFA3 in samples of this trial was not quantifiable. In SAT tissue, a time effect was evident with decreasing *mRNA* abundance of HCA_2 from 21 d before compared with day 105 ($P < 0.01$), 196 ($P < 0.005$), and 252 ($P < 0.01$) after parturition. The *mRNA* abundance of HCA_2 in liver showed no changes over time and was affected by CLA only at one time point, that is, on day 105 p.p., the *mRNA* abundance of HCA_2 was less in CLA treated cows than in the control cows.

Trial 2

In trial 2, the coefficients of correlation for comparisons between FFA1, FFA2, FFA3, and HCA_2 *mRNA* abundance were not significant at the level of $P < 0.01$ (data not shown).Table 3contains the *mRNA* abundances of FFA1, FFA2, FFA3, and HCA_2 in different tissues of primiparous cows supplemented with or without CLA; in case of absence of differences between CLA and control group, pooled data were used. Because, time and treatment effects only occurred in few cases, we pooled the data across time and treatment to provide a comparison of the *mRNA* abundances in the different tissues tested (Fig. 3). In general, the abundances of the receptors were highest in the AT depots, with exception of FFA1 that had the highest abundance in liver. Comparing SAT and VAT for FFA2 and FFA3 expression revealed higher *mRNA* abundance in the VAT depots.

For FFA1 *mRNA* abundance, we observed differences between CLA and control group in omental and retroperitoneal AT. In both tissues, greater values ($P < 0.05$) were recorded on day 105 and in retroperitoneal AT additionally on day 42 in the CLA group than in the control group. In the control group, the *mRNA* abundance in 2 VAT, that is, in mesenterial and retroperitoneal AT, showed time-related changes. The highest *mRNA* abundance of FFA1 was found in liver and the lowest in muscle. Among all AT, the FFA1 *mRNA*

abundance was highest in SAT from withers. In the mammary gland, FFA1 *mRNA* was not detectable with the protocol used herein.

Time-related changes in FFA2 *mRNA* abundance occurred in SAT from withers with lower *mRNA* abundance on day 1 in comparison with day 105. The *mRNA* abundance of FFA2 was highest in mesenterial and second highest in omental AT, both belonging to the VAT depots. We observed time effects in mesenterial, retroperitoneal AT, and SAT from withers with higher FFA3 *mRNA* abundance on day 1 vs day 105. In addition, the abundance in mesenterial AT and SAT from withers was higher on day 1 than on day 42. As mentioned before for trial 1 (SAT and liver), the *mRNA* encoding for FFA3 was also not quantifiable in liver, in mammary gland, and in muscle from trial 2 with the assay protocol we used. The FFA3 *mRNA* abundance was higher in the analyzed VAT, whereby retroperitoneal AT had the highest abundance. In SAT from sternum, the lowest values over all tissues were observed. In mammary gland and in liver, time-related changes were seen with higher HCA_2 *mRNA* abundance on day 1 in comparison with day 42 and 105 in mammary gland and an inverse time-effect in liver, respectively. The expression of HCA_2 was higher in the analyzed AT than in the non-AT, with lowest HCA_2 *mRNA* abundance in muscle.

Discussion

Regulation of FFA1, FFA2, and FFA3

We observed the highest abundance of FFA1 *mRNA* in liver. Up to now, physiological roles of FFA1 are only shown in pancreatic β-cells and enteroendocrine cells. In both cell types, activation of FFA1 leads to stimulation of the GSIS by pancreatic β-cells, whereby in response to FFAs, enteroendocrine cells stimulate GSIS indirectly by modulating the secretion of incretin hormones [24,25]. Besides, it was shown that glucagon secretion is in part dependent on FFA1 activation [26,27]. In humans, the *mRNA* abundance is very low in liver compared with AT and muscle [28]. Based on this observation, we speculate that the high *mRNA* abundance we detected in liver might be related to the peculiarities of ruminant physiology in general or the specific adaptation to pregnancy and lactation. We could not confirm with our data that FFA1 *mRNA* is present in the bovine mammary gland as reported by Yonezawa et al [29]; this discrepancy is possibly due to the limited amount of total RNA used for cDNA synthesis in our protocol.

It has already been reported that long-chain CLA isomers are able to activate FFA1 [14]. The abundance of the FFA1 receptor *mRNA* was increased by CLA treatment in trial 2, but this difference was limited to only 2 visceral AT depots (omental and retroperitoneal). A

study on primary cultured chicken hepatocytes provided evidence that linoleic acid increases FFA1 expression in vitro [30]. In these cells [30], it was shown that activation of FFA1 stimulated PPARα,β,δ signaling, a mechanism which might be involved in the upregulation of FFA1 by CLA in the 2 visceral AT.

As discussed before [4], shown also in cattle [9], and confirmed by our present data, the expression patterns of FFA2 are tissue specific. In addition, our data point to a regulation of the receptor *mRNA* during the peripartal period in SAT of tail head in trial 1 and in SAT of withers in primiparous cows in trial 2. Recent studies focusing on FFA2 demonstrate functions of this receptor in anti-inflammatory processes [31]. Functionality of bovine FFA2 was demonstrated in vitro [9]. In dairy heifers propionate, one of the main ligands of FFA2 induces granule release from neutrophils [32], but Yonezawa et al [33] were not able to confirm short-chain fatty acids signaling via FFA2 in contrast to FFA3 in bovine mammary epithelial cells. Therefore, mediation of short-chain fatty acids signaling in ruminants might be limited to specific tissues or cells and cannot be extended to all tissues. In mice, activation of FFA2 reduces lipolysis [34]. Therefore, the reduction of FFA2 *mRNA* from early to mid-lactation might be related to the observation of increasing basal lipolysis in pluriparous dairy cattle throughout lactation [35,36]. Contrary to this, the primiparous animals from trial 2 had less FFA2 *mRNA* around parturition as compared with mid-lactation only in SAT from withers, pointing to differential gene regulation between different adipose depots.

In contrast to previous studies [9], we detected FFA2 *mRNA* expression in bovine AT with greatest *mRNA* abundance in omental and the second highest in mesenterial AT. These AT are the only ones draining directly through the portal vein into the liver [37] and, therefore, these VAT may react more directly to stimulated lipolysis than SAT [38,39]. Thus, we assume that in consequence of lipolysis, AT derived metabolites of omental and mesenteric AT depots affect liver metabolism more potently than retroperitoneal AT and the SAT depots. The higher abundance of the FFA2 receptor *mRNA*, provided its translation to the functional protein, might be involved in limiting lipolysis especially from these 2 adipose depots. FFAs reduce hepatic insulin sensitivity as demonstrated in rats [40]. Therefore, the regulation of FFA2 expression might be important for mitigating the stimulated NEFA flux to the liver to support maintenance of insulin sensitivity. We observed lower *mRNA* expression of FFA2 in muscle vs liver, which has been reported in pigs before [41].

The FFA3 *mRNA* was present in all analyzed AT from trial 2. In humans, FFA3 expression was highest in AT [5]. However, obversefindings were also reported, that is absence of FFA3 *mRNA* in human AT or adipocytes [7] or bovine AT [9]. The detection of FFA3 *mRNA*

substantiates former studies from our group demonstrating FFA3 *mRNA* in bovine SAT and in differentiated bovine preadipocytes [42,43]. The higher FFA3 abundance in VAT vs SAT in sheep previously reported from our group [44] was herein confirmed for dairy cattle.
With this study we could not confirm the presence of FFA3 *mRNA* in mammary gland [29], which might be due to the assay as discussed for FFA1. Based on the greater FFA3 *mRNA* abundance in two VAT (mesenterial and retroperitoneal) and one SAT (withers) on day 1 compared to day 105 after parturition, FFA3 seems to be down-regulated from early to mid-lactation at least in discrete AT of primiparous cows.
The correlation between FFA1 and FFA2 *mRNA* abundances in SAT suggests concordant regulation of these receptors expression at least in pluriparous cows. The positive correlation of FFA2 *mRNA* in SAT with plasma NEFA concentration might indicate transcriptional regulation resulting in a negative feedback mechanism to down-regulate lipolysis, which may help to fine-tune lipolysis throughout lactation.

Regulation of HCA_2

We observed highest abundance of the HCA_2 *mRNA* in AT which is different from Titgemeyer et al [45]. In their study, notably more HCA_2 *mRNA* was observed in liver than in AT of steers. Nevertheless, these results were not supported by likewise measured HCA_2 protein abundance. We speculate that the differences between our results and the results of Titgemeyer et al [45] might be related to the different sex of the animals. However, similar to their results, we also found low abundance in muscle. Muscle, as a non-AT, is only of minor relevance in providing FFAs by lipolysis [46]. Generally, the lower HCA_2 *mRNA* abundance in the analyzed non-AT vs the analyzed AT may argue for a relatively higher physiological relevance of this receptor in AT. However, the only time-related changes of HCA_2 *mRNA* abundance in AT were observed in trial 1 with decreasing values from day 21 to day 105 relative to parturition. This decrease might partly counteract the increase of the circulating concentrations of BHB, the endogenous ligand of HCA_2 occurring during the transition from pregnancy to lactation and thus dampen the antilipolytic effects of BHB.
The function of HCA_2 in liver in which increasing *mRNA* abundance with time of lactation was observed (trial 2) is not entirely clarified yet. Li et al [47] provide first indications that HCA_2 is involved in reduction of hepatic cholesterol release. However, cholesterogenesis in liver is less important in ruminants when compared with AT and the intestine as shown in goats [48]. De novo synthesis of cholesterol might be crucial for milk synthesis, yet the

expression of the liver synthesis machinery is not activated until week 2 p.p.[49]and the functional interrelationship with hepatic HCA_2 expression remains to be further elucidated. Lemor et al [42] already reported a trend for decrease of HCA_2 *mRNA* in SAT during the transition from late pregnancy to early lactation. The present study supports decreasing values from late pregnancy to mid and late lactation. This corroborates that HCA_2 with its lipolysis adjusting impact seems to be relevant during the considered period, at least in SAT. Correlations between HCA_2 and peroxisome proliferator–activated receptor gamma (PPARG) *mRNA* abundance have been reported for murine epididymal AT recently [50]. Activation of the nuclear receptor PPARG improves, for example, insulin sensitivity and promotes AT differentiation [51]. Jeninga et al [52] provided evidence that PPARG directly regulates HCA_2 gene expression in murine adipocytes. Our findings suggest comparable regulation in the bovine [20]. The stronger positive correlation between PPARG and HCA_2 in SAT vs liver is in support of PPARG having a greater impact on the regulatory mechanism of the anti-lipolytic receptor HCA_2 in AT than in non-AT in dairy cows. The PPARG2 isoform is most abundant in adipocytes [53] and we also observed high correlation between PPARG2 and HCA_2 *mRNA* abundance in SAT; we thus assume that the regulatory link also applies for the PPARG2 isoform.

Conclusion

The present longitudinal and tissue-specific characterization of FFA1/2/3 and HCA_2 *mRNA* expression in dairy cows provides a first insight into their regulation at the level of transcription throughout lactation and the relation with the concomitant metabolic and hormonal changes, in particular with regard to fat metabolism. The correlation established between PPARG likewise PPARG2 and HCA2 points to a functional interplay particularly in AT. Nevertheless, our understanding of the physiological roles of FFA1/2/3 and HCA_2 in lactating cows but also in other physiological states and other species is just at the beginning.

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Table 1

Sequences of the primer and real-time polymerase chain reaction conditions used for quantification of the target genes

Gene	Forward Primer Sequence (5'-3') Reverse Primer Sequence (5'-3')	Acc. no.[a]	bp[b]	Con. (nM)[c]	Mean Cq[d]	Annealing (s/℃)[e]	Elongation (s)[f]
FFA1[g]	AATTCCACCAGCTCCTTGGGCAT GGCCGCCTTTAGCTTCCGTCT	XM_870502	213	800	31.4	60/60	60
FFA2[h]	CGCTCCTTAATTTCCTGCTG CAAAGGACCTGCGTACGACT	NM_001163784	174	800	29.9	60/60	60
FFA3[i]	ACCTGATGGCCCTGGTG GGACGTGAGATAGATGGTGG	NM_001145233	215	1200	28.7	40/60	30
HCA_2[j]	GGACAGCGGGCATCATCTC CCAGCGGAAGGCATCACAG	XM_002701703	140	200	27.6	30/61	30
PPARG[k]	AGGATGGGGTCCTCATATCC GCGTTGAACTTCACAGCAAA	Y12420	121	800	23.6	60/61	60
PPARG2[l]	ATTGGTGCGTTCCCAAGTTT GGCCAGTTCCGTTCAAAGAA	Y12420	57	400	22.0	60/60	60

[a] NCBI Accession Number.
[b] Base pairs.
[c] Concentrations for each primer.
[d] Mean quantification cycle from trial 1 and 2 (except FFA3 which was not quantifiable in trial 1).
[e] Initial denaturation for 10 min at 90℃; denaturat ion for 30 s at 95℃.
[f] Extension at 72℃.
[g] Free fatty acid receptor 1.
[h] Free fatty acid receptor 2 [43].
[i] Free fatty acid receptor 3.
[j] Hydroxy-carboxylic acid receptor 2 [43].
[k] Peroxisome proliferator-activated receptor gamma [43].
[l] Peroxisome proliferator-activated receptor gamma 2 [20].

Table 2

Coefficients of correlation (Spearman) for comparisons between FFA1, FFA2, and HCA_2 *mRNA* abundance in SAT and liver, PPARG/2 *mRNA* abundance in SAT as well as for comparisons of receptors *mRNA* with body condition and blood variables in pluriparous cows (trial 1)

Tissue		SAT			Liver		
	Gene	**FFA1**	**FFA2**	**HCA_2**	**FFA1**	**FFA2**	**HCA_2**
SAT	FFA1	---	0.478	0.323	---	ns	ns
	FFA2		---	0.314		---	ns
	HCA_2			---			---
	PPARG[a]	ns	ns	0.782	ns	ns	ns
	PPARG2[a]	ns	ns	0.712	ns	ns	0.294
Liver	FFA1	---	ns	0.350	---	0.456	ns
	FFA2		---	ns		---	0.382
	HCA_2			---			---
	PPARG[a]	ns	ns	ns	ns	ns	0.319
BCS[a]		ns	ns	0.435	ns	ns	ns
BFT[a]		ns	ns	0.429	ns	ns	ns
NEFA[a]		0.340	0.300	ns	ns	ns	ns
BHB[a]		ns	ns	ns	ns	ns	ns

Abbreviations: BCS, body condition score; BFT, back fat thickness; BHB, β-hydroxybutyrate; FFA, free fatty acid receptor; HCA, hydroxy-carboxylic acid receptor; ns, not significant; PPARG, peroxisome proliferator–activated receptor gamma; NEFA, non-esterified fatty acids; SAT, subcutaneous adipose tissue.

The table shows Spearman correlation coefficients ($P < 0.01$). Pooled data from the control group, the group supplemented with CLA, and all sampling dates were used for analysis.

[a] Data for PPARG, PPARG2, BCS, BFT, NEFA, and BHB were published earlier [16,20].

Table 3
FFA1, FFA2, FFA3 and HCA_2 *mRNA* abundance in different tissues of primiparous cows (trial 2) supplemented with or without CLA and sampled at day 1, 42 or 105 after parturition

D	TR	Tissue *mRNA* abundance (A.U.)								
		VAT omental	VAT mesenterial	VAT retroperitoneal	SAT tail head	SAT withers	SAT sternum	Muscle	Mammary gland tissue	Liver
FFA1										
1	---	0.96±0.40	0.56±0.05^{a}	0.68±0.16^{ab}	0.82±0.24	1.04±0.17	0.59±0.38	0.98±0.24	ND	0.96±0.31
42	CON	2.37±0.69	1.40±0.12^{b}	**1.27±0.22**^{a}	1.50±0.52	2.33±0.55	1.58±0.48	1.13±0.31		1.04±0.34
	CLA	1.38±0.29	1.18±0.30	**1.89±0.37**	1.32±0.39	1.91±0.42	1.00±0.34	0.72±0.06		1.14±0.30
105	CON	**0.80±0.26**	0.92±0.38^{ab}	**0.57±0.19**^{b}	0.84±0.35	2.04±0.92	2.24±0.61	1.46±0.35		1.19±0.18
	CLA	**1.77±0.09**	0.90±0.19	**1.45±0.33**	1.20±0.34	2.44±0.60	0.66±0.22	1.20±0.43		2.00±0.25
FFA2										
1	---	6.22±1.42	2.48±0.55	1.23±0.39	0.52±0.12	0.40±0.17^{a}	0.29±0.08	0.58±0.15	2.47±2.10	1.67±0.99
42	P	6.13±1.52	2.76±0.46	0.81±0.18	0.96±0.17	0.90±0.26^{ab}	0.72±0.20	0.79±0.21	1.22±0.29	1.11±0.15
105	P	3.19±0.81	2.01±0.39	0.52±0.12	0.70±0.16	1.06±0.21^{b}	0.76±0.12	1.49±0.24	1.78±0.52	1.20±0.20
FFA3										
1	---	1.96±0.61	3.16±0.68^{a}	5.33±0.79^{a}	1.70±0.47	1.47±0.22^{a}	0.61±0.11	ND	ND	ND
42	P	1.77±0.46	1.77±0.19^{b}	4.80±0.67^{a}	0.97±0.26	0.75±0.20^{b}	0.45±0.09			
105	P	1.10±0.47	0.84±0.22^{b}	1.73±0.41^{b}	0.93±0.34	0.40±0.09^{b}	0.35±0.16			
HCA_2										
1	---	0.75±0.15	1.09±0.30	1.67±0.41	1.66±0.20	3.22±0.89	0.97±0.18	1.47±0.35	5.98±3.09^{a}	0.43±0.12^{a}
42	P	1.45±0.33	0.82±0.16	1.81±0.26	1.62±0.49	1.81±0.57	1.12±0.30	0.92±0.21	0.72±0.18^{b}	1.04±0.18^{b}
105	P	1.02±0.27	0.97±0.21	1.81±0.29	1.43±0.23	2.37±0.77	1.05±0.28	0.57±0.12	0.65±0.11^{b}	1.41±0.41^{b}

Abbreviations: CLA, CLA group; CON, control group; D, days relative to calving; FFA, free fatty acid receptor; HCA, hydroxy-carboxylic acid receptor; ND, not detectable; SAT, subcutaneous adipose tissue; TR, treatment; VAT, visceral adipose tissue; P, pooled data from both groups.
The shown values are mean values (mean ± standard error of the mean). Significant differences ($P < 0.05$) between CON and CLA are depicted by bold values. If CLA and CON were not different, pooled data (P) are displayed. Significant differences between days per tissue are indicated by different superscript letters ($P < 0.05$). Data are normalized based on the geometric mean of Eukaryotic translation initiation factor 3 (EIF3K), Lipoprotein receptor–related protein 10 (LRP10), RNA polymerase II (POLR2A), Emerin (EMD), Marvel domain containing 1 (MARVELD1), and Hippocalcin-like 1 (HPCAL1) for each SAT and mesenterial VAT; EIF3K, LRP10, POLR2A, EMD, and MARVELD1 for omental and retroperitoneal VAT; HPCAL1, LRP10, POLR2A, EIF3K, glyceraldehyde-phosphate dehydrogenase (GAPDH) for liver; LRP10, EMD, POLR2A, EIF3K for muscle, and MARVELD1, EMD, LRP10, EIF3K, POLR2A, HPCAL1 for mammary gland tissue.

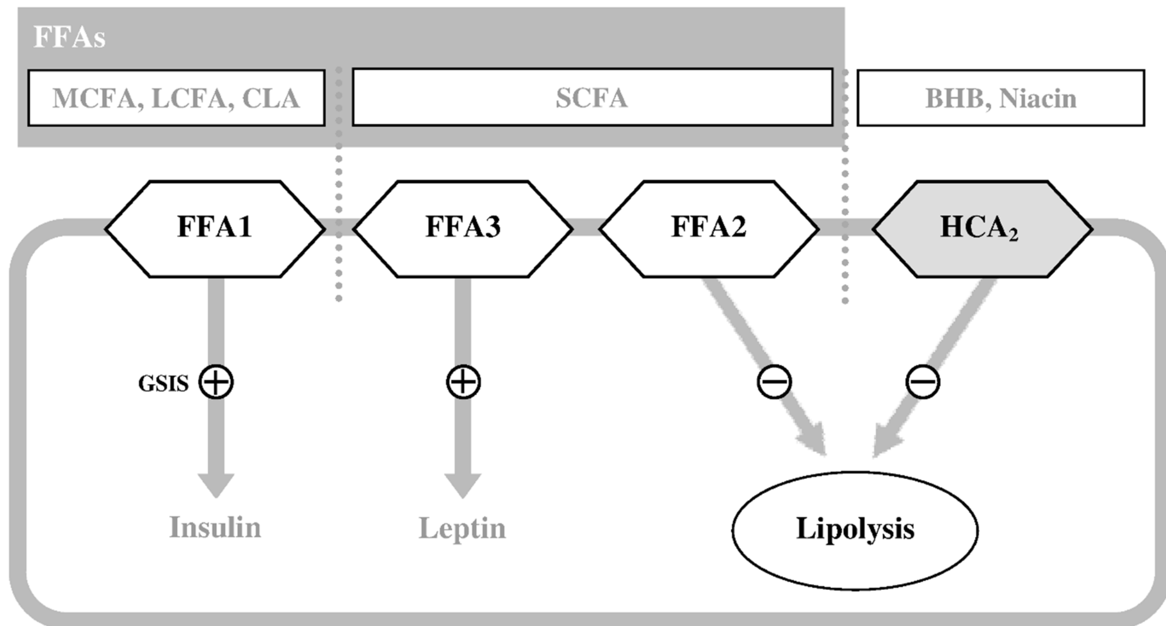

Fig. 1. Schematic model illustrating the presently known functions of the ligands via their corresponding receptors. BHB, b-hydroxybutyrate; CLA, conjugated linoleic acid; FFA, free fatty acids; FFA, free fatty acid receptor; GSIS, glucose-stimulated insulin secretion; HCA, hydroxy-carboxylic acid receptor; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; SCFA, short-chain fatty acids. Adapted from Stoddart et al [4], Brown et al [5,6], Hong et al [7], Xiong et al [8], and Offermanns et al [11].

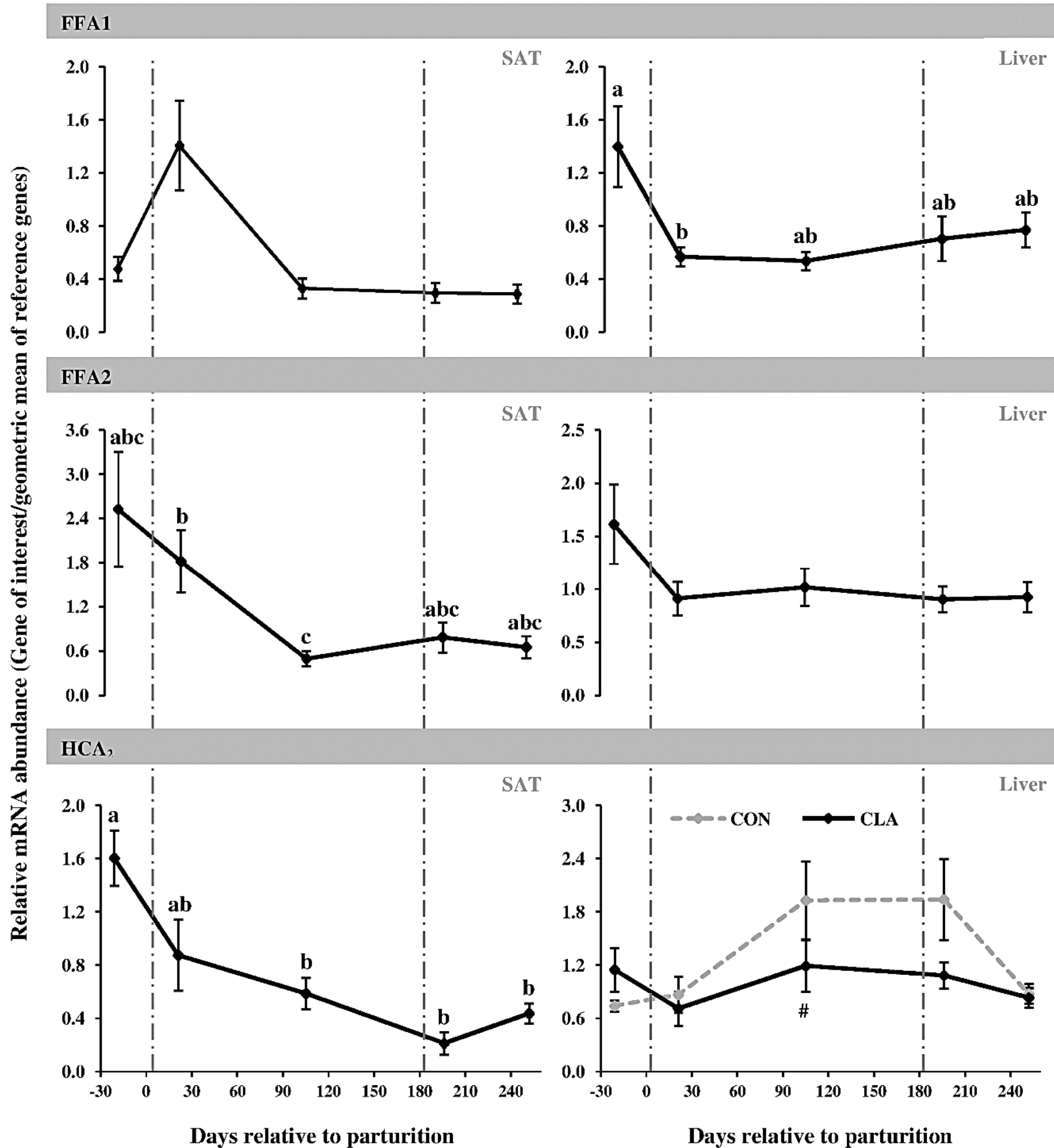

Fig. 2. Longitudinal *mRNA* expression of FFA1/2 and HCA_2 in SAT from tail head and liver from pluriparous cows (trial 1). Except for HCA_2 mRNA abundance in liver, pooled data from control and CLA group is shown (means ± standard error of the mean) because no CLA effect was observed. Different letters indicate significant differences between the sampling dates ($P < 0.05$). CLA effects are defined using number sign for time-matched comparisons ($P < 0.05$). The dashed lines indicate the supplementation period (from day 1 to 182 after parturition). CON, control group; CLA, group supplemented with conjugated linoleic acids; FFA, free fatty acid receptor; HCA, hydroxy-carboxylic acid receptor; SAT, subcutaneous adipose tissue.

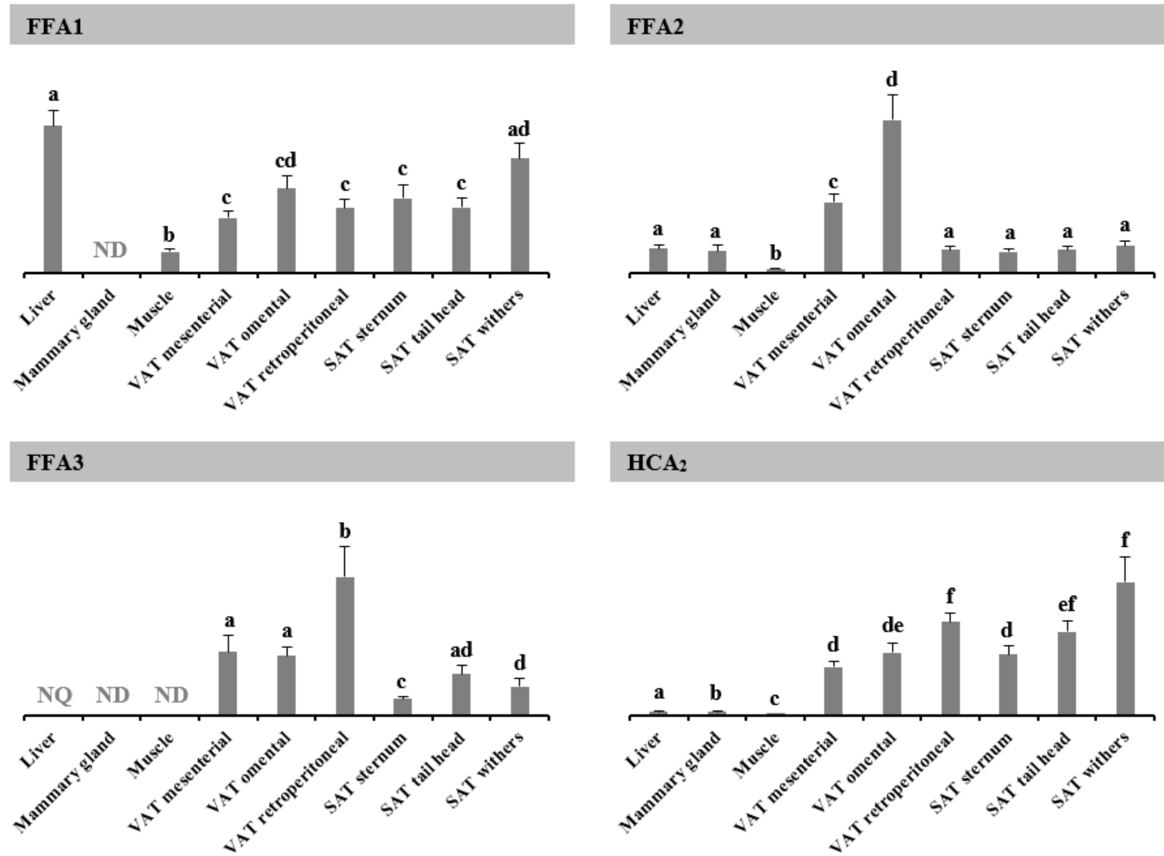

Fig. 3. Comparison of the *mRNA* abundance of FFA1/2/3 and HCA_2 in different tissues of primiparous cows (trial 2). Data were pooled from all groups and sampling dates (means ± standard error of the mean). Significant differences between tissues are indicated by different letters. Data are normalized based on the geometric mean of Eukaryotic translation initiation factor 3 (EIF3K), Lipoprotein receptor–related protein 10 (LRP10), RNA polymerase II (POLR2A), and Hippocalcin-like 1 (HPCAL1) for each FFA2 and HCA_2; EIF3K, LRP10, POLR2A, HPCAL1, and Emerin (EMD) for FFA1; EIF3K, LRP10, POLR2A, HPCAL1, EMD, and Marvel domain containing 1 for FFA3. FFA, free fatty acid receptor; HCA, hydroxy-carboxylic acid receptor; ND, not detectable; NQ, not quantifiable; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

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Expression of metabolic sensing receptors in adipose tissues of periparturient dairy cows with differing extent of negative energy balance

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Short title: Metabolic sensing receptors in dairy cows

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Abstract

We recently showed that the mRNA expression of genes encoding for specific nutrient sensing receptors, namely the free fatty acid receptors (FFAR) 1, 2, 3, and the hydroxycarboxylic acid receptor (HCAR) 2, undergo characteristic changes during the transition from late pregnancy to lactation in certain adipose tissues (AT) of dairy cows. We hypothesised that divergent energy intake achieved by feeding diets with either high or low portions of concentrate (60% v.30% concentrate on a dry matter basis) will alter the mRNA expression of *FFAR 1, 2, 3,* as well as *HCAR2* in subcutaneous (SCAT) and retroperitoneal AT (RPAT) of dairy cows in the first 3 weeks *postpartum* (*p.p.*). For this purpose, 20 multiparous German Holstein cows were allocated to either the high concentrate ration (HC, n=10) or the low concentrate ration (LC, n=10) from day 1 to 21 *p.p.* Serum samples and biopsies of SCAT (tail head) and RPAT (above the peritoneum) were obtained at day –21, 1 and 21 relative to parturition. The mRNA abundances were measured by quantitative PCR. The concentrations of short-chain fatty acid (SCFA) in serum were measured by gas chromatography-flame ionisation detector. The *FFAR1* and *FFAR2* mRNA abundance in RPAT was higher at day –21 compared to day 1. At day 21 *p.p.* the *FFAR2* mRNA abundance was 2.5-fold higher in RPAT of the LC animals compared to the HC cows. The *FFAR3* mRNA abundance tended to lower values in SCAT of the LC group at day 21. The *HCAR2* mRNA abundance was neither affected by time nor by feeding in both AT. On day 21 *p.p.* the HC group had 1.7-fold greater serum concentrations of propionic acid and lower concentrations of acetic acid (trend: 1.2-fold lower) compared with the LC group. Positive correlations between the mRNA abundance of *HCAR2* and peroxisome proliferator-activated receptorγ-2 (*PPARG2*) indicate a link between *HCAR2* and *PPARG2* in both AT. We observed an inverse regulation of *FFAR2* and *FFAR3* expression over time and both receptors also showed an inverse mRNA abundance as induced by different portions of concentrate. Thus, indicating divergent nutrient sensing of both receptors in AT during the transition period. We propose that the different manifestation of negative EB in both groups at day 21 after parturition affect at least *FFAR2* expression in RPAT.

Keywords: transition period, bovine adipose tissue, metabolic sensing

Implications

At the onset of lactation, the feed intake does not increase to the same extent as the energy requirements for milk synthesis resulting in a negative energy balance in high-yielding dairy cows. To cover the energy requirements of increasing milk synthesis, the mobilisation of energy from adipose tissue is crucial during the peripartal period. We studied the effects of two different concentrate portions in the feeding ration on the transcriptional regulation of four different nutrient sensing receptors, involved in adipose tissue metabolism. We used adipose tissue from a subcutaneous and a visceral localisation. The findings of our study may help to understand, in parts, how the energy density of the ration may affect the metabolism of adipose tissue due to the expression of the investigated receptors.

Introduction

One of the most challenging times for high-yielding dairy cows is the transition from late pregnancy to early lactation. The energy requirements in early lactation cannot be entirely met by voluntary feed intake and the animals therefore enter a state of negative energy balance (EB) during which lipolysis increases to provide non-esterified fatty acids (NEFA) from adipose tissue (AT) as energy substrates for other organs or precursors for milk fat synthesis (Drackley, 1999). In a previous study we observed differential expression of particular G protein-coupled receptors (GPCR) mRNAs, which are involved in energy and metabolic sensing, due to lactation induced changes in EB (Friedrichs et al., 2014). These GPCRs belong to the family of free fatty acid receptors (FFAR) 1, 2, and 3 (also known as GPCR40, GPCR43 and GPCR41, respectively). They enable free fatty acids (FFA), as their ligands, to act as signaling molecules (Stoddart et al., 2008). The FFAR1 is a target for saturated and unsaturated medium and long chain fatty acids (LCFA) (Brown et al., 2005). The FFAR1 is most abundant in insulin-producing pancreatic β-cells, but it is also expressed in other tissues; however, its physiological role in adipose is not clear. In contrast to FFAR1, the physiological functions of FFAR2 and FFAR3 have been identified and comprise an inhibition of lipolysis in adipocytes through activation of FFAR2 (Honget al., 2005) and increased leptin secretion by activation of FFAR3 (Xiong et al., 2004). The receptors FFAR2 and FFAR3 are activated by short-chain fatty acids (SCFA). In cattle the affinity of FFAR2 and FFAR3 for FFA is different to human or murine receptors with preference for FFA with a longer carbon backbone: FFAR2 displaying affinity C6 > C5 > C4 = C7 > C3 = C8 > C2 = C9 and the bovine FFAR3 displays no affinity for C1 (Hudson et al., 2012). Besides serving as major substrates for energy production in ruminants, SCFA have various other

regulatory effects (Bergman, 1990). They may increase blood insulin and glucagon concentrations in ruminants (Harmon, 1992), regulate gene expression in vitro (Li et al., 2007) and exert immune-modulatory effects; for example FFAR2 is involved in granule release from bovine neutrophils induced by propionate (Carretta et al., 2013). It is well documented that the energy density of the diet influences the microbial SCFA production in the rumen, for example a high energy content or high concentrate proportion in the diet increases the production of propionate (Rabelo et al., 2003). The hydroxycarboxylic acid receptor (HCAR) 2 (previously termed GPCR109A) is mainly expressed in adipocytes and can be activated by niacin and β-hydroxybutyrate (BHBA) as an endogenous ligand. Due to its function as metabolic sensor suppressing lipolysis during starvation, HCAR2 is an important target for a group of antilipolytic drugs (Offermanns et al., 2011). We recently reported that the HCAR2 ligand niacin stimulates the expression of HCAR2 in differentiated bovine preadipocytes in vitro (Kopp et al., 2014). Comparable to other mammalian species, the retroperitoneal AT (RPAT) from dairy cattle seems to have a higher lipolytic activity than subcutaneous AT (SCAT) due to the higher expression of hormone-sensitive lipase and the greater lipolytic response to adrenergic stimulation in RPAT (Locher et al., 2011; Kenéz et al., 2013). In dairy cattle two isoforms of the transcription factor peroxisome proliferator-activated receptor γ (PPARG), namely PPARG and its isoform PPARG-2 were described (Sundvold et al., 1997). Both members of the nuclear-receptor family bind various fatty acids and their activation is associated with the improvement of insulin sensitivity by increasing glucose and fatty acid uptake. The isoform PPARG2 is the leading isoform in adipose tissue and is involved in the regulation of adipocyte differentiation (Hammarstedt et al., 2005; Tyagi et al., 2011). The mRNA abundance of *PPARG* in mice and of both receptors in SCAT of dairy cows were shown to be positively linked with *HCAR2* (Wanders et al., 2012; Friedrichs et al., 2014).

Therefore, we hypothesised that differing portions of concentrate in and niacin supplementation to the diet of dairy cattle in the first 3 weeks *postpartum* (*p.p.*) will alter the expression of metabolic sensing receptors in two adipose depots. In this study we describe the mRNA expression of genes encoding *FFAR1, FFAR2, FFAR3* and *HCAR2* in a SCAT and RPAT of cows in the transition period as influenced by time and the diet fed *p.p.*

Material and methods

Animals, Feeding and Sample Collections

This study was conducted at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany. All animal experiments were conducted according to the European Community regulations concerning the protection of experimental animals and were approved by the Lower Saxony state office for consumer protection and food safety (LAVES, Oldenburg, Germany). The experimental design has been described in detail elsewhere (Locheret al., 2011). Briefly, 20 multiparous pregnant German Holstein cows, dried off 8 weeks before calving, were fed according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). On day 1 *p.p.* one half of the cows (n=10) was allocated to either the high-concentrate (HC) group receiving a diet with a 60 : 40 concentrate-to-roughage ratio (on a dry matter (DM) basis) or the low-concentrate (LC) group receiving a diet with a 30 : 70 concentrate-to-roughage ratio. The HC diet comprised 24% and 16% and the LC diet 42% and 28% corn and grass silage, respectively. One half of each dietary group also received as part of the pelletized concentrate 24 g powdered niacin per day which contained at least 99.5% nicotinic acid (Lonza Ltd, Basel, Switzerland). All diets were fed individually as a total mixed ration. The detailed composition of the diet as well as nutrient, fiber, and energy content of the different feed ingredients fed *p.p.* is provided in Supplementary Table S1 and elsewhere (Locher et al., 2011). Milk yield was recorded daily by a milk meter (Lemmer-Fullwood GmbH, Lohmar, Germany) and milk composition was analysed twice a week by a milk analyser based on Fourier transform infrared spectroscopy (Milkoscan FT 6000; Foss Electric, Hillerød, Denmark). The individual DM intake was recorded by a computerised feeding system (Insentec BV, Marknesse, the Netherlands). Data for milk yield, milk composition and DM intake were pooled for each week of lactation. Energy balance was calculated as follows: EB = NE_L intake – energy in milk – NE_M (GfE, 2001). On day –21, 1, and 21 relative to parturition, blood and AT samples were obtained. Blood samples were drawn from the jugular vein in the morning and centrifuged at 2000 × **g** for 10 min to separate serum and plasma. The AT samples were obtained by biopsy as described previously (Locher et al., 2011), whereby the SCAT samples were taken from the tail head region and the RPAT samples were taken directly above the peritoneum each time alternating from the left and right flank. The AT samples were snap-frozen in liquid nitrogen. The serum, plasma and AT samples were stored at −80°C until further processed.

Measurement of SCFA in serum

The concentrations of serum SCFA were determined according to the method described by Kristensen (2000). Briefly, 450 µl plasma was treated an internal standard solution (50 µl 2-ethyl butyrate, 75.45 µmol/10 ml), 700 µl 2-chloroethanol and 700 µl acetonitrile. Samples were centrifuged (15 min, 4°C, 3000 × **g**) and 1600 µl of the supernatant was combined with 20 µl 0.5 N NaOH and 1600 µl heptane. After mixing for 30 s, the aqueous phase (1300 µl) was removed and treated with 10 µl 37% HCl, 100 µl pyridine and 50 µl 2-chloroethyl chloroformate. After 5 min at RT, the reaction mixture was extracted with 2500 µl H_2O and 100 µl chloroform and the organic phase was separated after centrifugation (3 min, RT, 3000 × **g**). The organic phase was dried with Na_2SO_4 (15 mg) for 30 min and 1 µl injected into a gas chromatograph equipped with a flame ionisation detector (Series 17A; Shimadzu Corp., Kyoto, Japan). Separation was achieved on a free fatty acid phase column (length 25 m, internal diameter 0.25 mm, particle diameter 0.25 µm) (Kristensen, 2000).

Relative quantification of mRNA

After homogenisation of the AT samples with the Precellys®24 system (peQLab Biotechnology, Erlangen, Germany), total RNA was extracted from each sample using Trizol (Invitrogen, Karlsruhe, Germany) with a subsequent DNase (Qiagen, Hilden, Germany) treatment in solution and purified using spin columns (RNeasy® Mini Kit, Qiagen, Hilden, Germany). From 1 µg total RNA a reverse transcription with RevertAid™ (Fermentas, St. Leon-Rot, Germany) in a Multicycler PTC 200 (MJ Research, Watertown, MA) was performed resulting in an 80 µl cDNA reaction volume. The real-time PCR mixes, with a total volume of 10 µl consisting of 2 µl cDNA (diluted 1 : 4) as template, 1 µl primer mix, 2 µl water and 5 µl SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Nümbrecht, Germany), were performed in an Mx3000P (Agilent, Santa Clara, CA, USA) in three replicates. The sequences of the primers used and the conditions used in qPCR are provided in Table 1. All PCR products were confirmed by sequencing. Relative quantification of the target genes using efficiency corrected data was performed with standard curves diluted from cDNA except in case of *FFAR3*, for which a dilution series based on the purified amplicon was used. The qPCR efficiency of the target genes are also provided separately in Table 1 and those of the reference genes were in the range from 98.3% to 103.1%.

Reference gene stability and data analysis

To determine the most stably expressed genes for subsequent data normalisation, a set of seven genes was tested with qBASE^{plus} 2.0 (Biogazelle, Ghent, Belgium) separately for each tissue and for both tissues combined. Based on the stability of their expression as final reference genes, low density lipoprotein receptor-related protein 10, RNA Polymerase II and emerin were used for RPAT and comparison of both tissues; for SCAT, marvel domain containing 1 was additionally used for normalisation. Data are presented as ratio of the mRNA abundance of the gene of interest and the geometric mean of the corresponding reference genes. The characteristics of the primers and their real-time PCR conditions used for the reference genes are described elsewhere (Saremi et al., 2012).

Statistical analyses

The statistical analyses were performed with the software package SPSS (version 21.0, SPSS Inc., Chicago, IL, USA). All data are presented as arithmetic means ± SEM, significance was set at $P < 0.05$ and a trend was noted when $0.05 < P < 0.10$. Data were tested for normal distribution using the Kolmogorov–Smirnov test and homogeneity of variances was tested using the Levene's test. Accordingly, the general linear model or Mann–Whitney U test was used for comparing the HC *v.* the LC group or the groups with and without niacin supplementation. To test for differences between the sampling dates, the ANOVA or the Wilcoxon signed-rank test followed by Bonferroni correction was used. Parametric testing was performed for *FFAR1* mRNA abundance in RPAT and *HCAR2* mRNA abundance in both tissues. For correlation analyses, the Spearman's rank correlation coefficient (two-tailed) was calculated.

Results

Temporal effects

As shown in Figure 1, the mRNA abundance of *FFAR1* and *FFAR2* in SCAT remained unchanged during the transition period. In contrast, an effect of time with greater abundance of *FFAR1* and *FFAR2* mRNA on day −21 in comparison to day 1 relative to calving ($P < 0.01$) was observed in RPAT. The mRNA abundance of *FFAR3* was lower at day −21 compared to day 1 relative to calving in both tissues (SCAT: $P < 0.05$; RPAT: $P < 0.05$); in RPAT day −21 was also lower compared to day 21 *p.p.* ($P < 0.05$). The *HCAR2* mRNA was neither affected by time nor by treatment.

At day 1 after parturition propionic acid, n-butyric acid, n-valeric acid, and n-caproic acid were detectable albeit at low levels in <50% of the samples. For these SCFA we compared only day –21 with day 21 relative to calving (Figure 2). Except for n-butyric acid and n-valeric acid, all SCFA and total SCFA concentrations were lower on day –21 than on day 21; acetic acid ($P < 0.01$) and total SCFA ($P < 0.01$) in serum were also higher at day 21 compared with day 1 after parturition. The concentrations of n-butyric acid did not change with time and n-valeric acid was higher at the beginning compared to the end of the transition period ($P < 0.05$). Acetic acid formed the highest portion of total SCFA.

Dietary effects

In this study we found no effect of supplementing niacin on any of the variables investigated. For this reason, data of the subgroups (with or without niacin) were each pooled within the HC and the LC group for all further analyses.

With the exception of *FFAR2* in RPAT, we detected no differences between the HC and the LC group (Table 2); in RPAT the mRNA abundance of *FFAR2* was greater in the LC animals than in the HC cows at day 21 relative to calving ($P < 0.01$). The abundance of *FFAR3* mRNA tended to lower values in SCAT of LC *v*. HC animals ($P < 0.1$). On day 21 after calving the LC group, compared to the HC group, had 1.7-fold lower serum concentrations of propionic acid ($P < 0.01$) and 1.2-fold higher concentrations of acetic acid serum as a trend ($P < 0.01$; Figure 2). In Table 3 the performance data and the concentrations of particular and total SCFA in serum from day 21 *p.p.* are listed separately for the LC and the HC group.

Location effects

When comparing both tissues, we observed a trend for a lower *FFAR1* mRNA abundance in RPAT compared to SCAT at day 1 ($P < 0.1$) and 21 ($P < 0.1$) after parturition. The expression of *FFAR2* was not different between SCAT and RPAT at the different sampling dates. In RPAT, *FFAR3* mRNA abundance was greater compared to *FFAR3* mRNA abundance in SCAT at day –21 relative to calving ($P < 0.05$). Also HCAR2 mRNA abundance was higher in RPAT compared to SCAT at day –21 ($P < 0.05$) and also at day 21 ($P < 0.05$) relative to calving.

Results of the correlation studies

As summarised in Table 4 (*r* values reported in the table), in SCAT the mRNA abundance of *FFAR1* and *FFAR2* was positively correlated ($P < 0.01$), but not in RPAT. In both AT the mRNA abundance of *HCAR2* was positively correlated with *FFAR3* (SCAT: $P < 0.01$; RPAT: $P < 0.01$) and peroxisome proliferator-activated receptorγ-2 (*PPARG2*; SCAT: $P < 0.01$; RPAT: $P < 0.01$). The *HCAR2* mRNA abundance in SCAT was related to the one in RPAT ($P < 0.01$); HCAR2 and PPARG2 were interrelated among RPAT and SCAT ($P < 0.05$). The independent analysis of both feeding groups revealed partly higher correlation coefficients which was associated at least partly with the observation that the correlation between the receptor mRNAs exist only within the LC group. This was relevant for *FFAR3* compared to *FFAR1* ($r = -0.516$; $P < 0.05$) as well as compared to *FFAR2* ($r = -0.598$; $P < 0.01$), *HCAR2* and *FFAR1* ($r = -0.654$; $P < 0.01$) within SCAT. In RPAT *HCAR2* correlated with *FFAR2* only within the LC group ($r = 0.494$; $P < 0.01$) but with *FFAR3* only within the HC group ($r = 0.606$; $P < 0.01$).

We observed a positive correlation between *FFAR1* mRNA abundance in SCAT and n-caproic acid concentration in serum ($r = 0.322$; $P < 0.05$) and a negative correlation between *FFAR3* mRNA abundance in SCAT and n-butyric concentration in serum ($r = -0.369$; $P < 0.05$). The *FFAR2* mRNA abundance in SCAT was correlated with the glucose concentrations in serum ($r = 0.346$; $P < 0.01$). The target mRNA measured in RPAT showed no correlation with the measured SCFA at all. The *FFAR2* mRNA abundance in RPAT and the BHBA concentration in the circulation were correlated ($r = 0.307$; $P < 0.05$). In RPAT, the *FFAR3* mRNA abundance was negatively correlated with the triglyceride concentrations in serum ($r = -0.406$; $P < 0.01$) and positively with the ones of NEFA in serum ($r = 0.287$; $P < 0.05$), respectively. We also observed a negative correlation between *FFAR3* mRNA abundance in RPAT and EB ($r = -0.585$; $P < 0.05$).

Discussion

Changes in FFAR1 expression

Less information is available about the influence of dietary components or metabolites on *FFAR1* expression (Kebede et al., 2012). In the present study in dairy cows, the concentrate portion in the diet had no effect on the *FFAR1* mRNA expression in both AT depots. A recent study in mice showed that the digestibility of fibre affects *FFAR1* expression in AT, that is, soluble fibre compared to insolublefibre intake was accompanied with an increased SCFA production in the colon and was associated with increasing *FFAR1* mRNA abundance

in the epididymal AT (Isken et al., 2010). However, this link could not be confirmed with our results for dairy cows. The reasons for the downregulation of this receptor from late pregnancy to early lactation observed herein and its physiological consequences in RPAT remain unknown as does the function of this receptor in adipocytes. Generally, the *FFAR1* gene expression in AT is very low compared to pancreas and brain in humans (Itoh et al., 2003), and lower in AT compared to liver in cattle (Friedrichs et al., 2014). Therefore, the importance of the regulation of *FFAR1* mRNA or the corresponding protein should be verified in future using adequate models.

Changes in FFAR2 and FFAR3 expression related to SCFA content in blood

In differentiated murine adipocytes, acetic and propionic acid were demonstrated to stimulate the gene expression of *FFAR2 in vitro* and both fatty acids also affected adipogenesis and adipocyte differentiation (Hong et al., 2005). In the current study, the LC diet expectedly resulted in decreased concentrations of propionic acid in serum and in a trend for increased acetic acid concentrations. Concomitantly the mRNA abundance of *FFAR2* was increased in RPAT. Even though acetic acid is the most abundant SCFA in the circulation, the pEC_{50} potency values for the bovine FFAR2 and FFAR3 reported by Hudson et al. (2012) are in the millimolar range and let us assume that the circulating concentrations of acetic acid recorded in the cows of the present study were not able to activate either receptor. Based on their characterised affinities for SCFA (Hudson et al., 2012) we speculate that the plasma concentration of butyric acid may stimulate both receptors.

It remains open as to whether the observed changes of the abundance of *FFAR2* mRNA in RPAT were triggered by the altered circulating SCFA concentrations in the animals or not. An *in vitro* study on bovine adipose tissue explants showed no effect of propionic acid on *FFAR2* and *FFAR3* expression (Hosseini et al., 2012) and a recent study on human cell culture explants from omental AT showed no effect of neither acetic acid nor propionic acid on *FFAR2* expression during differentiation (Dewulf et al., 2013). The previously reported link between peroxisome proliferator-activated receptor γ (PPARG) and FFAR2 in mice (Dewulf et al., 2013) could not be confirmed by our study. Thus, the observed weak correlation between the mRNA abundance of *PPARG2*, the most prominent PPARG isoform in AT (Tyagi et al., 2011) and *FFAR2* in cattle, point to species specificity. As reported previously (Locher et al., 2011), animals from the LC group underwent a more negative EB in the third week of lactation, showed higher BHBA concentrations and had numerically higher NEFA concentrations (although there was no significant dietary effect), at day 21

after parturition, indicating a more extensive fat mobilisation in LC animals. Expressional up-regulation and activation of *FFAR2* in these animals seems to counteract extensive lipolysis in RPAT due to its role in inhibition of lipolysis in adipocytes (Hong et al., 2005) supported by the negative correlation between *FFAR2* mRNA abundance and EB. However, the herein observed decline in *FFAR2* mRNA abundance in RPAT from late pregnancy to the onset of lactation might be associated with the declining EB during this time period to reduce the inhibitory effects of FFAR2 on lipolysis and thus to enable the necessary *p.p.* catabolism. In previous works, we also observed no differences of *FFAR2* mRNA abundance in SCAT comparing day −21, 1 and 21 relative to calving, but differential expression between the different adipose depots (Friedrichs et al., 2014). The expression of *FFAR3* mRNA was inversely related to *FFAR2* mRNA with higher *FFAR3* mRNA abundance in late pregnancy compared to the onset of lactation in RPAT. Another indication for the inverse expressional regulation of both receptors is the detected trend with lower values for *FFAR3* mRNA abundance in the SCAT of animals from the LC group and the negative correlation between *FFAR2* and *FFAR3* mRNA abundance in SCAT. The negative correlation between *FFAR2* and *FFAR3* mRNA abundance in SCAT occurred solely in the LC group which might indicate a greater importance of these receptors in the animals that underwent a more severe negative EB.
Based on the higher responsiveness of RPAT *v.* SCAT towards lipolytic stimuli in cattle (Locher et al., 2011; Kenéz et al., 2013) and the herein observed changes with time and energy intake, the differential expression of *FFAR2* in RPAT suggests that energy sensing might be more important in RPAT than SCAT. In addition, the circulating concentrations of propionic acid achieved by the feeding regime using either high or low portions of concentrate were related to the mRNA abundance of *FFAR2* in RPAT, but not in SCAT, also indicating that metabolic sensing through FFAR2 to adapt lipolysis might be more important in RPAT than SCAT. We also provide evidence for an inverse differential expression of both receptors in RPAT of dairy cattle in the periparturient period. With the results for the correlation between *FFAR1* and *FFAR2* mRNA abundance we can confirm the previously suggested concordant regulation of these receptors expression in SCAT (Friedrichs et al., 2014).

Changes in HCAR2 expression

The circulating concentrations of BHBA, the endogenous ligand of HCAR2, were different between HC and LC animals, but the mRNA abundance of *HCAR2* in both AT was not

affected by the diet. In an *in vitro* study on bovine SCAT and RPAT explants the lack of BHBA-induced change on *HCAR2* expression has already been described, attributed the lack to the short duration of the treatment (Hosseini et al., 2012). Based on our previous and present results we speculate that BHBA is not regulating the transcription of *HCAR2* in bovine AT. However, in our study the *HCAR2* expression did not differ between the animals fed a diet supplemented with niacin compared to the animals fed a diet without niacin. A possible explanation for the missing effect could be the ruminal degradation of the niacin, as the supplement used herein was not rumen-protected.

In another study we found a decrease of the receptor mRNA abundance from late pregnancy (day −21) to mid- (day 105) and late-lactation (day 252 relative to calving), but also not in the time interval considered in the current study (Lemor et al., 2009; Friedrichs et al., 2014). We thus assume that the expressional regulation of *HCAR2* and the feedback of BHBA on lipolysis via this receptor are only relevant if BHBA concentrations are in a range observed during ketosis. Effects of high BHBA concentrations on lipolysis were shown by Kenéz et al. (2014) in vitro but not evident in the current study at least at sampling on day 21 *p.p.* Alternatively or in addition it might be that up-regulation is more relevant in stages of positive EB to limit lipolysis. Irrespective of time, in our study the mRNA abundance of *HCAR2* was higher in RPAT than in SCAT, whereas a recent report describes greater HCAR2 protein abundance in SCAT than RPAT (Kenéz et al., 2014). However, a study in rumen-fistulated Holstein steers showed no difference in HCAR2 protein as well as mRNA abundance between SCAT and RPAT, respectively (Titgemeyer et al., 2011).

A correlation between *HCAR2* and *PPARG2* in SCAT has been reported for cattle previously (Friedrichs et al., 2014) and this association can be extended to bovine RPAT based on our present results. Similarly, *HCAR2* was shown to be positively associated with *PPARG* in mice (Wanders et al., 2012). Feeding a high-fat diet reduced *HCAR2* expression in murine epididymal adipose tissue and correlated with a decline in *PPARG* expression (Wanders et al., 2010). In both AT investigated the mRNA abundance of *PPARG2* was not affected by the concentrate portion fed to the animals; considering the potential regulatory impact of PPARG on *HCAR2* expression, this might explain the unaltered *HCAR2* mRNA abundance. Thus we assume that *HCAR2* is regulated through PPARG rather than by its endogenous ligand BHBA. This assumption is in line with the study of Kopp et al. (2014) in which the mRNA abundance of *HCAR2* was not downregulated in bovine adipocytes in vitro by uncoupling of G-protein signalling using pertussis toxin.

Conclusions

Energy balance after parturition affects the mRNA abundance of *FFAR* an *HCAR2* in RPAT and SCAT differently. Based on our results divergent nutrient sensing by FFAR2 and FFAR3 in AT of dairy cows is indicated. The adjustment of lipolysis by *HCAR2* mRNA abundance may take place only in case of subclinical or clinical BHBA concentrations during the transition period, alternatively in case of positive EB to limit lipolysis.

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Table 1. Sequences of the primer and real-time PCR conditions used for the quantification of the target genes in the adipose tissue of dairy cows

Gene[1]	Forward Primer Sequence (5'-3') Reverse Primer Sequence (5'-3')	Acc. no.[2]	bp	Con. (nM)[3]	Mean Cq[4]	Annealing (s|°C)[5]	Elongation (s)[6]	Efficiency
FFAR1	AATTCCACCAGCTCCTTGGGCAT GGCCGCCTTTAGCTTCCGTCT	NM_001309646	213	800	35.7	60|60	60	100.2%
FFAR2	CGCTCCTTAATTTCCTGCTG CAAAGGACCTGCGTACGACT	NM_001163784	174	800	34.6	60|60	60	109.6%
FFAR3	ACCTGATGGCCCTGGTG GGACGTGAGATAGATGGTGG	NM_001145233	215	200	35.0	40|60	30	104.0%
HCAR2	GGACAGCGGGCATCATCTC CCAGCGGAAGGCATCACAG	XM_010823378	140	200	28.9	30|61	30	100.5%
PPARG2	ATTGGTGCGTTCCCAAGTTT GGCCAGTTCCGTTCAAAGAA	Y12420	57	400	26.2	60|60	60	108.0%

[1]FFAR1 = free fatty acid receptor 1 (Friedrichs *et al.*, 2014); FFAR2 = free fatty acid receptor 2 (Hosseini *et al.*, 2012); FFAR3 = free fatty acid receptor 3 (Friedrichs et al., 2014); HCAR2 = hydroxycarboxylic acid receptor 2 (Lemor et al., 2009); PPARG2 = peroxisome proliferator-activated receptor gamma 2 (Saremi et al., 2014).
[2]Acc. No. = NCBI Accession Number.
[3]Concentrations for each primer.
[4]Mean quantification cycle from SCAT and RPAT.
[5]Initial denaturation for 10 min at 90℃; denaturati on for 30 s at 95℃.
[6]Extension at 72℃.

Table 2. Relative tissue mRNA abundance of free fatty acid receptor 1, 2, 3, and hydroxycarboxylic acid receptor 2 in subcutaneous and retroperitoneal adipose tissue of dairy cows fed either high or low portions of concentrate

Gene[1]	d	F[2]	Relative tissue mRNA abundance[3]		SEM[4]
			SCAT	RPAT	
FFAR1	-21	-	1.18	1.17	0.20
	1	-	1.11	1.06	0.17
	21	HC	0.96	1.53	0.25
		LC	1.93	1.20	0.31
FFAR2	-21	-	1.12	1.53	0.13
	1	-	0.77	0.72	0.10
	21	HC	2.11	0.74*	0.34
		LC	0.98	1.85*	0.26
FFAR3	-21	-	0.64	0.73	0.12
	1	-	4.28	4.91	1.74
	21	HC	6.03†	3.16	1.25
		LC	1.20†	3.09	1.18
HCAR2	-21	-	1.43	1.59	0.15
	1	-	1.25	1.17	0.20
	21	HC	1.47	1.34	0.20
		LC	1.40	1.09	0.19

*Values within a column marked with a star differ significantly at $P<0.05$ between the HC and LC group at d 21 after parturition.
†Values within a column marked with a cross differ at $P<0.10$ (trend) between the HC and LC group at d 21 after parturition.
[1]FFAR1 = free fatty acid receptor 1; FFAR2 = free fatty acid receptor 2; FFAR3 = free fatty acid receptor 3; HCAR2 = hydroxycarboxylic acid receptor 2.
[2]F = feeding: HC = high concentrate group fed a diet with 60 : 40 concentrate-to-roughage ratio; LC = low concentrate group fed a diet with 30 : 70 concentrate-to-roughage ratio. Both diets were fed from d 1 to d 21 relative to calving.
[3]SCAT = s.c. adipose tissue; RPAT = retroperitoneal adipose tissue.
Given are means presented as ratios of the mRNA abundance of the gene of interest and the geometric mean of the corresponding reference genes. For SCAT, lipoprotein receptor-related protein 10, RNA Polymerase II, emerin and marvel domain containing 1 were used for normalization. For RPAT, lipoprotein receptor-related protein 10, RNA Polymerase II and emerin were used for normalization. The values for d 21 are shown separately concerning to feeding on a high concentrate vs. on a low concentrate diet.
[4]SEM = standard error of the mean. The pooled standard error of the mean relative mRNA abundance in SCAT and RPAT is given.

Table 3. Performance data and serum concentrations of metabolites and short chain fatty acids of dairy cows fed either high or low portions of concentrate.

Item	HC[1]	LC[2]	SEM[3]	*P*-value
DMI (kg/d)[4]	18.93[a]	16.32[b]	0.58	< 0.01
NE_L (MJ/d)[4]	141.7[a]	113.7[b]	4.14	< 0.01
Milk yield (kg/d)[4]	37.0	31.4	1.82	<0.10
Milk fat (%)[4]	4.3[a]	5.0[b]	0.19	< 0.05
Milk protein (%)[4]	3.4[a]	3.1[b]	0.07	< 0.05
ECM (kg/d)	40.6	37.9	2.39	0.44
EB (MJ/d)[4]	-15.3[a]	-33.7[b]	5.85	< 0.05
BHBA (mmol/L)[4]	0.47[a]	0.76[b]	0.08	< 0.05
Triglyceride (µmol/L)	0.13[a]	0.22[b]	0.03	< 0.05
NEFA (µmol/L)	674.5	848.8	170.79	0.29
Acetic acid (µmol/L)	710.5	841.2	52.15	< 0.10
Propionic acid (µmol/L)	40.9[a]	24.3[b]	3.38	< 0.01
n-Butyric acid (µmol/L)	32.2	31.0	1.78	0.66
n-Valeric acid (µmol/L)	21.9	18.4	1.54	0.14
n-Caproic acid (µmol/L)	32.5	32.7	2.28	0.95
Total SCFA (µmol/L)	867.2	957.5	54.63	0.26

[a,b]Values within a row with different superscript letters differ significantly at $P<0.05$ between the HC and LC group at d 21 after parturition or in the third week of lactation, respectively.
[1]HC = high concentrate group fed a diet with 60 : 40 concentrate-to-roughage ratio.
[2]LC = low concentrate group fed a diet with 30 : 70 concentrate-to-roughage ratio.
Both diets were fed from d 1 to d 21 relative to calving.
Given are means. Performance data are given as means for the third week of lactation; all variables assessed in serum were obtained on d 21 postpartum.
[3]SEM = standard error of the mean. The pooled standard error of the mean values from the HC group and LC group is given.
[4]Data has been published previously by Locher *et al.* (2011).

Table 4. Coefficients of correlation (Spearman) between relative mRNA abundance of receptors involved in nutrient sensing and peroxisome proliferator-activated receptor γ-2 in subcutaneous and retroperitoneal adipose tissue (SCAT and RPAT, respectively) of dairy cows

Tissue		**SCAT**				**RPAT**
	Gene[1]	***FFAR1***	***FFAR2***	***FFAR3***	***HCAR2***	***HCAR2***
SCAT	*FFAR1*	---	0.40^{**}	ns	-0.29^{*}	ns
	FFAR2		----	-0.38^{*}	-0.31^{*}	ns
	FFAR3			---	0.50^{**}	ns
	HCAR2				----	0.44^{**}
	PPARG2	-0.32^{*}	ns	ns	0.54^{**}	0.30^{*}
RPAT	*FFAR1*	ns	ns	ns	ns	ns
	FFAR2	ns	ns	ns	ns	ns
	FFAR3	ns	ns	ns	ns	0.46^{**}
	HCAR2	ns	ns	ns	0.44^{**}	-
	PPARG2	ns	ns	ns	ns	0.45^{**}

$^{**}P < 0.01$; $^{*}P < 0.05$.

[1]FFAR1 = free fatty acid receptor 1; FFAR2 = free fatty acid receptor 2; FFAR3 = free fatty acid receptor 3; HCAR2 = hydroxycarboxylic acid receptor 2; PPARG2 = peroxisome proliferator-activated receptor γ-2.

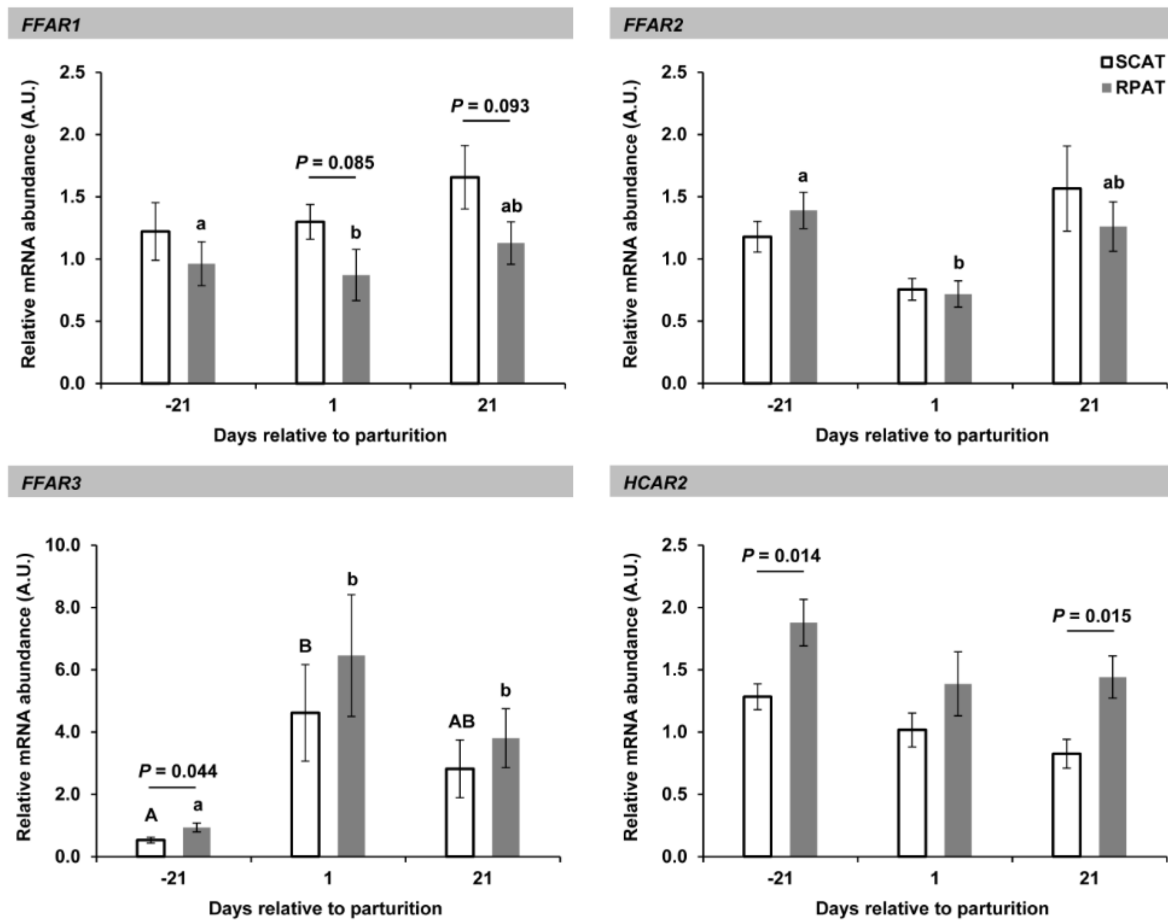

Fig. 1. Relative mRNA abundance of the free fatty acid receptor 1, 2, and 3 and hydroxycarboxylic acid receptor 2 at d -21, 1 and 21 relative to parturition in subcutaneous (SCAT, white bars) and retroperitoneal adipose tissue (RPAT, grey bars) of dairy cows. Pooled data from the cows fed either low or high portions of concentrate low are shown (means ± SEM) to compare the mRNA abundances between both AT. A line indicates a difference between the tissues with the corresponding *P*-value written above. Different small letters and different capital letters indicate significant differences in between the sampling dates in SCAT and in RPAT ($P < 0.05$), respectively. For normalisation enabling a comparison of both tissues, the ratio of the mRNA abundance of the gene of interest and the geometric mean of the mRNA abundance of low density lipoprotein receptor-related protein 10, RNA Polymerase II and emerin was used.

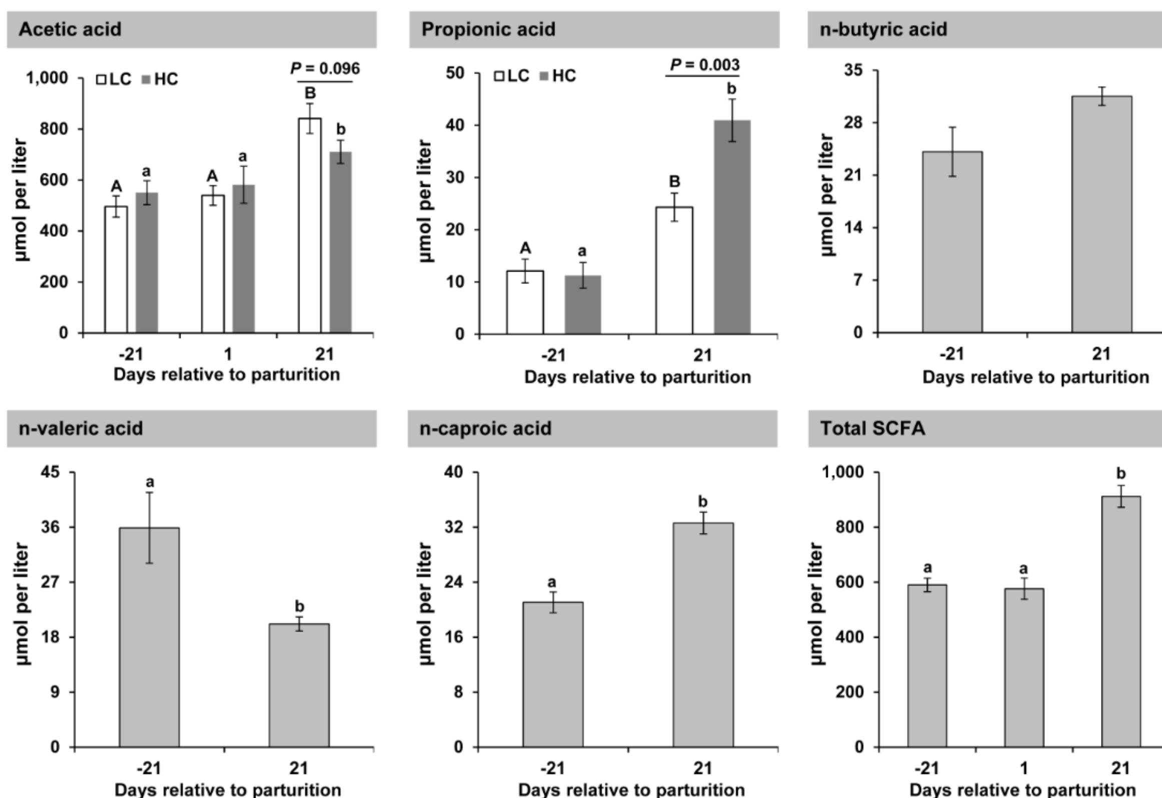

Fig. 2. Specific short-chain fatty acids (SCFA) and total SCFA in serum of dairy cows on day−21 and 21 relative to calving, for acetic acid and total SCFA additionally at day 1 relative to calving. Except for acetic and propionic acid, pooled data from cows fed either low (LC; fed a diet with 30 : 70 concentrate-to-roughage ratio; white bars) or high portions of concentrate (HC; fed a diet with 60 : 40 concentrate-to-roughage ratio; dark grey bars) are shown (means ± SEM; light grey bars), because no effect of different portions of concentrate in the diet were observed. Different letters indicate significant differences between the sampling dates ($P<0.05$), whereas in the first two graphs capital letters indicate differences in the LC group and small letters indicate differences in the HC group. The effects of different portions of concentrate in the diet are defined using a line with the corresponding P-value written above.

5 General discussion and conclusions

This thesis compiles the results from three conducted trials, of which two investigated the influence of supplementation with CLA (CLA-1 trial: multiparous cows from d -21 up to d 252 relative to calving; CLA-2 trial: primiparous cows from d 1 up to d 105 relative to calving) and one trial with a 2 x 2 factorial design investigated the influence of NA supplementation as well as the modulation of energy density of the post-fresh diet (NA trial: multiparous cows from d -21 up to d 21 relative to calving).

Changes during the transition from pregnancy to lactation

Except for *HCAR2*, the expression of all receptors studied herein changed in RP AT during the transition period, suggesting a role of these three fatty acid binding receptors in nutrient sensing during the transition period in RP AT. During the transition period, the expression of *HCAR2* changed neither in SC AT nor in RP AT, and among all receptors in SC AT only *FFAR3* mRNA was altered during this time. The two investigated receptors for SCFA, *FFAR2* and *FFAR3*, were inversely regulated during the transition period up to mid lactation in RP AT, whereas *FFAR2* seemed to be downregulated from late pregnancy to calving and afterwards upregulated. The *FFAR3* mRNA expression showed a *vice versa* regulation in RPAT. Both receptors and their downstream targets seem to exert different actions on lipid and glucose metabolism: Activation of FFAR2 has been shown to suppress lipolysis (Ge et al., 2008) and insulin signaling (Kimura et al., 2014); in contrast, FFAR3 stimulates the secretion of leptin (Xiong et al., 2004), a possible promoter of lipolysis (Rodríguez et al., 2003), and has been shown to enhance the insulin responsiveness (Han et al., 2014). The herein observed inverse regulation of both receptors' expression is possibly a complementary adaptation of adipocytes' metabolism. In consideration of their actions, the observed expressional changes of both receptors from late pregnancy to the onset of lactation were unexpected as they would account for an improved insulin signaling at least in RP AT at the beginning of lactation. However, the effect of FFAR2 and FFAR3 on the insulin signaling pathway may be small compared to their activity on lipid metabolism.

Another feature of the murine FFAR3 is its ability to interact with BHBA, albeit contradictory interactions indicating BHBA functions as antagonist (Kimura et al., 2011) or agonist (Won et al., 2013) for FFAR3 expressed by neurons were reported. The circulating concentrations of NEFA and in turn BHBA often increase as a result of intensive lipolysis due to the energy demands in early lactation. In this regard, reconnoitering the link between FFAR3 and BHBA would improve our understanding of these receptors' physiological role

in times of energy deficits. In considering the expression of *HCAR2*, an important target for BHBA, remaining unchanged during the transition period, the regulation of *FFAR3* seems even more interesting. However, in a short-term experiment with explants of SC AT (sternum) and RP AT, BHBA treatment had no effect on the mRNA abundance of either *FFAR3* or *HCAR2* in either tissue (Hosseini et al., 2012).

As already mentioned, the circulating NEFA concentrations normally increase with the onset of lactation and the downregulation of *FFAR1* mRNA in RP AT from -21 to 1 d relative to calving might be a feedback mechanism to avoid overstimulation of the FFAR1-associated signaling pathways by its ligands. Activation of the FFAR1 has been shown to decrease the hepatic lipid accumulation in mice fed a high-fat diet (Ou et al., 2014). Thus the observed decline of hepatic *FFAR1* mRNA abundance from d -21 to 21 relative to calving may account for a rise in lipid accumulation during this time in dairy cows.

Comparison of VC versus SC AT depots

The animals studied in the CLA-2 trial had 30.0 kg total AT as a sum of RP, omental, mesenteric, and SC adipose depots, accounting for 6.7 % of the empty body weight directly after calving (von Soosten et al., 2011). At 42 DIM the weight of the total AT showed an expected decrease to 23.0 kg (von Soosten et al., 2011). With regard to the distribution of weight among the investigated AT, omental AT had the most weight (7.4 to 11.0 kg) and the whole SC adipose fraction the least weight (3.0 to 4.9 kg) at 1, 42, and 105 DIM (von Soosten et al., 2011). Despite their different extents in dairy cows, the total mass of all VC AT studied was negatively correlated with the circulating ADIPOQ concentrations as already shown for humans (Cnop et al., 2003; Saito et al., 2012), whereas SC AT was not associated with the ADIPOQ concentrations in serum in cows (Singh et al., 2014a). In addition, the mass of each individual VC AT depot, mesenterial, omental, and RP AT, was negatively correlated with the ADIPOQ concentration in the serum of dairy cows (Singh et al., 2014a). Generally, *FFAR2* and *FFAR3* were more highly expressed in the VC AT than SC AT, suggesting a more important function of the VC AT in the sensing of SCFA by these receptors.

Among the different AT studied herein, the RP AT seems to have the most crucial role in allocating the energy. It seems that the RP AT reacts most sensitively to the advancement of lactation, as it was the only AT studied in the CLA-2 trial with a decrease of its depot weight when comparing the first day in milk (DIM) with 42 DIM and 105 DIM (von Soosten et al., 2011). Moreover, RP AT was the only fat depot showing alterations in adipocyte sizes related to the duration of the lactation. The adipocyte size in RP AT of the animals that

received a diet supplemented with a control fat (mainly containing stearic acid) was 1.2-fold smaller at 105 DIM than at 1 DIM (Akter et al., 2011). The decreased depot weight and the decreased adipocyte size limited to RP AT suggest a preferential mobilization of this AT (Akter et al., 2011).

A further unique feature of RP AT seems to be its low concentration of ADIPOQ, an adipokine suppressing lipolysis (Tao et al., 2014). Among all the bovine AT investigated, RP AT had the lowest ADIPOQ concentration and, considering the wet tissue weight, the lowest amount of total ADIPOQ (Singh et al., 2014a). One of the receptors studied in this work, the *FFAR3*, showed the highest gene expression in RP AT and an activation of FFAR3 stimulates the secretion of leptin (Xiong et al., 2004), which in turn has been shown to exert lipolytic effects on the AT of rodents. This is supported by observations by Saremi et al. (2014), showing the numerically highest expression of leptin and its receptor in RP AT in the animals of the CLA-2 trial. Both, low ADIPOQ concentrations and high leptin concentrations in RP AT, would also account for a higher lipolytic rate in this depot based on the paracrine effects of leptin and ADIPOQ.

The studies available from monogastric species comparing VC AT from other different sites with RP AT are limited to rats. Compared to mesenteric VC AT, RP AT from rats has larger adipocyte sizes and higher expression of lipolytic enzymes, such as *HSL* and adipose TAG lipase (Palou et al., 2009). In the same animal cohort, *PPAR-γ2* and *SREBP1* expression, two transcription factors relevant in lipogenesis, decreased rapidly after 4 h of fasting and adipose TAG lipase increased after 24 h of fasting in RP AT, but not in mesenteric AT (Palou et al., 2010).

Influence of CLA supplementation to the diet

The animal experiments studied herein confirmed the reported reduction of milk fat by CLA. In the primiparous animals from the CLA-2 trial the milk fat percentage decreased by 14 % from week 1 to week 6 of lactation, but only after 28 DIM in the group that received 100 g/d of rumen-protected CLA (providing 6.0 g/d of the *t*10,*c*12 CLA and 5.7 g/d of the *c*9,*t*11 CLA) compared to the group that received an isocaloric control fat supplement (von Soosten et al., 2011). In the period from week 6 to week 26 of lactation, the milk fat percentage was 12 % lower in the multiparous animals from the CLA-2 trial within the CLA group than in the control group (Pappritz et al., 2011b). Surprisingly, both experiments resulted in different alterations of the milk fatty acid pattern. In the multiparous animals (CLA-1 trial), CLA supplementation reduced significantly the portion of C16:0 and C16:1 in total in milk

(Pappritz et al., 2011b). In the primiparous animals (CLA-2 trial), the decrease in milk fat was characterized by a significant decrease in single *de novo* synthesized fatty acids (C8:0, C10:0 and C12:0) as well as C16:0 over the whole supplementation period (von Soosten et al., 2013). These disaccording observations might be due to the different duration of supplementation (26 weeks *versus* 15 weeks). Possibly, the decrease of *de novo* synthesized fatty acids does not appear in later stages of lactation and thus overlap the alteration in mid lactation as the data for the supplementation period have been pooled for analysis. Another possible explanation could be an effect of parity. However, the proportion of *t*10,*c*12 CLA in the milk of multiparous animals (CLA-1 trial) was slightly increased in the CLA group, indicating a transfer to the mammary gland and milk fat throughout the supplementation period, whereas the portion of *c*9,*t*11 CLA in milk remained unaffected (Pappritz et al., 2011b). Investigations of the mammary gland imply a transfer of both isomers towards the mammary gland, whereas transfer efficiency seems to be less than 0.1 % of the consumed total CLA (von Soosten et al., 2013). At 42 and 105 DIM the *PPAR-γ2* mRNA abundance in the mammary gland was lower in the animals treated with CLA than in the cows receiving the control fat supplement (Saremi et al., 2014). As fatty acids cannot bind to SREBP1, this transcription factor regulates parts of the MFD caused by the *t*10,*c*12 CLA isomer indirectly (Kadegowda et al., 2009). In contrast, PPAR-γ is a target for CLA and *t*10,*c*12 CLA has been shown to inhibit *PPAR-γ* expression and its downstream genes regulating lipogenesis (Kadegowda et al., 2013). Thus an involvement of PPAR-γ in the mammary lipid metabolism seems to be likely.

In the multiparous animals (CLA-1 trial), the CLA supplementation to the diet also decreased the mRNA abundance of *PPAR-γ* at 105 DIM in the liver of CLA cows compared to the controls (Saremi et al., 2014). The mRNA abundance of *PPAR-γ* decreased in liver tissue of the cows receiving a diet supplemented with CLA even beyond the supplementation period at 196 DIM, and the same was evident for *PPAR-γ2* in SCAT (Saremi et al., 2014). The results of our correlation studies indicate of an expressional link between *PPAR-γ* and *HCAR2* in the liver (CLA-1 trial) as well as *PPAR-γ2* and *HCAR2* in the AT (CLA-1 trial and NA trial) of cows. In accordance with the decreased *PPAR-γ* expression, we observed herein a decreased mRNA of *HCAR2* in the liver of the CLA animals in the CLA-1 trial. However, we did not observe a CLA effect on the expression of *HCAR2* in SC AT from the tail head of the multiparous animals, even if the *PPAR-γ2* expression had been decreased by supplementation of CLA to the diet. Differences in the mRNA abundance of the investigated receptors in the AT of CLA-fed animals and in the AT of control animals were scarce and

limited to *FFAR1*. We observed higher expression of *FFAR1* in omental and RP AT of the primiparous animals fed a diet supplemented with CLA at 105 DIM and in RP AT additionally at 42 DIM (CLA-2 trial). Up to now, the specific functions of FFAR1 in adipocytes or AT have been unknown. Possibly, there is an effect of a single nucleotide polymorphism (SNP) of the *FFAR1* gene region on adipocytes' lipid metabolism reflected by different fasting plasma NEFA concentrations in human subjects with different SNP of the *FFAR1* gene (Walker et al., 2011). Another recent study reported a decreased hepatic lipid accumulation accompanied with a decreased hepatic mRNA expression of lipogenesis-related proteins as well as *SREBP1* mRNA expression in response to the activation of FFAR1 in mice (Ou et al., 2014). It has been shown that CLA are potent ligands of the murine FFAR1 (Schmidt et al., 2011). The observed expressional upregulation in two VC AT in response to CLA could in turn result in a decreased expression of *SREBP1*, a decreased rate of lipogenesis and reduced lipid accumulation. Indeed, a study in human adipocytes provided evidence of a suppression of transcription factors (e.g. SREBP1 and PPAR-γ) by the *t*10,*c*12 CLA isomer, but not the *c*9,*t*11 CLA, which resulted in a decreased *de novo* lipid synthesis followed by a decreased lipid accumulation (Obsen et al., 2012). Nonetheless, the involvement of FFAR1 was not investigated in this study (Obsen et al., 2012). In consideration of FFAR1 acting as a metabolic sensor for the extracellular availability of LCFA in AT, a response to a higher availability of its ligands with a decreased synthesis of fatty acids would be reasonable. Stearic acid is also a ligand of FFAR1 (Itoh et al., 2003) and was used in the control fat supplement to replace CLA for providing isoenergetic rations. Thus an effect of the control fat supplement could not be excluded, but seems unlikely as it binds with 100-fold less affinity than linoleic acids at least to the human and rodent FFAR1 (Itoh et al., 2003). However, the available data do not allow at present a conclusive appraisal; further research is needed to identify the role of FFAR1 in adipocytes. Furthermore, the role of FFAR4, another receptor for LCFA highly expressed in AT and a possible target for CLA (Oh et al., 2010; Shen et al., 2013), should be investigated.

A possible explanation for the limited CLA effects on AT observed herein may be the very low transfer efficiency of supplemented *t*10,*c*12 CLA into the AT fractions of the cows at 105 DIM after consuming a CLA-supplemented diet. Approximately 0.03 to 0.06 % of total consumed *t*10,*c*12 CLA was transferred to the single VC AT depots and 0.01 % of total consumed *t*10,*c*12 CLA to the whole SC AT fraction (von Soosten et al., 2013). Due to an increased incorporation of the *c*9,*t*11 and the *t*10,*c*12 CLA isomer in adipocyte membrane phospholipids of obese rats in response to CLA supplementation, the fluidity and

permeability of the adipose membranes have been shown to be reduced (Martins et al., 2010). However, further research is needed to point out whether the membrane incorporation of the CLA isomers supports the CLA-mediated effects by altering the properties of the membranes.

Influence of nicotinic acid supplementation to the diet and rumen-protection

The supplementation of NA to the diet of dairy cows neither influenced cows' performance, nor the concentration of blood metabolites or hormones (Locher et al., 2011) nor the mRNA abundance of the receptors studied herein (NA trial). Conceivably, the adequacy of the supplemented NA in a non-rumen-protected form is questionable, as this form has a poor stability in the rumen because of its microbial degradation (Erickson et al., 1991) and thus approximately only 5 % of the supplemented NA reaches the duodenum, where it is mainly absorbed (Santschi et al., 2005). A more effective form of NA supplementation, if orally applied, is provided by rumen-protected, encapsulated supplements containing NA surrounded by several layers of lipids, which are insoluble in the rumen and thus prevent microbial degradation of the NA (Morey et al., 2011). Results from other studies investigating the effect of niacin supplementation on blood NA concentrations are not consistent, whereas blood NAM concentrations seem to increase in response to niacin supply (Niehoff et al., 2009).

However, the CLA supplement used herein is thought to be rumen-protected, and in spite of lipid encapsulation of the CLA in the supplement, just a small portion (5–8 %) escaped ruminal biohydrogenation and only 0.4 g of the daily consumed 8 g *t*10,*c*12 CLA reached the duodenum (Pappritz et al., 2011a).

Influence of modulating the dietary energy density

The concentrate fed to the animals in the post-fresh diet (NA trial) mostly consisted of wheat grain (50 %), soybean meal (27 %) and corn (21 %) and thus provided more NFC to the animals than the fed roughage. As estimated, the concentrate provided more metabolizable energy (12.9 to 13.1 MJ/kg of DM) than both silages (10.7 to 10.8 MJ/kg of DM) fed to the multiparous animals (Locher et al., 2011).

The increased energy density directly after calving resulted in a higher DMI, an increased milk protein content, and a decreased milk fat content in the animals fed the high-concentrate (HC; 60:40 concentrate:roughage ratio) diet compared to the animals fed the low-concentrate (LC; (HC; 30:70 concentrate:roughage ratio) diet (Locher et al., 2011). Additionally, the HC

animals tended toward a higher milk yield in the third week of lactation compared to the LC animals (Locher et al., 2011). Due to the higher DMI, the higher metabolizable energy of the concentrate and the lower milk fat content, the animals fed the HC diet underwent a less negative EB (−15.3 ± 6.0 MJ/d *versus* −33.8 ± 7.7 MJ/d) during this time (Locher et al., 2011). The TAG and BHBA blood concentrations were also lower in the animals fed the HC diet than in the animals fed the LC diet at d 21 after calving, whereas NEFA concentrations were numerically lower without reaching the level of significance (Locher et al., 2011). This is in accordance with the observations of Rabelo et al. (2005) regarding lipid metabolism in a study investigating the effect of different portions of NFC (47 % *versus* 41 % NFC based on DM). The multiparous cows fed a diet with increased dietary energy density immediately after calving for the first 3 weeks of lactation had lower hepatic TAG and lower blood BHBA concentrations than the multiparous cows fed a diet lower in energy density, whereas the blood concentrations of NEFA did not differ between the two groups (Rabelo et al., 2005). The lower BHBA and TAG could reflect a lower mobilization of AT as the energy demands at the onset of lactation are more adequately covered by the energy provided by the diet higher in energy density. In contrast to Rabelo et al. (2005), who observed higher glucose and insulin concentrations in the animals fed the diet higher in energy density, neither of the portions of concentrate resulted in different glucose and insulin concentrations in the animals studied in the NA trial (Locher et al., 2011). The animals fed with a diet containing a lower portion of concentrate had a higher *FFAR2* mRNA abundance in RP AT and tended to have a lower *FFAR3* mRNA abundance in SC AT at 21 d after calving. As already mentioned, activation of *FFAR2* by SCFA mediates the suppression of lipolysis (Hong et al., 2005; Ge et al., 2008). In addition, activation of the receptor also suppresses insulin signaling, leading to the inhibition of fat accumulation in AT and promotion of a lipid and glucose utilization in muscle tissue (Kimura et al., 2014). A higher suppression of the insulin signaling leads to higher rates of lipolysis and decreased rates of fatty acid and TAG synthesis as well as decreased TAG and glucose uptake (Dimitriadis et al., 2011). However, as insulin is known to decrease lipolysis (Dimitriadis et al., 2011), further studies need to investigate the discrepancy between the demonstrated suppression of lipolysis and the demonstrated suppression of insulin signaling by activation of FFAR2 in AT.

In conclusion, the effects of the different nutritional strategies on the expression of the nutrient sensing receptors investigated herein were rare or, in case of the NA, absent. The effects of CLA were limited to the mRNA abundance of *FFAR1* in omental and RP AT. The

effects of different energy densities of the diet fed post-fresh were limited to the expression of *FFAR2* in RP AT. The observed effects indicate a higher expressional responsiveness of VC AT to nutritional factors compared to SC AT of dairy cows, at least at the molecular level. Another hint regarding the higher responsiveness of RP AT compared to SC AT to the energy status is provided by the observed expressional changes of *FFAR1* and *FFAR2* just in RP AT and not in SC AT in the transition period. In contrast, the expression of *FFAR3* showed alterations in SC and RP AT. The expressional regulation of *HCAR2* in AT was not influenced either by the different applied nutritional strategies or by the progress of the transition period. This points to a higher relevance of metabolites and nutrients in the expressional regulation of the investigated *FFAR* than *HCAR2* in the AT of dairy cows, at least in the current studies. However, a better understanding of the mechanisms and actions of FFAR activation would help to identify the consequences of signal transduction over these receptors for the whole metabolism.

6 Summary

With the onset of lactation, the energy requirements of dairy cattle for milk production and maintenance increase considerably while the energy intake by voluntary feed intake does not, thus resulting in a negative energy balance (NEB). The adaption to the requirements of lactation is often related to metabolic disorders, reduced fertility, lameness and various infectious diseases in the periparturient period accompanied with restrictions in the effectiveness of milk production. To minimize the disease incidence during this time, different management and nutritional strategies are used in practice, e.g. feeding of nutritional supplements or increasing the energy content in the diet. Adipose tissue (AT) is an important energy store. Therefore, the physiological processes and changes in this organ are of particular importance during this period of comprehensive NEB. Due to their ability to sense nutrient and energy availability and accordingly adapt cells' metabolism, a pivotal role in the coordination of metabolic processes is taken by nutrient sensing G protein-coupled receptors (GPCR). However, there has been very little investigation on the regulation of mRNA expression of nutrient sensing GPCR in the AT of dairy cattle. Thus, the aim of this thesis was to study the mRNA expression of selected GPCR involved in nutrient sensing (free fatty acid receptors [*FFAR*] *1*, *2*, and *3*, and hydroxycarboxylic acid receptor [*HCAR*] *2*) in the AT of dairy cattle in the transition period (d -21 to d 21 relative to calving), and to compare the expression of these genes in AT from different subcutaneous (SC) and visceral (VC) locations. The main focus of this thesis was to study potential effects on the mRNA expression of the aforementioned receptors of different nutritional strategies, namely supplementing conjugated linoleic acids (CLA) or nicotinic acid (NA) to the diet of the dairy cows as well as feeding diets with different energy densities. The mRNA abundance of *FFAR 1*, *2*, and *3*, and *HCAR2* was quantified by qPCR.

In **Manuscript 1**, the possible changes in mRNA expression of *FFAR 1*, *2*, and *3*, and *HCAR2* depending on the metabolic changes occurring during the lactation cycle and on supplementing CLA to the diet of dairy cows were tested in AT from different SC and VC locations. For this purpose two separate feeding trials (CLA-1 and CLA-2 trial) were conducted. In both trials, German Holstein cows were allocated to a CLA group receiving 100 g/d of a rumen-protected CLA supplement (1:1 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12) or a control group receiving 100 g/d of a control supplement (in which CLA was substituted by stearic acid) on the day of calving. The CLA-1 trial was carried out in 21 multiparous cows (CLA group: n = 11; control group: n = 10) and the respective supplements

were fed from d 1 until d 182 after calving. From both feeding groups, biopsies were collected from SC AT from the tail head region at d -21, 21, 105, 196, and 252 relative to calving. In the CLA-2 trial, from 25 primiparous cows, 5 animals were slaughtered at d 1 and the remaining animals were allocated randomly to either CLA (n = 10) or control supplement (n = 10) starting from day 1 after calving. Five cows per group were slaughtered at d 42 and 105 p.p. Samples were collected from 3 VC AT (omental, mesenterial, and retroperitoneal [RP]) and 3 SC AT (tail head, withers, and sternum) after slaughter. In the CLA-1 trial, the *FFAR2* mRNA abundance in SC AT was higher at d 21 than at d 105 after calving. A time effect was also evident for *HCAR2* expression with decreasing mRNA abundance of *HCAR2* from 21 d to d 105 after parturition. The observed correlation between mRNA abundance of *HCAR2* and peroxisome proliferator-activated receptor (*PPAR*)-*γ* likewise *PPAR-γ2* in SC AT indicates a link between *HCAR2* and *PPAR-γ* gene expression. In the CLA-2 trial, a comparison of SC AT and VC AT revealed a higher mRNA abundance of *FFAR2* and *FFAR3* in the VC AT depots, pointing to a higher adaptability of the VC AT to metabolite and nutrient availability compared to SC AT. Furthermore, the findings of this study substantiate the presence of *FFAR2* and *FFAR3* mRNA in the AT of dairy cows. In the CLA-2 trial, we observed differences between the CLA and the control group in omental and RP AT, with higher values of *FFAR1* mRNA abundance on d 105 and in RP AT additionally on d 42 in the CLA group than in the control group. However, to interpret such effects, further studies are needed to identify the role of FFAR1 in adipocytes.

In **Manuscript 2**, the mRNA expression of genes encoding for *FFAR1*, *FFAR2*, *FFAR3*, and *HCAR2* in SC AT and VC AT of cows in the transition period as influenced by time and NA supplementation to the diet or divergent portions of concentrate in the diet was described. For this aim, 20 multiparous German Holstein cows were allocated either to a high-concentrate (HC) group receiving a diet with a 60:40 concentrate:roughage ratio (on a dry matter basis; n = 10) or a low-concentrate (LC) group receiving a diet with a 30:70 concentrate:roughage ratio (n = 10) on d 1 p.p. One half of each group received 24 g of non-rumen-protected NA (NA trial). On d -21, d 1, and d 21 relative to parturition, serum samples were taken and AT were obtained by biopsy, with SC AT samples being taken from the tail head region and RP AT samples being taken directly above the peritoneum each time alternating from the left and right flank. In serum the short-chain fatty acids (SCFA) were quantified via GC-FID. In this study we found no effect of supplementing NA on the receptors' mRNA investigated. Possibly as a mechanism to counteract extensive lipolysis in

RP AT, the mRNA abundance of *FFAR2* in RP AT was higher in the LC than in the HC cows at d 21 after calving. In addition, the abundance of *FFAR3* mRNA tended to lower values in SC AT of LC *versus* HC animals. In RP AT, an effect of time with greater abundance of *FFAR1* and *FFAR2* mRNA on d -21 in comparison to d 1 relative to calving was observed. The mRNA abundance of *FFAR3* was lower at d -21 than at d 1 relative to calving in SC and RP AT, and in RP AT additionally lower than at to 21 d after calving. The mRNA abundance of *FFAR3* changed inversely as compared to *FFAR2* in RP AT, a finding that is further supported by the negative correlation between *FFAR3* and *FFAR2* in SC AT. The observed inverse regulation of *FFAR2* and *FFAR3* in RP AT might indicate that the signal transduction exert different physiological effects between the two receptors. Based on the potency values for the different SCFA found in the literature for the bovine FFAR2 and FFAR3, we assume that both receptors are activated by the measured concentrations of butyric acid rather than by acetic acid in the circulation. In this study, the *HCAR2* mRNA abundance was neither affected by treatment nor by time. Thus, we speculate that the expressional regulation of *HCAR2* is more relevant in times of positive energy balance for limiting lipolysis. The results from correlation studies provide further hints of an expressional regulation of *HCAR2* over *PPAR-γ2* in SC AT, but also in RP AT of cows.

Besides providing information about the longitudinal mRNA expression of certain GPCR that are involved in nutrient sensing in AT from different locations, the present thesis contributes some initial insights into the adjustments of these modulatory factors in response to different nutritional strategies. Moreover, the dissertation serves as a basis for further studies exploring the role of FFAR1 in adipocytes as well as the role of FFAR2 and FFAR3 in lipid metabolism. Further studies should also investigate other nutrient sensing GPCR, such as FFAR4, and include the expression of the investigated receptors in AT at the protein level. Identification of FFAR participation in different pathways, not only in AT, could help to identify the importance and consequences of signal transduction via these receptors for the whole metabolism in cattle and in other species.

7 Zusammenfassung

Mit beginnender Laktation, steigt der Energiebedarf von Milchkühen für die Milchproduktion deutlich an, wohingegen die Energiezufuhr über die freiwillige Futteraufnahme nur langsam ansteigt, was eine negative Energiebilanz (NEB) zur Folge hat. Die Anpassung an die Anforderungen der Laktation geht daher häufig mit Stoffwechselstörungen, verminderter Fruchtbarkeit, Lahmheit und verschiedenen Infektionskrankheiten im peripartalen Zeitraum einher, was wiederum zu einer Minderung der Effektivität der Milchproduktion führt. Um die Erkrankungsinzidenz während dieser Zeit zu minimieren, finden verschiedene Management- und Fütterungsstrategien, wie z.B. der Einsatz von Nahrungssupplementen oder die Erhöhung des Energiegehaltes der Futterration, in der Praxis Anwendung. Da das Fettgewebe (AT) ein wichtiger Energiespeicher ist, sind die physiologischen Prozesse und Veränderungen in diesem Organ in Zeiten einer ausgeprägten NEB von besonderer Bedeutung. Aufgrund ihrer Fähigkeit den Zellstoffwechsel der Verfügbarkeit von Nährstoffen und Metaboliten im Energiestoffwechsel anzupassen, nehmen *nutrient sensing* G-Protein-gekoppelte Rezeptoren (GPCR) eine zentrale bei der Koordinierung von Stoffwechselprozessen ein. Bislang liegen nur wenige Studien zur Untersuchung der Regulation der mRNA-Expression von *nutrient sensing* GPCR im AT von Milchkühen vor. Ein Ziel der vorliegenden Arbeit war daher die Untersuchung der mRNA-Expression ausgewählter *nutrient sensing* GPCR, der freie Fettsäuren-bindenden Rezeptoren (*FFAR*) *1*, *-2*, *-3*, und des Hydroxycarbon-säuren-bindenden Rezeptors (*HCAR*) *2*, im AT von Milchkühen in der Transitphase (d 21 *ante partum* [a.p.] bis d 21 *post partum* [p.p.]). Zudem wurde die Expression dieser Gene im AT unterschiedlicher subkutaner (SC) und viszeraler (VC) Lokalisationen verglichen. Das Hauptaugenmerk dieser Arbeit lag jedoch auf der Untersuchung des Einflusses verschiedener Fütterungsstrategien auf die mRNA-Expression der oben genannten Rezeptoren, wobei ein möglicher Einfluss einer Supplementation von konjugierten Linolsäuren (CLA), einer Supplementation von Nikotinsäure (NA) oder einer Fütterung von Rationen mit unterschiedlicher Energiedichte untersucht wurde. Die mRNA-Menge von *FFAR1*, *-2*, *-3* und *HCAR2* wurde mittels qPCR bestimmt.

In **Manuskript 1**, wurden mögliche Einflüsse der Stoffwechselveränderungen während der Laktation und der Einfluss einer Supplementation von CLA auf die *FFAR1*, *-2* , *-3* und *HCAR2* mRNA-Expression im AT von Milchkühen verschiedener SC und VC Lokalisationen untersucht. Zu diesem Zweck wurden zwei separate Fütterungsversuche

durchgeführt (CLA 1- und CLA 2-Versuch). In beiden Versuchen wurden Holstein-Frisean-Milchrinder entweder einer CLA-Gruppe, in welcher die einzelnen Tiere 100 g/d eines pansengeschützten CLA-Supplements (1:1-Mischung von *cis*-9,*trans*-11 und *trans*-10,*cis*-12) erhielten, oder einer Kontrollgruppe, in welcher die Tiere 100 g/d eines Kontrollsupplements (in dem CLA wurde durch Stearinsäure ersetzt wurde) erhielten, zugeteilt. Alle Supplemente wurden ab dem Tag der Abkalbung gefüttert. Der CLA 1-Versuch wurde an 21 pluriparen Kühen (CLA-Gruppe: n=11; Kontrollgruppe: n=10) durchgeführt, wobei die entsprechenden Supplemente von d 1 bis d 182 p.p. gefüttert wurden. Von beiden Gruppen wurden Biopsate an d 21 a.p., sowie an d 21, 105, 196 und 252 p.p. vom SC AT aus der Region um den Schwanzansatz entnommen. Im CLA 2-Versuch wurden von 25 primiparen Kühe, fünf Tiere an d 1 p.p. geschlachtet, den verbleibenden 20 Tieren wurde von d 1 p.p. an entweder das CLA- (n=10) oder das Kontrollsupplement (n=10) verabreicht. An d 42 p.p. bzw. d 105 p.p. wurden je fünf Kühe pro Gruppe geschlachtet, wobei Gewebeproben aus drei VC Fettdepots (omental, mesenterial und retroperitoneal [RP]) und drei SC Fettdepots (vom Schwanzansatz, vom Brustbein, vom Widerrist) entnommen wurden. Im CLA 1-Versuch war die *FFAR2* mRNA-Menge in SC AT höher an d 21 p.p. im Vergleich zu d 105 p.p. Die *HCAR2* mRNA-Menge zeigte einen Abfall im Verlauf der Laktation mit niedrigerer mRNA-Menge an d 105 p.p. im Vergleich zu d 21 p.p. Die Korrelation zwischen der mRNA-Menge von *HCAR2* und der mRNA-Menge von dem Peroxisom-Proliferator-aktivierten Rezeptor (*PPAR*)-*γ*, sowie dessen Isoform *PPAR-γ2*, im SC AT deutet auf einen regulatorischen Zusammenhang zwischen der *HCAR2*- und der *PPAR-γ/2*-Genexpression hin. Ein Vergleich der SC und VC Fettdepots zeigt höhere *FFAR2* und *FFAR3* mRNA-Mengen in den VC Depots im CLA 2-Versuch, was ein Hinweis auf eine höhere Anpassungsfähigkeit des VC AT an die Verfügbarkeit von Nährstoffen im Vergleich zum SC AT sein könnte. Zudem belegen die Ergebnisse dieser Studie das Vorkommen von *FFAR2* und *FFAR3* mRNA im bovinen AT. Im CLA 2-Versuch, zeigten sich Unterschiede zwischen der CLA- und der Kontrollgruppe im omentalen und RP AT hinsichtlich der *FFAR1* mRNA-Menge, wobei die mRNA-Menge des *FFAR1* höher an d 105 p.p. und im RP AT zusätzlich an d 42 p.p. in der CLA-Gruppe im Vergleich zur Kontrollgruppe war. Für eine Interpretation dieser Effekte sind allerdings weitere Studien nötig, welche die Auswirkungen einer FFAR1-Aktivierung auf den Fettzellstoffwechsel untersuchen.

In **Manuskript 2**, erfolgte die Darstellung der mRNA-Expression von *FFAR1*, *-2*, *-3*, und *HCAR2* in einem SC AT und einem VC AT in der Transitphase. Zudem wurde der Einfluss einer Supplementation von Nikotinsäure (NA) und von Futterrationen mit unterschiedlichem Kraftfutteranteil auf die mRNA-Expression dieser Rezeptoren untersucht. Hierfür wurden 20 pluripare Holstein-Frisean-Milchrinder entweder einer Gruppe zugeteilt, die eine Futterration mit einem hohen Kraftfutteranteil von 60 % und einem niedrigen Raufutteranteil von 40 % (HC; n=10) erhielt; oder einer Gruppe, die eine Futterration mit einem niedrigen Kraftfutteranteil von 30 % und einem hohen Raufutteranteil von 70 % (LC; n=10) erhielt (bezogen auf die Trockenmasse). Je die Hälfte der Tiere beider Gruppe erhielt 24 g/d eines nicht-pansengeschützen NA-Supplements. Die Fütterung des Supplements bzw. der verschiedenen Rationen erfolgte von d 1 bis d 21 p.p. An d 21 a.p., d 1 und d 21 p.p. wurden Serumproben und Biopsate des AT gewonnen. Das SC AT wurde dabei im Bereich des Schwanzansatzes und das RP AT direkt über dem Bauchfell alternierend über die linke oder rechte Flanke entnommen. Im Serum wurden die kurzkettigen Fettsäuren (SCFA) mittels GC-FID gemessen. Die NA-Supplementierung des Futters zeigt in diesem Versuch keinen Einfluss auf die mRNA-Menge der untersuchten Rezeptoren und die Serumkonzentrationen der SCFA. An d 21 p.p. war die *FFAR2* mRNA-Menge im RP AT der Tiere aus der LC-Gruppe höher als im RP AT der Tiere aus der HC-Gruppe, was eventuell einen Mechanismus darstellt um einer gesteigerten Lipolyse im RP AT entgegenzuwirken. Ebenfalls, zeigte sich ein Trend mit niedrigeren mRNA-Mengen des *FFAR3* im SC AT der Tiere aus der LC-Gruppe *versus* der HC-Gruppe. Im RP AT konnte ein Zeiteffekt beobachtet werden mit größerer *FFAR1* und *FFAR2* mRNA-Menge an d 21 a.p. im Vergleich zu d 1 p.p. Die *FFAR3* mRNA-Menge hingegen war niedriger an d 21 a.p. im Vergleich zu d 1 p.p. im SC und RP AT; im RP AT war diese ebenfalls niedriger an d 21 a.p. im Vergleich zu d 21 p.p. Das Gen für *FFAR3* zeigte eine umgekehrt proportionale Expression im Vergleich zu *FFAR2*, was sich ebenfalls in der negativen Korrelation zwischen der *FFAR2* und *FFAR3* mRNA-Menge im SC AT wiederspiegelte. Die beobachtete inverse Regulation von *FFAR2* und *FFAR3* im RP AT könnte darauf hindeuten, dass eine Signaltransduktion über einen der beiden Rezeptoren jeweils unterschiedliche physiologische Effekte hat. Aufgrund der aus der Literatur bekannten Konzentrationen der verschiedenen SCFA die für eine Aktivierung des bovinen FFAR2 bzw. FFAR3 nötig sind, kann davon ausgegangen werden dass die gemessenen Buttersäure-Konzentrationen eher in Lage sind beide Rezeptoren zu aktivieren als die gemessenen Essigsäure-Konzentrationen. Die mRNA-Menge von *HCAR2* in den untersuchten AT wurde weder von den verschiedenen Fütterungsstrategien beeinflusst, noch

zeigte diese Veränderung im Verlauf der Transitphase. Das lässt vermuten, dass die Regulation der *HCAR2*-Expression relevanter für die Restriktion der Lipolyse in Zeiten einer positiven Energiebilanz ist. Die Ergebnisse der durchgeführten Korrelationsstudien liefern weitere Hinweise für Regulation der *HCAR2*-Expression über *PPAR-γ2* im bovinen SC AT, sowie im RP AT.

Neben der Charakterisierung der mRNA-Expression von einigen *nutrient sensing* GPCR in unterschiedlich lokalisierten AT, gibt die vorliegende Arbeit einen ersten Einblick in die Anpassungen dieser modulierenden Faktoren im bovinen AT an verschiedene Fütterungsstrategien. Darüber hinaus dienen die hier gezeigten Ergebnisse als Basis für weitere Untersuchungen zur Aufklärung der Funktion des FFAR1 in Adipozyten, sowie des Einflusses einer FFAR2- bzw. FFAR3-Aktivierung auf den Fettstoffwechsel. Nachfolgende Studien sollten weitere *nutrient sensing* GPCR, z.B. FFAR4, einschließen und auch die Proteinexpression dieser Rezeptoren im AT erfassen. Ein Identifizieren der Beteiligung der FFAR-Aktivierung an einzelnen Stoffwechselprozessen nicht nur im AT, könnte helfen die Bedeutung und die Auswirkungen einer Signaltransduktion über diese Rezeptoren für den gesamten Stoffwechsel von Milchrindern, aber auch von anderen Spezies, zu bestimmen.

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