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Abstract

With the onset of lactation, dairy cows have to mobilize body reserves, mainly in form of fat, to cover the energy output via milk. Homeorhetic adaptations, achieved via the orchestrated actions of hormones, are necessary to ensure the nutrient drain towards the mammary gland. In contrast to the well examined hormones, e.g. growth hormone and prolactin, which are knowingly involved in the metabolic control during the transition period, the role of messenger molecules originating from adipose tissue (AT) (i.e. adipokines), their receptors as well as the role of other receptors and enzymes present in different adipose tissue depots was only scarcely investigated in dairy cows. The aim of this thesis was to characterize the serum concentration of three adipokines as well as the mRNA abundance and the protein expression of related factors in two different adipose tissue depots which are potentially involved in glucose and lipid metabolism during late gestation and early lactation. In addition, different amounts of concentrate as well as a dietary supplementation with nicotinic acid were tested for potential effects on the target variables. Using blood samples as well as samples of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) obtained from 20 pluriparous cows, the time course of 3 adipokines and of the mRNA abundance of eight different target genes as well as their potential relationship were characterized. Neither the amount of concentrate, nor the supplementary niacin had any influence on the variables examined herein. The adipokine apelin remained fairly constant and might thus not be suitable to predict insulin sensitivity during the transition period. Resistin increased towards parturition and returned to pre-calving levels within one week after parturition. Since this observation substantiates recently published results, resistin may qualify as a biomarker for metabolic adaptations. Receptors involved in the regulation of lipolysis, i.e. hydroxycarboxylic acid receptor 1 (HCA_1, a receptor for lactate) and 2 (HCA_2, a receptor for BHBA and niacin) and tumor necrosis factor α receptor 1 (*TNFR1*), showed different mRNA expression patterns between SCAT and RPAT and point to an AT depot-specific lipolytic regulation. The serum lactate concentrations were likely too low to activate the receptor and no correlation was observed between lactate and HCA_1 mRNA abundance. The decreased HCA_2 mRNA after parturition points to a lipolytic regulation at the level of receptor quantity, rather than via its endogenous ligand BHBA. The mRNA of the adiponectin receptors 1 (*AdipoR1*) and 2 (*AdipoR2*) decreased after parturition supporting a link to the reduced insulin sensitivity characteristically observed around calving. The higher *AdipoR1* mRNA abundance and the higher correlation between *AdipoR1* and *AdipoR2* mRNA in RPAT point to a tissue-specific regulation of insulin sensitivity. The decreased mRNA of nicotinamide phosphoribosyltransferase (*Nampt*) and the trend for a decreasing mRNA abundance of the NAD-dependent enzyme Sirtuin-1 (*SIRT1*) as well as the observed protein expression might contribute to the decreasing insulin sensitivity after parturition. PPARγ-co activator 1α protein expression at d 21 after parturition was higher in the animals receiving a low amount of concentrate. The highest correlation was observed between the mRNA abundances of *SIRT1* and *AdipoR1*. This indicates a functional relationship between these factors and point to a regulatory role in glucose metabolism during the transition period. The present dissertation serves as a basis for further studies elucidating the complex regulation of the metabolic adaptations during the transition period. To substantiate the present results, the variables investigated on the level of transcription herein should be further examined on the level of protein expression or, as for SIRT1, the enzyme activity should be assessed.

Kurzzusammenfassung

Zur Kompensation des Energieaustrags über die Milch sind Milchkühe zu Beginn der Laktation auf körpereigene Energiereserven angewiesen und mobilisieren diese hauptsächlich in Form von Fett. Um den Nährstofffluss zur Milchdrüse zu gewährleisten, finden komplexe, hormonregulierte Anpassungsreaktionen des Stoffwechsels statt. Im Gegensatz zu den „klassischen" Hormonen wie Wachstumshormon und Prolaktin, die bekannterweise an diesen Anpassungsreaktionen beteiligt sind, wurde die Regulation der aus dem Fettgewebe stammenden Signalmoleküle, der Adipokine, deren Rezeptoren, sowie weiterer Enzyme und Rezeptoren in verschiedenen Fettdepots von Milchkühen bislang kaum untersucht. Im Rahmen der vorliegenden Dissertation wurden die Serumprofile dreier Adipokine, sowie die mRNA- und Protein-Expression verschiedener, am Glukose- und Fettstoffwechsel beteiligter Variablen in zwei verschiedenen Fettdepots von der späten Trächtigkeit bis zur frühen Laktation charakterisiert. Zusätzlich wurden potentielle Effekte des Kraftfutteranteils in der Ration sowie einer Niacinsupplementation untersucht. Hierzu wurde anhand von Blutproben sowie Bioptaten von subkutanem (SCAT) und retroperitonealem (RPAT) Fettgewebe von 20 pluriparen Milchkühen die Serumkonzentration der Adipokine, die mRNA-Expression von 8 Zielgenen, die Proteinexpression zweier Zielgene, sowie mögliche Zusammenhänge zwischen den einzelnen Variablen untersucht. Weder der Kraftfutteranteil noch die Niacinsupplementation hatten einen Einfluss auf die Zielvariablen. Die Serum Apelin-Konzentrationen blieben weitgehend unverändert und eignen sich daher nicht zur Einschätzung der Insulinsensitivität während der Transitphase. Die Serum-Resistinkonzentrationen stiegen zur Kalbung hin auf den Höchstwert an und sanken innerhalb einer Woche zurück auf das vorgeburtliche Niveau. Die Korrelation mit den Plasma-NEFA Werten deutet auf eine Rolle in der lipolytischen Regulation hin, entsprechend könnte Resistin als Biomarker für die Stoffwechselreaktionen im geburtsnahen Zeitraum herangezogen werden. Verschiedene an der Regulation der Lipolyse beteiligte Rezeptoren, wie die Hydroxycarboxylsäure-Rezeptoren 1 (HCA_1, ein Rezeptor für Laktat) und -2 (HCA_2, ein Rezeptor für BHBA/Niacin) sowie Tumornekrosefaktor α Rezeptor 1 (TNFR1), zeigten unterschiedliche mRNA-Expressionsmuster zwischen SCAT und RPAT und weisen auf eine depot-spezifische Regulation der Lipolyse hin. Die gemessenen Laktatwerte waren vermutlich zu gering zur Aktivierung des HCA_1-Rezeptors und es wurde keine Korrelation zwischen HCA_1 und Laktat beobachtet. Die verminderte HCA_2-mRNA nach der Kalbung deutet eher auf eine lipolytische Regulation auf Ebene der Rezeptorquantität, als auf Ebene des endogenen Liganden BHBA hin. Die mRNA-Expression der Adiponektinrezeptoren 1 (*AdipoR1*) und 2 (*AdipoR2*) zeigte eine Verringerung nach der Kalbung. Dies bekräftigt einen Zusammenhang mit der in diesem Zeitraum verminderten Insulinsensitivität (IS). Die höhere *AdipoR1* mRNA-Expression sowie die höhere Korrelation zwischen *AdipoR1* und *AdipoR2* in RPAT weisen auf eine depot-spezifische Regulation der Insulinsensitivität hin. Die mRNA-Verringerung der Nikotinamidphosphoribosyltransferase (*Nampt*) sowie der Trend für eine verminderte Expression des NAD-abhängigen Enzyms Sirtuin-1 (*SIRT1*) tragen möglicherweise ebenfalls zu der verminderten IS nach der Kalbung bei. Die PPARγ-co activator 1α Protein-Expression zeigte einen Trend hin zu einer postpartalen Erhöhung und könnte die Verringerung der IS abmildern. Die höchste Korrelation wurde zwischen SIRT1 und AdipoR1 ermittelt. Dies weist auf eine funktionelle Beziehung dieser Faktoren hin und deutet auf eine regulatorische Rolle im peripartalen Glukosestoffwechsel hin. Die vorliegende Dissertation kann als Basis für weiterführende Studien dienen, welche die komplexe Regulation des Stoffwechsels in der Transitphase erforschen. Zur Bestätigung der vorliegenden Ergebnisse und Hypothesen sollten die auf mRNA-Ebene untersuchten Variablen zukünftig auf Proteinebene untersucht werden, und insbesondere für SIRT1, die Enzymaktivität gemessen werden.

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List of abbreviations

AC	adenylyl cyclase
ADIPOQ	adiponectin
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
AMPK	Adenosine monophosphate-activated protein kinase
a.p.	ante partum
AT	adipose tissue
BCS	body condition score
BHBA	beta-hydroxybutyrate
cAMP	cyclic adenosine monophosphate
CAP1	adenylyl cyclase-associated protein 1
3,5-DHBA	3,5-dihydroxybenzoic acid
DMI	dry matter intake
eNAMPT	extracellular nicotinamide phosphoribosyltransferase
FFA	free fatty acids
GH	Growth hormone
GPR	G-protein coupled receptor
HCA_1	hydroxycarboxylic acid receptor 1
HCA_2	hydroxycarboxylic acid receptor 2
HMW	high molecular weight
HSL	hormone-sensitive lipase
IGF	insulin-like growth factor
IL-6	interleukin-6
iNAMPT	intracellular nicotinamide phosphoribosyltransferase
IR	insulin resistance
IS	insulin sensitivity
LMW	low molecular weight
MMW	middle molecular weight
NA	nicotinic acid

NAD	nicotinamide adenine dinucleotide
NAM	nicotinamide
NAMPT	nicotinamide phosphoribosyltransferase
NEB	negative energy balance
NEFA	non-esterified fatty acids
NF-κB	nuclear factor-kappa B
PBEF	pre-B-cell colony-enhancing factor
p.p.	post partum
PKA	Protein kinase A
PPARγ	peroxisome proliferator-activated receptor γ
PPARGC1A	PPARγ co-activator 1α
ROR 1	receptor tyrosine kinase-like orphan receptor 1
RPAT	retroperitoneal adipose tissue
SCAT	subcutaneous adipose tissue
SIRT1	Sirtuin-1
TLR4	Toll like receptor 4
TNF-α	Tumour necrosis factor α
TNFR1	Tumour necrosis factor α receptor 1
TNFR2	Tumour necrosis factor α receptor 2
VAT	visceral adipose tissue

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1. Introduction

Over the last decades, the milk yield of an average dairy cow increased threefold based on factors such as genetic selection and improved feeding strategies. This higher milk production is often associated with an impaired immune response (Hoeben et al., 1997) as well as with metabolic diseases such as milk fever and mastitis (Fleischer et al., 2001). Most of these diseases occur during the first two weeks of lactation (Burvenich et al., 2007) and may cause a decreased milk production, high veterinary treatment costs and may thus result in a reduced profitability. Special feeding strategies might help to improve the cow's health status in the periparturient period and to ensure the dairy farm's economic efficiency. Nicotinic acid has long since been used as an anti-lipolytic agent with the aim to release the metabolic pressure in periparturient dairy cows (Fronk and Schultz, 1979, Dufva et al., 1983). In humans, nicotinic acid is used in the treatment of atherosclerosis because of its lipid-lowering effects (Taylor et al., 2004). Dietary nicotinic acid supplementation was further reported to affect the secretion of several adipokines (Westphal et al., 2007) and to improve insulin sensitivity (IS) of subcutaneous adipocytes (Linke et al., 2009). Additionally nicotinic acid was reported to possess immune-modulating and anti-inflammatory properties (Yu and Zhao, 2007). Furthermore, dietary non-fiber carbohydrate supply during the prepartum period was reported to ameliorate metabolic parameters during the transition from late gestation to lactation and to improve lactation performance (Minor et al., 1998). Thus combining a dietary niacin supplementation and a non-fiber carbohydrate supply might present a feeding strategy to improve the metabolic situation and the immune status of high yielding dairy cows.

1.1. The transition period: from late gestation to early lactation

The transition period of the dairy cow is defined as the time from three weeks before until three weeks after parturition, which is characterized by major changes in energy metabolism (Grummer, 1995). It is the most critical time for dairy cows: the energy requirements increase up to 4 fold in the short time from late pregnancy to early lactation (Bell and Bauman, 1997). A successful transition determines the profitability of dairy cows, since most health disorders occur during this time and may result in reduced milk production which often extend over the whole lactation (Drackley, 1999).

1.1.1. Homeorhetic adaptations around parturition

During the transition period, the nutrient requirements are increased in order to support fetal growth and milk synthesis (Grummer, 1995). The fetal nutrient requirements are maximized in the last weeks before calving, whereas the cows' voluntary feed intake decreases during the respective period (Bell, 1995), reaching a low-point in the final week before parturition (Grummer, 1995). Consequently, the animals enter a state of negative energy balance (NEB), i.e. the energy consumed is lower than the energy required (Grummer, 2007) and thus have to mobilize body reserves, mainly in form of fat, to at least partially meet their energy requirements (Grummer, 1995, Bell and Bauman, 1997).

The initiation of lactation further changes metabolism and induces homeorhetic adaptations which are necessary to ensure nutrient partitioning towards the mammary gland (Bauman and Currie, 1980). Increasing growth hormone (GH) concentrations post partum (p.p.) (Accorsi et al., 2005) lead to reduced adipogenesis and enhanced gluconeogenesis in liver. Further, GH indirectly increases the utilization of non-esterified fatty acids (NEFA) in the mammary gland and muscle (Renaville et al., 2002). These adaptations are accompanied by reduced insulin sensitivity (IS) of peripheral tissues (Bell and Bauman, 1997), allowing to direct glucose towards the mammary gland (Bell, 1995). As a response, the peripheral tissues start to increase fatty acid oxidation and decrease glucose uptake and utilization (Butler et al., 2003). The high ratio of GH to insulin in serum after parturition further enhances triglyceride lipolysis in adipose tissue and leads to elevated serum NEFA concentrations (Drackley et al., 2005). This in turn facilitates the hepatic NEFA uptake (Bobe et al., 2004), which is directly related to the plasma NEFA concentrations (Bell, 1979). When the hepatic capacity to export NEFA as very low density lipoproteins is exceeded, NEFA are stored as triglycerides (Katoh, 2002), resulting in a risk for developing fatty liver (Bobe et al., 2004). Further, due to incomplete hepatic NEFA oxidation, the acetyl CoA which is not utilized in the Krebs cycle is converted into ketone bodies (Adewuyi et al., 2005), resulting in increased concentrations of ketone bodies like β-hydroxybutyrate (BHBA), acetone and acetoacetate (LeBlanc, 2010). Together with the elevated plasma NEFA concentrations, this leads to a higher incidence of ketosis and may result in infectious diseases like mastitis, due to compromised immune function (Drackley, 1999). Thus, the understanding of adipose tissue (AT) metabolism and its interaction with other organs is crucial to develop strategies currently lacking to support the cows' health during the transition period and, in consequence, the farms profitability. Important instruments to ensure a successful transition are the periparturient nutritional

management strategies, which should support the aforementioned metabolic adaptations (Esposito et al., 2014).

1.1.2. Feeding strategies to influence and ameliorate the periparturient metabolic situation

Dairy cows experience the most severe NEB within one to two weeks after calving (Grummer, 2007). Physiological conditions associated with insufficient energy supply predispose dairy cows to metabolic and infectious disorders such as milk fever, ketosis, displaced abomasum, mastitis and metritis. Thus one strategy to support a successful transition of dairy cows is to increase the energy intake (Esposito et al., 2014). A second strategy to reduce the metabolic pressure is the use of feed additives to counterbalance adipose tissue lipolysis.

1.1.2.1. Adjustment of the dietary energy supply

A possible approach to increase the energy balance before calving is to increase the prepartum concentrate intake. Minor et al. (1998) showed that feeding a high concentrate diet prepartum resulted in a higher dry matter intake (DMI) and thus in a higher energy balance as compared to cows fed a standard diet. Further the body condition score (BCS) should be monitored, since the optimum calving BCS should be between 3.0 to 3.25 (Roche et al., 2009). Lower calving BCS is associated with decreased production and reproduction, whereas calving BCS above 3.5 is associated with reduced DMI and milk production in early lactation and an increased risk of metabolic disorders (Roche et al., 2009). Recently published results showed that overconditioning of non-pregnant, non-lactating cows resulted in changes in the protein expression of key regulators of AT metabolism as well as an impaired IS (Locher et al., 2015). Minor et al. (1998) further reported significantly lower NEFA and BHBA concentrations for cows receiving high amounts of concentrate. The authors concluded that the high amount of concentrate in the prepartum diet improved the metabolic variables during the transition period and subsequently enhanced the lactation performance. In addition, the high non-fiber carbohydrate supply stimulates ruminal propionate production leading to increased hepatic gluconeogenesis and a rise in plasma insulin concentrations. The anti-lipolytic effects of insulin further lead to a decline in plasma NEFA and a reduced hepatic BHBA formation (Minor et al., 1998). The study of Rabelo et al. (2003) further supported the positive effects of feeding high energy diets in the prepartum period, i.e. a higher DMI. The observation that the prepartum feeding regimen exerted only minor effects on the overall performance post partum, is in accordance with other studies observing the effect of a

different energy supply in the periparturient period only (Vandehaar et al., 1999, Holcomb et al., 2001, Keady et al., 2001). Rabelo et al. (2005) further confirmed only a slight influence of the prepartum diet on the metabolic status of dairy cows post partum, but showed that feeding a diet with a high energy density directly after parturition until d 70 p.p. can improve the metabolic profile post partum with higher plasma glucose and lower BHBA concentrations.

1.1.2.2. Use of Nicotinic acid as an anti-lipolytic dietary supplement

Niacin, either occurring as nicotinic acid (NA) or nicotinamide (NAM), functions as precursor for the coenzyme nicotinamide adenine dinucleotide (NAD). Although NA and NAM are chemically similar and nutritionally equivalent, they exert different modes of action, i.e. NA is a powerful anti-lipolytic agent at supraphysiological doses whereas this function is not shared by NAM (Carlson, 2005). In dairy science, NA was suggested as a dietary supplement to counterbalance catabolic metabolism in the periparturient period by alleviating lipolysis (Pires and Grummer, 2007, Pires et al., 2007).

Nicotinic acid, in pharmacological doses, exerts anti-lipolytic effects via the receptor HCA_2, formerly named GPR109A (Offermanns et al., 2011). In 2005, BHBA was identified as endogenous ligand (Taggart et al., 2005). As early as 1972, BHBA was shown to inhibit lipolysis *in vitro* in adipocytes of subcutaneous AT (SCAT) in cattle (Metz and van den Bergh). Two years later the authors confirmed the anti-lipolytic effect of BHBA *in vivo* (Metz et al., 1974). In 2013, the inhibitory effect of BHBA on lipolysis *in vitro* was shown to be lower in the first week of lactation compared to late pregnancy in dairy cows (van der Drift et al., 2013). The HCA_2 mRNA as well as the protein have been shown to be expressed in several tissues of cattle, including adipose tissue (Titgemeyer et al., 2011, Friedrichs et al., 2014, Kenez et al., 2014). Further HCA_2 mRNA expression was reported in different brain regions (Titgemeyer et al., 2011). On a molecular level, NA was shown to decrease NEFA and triglyceride release via dephosphorylating hormone-sensitive lipase (HSL) in bovine adipose tissue (Kenez et al., 2014).

Results obtained from *in vivo* studies are contradictory. Decreasing serum NEFA concentrations were reported following an oral niacin supplementation of 12 g/d (Fronk and Schultz, 1979). Dufva et al. (1983) further reported a decline in plasma NEFA concentrations in two experiments differing in duration of the treatment and daily amount of niacin. In one experiment, the animals were supplemented with 6 g niacin / day two weeks before parturition and with 12 g / day for 4 weeks after calving. In the second experiment, the animals either received 3, 6 oder 12 g niacin / day for 10 weeks post partum. Studies

administering niacin orally as a bolus dose of 4 x 40 g over 2 intervals (Waterman and Schultz, 1972, Waterman et al., 1972) observed decreasing plasma NEFA concentrations followed by a rebound. Using abomasal infusion of niacin, a minimum of 3 mg/kg body weight/h was reported to decrease plasma NEFA followed by a rebound (Pescara et al., 2010). Pires and Grummer (2007) administered either 3, 6 or 60 mg/kg body weight/day as an abomasal bolus dose and reported the maximal anti-lipolytic response with the lowest niacin dose, since the two higher doses also induced the greatest rebound in plasma NEFA. On the other hand, a number of studies administering orally 6 to 12 g niacin/day, showed no dose-dependent decrease of serum NEFA concentrations (Jaster et al., 1983, Campbell et al., 1994, Minor et al., 1998). In addition to the effects of niacin on plasma NEFA concentrations, effects on AT mRNA and protein expression were reported. In humans, a sixth months niacin treatment was reported to decrease the mRNA abundance of hormone-sensitive lipase as well as of the niacin receptor HCA_2 (Linke et al., 2009). In bovine differentiated adipocytes, nicotinic acid was shown to increase the protein expression of GPR109A as well as AMP-activated kinase (Kopp et al., 2014).

1.2. Adipose Tissue

Adipose tissue is a highly complex organ consisting of adipocytes, and other cells summarized as the stromovascular fraction, e.g. connective tissue matrix, nerve tissue, immune cells (Kershaw and Flier, 2004) as well as e.g. multipotent stem cells (Zuk et al., 2002). The traditional view of AT as a passive energy storage has changed with the discovery of leptin (Zhang et al., 1994). Since then, AT was recognized as an active endocrine organ, playing a role in the regulation of various physiological processes (Trayhurn and Beattie, 2001).

In mammals, two types of AT have traditionally been distinguished, i.e. white and brown adipose tissue. Recently a new type of brown-like adipocytes residing in white AT was discovered, showing distinct gene expression patterns from both white and brown AT. These novel adipocytes have been termed beige or inducible brown adipocytes (Wu et al., 2012). Brown adipose tissue is abundant in small mammals or in newborns and is required for heat production, known as non-shivering thermogenesis. In adults, high amounts of brown adipose tissue are associated with lower body weight (Saely et al., 2012). White adipose tissue plays a role in metabolic regulation and physiological homeostasis (Trayhurn and Beattie, 2001) and can be divided into subcutaneous (SCAT) and visceral (VAT) adipose tissue (Bjorndal et al., 2011). Visceral AT surrounds the inner organs and can further be divided into omental

(surrounding the intestine), mesenteric (the more deeply buried depot surrounding the intestine), retroperitoneal (RPAT, surrounding the kidney) and pericardial AT (Bjorndal et al., 2011). Subcutaneous AT is the depot below the skin and can be separated into a deep and a superficial layer by the *fascia superficialis* (Wajchenberg et al., 2002). The individual AT depots can be further subdivided according to their relation to the portal circulation (Bjorntorp, 1990). Mesenteric and omental AT depots drain to the portal vein, whereas subcutaneous and retroperitoneal AT display the non-portal draining AT depots (Rebuffe-Scrive et al., 1989). Retroperitoneal AT contains more lipogenesis and lipolysis-related genes, whereas genes related to fatty acid β-oxidation are more abundant in the portal mesenteric AT (Rebuffe-Scrive et al., 1989, Palou et al., 2009). Studies in humans and rodents have shown that AT from different sites differ in their metabolic activity (Smith and Zachwieja, 1999). Table 1 will give an overview about functional differences between SCAT and VAT in humans and dairy cows.

Table 1. Functional differences of subcutaneous (SCAT) and visceral adipose tissue (VAT) in humans and dairy cows

Characteristics	VAT > SCAT	References
Adipocyte size	VAT > SCAT	Ibrahim 2010[*], Akter et al. 2011[‡]
Mitochondrial DNA content	VAT > SCAT	Kraunsoe et al. 2010[*], Laubenthal et al. 2014[‡]
Metabolic activity	VAT > SCAT	von Soosten et al. 2011 [‡]
mitochondrial respiration	VAT > SCAT	Kraunsoe et al. 2010[*]
Sensitivity to catecholamine-induced lipolysis	VAT > SCAT	Hellmer et al. 1992[*], Weber et al. 2013 [‡]
Expression of proteins related to lipolysis (HSL, AMPK)	VAT > SCAT	Locher et al. 2011[‡], Locher et al. 2012[‡]
Glucocorticoid receptors	VAT > SCAT	Pedersen et al. 1994[*], Friedauer et al. 2014[‡]
Adipose tissue mobilization during lactation	VAT > SCAT	von Soosten et al. 2011[‡]
Expression of proteins related to anti-lipolysis (HCA_2)	SCAT > VAT	Kenez et al. 2014[‡]
Insulin-induced anti-lipolytic effect	SCAT > VAT	Wajchenberg 2000[*]
Correlation with insulin resistance	VAT > SCAT	Goodpaster et al. 1999[*]
Uptake of dietary fatty acids	VAT > SCAT	Jensen 2008[*]
Genes related to fatty acid β-oxidation	SCAT > VAT	Palou et al. 2009[*]

Studies in [*]humans, [‡]dairy cows. AMPK: Adenosine monophosphate-activated protein kinase; HCA_2: hydroxycarboxylic acid receptor 2; HSL: Hormone-sensitive lipase; SCAT: subcutaneous adipose tissue; VAT: visceral adipose tissue

1.3. Adipokines as important metabolic regulators

Adipokines are biologically active proteins synthesized and secreted from adipocytes (Trayhurn and Wood, 2004). They can act either at a local (auto-/ paracrine) and/or on a systemic (endocrine) level (Bluher, 2012) and function as regulators of body homeostasis (Ouchi et al., 2011). Adipocytes from different localizations, i.e. visceral and subcutaneous adipose tissue, display unique adipokine expression profiles (Samaras et al., 2010). In states

of obesity the secretory status of an AT depot can be modified by changes in the cellular composition of the tissue, i.e. modifications in the number, phenotype and localization of immune cells as well as vascular and structural cells. The changing adipokine secretion can thus influence the function of the associated tissue (Ouchi et al., 2011). Adipokines vary in terms of their protein structure as well as in their putative function. Several groups can be distinguished: cytokines, growth factors, proteins of the alternative complement system, as well as proteins involved in vascular homeostasis, in the regulation of blood pressure, in lipid metabolism, glucose homeostasis, angiogenesis and proteins associated with acute phase and stress responses (Trayhurn and Wood, 2004). Figure 1 displays an overview about the autocrine, paracrine and endocrine functions of the four selected adipokines, i.e. adiponectin (AdipoQ), apelin, resistin and visfatin. Details about the functional involvement in glucose and lipid metabolism will be described in paragraph 1.3.5.

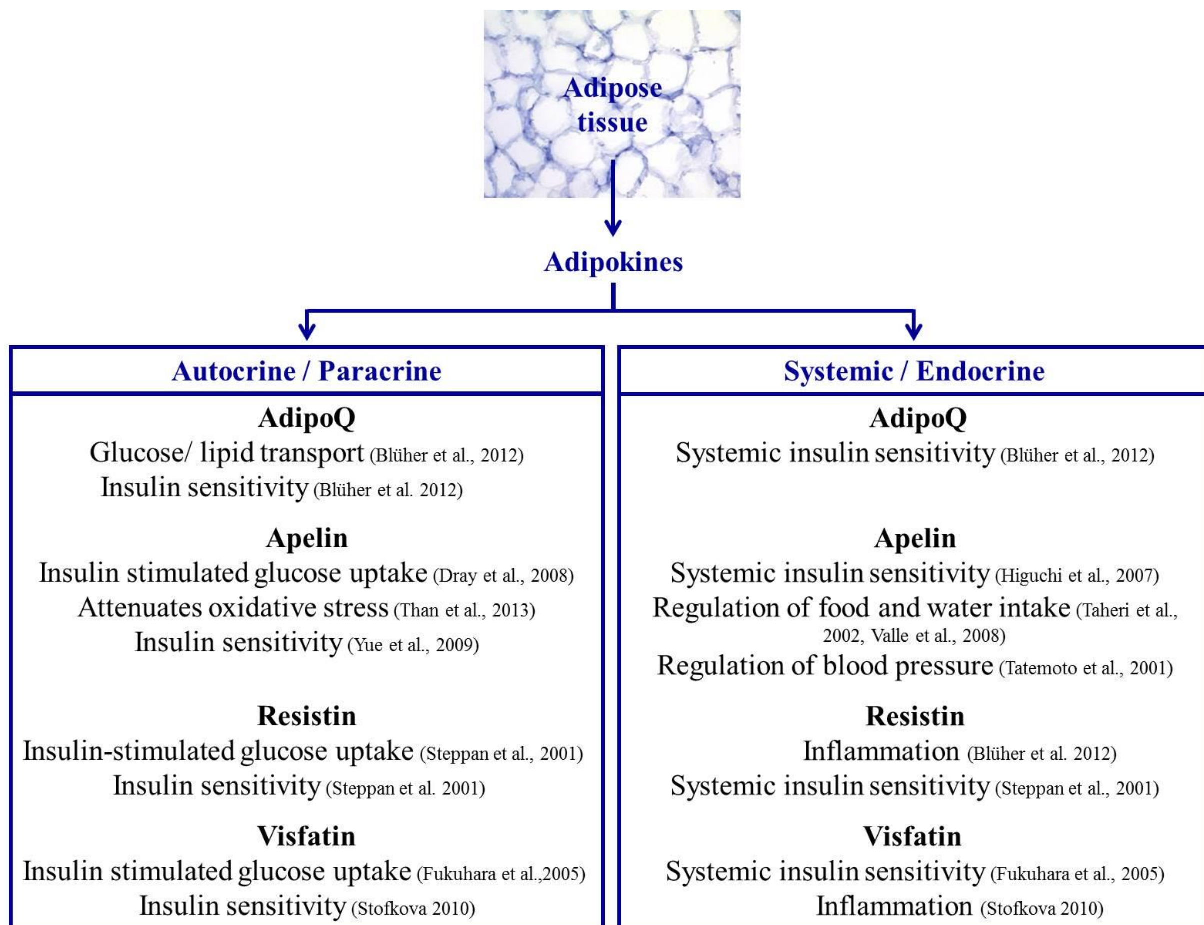

Figure 1. Autocrine, paracrine and endocrine effects of the selected adipokines adiponectin, apelin, resistin and visfatin. AdipoQ: adiponectin (modified according to Blüher 2012).

1.3.1. Adiponectin and adiponectin receptors

Adiponectin is one of the most abundant adipokines in circulation (Hotta et al., 2001). It was initially characterized in the 1990s by four different research groups and named as Acrp30 (adipocyte complement-related protein of 30 kDa) (Scherer et al., 1995), apM1 (adipose most abundant gene transcript 1) (Maeda et al., 1996), adipoQ (Hu et al., 1996) and GBP28 (gelatin-binding protein of 28 kDa) (Nakano et al., 1996). Adiponectin is expressed by differentiated adipocytes with circulating concentrations in the µg/ ml range and accounts for approximately 0.01 % of total plasma protein in humans (Arita et al., 1999). In dairy cows, the AdipoQ concentration in serum was reported to decrease from late pregnancy to a nadir at parturition and to gradually incline thereafter (Giesy et al., 2012, Mielenz et al., 2013, Singh et al., 2014). Adipocytes synthesize adiponectin as a 28 kDa monomer and secrete it as full length adiponectin in three major isoforms, i.e. low molecular weight (LMW) consisting of three monomers that form a trimer, middle molecular weight (MMW), composed of two trimers and in the high molecular weight form (HMW) which comprises 12-18 monomers (Waki et al., 2003). The half-life of the MMW and HMW in mice is 4.5 and 9 h, respectively and neither MMW nor HMW are interconverted in circulation (Pajvani et al., 2003). Proteolytic cleavage of the adiponectin molecules results in the globular head domain that still remains biologically active (Fruebis et al., 2001). The distribution of the individual AdipoQ isoforms differs among species. In healthy women HMW adiponectin accounts for 55 % of total adiponectin, MMW and LMW add up to 28 and 17 % respectively. In mice, HMW and MMW adiponectin occur at a ratio of 2:1 (Schraw et al., 2008). In plasma of Holstein dairy cows, adiponectin occurs mainly as HMW complexes and the distribution is unaffected by pregnancy or lactation (Giesy et al., 2012). As opposed to the adipokines leptin and visfatin, adiponectin is inversely correlated to body mass and insulin sensitivity (Arita et al., 1999). So far, three receptors haven been identified to bind adiponectin with different affinities: adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2) and T-cadherin (Yamauchi et al., 2003). Based on its structure and the fact, that it does not exert a direct effect on AdipoQ cellular signaling and function, T-cadherin was recently supposed to rather function as AdipoQ binding protein (Yamauchi et al., 2014). AdipoR1, which is abundantly expressed in muscle acts as a receptor for globular and full-length adiponectin isoforms, whereas AdipoR2 which is most abundantly expressed in liver, has a higher affinity for full-length adiponectin isoforms (Kadowaki et al., 2006) (Fig.2). In dairy cows, AdipoR1 and 2 mRNA abundance was shown to decrease in SCAT during the transition period (Lemor et al.,

2009, Saremi et al., 2014). Comparing SCAT from the tailhead region and RPAT, no differences were observed both receptors (Saremi et al., 2014).

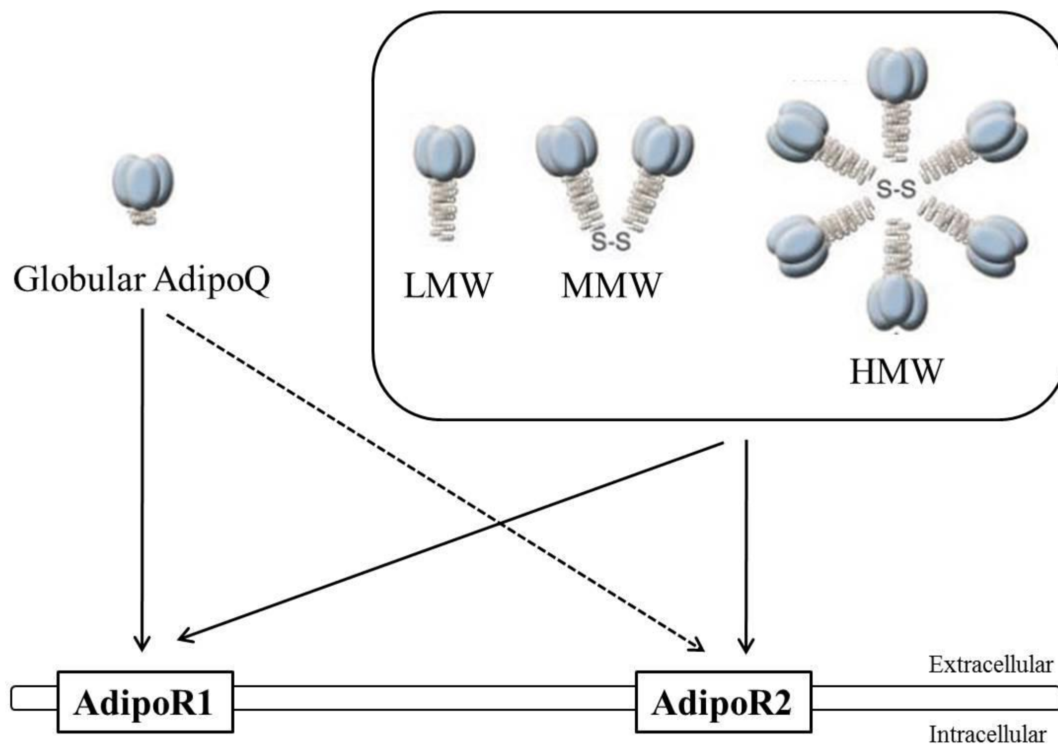

Figure 2. Adiponectin and its receptors. AdipoQ: adiponectin; AdipoR1: adiponectin receptor 1; AdipoR2: adiponectin receptor 2; HMW: high molecular weight adiponectin; LMW: low molecular weight adiponectin; MMW: middle molecular weight adiponectin (modified from Kadowaki et al., 2006).

1.3.2. Apelin

Apelin is a 77-amino acid pre-proprotein with a signal peptide in the N-terminal region. After cleavage of the signal peptide, the 55-amino acid proprotein, can be cleaved into several active forms with 12 (apelin-12) to 36 (apelin-36) amino acids in the C-terminal part (Tatemoto et al., 1998, Lee et al., 2000). Additionally the N-terminal glutamate of apelin- 13 can be post-translationally modified resulting in the pyroglutamate apelin-13 which is more protected from exopeptidase degradation (Habata et al., 1999) (Figure 3). These peptides show distinct affinities for the apelin receptor APJ, i.e. apelin-13 binds more effectively than apelin-36 (Hosoya et al., 2000). The amino-acid sequence of apelin-13 is highly conserved and identical in all mammals (Pitkin et al., 2010). The cleavage mechanism of the different peptides has not been fully clarified. Initially it was suggested, that apelin-36 might function as a precursor which is further cleaved into the more active shorter forms (Kleinz and

Davenport, 2005). Recently a specific endopeptidase was characterized which specifically cleaves the proapelin peptide into apelin-13 without evidence for production of apelin-36 (Shin et al., 2013).

Apelin mRNA is expressed in adipose tissue (Boucher et al., 2005) as well as in a variety of other tissues including lung, heart, skeletal muscle, kidney, brain and liver (O'Carroll et al., 2000). Among the tissues, the distribution of the apelin peptides is variable. The long forms of apelin close to apelin-36 were found to be the major forms in lung, testis and uterus, in mammary gland long and short forms (close to apelin-13) were also detected (Kawamata et al., 2001). Biologically active apelin and its mRNA have been shown to increase during pregnancy and lactation in the mammary gland of rats, reaching the highest level around parturition (Habata et al., 1999). Apelin was also found in human milk and was significantly lower in subjects with gestational diabetes than in healthy lactating women. Comparing colostrum and mature milk, apelin concentrations were higher in mature milk (Aydin, 2010). The function of apelin for infants is still unknown (Catli et al., 2014) Further, apelin serum levels were reported to depend on the nutritional status, i.e. a hypocaloric diet decreased adipose tissue and plasma apelin concentrations (Castan-Laurell et al., 2008). Considering different functions, apelin-13 was shown to be involved in the regulation of water and food intake (Taheri et al., 2002, Sunter et al., 2003, Valle et al., 2008). In addition, apelin peptides exert cardiovascular functions, i.e. a regulatory function on blood pressure (Lee et al., 2000, Tatemoto et al., 2001).

In cattle a large amount of apelin was found in colostrum as well as in processed milk (Habata et al., 1999). It was shown to be regulated in the bovine *corpus luteum* during the estrous cycle and pregnancy (Schilffarth et al., 2009). The authors suggested, that apelin is involved in the regulation of angiogenesis during follicle maturation. Nothing was known about the serum apelin concentrations in dairy cows during the time from late pregnancy to early lactation.

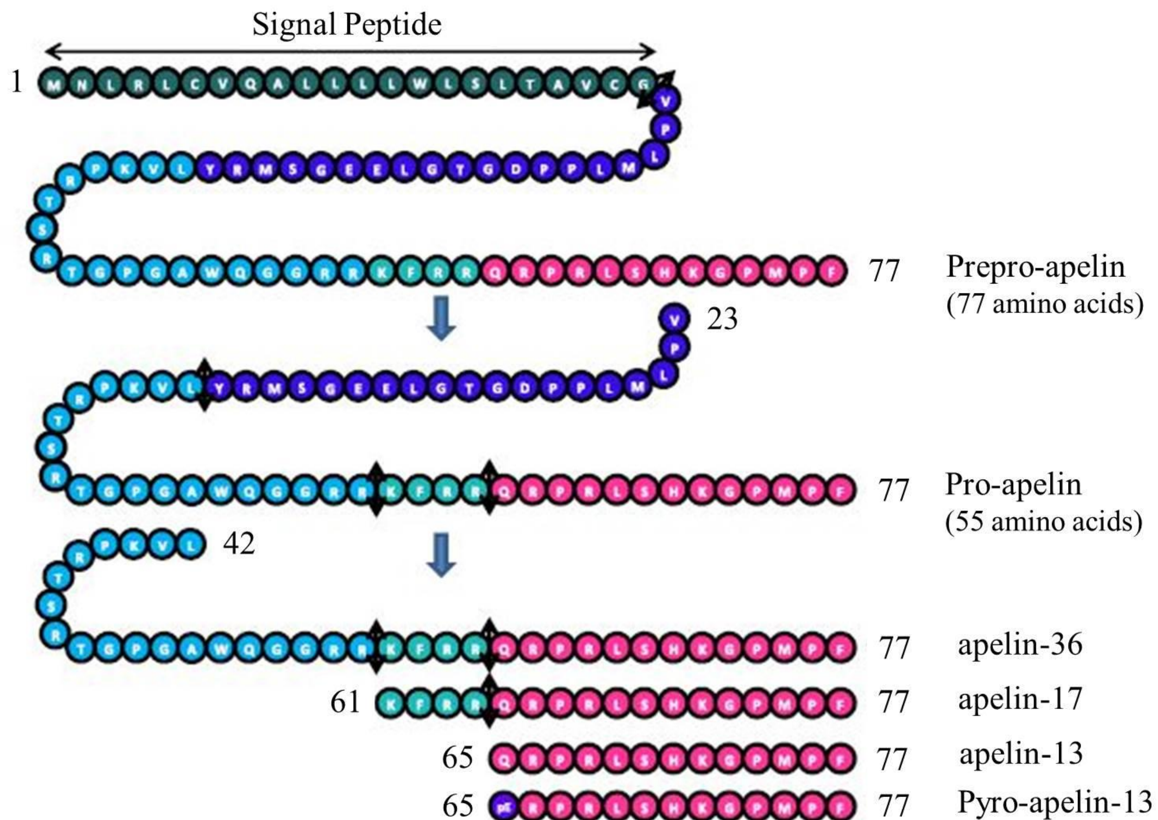

Figure 3. Schematic depiction of the different apelin fragments generated after proteolytic cleavage (according to Chavez-Almagro et al. 2015).

1.3.3. Resistin

Resistin, also called ADSF (adipose tissue-specific secretory factor) (Kim et al., 2001) or FIZZ3 (found in inflammatory zone) (Holcomb et al., 2000) was identified as a 12.5 kDa cysteine-rich protein (Kim et al., 2001). In mice, the resistin peptide is primarily expressed and secreted by adipose tissue and the expression is induced during adipocyte differentiation. (Steppan et al., 2001). Additionally it was shown to be expressed in mouse brain (Wiesner et al., 2006). In humans, resistin is primarily expressed in and secreted from monocytes (Savage et al., 2001, Patel et al., 2003). Like leptin, resistin levels are higher in women than in men, decrease during a fasting period and increase after refeeding (Rajala et al., 2004). The serum levels were shown to increase in genetic and diet induced obesity in mice and, like in humans, to decrease after a 48 h fasting period (Steppan et al., 2001).

To date, resistin has been shown to exert a number of functions. Central administration of resistin was shown to induce a short-term satiety in rats (Tovar et al., 2005). The authors also found resistin mRNA in the hypothalamus and therefore hypothesized that brain derived resistin probably acts as neuropeptide which is involved in the regulation of energy

homeostasis. Besides, resistin was reported to induce angiogenesis (Mu et al., 2006), to promote muscle cell proliferation (Calabro et al., 2004) and to influence adipogenesis (Kim et al., 2001). Furthermore, resistin plays a role in inflammatory pathways via enhancing the secretion of the pro-inflammatory cytokines interleukin-6 (IL-6) and Tumor necrosis factor-α (TNF-α) as well as the nuclear translocation of nuclear factor-kappa B (NF-κB) transcription factor (Silswal et al., 2005). Treatment of human peripheral blood mononuclear cells with recombinant resistin increased TNF-α, IL-6 and resistin itself, showing that resistin induces a positive feedback mechanism on its release (Bokarewa et al., 2005).

Up to now, several receptors were found to be involved in resistin signaling. In murine white adipose tissue, an isoform of decorin was shown to bind resistin, resulting in proliferation and migration (Daquinag et al., 2011). Decorin, a stromal proteoglycan, is mainly sysntesized by fibroblasts and involved in many processes, e.g. regulation of the collagen synthesis and the regulation of a variety of receptors like the insulin-like growth factor receptor 1 (Neill et al., 2012). Additionally, the mouse receptor tyrosine kinase-like orphan receptor (ROR) 1 has been shown to modulate resistin-dependent effects on adipose tissue and glucose metabolism (Sanchez-Solana et al., 2012). In human rheumatoid arthritis, resistin was shown to interfere with the insulin-like growth factor (IGF) signaling pathway (Bostrom et al., 2011). Furthermore, resistin induces pro-inflammatory effects in rats via binding to the hypothalamic Toll like receptor 4 (TLR4) (Benomar et al., 2013). Recently, the adenylyl cyclase-associated protein 1 (CAP1) has been identified as a receptor for resistin. Binding of resistin to CAP1 in monocytes increases the NF-κB-dependent transcription of inflammatory cytokines, thus leading to inflammation in humans (Lee et al., 2014). The authors also assessed the role of the three earlier mentioned receptors decorin, ROR1 and TLR4. According to their results, all three receptors only exerted marginal effects on pro-inflammatory actions of resistin. Thus they concluded, that CAP1 is the most important resistin receptor in humans.

In dairy cows, resistin mRNA in AT was shown to be more abundant in lactating than in non-lactating animals. In the mammary gland, the finding was reverse (Komatsu et al., 2003). Recently, serum resistin concentrations in primiparous dairy cows were shown to increase towards parturition and to return to pre-calving levels within 6 weeks of lactation (Reverchon et al., 2014).

1.3.4. Visfatin

Visfatin was initially characterized as the human pre-B-cell colony-enhancing factor (PBEF), a cytokine acting on precursor B-cells which is mainly expressed in bone marrow, muscle and liver (Samal et al., 1994). Eight years later, this cytokine has been characterized as a nicotinamide phosphoribosyltransferase (NAMPT), i.e. an enzyme which is involved in the biosynthesis of nicotinamide adenine dinucleotide (NAD) (Rongvaux et al., 2002). Two forms of NAMPT have been described: intracellular (iNAMPT) and extracellular NAMPT (eNAMPT) (Revollo et al., 2007), with iNAMPT displaying the rate-limiting component in the NAD biosynthesis (Revollo et al., 2004). The extracellular form which was characterized as visfatin/ PBEF, also exerts NAD-biosynthetic functions and is physiologically important for the regulation of glucose metabolism (Revollo et al., 2007). The name visfatin was introduced in 2005 for a protein corresponding to PBEF. This protein was shown to be highly expressed in VAT of humans and mice and to bind and activate the insulin receptor. Thus the authors identified it as an adipokine and termed it visfatin (Fukuhara et al., 2005). In 2007 visfatin has additionally been shown to act as a pro-inflammatory cytokine (Moschen et al., 2007).

As demonstrated in primiparous Holstein cows, visfatin is expressed in the mammary gland and is secreted into milk (Yonezawa et al., 2006). Visfatin mRNA was also shown to be expressed in SCAT of Holstein cows and tended to decrease after parturition (Lemor et al., 2009). In sheep, visfatin mRNA was observed in subcutaneous and visceral AT depots without any difference between them (Lemor et al., 2010). Information about a visfatin receptor is currently lacking.

1.3.5. Interrelationship between adipokines and glucose as well as lipid metabolism

During the periparturient metabolic adaptation processes, the aforementioned adipokines might play important roles in the regulation of glucose and adipose tissue metabolism in cattle.

Adiponectin, showing a decrease from late pregnancy with a nadir at parturition and a gradually incline thereafter in dairy cows (Singh et al., 2014), was shown to act as an insulin-sensitizing adipokine (Fruebis et al., 2001, Yamauchi et al., 2001, Fu et al., 2005). A decrease in IS was accompanied by decreasing plasma AdipoQ concentrations in Rhesus monkeys (Hotta et al., 2001) as well as in humans and mice (Maeda et al., 2001). Furthermore, AdipoQ was shown to stimulate glucose uptake and fatty acid oxidation in myocytes and to decrease

hepatic gluconeogenesis (Yamauchi et al., 2002). Thus the decreased serum AdipoQ concentrations around parturition were suggested to display a physiological mechanism which improves gluconeogenesis and facilitates the glucose supply to the mammary gland for milk production (Saremi et al., 2014). Considering the different isoforms, HMW AdipoQ, the most abundant isoform in dairy cows (Giesy et al., 2012), was shown to be the biologically most active form associated with an improvement of IS (Pajvani et al., 2003). In mice, HMW AdipoQ was lower in obese and insulin resistant individuals, than in lean insulin sensitive litter mates (Schraw et al., 2008). In humans and mice, AdipoQ was additionally shown to inhibit lipolysis (Qiao et al., 2011, Wedellova et al., 2011).

The adipokine **apelin** was shown to be up-regulated by insulin and obesity (Boucher et al., 2005). Furthermore, apelin was able to decrease hyperglycemia in mice. Thus it was suggested to play a physiological role in glucose metabolism (Dray et al., 2008). In human AT explants, apelin was shown to stimulate glucose transport in an Adenosine monophosphate-activated protein kinase (AMPK) dependent way (Attane et al., 2011). Apelin further seems to play a role in intestinal glucose absorption. It was suggested that glucose induces its own absorption by inducing the paracrine secretion of apelin (Bertrand et al., 2015). Besides, apelin was shown to improve IS by increasing the circulating serum AdipoQ concentrations (Higuchi et al., 2007). Yue et al. (2009) detected that apelin knock-out mice showed increased insulin as well as decreased AdipoQ levels. Thus the authors concluded that apelin is necessary for the maintenance of IS. In addition to that, apelin was shown to influence energy expenditure and to decrease the triglyceride content in AT of mice and thus appears to regulate adiposity and lipid metabolism (Higuchi et al., 2007). In isolated adipocytes as well as in 3T3-L1 adipocytes, apelin was reported to inhibit isoproterenol stimulated lipolysis through a pathway involving AMPK (Yue et al., 2011). These results were confirmed by Than et al. (2012), who reported that apelin decreased the free fatty acid release of 3T3-L1 adipocytes via AMP and by increasing the amount of perilipin, a protein surrounding the lipid droplets, and thus increasing their resistance to lipases. Apelin was also shown to increase fatty acid oxidation in muscle through AMPK activation (Attane et al., 2012).

Resistin was initially characterized to affect glucose metabolism antagonistically to insulin. Due to that fact the name was chosen for “resistance to insulin” (Steppan et al., 2001). Systemic treatment or overexpression of resistin in rats decreased the anti-gluconeogenetic capacity of insulin (Rajala et al., 2003, Satoh et al., 2004). A loss of resistin function leads to

increased body weight and body fat content as well as improved IS (Kim et al., 2004). The authors related this finding to a higher capacity of the AT to secrete adipokines like e.g. AdipoQ and leptin. Human resistin has been shown to enhance the proliferation of preadipocytes as well as to induce lipolysis in mature adipocytes. Besides activating triglyceride lipolysis, it was shown to activate reesterification of fatty acids und might thus increase metabolic rate (Ort et al., 2005). In dairy cows, resistin was reported to be low before parturition and to subsequently increase until one week after calving und return to pre-calving levels within 6 weeks post partum. Additionally recombinant resistin was shown to promote lipid mobilization in bovine adipose tissue explants. The authors hypothesized that resistin might contribute to the decreasing insulin sensitivity as well as it might be involved in the mobilization of adipose tissue during the transition period (Reverchon et al., 2014).

The role of **visfatin** in the context of glucose homeostasis is still unclear. Visfatin was first identified to be highly expressed in VAT and thought to bind and activate the insulin receptor and thus to exert insulin-mimicking effects (Fukuhara et al., 2005). As opposed to this, Berndt et al. (2005) did not find differences in the visfatin mRNA abundance between visceral and subcutaneous adipose tissue. Furthermore, the authors could not confirm a correlation between visfatin and parameters of IS. Pagano et al. (2006) also did not find a relation between visfatin and IS, whereas Sun et al. (2009) reported an improvement of whole body IS as well as a correlation of visfatin mRNA abundance with IS. Revollo et al. (2007) also found no insulin-mimetic activity of extracellular NAMPT, but they observed that the eNAMPT mediated systemic NAD synthesis is critical for β-cell function, i.e. insulin secretion. The authors concluded that eNAMPT´s NAD biosynthetic activity is physiologically important for the regulation of glucose metabolism and not the insulin-mimicking effects of visfatin as originally stated. Because of these inconsistent results, Fukuhara et al. (2007) withdrew their initial visfatin paper describing the insulin-mimicking effects on request of the Committee for Research Integrity (CRI) of Osaka University Graduate School of Medicine. Using a cell culture model, visfatin was shown to have no direct influence on beta cell survival but to potentiate glucose stimulated insulin secretion (Spinnler et al., 2013). Recently, visfatin was identified as an adipokine secreted upon cellular stress (Lin et al., 2015). The authors further reported that NAMPT activated NF-κB gene expression and suggested that visfatin contributes to cellular inflammation processes. Figure 4 gives an overview about the effects of AdipoQ, apelin, resistin and visfatin on IS and Lipolysis.

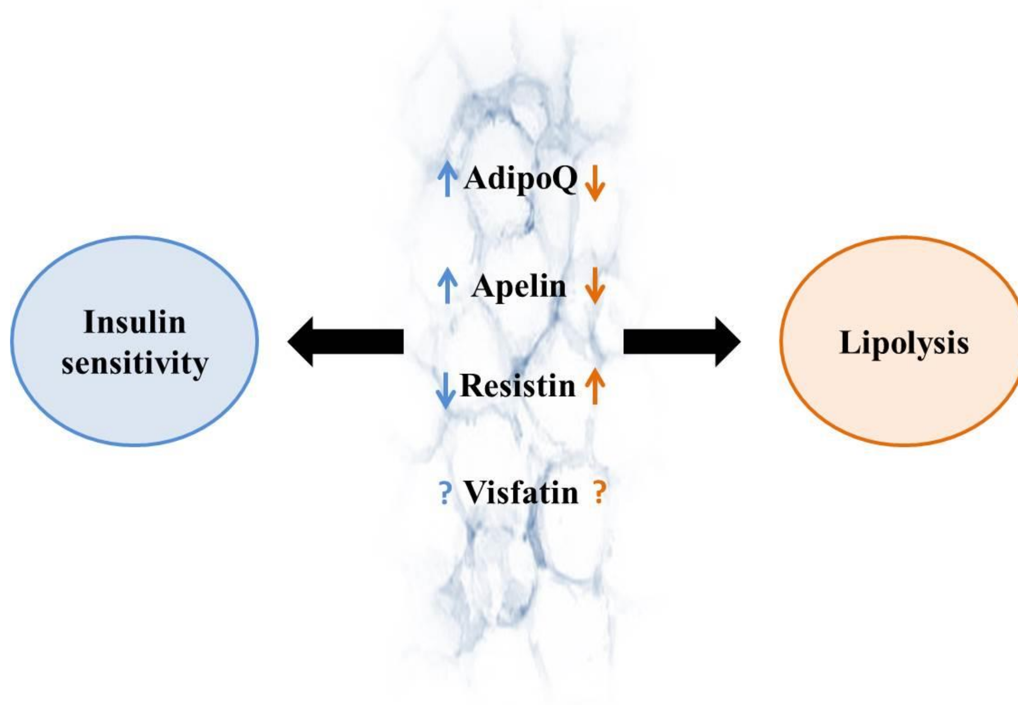

Figure 4. Influence of the adipokines adiponectin, apelin, resistin and visfatin on insulin sensitivity and lipolysis. AdipoQ: adiponectin.

1.4. Receptors linking energy uptake and glucose metabolism

1.4.1. Hydroxycarboxylic acid receptors

The hydroxycarboxylic acid receptors 1 (**HCA_1**) and 2 (**HCA_2**) are members of the G-Protein coupled receptor (GPR) family which is activated by hydroxycarboxylic acids (Offermanns et al., 2011). In humans, the lactate receptor HCA_1, formerly known as GPR81 (Liu et al., 2009, Offermanns et al., 2011), is abundantly expressed in white and brown AT and at a lower level in other tissues, e.g. liver, kidney, skeletal muscle and brain. In mice and rats, HCA_1 is also highly expressed in subcutaneous and omental AT, as well as to a lower extent in brain and other tissues like in humans (Liu et al., 2009). The expression of HCA_1 was shown to be upregulated during adipocyte differentiation *in vitro* (Ge et al., 2008) and to be decreased during inflammation (Feingold et al., 2011). In dairy cows, HCA_1 mRNA expression in SCAT and visceral AT was reported to depend on the dietary energy supply, i.e. energy overfeeding resulted in a lower HCA_1 mRNA expression (Ji et al., 2014).

Lactate is generated from the anaerobic oxidation of glucose (Rooney and Trayhurn, 2011). It is mainly produced in skeletal muscle, further white adipose tissue contributes significantly to the circulating lactate concentrations (DiGirolamo et al., 1992, Ahmed et al., 2010) and the

production rates in adipose tissue were reported to correlate with the fat cell size (Crandall et al., 1983). In ruminants, lactate also occurs as an intermediate during fermentation processes in the rumen (Waldo and Schultz, 1956). Binding of lactate to HCA_1 induces anti-lipolytic effects (Cai et al., 2008) through a G_i-dependent inhibition of adenylyl cyclase (AC) (Ahmed et al., 2010). As normal plasma lactate concentrations as well as increased plasma lactate values during extensive exercise were reported to be too low to induce the anti-lipolytic effect via HCA_1, Ahmed et al. (2010) hypothesized that the anti-lipolytic effect is mediated in an autocrine manner. According to their idea, lactate which is released from adipocytes upon insulin-stimulated glucose uptake, binds to HCA_1 in order to save energy in forms of triglycerides during times when glucose is available as an energy source (Fig. 3).

The receptor **GPR109A** (also referred to as HM74A in humans and PUMA-G in mice) acts as receptor for pharmacological doses of nicotinic acid (Tunaru et al., 2003, Wise et al., 2003). In 2003, the receptor was deorphanized by identifying BHBA as endogenous ligand (Taggart et al., 2005). After deorphanization, Offermanns et al. (2011) suggested the new name HCA_2. The receptor HCA_2 shows the highest expression level in white and brown adipose tissue (Soga et al., 2003, Tunaru et al., 2003, Wise et al., 2003) and it was additionally shown to be expressed in various immune cells, e.g. neutrophils and macrophages (Schaub et al., 2001, Kostylina et al., 2008). Furthermore, HCA_2 was shown to be expressed in the human brain (Miller and Dulay, 2008). In dairy cows, HCA_2 mRNA was reported to be expressed in muscle, mammary gland and liver as well as in different AT depots, with a higher abundance in the adipose tissues (Friedrichs et al., 2014). During the transition period, a trend for a decreased post partum HCA_2 mRNA expression was observed (Lemor et al., 2009). In steers the highest HCA_2 mRNA abundance was found in liver (Titgemeyer et al., 2011). The authors likewise detected HCA_2 mRNA in different brain regions and hypothesized that HCA_2 might enable the central nervous system to monitor circulating BHBA concentrations. As the hepatic BHBA formation depends on the amount of free fatty acids delivered from adipocyte lipolysis, the subsequent activation of HCA_2 might display a negative feedback mechanism to control the lipolytic rate during starvation (Offermanns et al., 2011) (Fig. 3). The expression of both HCA_1 and HCA_2 is regulated via peroxisome proliferator-activated receptor γ (PPARγ) (Jeninga et al., 2009).

Activation of HCA_1 and HCA_2 leads to the inhibition of adenylyl cyclase and subsequently to a decreasing intracellular cyclic adenosine monophosphate (cAMP) concentration (Offermanns et al., 2011). The reduction in cAMP in turn inhibits protein kinase A (PKA)

which decreases lipolysis via a reduced activation of hormone sensitive lipase (HSL) (Duncan et al., 2007) (Fig. 5).

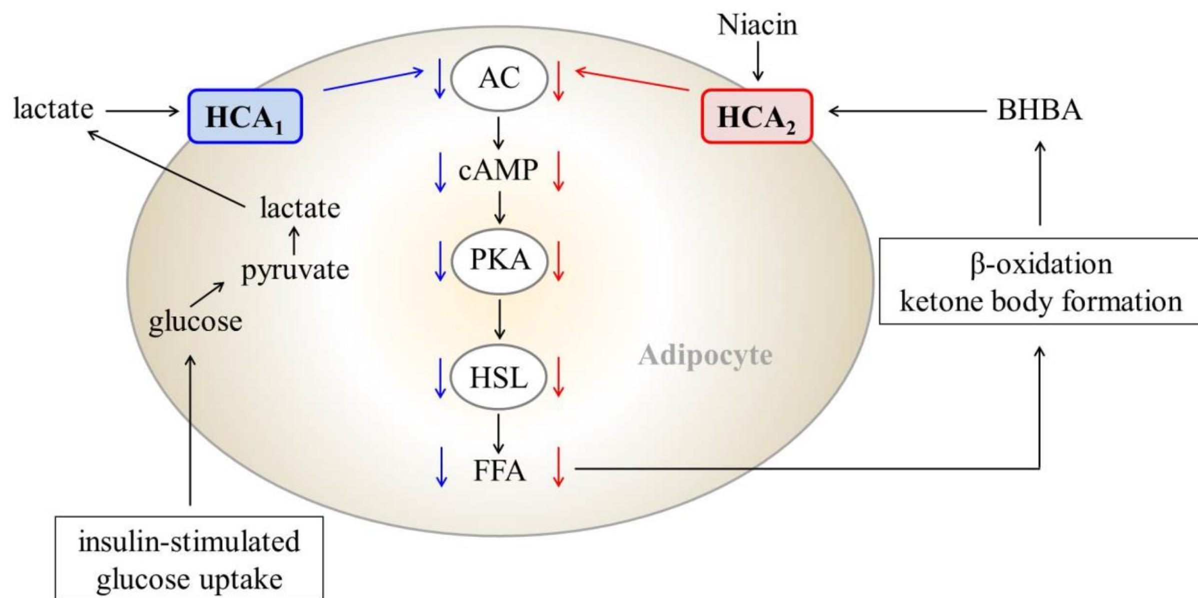

Figure 5. Anti-lipolytic activity of the receptors HCA_1 and HCA_2 in white adipose tissue. AC: adenylyl cyclase; BHBA: β-hydroxybutyrate; cAMP: cyclic adenosine monophosphate; FFA: free fatty acids; HCA_1: hydroxycarboxylic acid receptor 1; HCA_2: hydroxycarboxylic acid receptor 2; HSL: hormone-sensitive lipase; PKA: protein kinase A; (modified according to Offermanns et al. 2011).

1.4.2. Tumor necrosis factor-α and its receptor 1

Tumor necrosis factor-α is a pro-inflammatory cytokine mainly produced by lymphocytes and macrophages (Antuna-Puente et al., 2008) and further in adipose tissue (Hotamisligil et al., 1993). TNF-α is involved in the regulation of insulin sensitivity (Hotamisligil et al., 1993), inflammation, cell apoptosis and survival, cytotoxicity and the production of other cytokines (Ruan and Lodish, 2003). Furthermore TNF-α plays a role in adipose tissue metabolism, i.e. TNF-α alleviates fatty acid uptake via inhibition of lipoprotein lipase, decreases the expression of lipogenic genes and stimulates lipolysis (Sethi and Hotamisligil, 1999). TNF-α signaling is mediated via two ubiquitously expressed receptors, i.e. TNFR1 and TNFR2 (Tartaglia and Goeddel, 1992). The pro-lipolytic function of TNF-α as well as the inhibition of the insulin dependent glucose transport is mediated via TNFR1 (Sethi et al., 2000).

Plasma TNF-α was reported to decrease after parturition (Schoenberg et al., 2011). Furthermore, recombinant TNF-α was shown to induce insulin resistance (IR) in steers (Kushibiki et al., 2001) as well as in cows with fatty liver (Ohtsuka et al., 2001) indicating a role in the regulation of insulin sensitivity in cattle. Recently, a continuous TNF-α infusion into the subcutaneous adipose tissue depot at the tail head region was reported to have no influence on nutrient metabolism and insulin sensitivity (Martel et al., 2014). The authors related this finding to a compensatory mechanism, inducing tolerance to TNF-α and enhancing anti-inflammatory factors. For TNF-α mRNA abundance in bovine subcutaneous AT the results obtained are varying. Sadri et al. (2010) observed an increase from week 8 ante partum (a.p.) to the day of parturition without further change until 5 weeks thereafter in multiparous cows, whereas Saremi et al. (2014) reported constant mRNA expression when considering pluriparous cows from d 21 a.p. to d 252 in milk, or an increase in primiparous cows from d 1 to d 42 p.p. Information about the mRNA expression pattern of TNFR1 in bovine adipose tissue was lacking.

1.5. The Sirtuin1-PPARγ co-activator 1α axis

Sirtuin1 (SIRT1) belongs to a family of highly conserved NAD-dependent enzymes which deacetylate lysine residues on various proteins (Chaudhary and Pfluger, 2009). These enzymes were initially characterized in *Saccharomyces cervisiae* as promotors of longevity and named silent information regulator 2 (Sir2) (Ivy et al., 1986, Kaeberlein et al., 1999). The mammalian orthologous Sirtuin family encompasses seven members, with SIRT1 being nearly ubiquitously expressed in a variety of metabolically active tissues (Chaudhary and Pfluger, 2009). The activity of SIRT1 is dependent on the intracellular NAD/NADH ratio, i.e. a low energy level characterized by high NAD^+ and low NADH ratios leads to an activation of the enzyme (Imai et al., 2000, Chaudhary and Pfluger, 2009). During SIRT1 dependent reactions, NAD is consumed and nicotinamide is produced, which acts as an inhibitor of SIRT1 function (Kelly, 2010). In this context, SIRT1 has been discussed as a nutrient sensor being involved in the regulation of cellular metabolism (Leibiger and Berggren, 2006). In cattle, SIRT1 mRNA was reported to be expressed in AT of bulls and male Holstein calves and to increase with the animals´age (Ghinis-Hozumi et al., 2011, Liu et al., 2014). SIRT1 is involved in a wide range of biological processes such as oxidative stress (Luo et al., 2001, Brunet et al., 2004), the differentiation of muscle cells (Fulco et al., 2003) and adipose tissue metabolism, i.e. it induces an increased lipolysis via repressing PPARγ and modulates adipogenesis (Picard et al., 2004).

One of the target proteins of SIRT1 is the transcription coactivator PPARγ co-activator 1α (PPARGC1A) (Rodgers et al., 2005). A transcription coactivator is defined as a protein or protein complex, which increases the transcription rate via interacting with transcription factors. The coactivators themselves do not bind to specific DNA sequences (Puigserver and Spiegelman, 2003). PPARGC1A interacts with a variety of transcription factors, including PPARα, PPARδ and PPARγ, and is involved in biological processes like thermogenesis, mitochondrial biogenesis and glucose and fatty acid metabolism (Liang and Ward, 2006). Activation of PPARGC1A by SIRT1-dependent deacetylation induces gluconeogenic genes and inhibits glycolytic genes in liver (Rodgers et al., 2005). Further, PPARGC1A was reported to activate glycerol kinase via PPARα in human white AT (Mazzucotelli et al., 2007). The PPARGC1A protein was found to be expressed in liver, kidney and muscle of beef cows with no alterations related to the gestational status or lactation (Wood et al., 2013). The interaction between SIRT1 and PPARGC1A is known in the literature as SIRT1-PPARGC1A axis (Iwabu et al., 2010). The existence of the SIRT1-PPARGC1A axis was reported in adipose tissue of mice and supposed to play a role in the regulation of insulin resistance (de las Heras et al., 2013). Information about this regulatory system in dairy cows was not available.

1.6. Functional interplay between the variables considered herein

Visfatin/ NAMPT, the rate limiting enzyme in the mammalian NAD-biosynthesis pathway, is a regulatory element of SIRT1 function. Activation of NAMPT leads to an elevation of the intracellular NAD level and subsequently to the activation of SIRT1 (Revollo et al., 2004). This in turn deacetylates and activates PPARGC1A (Rodgers et al., 2005). Adiponectin and its receptor AdipoR1 were further reported to act as controlling elements in the regulation of SIRT1 activity. In muscle cells of mice, AdipoQ was shown to activate SIRT1 via signaling through AdipoR1 (Iwabu et al., 2010). Conversely, AdipoQ secretion was shown to be decreased after SIRT1 activation (Qiang et al., 2007).

Beyond that, SIRT1 inhibits the transcription of NF-κB (Yeung et al., 2004), an essential mediator of the effects of TNF-α on lipolysis in AT (Laurencikiene et al., 2007), which are mediated via TNFR1 (Sethi et al., 2000), a strong activator of $NF_{κB}$ (Puimege et al., 2014). Further, TNF-α was shown to up-regulate apelin expression (Daviaud et al., 2006). This adipokine is involved in glucose and adipose tissue metabolism (Higuchi et al., 2007, Dray et al., 2008). The receptors HCA_1 and HCA_2 are further involved in the regulation of lipolysis (Offermanns et al., 2011) and are both regulated via PPARγ (Jeninga et al., 2009). In 3T3-L1

adipocytes, TNF-α was reported to upregulate HCA_2 mRNA (Digby et al., 2010). Recently, the inhibition of NF-κB was reported to reduce the ability of LPS to increase HCA_2 expression by ~50% leading to the assumption that both NF-κB and non-NF-κB pathways are in involved in the mediation of the LPS effect. The prevention of the LPS-induced increase of HCA_2 resulted in increased TG accumulation and the expression of enzymes that catalyze TG synthesis (Feingold et al., 2014).

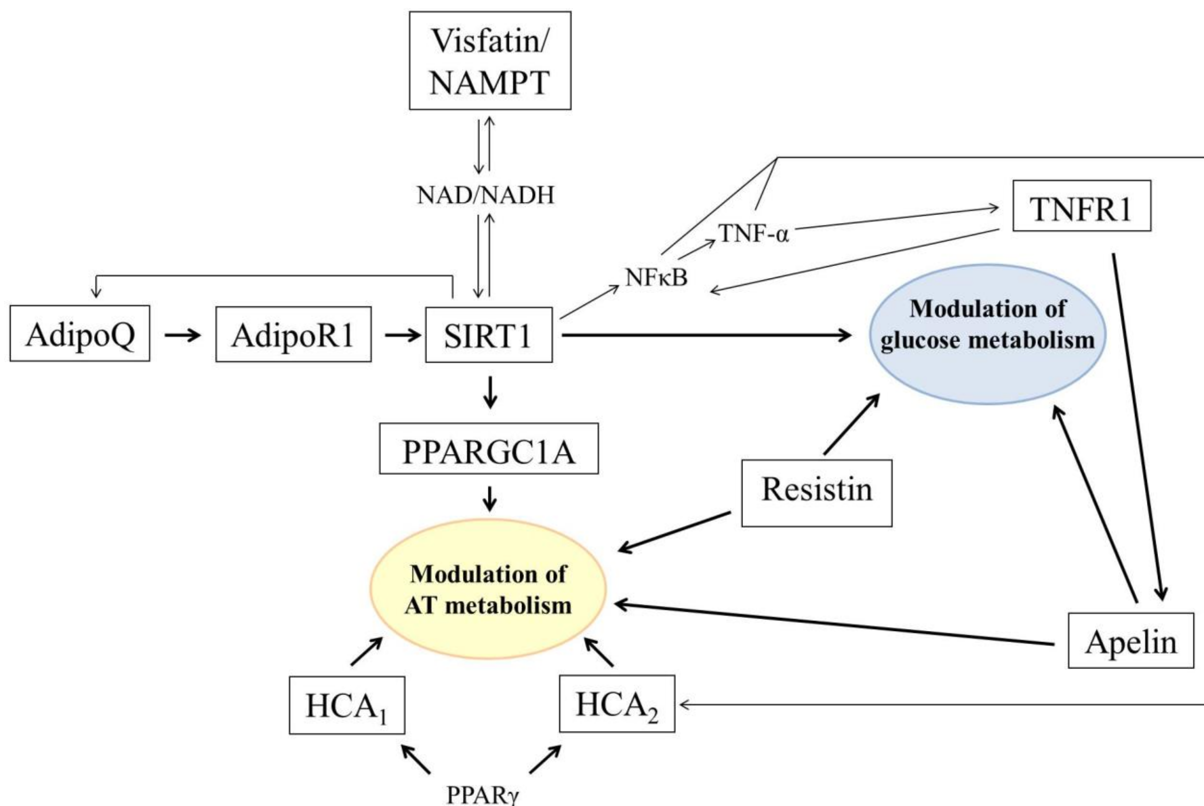

Figure 6. Relationship between the variables considered in this study. Stronger arrows indicate a direct relationship, whereas the thinner lines indicate effects via intermediates. AdipoQ: adiponectin; AdipoR1: adiponectin receptor 1; AdipoR2: adiponectin receptor 2; HCA_1: hydroxycarboxylic acid receptor 1; HCA_2: hydroxycarboxylic acid receptor 2; NAD: nicotinamide adenine dinucleotide; NAMPT: nicotinamide phosphoribosyltransferase; NF-κB: nuclear factor-kappa B; PPARγ: peroxisomal proliferator activated receptor gamma; PPARGC1A: PPARγ coactivator 1-α; SIRT1: sirtuin 1; TNF-α: Tumor necrosis factor-α; TNFR1: TNF-α receptor 1.

2. Objectives

The present experiment was designed to determine the metabolic and endocrine functions of adipose tissue in healthy dairy cows in the periparturient period. Further the effects of the proportion of dietary concentrate and a subsequent niacin supplementation were examined. The feeding strategy aimed to result in different degrees of postpartal lipomobilization in order to investigate the effects of the NA supplementation on the energy status more precisely. The variables observed herein were: the serum concentrations of the adipokines AdipoQ, apelin and resistin, as well as the mRNA abundance of the receptors HCA_1, HCA_2, AdipoR1, AdipoR2 and TNFR1, the transcriptional coactivator PPARGC1A, and of the enzymes SIRT1 and visfatin/NAMPT. Further the protein expression of SIRT1 and PPARGC1A was determined.

Based on the aforementioned strategy the objectives of the present study were:

1) to investigate the effects of two different portions of dietary concentrate (low vs. high concentrate) and a subsequent niacin supplementation on the target variables
2) to determine the serum adipokine concentrations
3) to compare the mRNA expression of the target genes between subcutaneous and retroperitoneal adipose tissue and
4) to discover possible relationships between the target genes and the adipokines.

We studied pluriparous cows from d 42 prepartum until d 100 post partum. The animals were allocated to two different feeding groups, receiving either a high portion (concentrate to roughage ratio 60:40) on a dry matter basis or a low portion (concentrate to roughage ratio 30:70) of dietary concentrate before parturition. Both groups were further subdivided into a niacin group receiving 24 g nicotinic acid per day and a control group. After parturition, a concentrate portion of 30% was fed to all cows and increased to 50% during 16 days for the low concentrate group and during 24 day for the animals of the high concentrate group.

We hypothesized, that feeding different portions of concentrate in the prepartum diet, resulting in differences in the energy balance and lipolysis post partum and that a subsequent niacin supplementation might influence the aforementioned variables.

3. Manuscript 1 (published in Journal of Dairy Science, 2016, 99: 1549-1559.)

Longitudinal changes in adipose tissue of dairy cows from late pregnancy to lactation, Part 1: The adipokines apelin and resistin and their relationship to receptors linked with lipolysis

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ABSTRACT

The transition from pregnancy to lactation is characterized by major changes in glucose and adipose tissue (AT) metabolism. Anti- and prolipolytic pathways mediated via the hydroxycarboxylic acid receptors 1 (HCAR1) and 2 (HCAR2) and tumor necrosis factor-alpha receptor 1 (TNFR1) as well as the adipokines apelin and resistin are likely involved in regulating these processes. This study aimed to determine the mRNA abundance of the aforementioned receptors in both subcutaneous and visceral adipose tissue, to characterize the adipokine concentrations in serum and to test the effects of feeding diets with either high or low portions of concentrate and a concomitant niacin supplementation from late gestation to

early lactation. Twenty pluriparous German Holstein cows were all kept on the same silage-based diet until d 42 antepartum (a.p.) when they were allocated to two feeding groups: until d 1 a.p., 10 animals each were assigned to either a high-concentrate (HC, 60:40 concentrate:roughage) or a low concentrate diets (LC, 30:70). Both groups were further subdivided into a control and a niacin group, the latter receiving 24 g/d nicotinic acid (NA) from d -42 until d 24. From d 1 postpartum (p.p.) to d 24 p.p., the concentrate portion was increased from 30 to 50% for all cows. Biopsies of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) were taken at d -42, 1, 21 and 100 relative to parturition. Blood samples were drawn along with the biopsies and on d -14, 3, 7, 14 and 42. The concentrations of the adipokines apelin and resistin in serum were measured via ELISA. The mRNA of the 3 receptors in AT was quantified as well as the protein abundance of HCAR2 by Western blot. The feeding regimen did not affect the variables examined. The concentrations of apelin remained fairly constant during the observation period whereas the resistin concentrations increased towards parturition and decreased to precalving levels within one week after calving. The mRNA abundance of *HCAR1*, *HCAR2* and *TNFR1* changed in SCAT and RPAT during the considered time period. For the HCAR2 protein, time-dependent changes were restricted to SCAT. The mRNA abundance of all receptors was greater in RPAT than in SCAT. The tissue specific correlations observed between the receptors point to a link between these factors and may indicate different regulatory roles in the respective tissues. This study provides insight into the complex metabolic adaptations during the transition period and supports a differential regulation of lipolysis among SCAT and RPAT in dairy cows.

Key words: dairy cow, transition period, hydroxycarboxylic acid receptor, apelin, resistin

INTRODUCTION

In dairy cows, the transition from late gestation to lactation is characterized by major metabolic changes. The requirements for nutrients increases rapidly after calving and not all animals can increase nutrient intake fast enough to meet those requirements (Grummer, 1995). Homeorhetic adaptations are necessary to facilitate the nutrient partitioning towards the mammary gland and to cover the energy demands for milk production (Bauman and Currie, 1980). These adaptations are accompanied by decreased peripheral insulin sensitivity (Bell and Bauman, 1997) and increased lipolysis (McNamara and Hillers, 1986).

As discussed by (Duncan et al. (2007) the hydrolysis of triacylglycerols displays the initial step of lipolysis. In adipose tissue of dairy cows this process is mediated via phosphorylation of hormone-sensitive lipase (HSL) under stimulation of catecholamines (McNamara and

Hillers, 1986, Sumner and McNamara, 2007, Locher et al., 2011). The increased lipolysis is at least partly mediated via an increased expression of the beta-adrenergic receptors, with the beta-2 receptors being suggested as the most important subtype (Sumner and McNamara, 2007, Sumner-Thomson et al., 2011). The adipocyte derived factors (adipokines) apelin and resistin, as well as the hydroxycarboxylic acid receptors 1 (HCAR1) and 2 (HCAR2) and the tumor necrosis factor-alpha receptor 1 (TNFR1) are likely relevant as regulatory elements in these processes.

Apelin is an adipokine which is up-regulated by insulin (Boucher et al., 2005) and was shown to increase glucose uptake by adipose tissue **(AT)** and skeletal muscle in mice (Dray et al., 2008). It is supposed to play a physiological role in glucose metabolism (Dray et al., 2008). In addition, apelin decreases lipolysis in mice by inhibiting the phosphorylation HSL (Yue et al., 2011). In dairy cows, apelin was demonstrated in serum and milk (Aydin, 2013) but the time-course of the serum apelin concentrations during lactation was unknown.

The pro-lipolytic tumor necrosis factor-alpha (TNF-α), whose main effects are mediated by its receptor TNFR1 (Sethi et al., 2000) was shown to up-regulate apelin expression (Daviaud et al., 2006). Thus a negative feedback loop has been suggested between these two factors (Yue et al., 2011). The abundance of *TNF-α* mRNA in bovine subcutaneous AT **(SCAT)** was reported to increase from week 8 antepartum (a.p.) to the day of parturition without further change until 5 weeks thereafter (Sadri et al., 2010), to remain unchanged when considering pluriparous cows from d 21 a.p. to d 252 in milk, or to increase in primiparous cows from d 1 to d 42 postpartum (p.p.) (Saremi et al., 2014). To our knowledge, the mRNA expression pattern of *TNFR1* in bovine adipose tissue is still unknown.

The adipokine resistin is associated with blood glucose concentrations and insulin sensitivity (Steppan et al., 2001). Recently, the plasma resistin concentrations in dairy cows were demonstrated to increase 1 week after calving; moreover recombinant resistin stimulated lipolysis in vitro (Reverchon et al., 2014).

The receptors HCAR1 and HCAR2 belong to the family of G-protein coupled receptors and are activated by hydroxylated carboxylic acids (Offermanns et al., 2011). The receptor HCAR1, which is mainly expressed on adipocytes (Wise et al., 2003) mediates insulin-induced antilipolytic effects via binding lactate (Cai et al., 2008), a quantitatively important substrate for gluconeogenesis next to propionate (Aschenbach et al., 2010). After parturition, the hepatic uptake of lactate increases without affecting the circulating concentrations (Larsen and Kristensen, 2009). Significant amounts of lactate are produced by the ruminal microbes when cows are fed a diet rich in starch (Aschenbach et al., 2010).

The receptor HCAR2 is activated by its endogenous ligand β-hydroxybutyrate (BHBA) (Gille et al., 2008), as well as by pharmacological doses of nicotinic acid and exerts antilipolytic effects after binding of its ligands (Tunaru et al., 2003). In humans and mice, HCAR2 is mainly expressed in adipocytes and immune cells (Tunaru et al., 2003, Wise et al., 2003). In cattle, HCAR2 was also found in different brain regions, including the hypothalamus; this might enable the central nervous system to sense the circulating BHBA concentrations (Titgemeyer et al., 2011). Recently, the mRNA abundance of *HCAR2* was shown to decrease from d 21 a.p. to d 105 p.p. in bovine subcutaneous AT (Friedrichs et al., 2014). Both, HCAR1 and HCAR2 are regulated via peroxisome proliferator-activated receptor γ (PPARγ) (Jeninga et al., 2009). During the transition period, AT is important for providing energy in the form of non-esterified fatty acids (NEFA) (Drackley, 1999).

Studies in humans and rodents have shown, that adipose tissues from different sites, i.e. visceral AT and subcutaneous AT (SCAT), differ in their metabolic activity (Smith and Zachwieja, 1999). Comparable to SCAT, retroperitoneal AT **(RPAT)** is not draining into the portal circulation (Rebuffe-Scrive et al., 1989, Bjorntorp, 1990). Further, RPAT contains more transcripts of genes related to lipogenesis and lipolysis, whereas the mRNA of genes related to fatty acid β-oxidation are more abundant in the portal draining mesenteric AT (Rebuffe-Scrive et al., 1989, Palou et al., 2009). By our knowledge, less data is available on retroperitoneal adipose tissue dynamic during the transition period of dairy cows. In primiparous German Holstein cows with rather low body weight and milk production, the mass of subcutaneous AT was shown to change only marginally during the first 105 days of lactation, whereas the RPAT depot decreased significantly during this time (von Soosten et al., 2011). Furthermore, the postpartum feeding regimen influences the phosphorylation pattern of HSL especially in RPAT (Locher et al., 2011). This suggests that RPAT might be more sensitive to periparturient challenges than SCAT (Locher et al., 2011). Therefore, the aim of the current study was to test whether feeding different amounts of concentrate in the prepartum period and a concomitant niacin supplementation, both aiming at inducing differences in energy balance, may affect the serum concentrations of apelin and resistin as well as the mRNA abundance of *HCAR1*, *HCAR2* and *TNFR1*. Furthermore we hypothesized that TNFR1 and HCAR1 are involved in the regulation of lipolysis in an AT depot-specific manner. Using serum samples and biopsies from SCAT and RPAT, we aimed to determine changes in serum concentrations and mRNA expression related to treatment and time from late pregnancy to lactation.

Materials & Methods

Animals and Feeding

The experiment was carried out between d 42 antepartum (a.p.) and d 100 postpartum (p.p.) at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany and approved by The Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany.

Twenty pluriparous German Holstein cows were kept in a free stall housing system with free access to water. Prior to the experiment, all cows received the same silage based diet as TMR. At d -42 the first biopsies were collected and afterwards the animals were randomly assigned to two feeding groups (n=10) differing in the concentrate to roughage ratio. Cows of the low concentrate (LC) group received a diet with a 30:70 concentrate to roughage ratio (on a dry matter basis), whereas the ratio for cows of the high concentrate (HC) group was 60:40. Both groups were further subdivided into a control and a niacin group, the latter receiving 24 g/d NA (Mianyang Vanetta Pharmaceutical Technology Co. Ltd, Sichuan, China) from d -42 until d 24. After parturition, a diet with a concentrate portion of 30% was fed to all cows and was increased to 50% within 16 days for the LC group and within 24 days for the HC group. The roughage mixture contained each 50% grass silage and 50% corn silage on DM basis. The nutrient contents of concentrate and roughage are presented in Table 1.

Blood Sampling and Analyses

Blood samples were drawn from the jugular vein on d -42, -14, 1, 3, 7, 14, 21, 42 and 100 relative to calving. After clotting, blood was centrifuged at 2000 *g* for 15 min at 15°C and serum was stored at -80°C. The total serum apelin concentrations were measured using a commercially available ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA) specific for bovine apelin (intra- and interassay coefficients of variation were 5.4 and 9.3 % respectively). The resistin concentrations in serum were determined with a commercially available ELISA kit (Uscn Life Science Inc., Wuhan, China). The intra- and interassay coefficients of variation were 9.1 and 12.5 % respectively. Serum lactate concentrations were also measured using a commercially available kit system (MAK-064, Sigma Aldrich, Nümbrecht, Germany).

Sampling of Adipose Tissue

Adipose tissue (SCAT and RPAT) was biopsied 4 times on d 42 a.p., d 1 after calving and on d 21 and 100 p.p. Samples were taken under aseptic conditions from the SCAT depot at the tail head and the RPAT depot below the lumbar vertebrae as described in detail by Locher et al. (2011). After rinsing in sterile saline solution to reduce surgically induced blood

contamination, the tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Western Blot Analysis

Details of the protein extraction and the Western Blot analysis are described by Kenez et al. (2014). In brief, the tissue samples were homogenized in a lysis buffer containing a protease inhibitor cocktail (CompleteMini, Roche Diagnostics GmbH, Mannheim, Germany), and a phosphatase inhibitor cocktail (PhosStop, Roche Diagnostics GmbH). After centrifugation supernatants were stored at -20°C until analysis. Ten µg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with a goat anti-HM74A (HCAR2) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C and subsequently with an anti-goat HRP secondary antibody (Sigma Aldrich) for 1 h at room temperature. Immunodetection was performed by incubating the membranes with SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL) and chemiluminescence was detected by a ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH, Munich, Germany). After immunodetection, Indian Ink staining was included as a loading control (an exemplary membrane showing the Indian Ink staining was included as supplemental figure 1). Bands were quantified by densitometry using Image Lab 4.0 software (Bio-Rad Laboratories GmbH).

RNA Extraction, Purification and Quality Control

The SCAT and RPAT biopsy samples (200 mg each) were homogenized in QIAzol (Qiagen, Hilden, Germany) using the Precellys 24 system (Peqlab Biotechnologie GmbH, Erlangen, Germany). Total RNA was extracted with Qiazol (Qiagen) and purification was done using spin columns, including a selective DNA removal during the lysis step (InviTrap Spin Universal RNA Mini Kit, Stratec Molecular GmbH, Berlin, Germany). The concentration and the purity of the RNA obtained were assessed with the Nanodrop 1000 (Peqlab Biotechnologie GmbH) by absorbance reading at 260 and 280 nm. RNA integrity was controlled by microcapillary electrophoresis using the Bioanalyzer 2100 (Agilent, Waldbronn, Germany) and the RNA 6000 Nano Kit (Agilent) to determine RNA integrity number (RIN) which was 6.4 ± 0.08.

cDNA synthesis

cDNA synthesis was performed with 500 ng total RNA per 40 µL reaction volume with Random Hexamer primers (250 pmol, Sigma Aldrich) and RevertAid™ Reverse Transcriptase (Thermo Fisher Scientific, Waltham, USA) using 2 µL of dNTP Mix (10 mM of

each dNTP, Thermo Fisher Scientific) on the Thermocycler Alpha-SC (Analytik Jena, Jena, Germany). Each run included a negative template control (NTC) and a no reverse transcriptase control (-RT). To alleviate the variation in cDNA synthesis, reverse transcription was performed in duplicates per tissue. Subsequently duplicates were pooled, diluted 1:4 and stored at -20°C until analysis.

Relative Quantification of mRNA

Relative quantification of the target genes was performed with standard curves based on purified amplicons, except in case of *HCAR1* where serial dilutions of cDNA were used. As controls, the no template control and no reverse transcriptase control of the cDNA synthesis were carried along, together with a no template control for each PCR run, containing 2µL water instead of diluted template. The reactions were performed in Mx3000P qPCR systems (Stratagene, Amsterdam, NL and Agilent, Santa Clara, CA) with a total volume of 10 µL, including 2 µL cDNA (diluted 1:4), 1 µL primer mix, 2 µL water and 5 µL SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Nümbrecht, Germany). The sequences of the utilized primers and their respective conditions for quantitative PCR are provided in Table 2. Specificity of the polymerase chain products was confirmed for each primer by sequencing.

Reference gene stability

Reference genes (RGs) were selected according to Saremi et al. (2012) and evaluated using qBase^{PLUS} 2.0 (Biogazelle, Ghent, Belgium). All subsequent calculations and data quality controls were done based on this software (Hellemans et al., 2007). As final reference genes (RG) lipoprotein receptor-related protein-10 and emerin were used for SCAT. For RPAT, lipoprotein receptor-related protein-10, emerin, RNA polymerase II, marvel domain containing 1 and eukaryotic translation initiation factor 3, subunit K appeared to be the most stable reference genes. For comparing both tissues, samples of SCAT and RPAT were normalized together using the RG of RPAT and additionally hippocalcin-like 1. Efficiency corrected data are presented as ratios of the target genes mRNA abundance and the geometric mean of the respective RGs.

Statistical Analyses

Statistical analyses were performed using SPSS (version 22.0, SPSS Inc., Chicago, USA) log-transformed mRNA data. No treatment effects were observed, therefore, data from the different feeding groups were merged for further analysis. The repeated comparison for the serum as well as the mRNA data between each time point was done using the mixed model procedure. The examined factors were considered as dependent variables; the level of

concentrate and the niacin supplementation were incorporated as fixed effects, sampling day as repeated effect and the respective interactions were included into the model. As covariance structure autoregressive (first-order autoregressive structure with homogenous variances) followed by Bonferroni correction was used. For apelin, day -42 values were considered as covariates. The Student´s t-test was applied for assessing differences between SCAT and RPAT at each individual time point. Pearson correlation coefficient was calculated for assessing correlations. Data are expressed as means ± SEM. Statistical significance was set at $P < 0.05$ and P-values $> 0.05 < 0.1$ were defined as a trend.

RESULTS

Energy balance and mobilization of body fat

The strategy of feeding different portions of concentrate, supplemented with niacin, did not affect lipomobilization postpartum as indicated by the BCS as well as BHBA and NEFA concentrations (Tienken et al., 2015). Also the energy balance was not affected by the feeding scheme postpartum (Tienken et al., 2015). Further, performance data and results of blood metabolite analysis during the dry period and during lactation are provided as supplemental table 1 and 2.

Apelin

For apelin, no treatment effects were observed and the serum concentrations remained unchanged from late pregnancy to lactation and were 1.21 ± 0.08 ng/mL in average (Fig.1 A).

Resistin

The serum resistin concentrations were not influenced by treatment, but changes related to time were observed. Serum resistin concentrations increased from d 42 a.p. until d 3 p.p. ($P < 0.05$) and decreased again to prepartum values until d 21 p.p. ($P < 0.01$). From d 21 until d 100 resistin remained on a constant level (Fig.1 B). Significant correlations with other variables assessed herein are presented in Table 3.

Lactate

The serum concentrations of lactate were not affected by treatment but showed a gradual decrease during the observed period ($P < 0.001$) as shown in Figure 2.

HCAR1, HCAR2, $TNFR_1$ mRNA

No differences were observed between the treatment groups, thus merged data were analyzed for time-dependent effects.

The expression profiles for all three genes differed between SCAT and RPAT as demonstrated in Figure 3. Comparing SCAT and RPAT, the *HCAR1* mRNA abundance was greater in RPAT than in SCAT on d 42 a.p. and 21 p.p. ($P < 0.001$).

The *HCAR2* mRNA abundance was significantly greater ($P < 0.05$) in RPAT than in SCAT at all sampling days (Fig.3).

When comparing both AT depots, the *TNFR1* mRNA abundance in RPAT was greater than in SCAT on d 42 a.p. ($P < 0.05$) and 21 p.p. ($P < 0.01$) (Fig.3).

The results are presented in detail in Figure 3. The correlations with other variables tested herein are reported in Table 3.

HCAR2 protein expression

For HCAR2 protein expression, no treatment- but time-dependent effects were observed. In SCAT, the HCAR2 protein was increased from d 42 a.p. to d 100 p.p. ($P < 0.001$) whereas in RPAT, no time-related changes were observed. Further details are presented in Figure 4.

DISCUSSION

In this study, the examined variables were mostly changing over time and showed adipose tissue depot-specific mRNA expression patterns. The prepartal feeding of different diets with regard to structure and energy content did not result in p.p. differences of energy balance, BCS and lipolysis as indicated by NEFA and BHBA concentrations in blood (Tienken et al., 2015). For the considered factors herein, no treatment effects were observed.

Apelin

Apelin improves muscular glucose uptake in mice (Dray et al., 2008) and is regarded as a biomarker which improves the prediction of type 2 diabetes in men (Ma et al., 2014). The apelin concentrations in serum from late pregnancy until d 100 of lactation in dairy cows remained at a fairly constant level. In consequence, apelin seems not suitable to assess the metabolic status of dairy cows during the time interval investigated herein which is knowingly associated with decreasing insulin sensitivity during early lactation (Bell, 1995). The constant serum concentrations we observed in cows are in accordance with results obtained in rodents, in which the apelin concentrations in plasma were not related to pregnancy and lactation (Kawamata et al., 2001). Apelin peptides are derived from a 55-amino acid pro-apelin, containing 23 amino acids in its C-terminal part, which are fully conserved between human and bovine (Lee et al., 2000). This proapelin is cleaved into different bioactive peptides with 13 (apelin-13) to 36 (apelin-36) amino acids in the C-

terminal part (Tatemoto et al., 1998). These peptides show distinct affinities for the apelin receptor APJ, i.e. apelin-13 binds more effective than apelin-36 (Hosoya et al., 2000). With the ELISA used herein, the total serum apelin concentrations were measured. It might be possible, that the proportion of the different apelin peptides change during the observed time, without affecting the overall apelin concentration in serum. As a preferential cleavage of pro-apelin to apelin-13 without production of longer isoforms was shown (Shin et al., 2013), we cannot exclude that the effects are mediated via apelin´s binding affinity to APJ rather than by changes in serum concentration. Comparing apelin blood and tissue concentrations, the apelin concentration observed in blood was markedly lower than those in different tissues, e.g. lung, testis and mammary gland, as observed in rat (Kawamata et al., 2001). Due to this observation, the authors speculated that apelin rather functions in a paracrine than an endocrine manner, which might be of importance also in dairy cows.

Resistin

The serum resistin concentrations measured herein in pluriparous cows confirm recently published results in primiparous cows, showing higher concentrations after parturition and a return to precalving levels within 6 weeks after calving (Reverchon et al., 2014). The authors likewise detected a correlation of resistin and NEFA as well as the stimulation of lipolysis by resistin in vitro. The high correlation between NEFA and resistin observed in our study thus supports the authors' thesis that resistin is likely involved in the lipolytic regulation during the periparturient period.

HCAR1 and lactate

It was not possible to confirm recently published result, where *HCAR1* mRNA expression in visceral and SCAT of dairy cows was shown to depend on dietary energy supply (Ji et al., 2014). However, considering the serum lactate concentrations measured herein which are in the physiologically normal range, it is arguable to which amount the receptor can be activated. The EC_{50} value for L-lactate on HCAR1 was reported to be 4.95 ± 0.42 mM in dairy cows (Liu et al., 2009). The lactate concentrations assessed in this study were 4 to 10-fold lower and thus may not or only marginally activate the receptor. The increased HCAR1 mRNA abundance in SCAT at day 100 after parturition compared to day 42 a.p. points to a regulatory role in times of established lactation. The increasing *HCAR1* mRNA expression from day 1 p.p. to day 21 p.p. in RPAT combined with the different abundance in SCAT and RPAT further support the idea of a tissue specific lipolytic regulation as well as a higher relevance for HCAR1 in RPAT in the periparturient period.

HCAR2

The observed decline of *HCAR2* mRNA abundance in SCAT from d 42 a.p. until d 21 p.p. is in accordance with results published in 2009, where a trend towards a decreasing mRNA abundance during the transition period was identified (Lemor et al., 2009). The *HCAR2* mRNA and HCAR2 protein both showed an increase from d 21 p.p. to d 100 p.p. in SCAT. To evaluate the influence on lipolysis by HCAR2, the serum BHBA concentrations have to be taken into account. The medium BHBA concentrations of 0.75 mM herein are below the threshold of 1.2 mM which is defined for ketonemic conditions (Enjalbert et al., 2001). In a recently published *in vitro* study, BHBA could induce an anti-lipolytic effect only at concentrations of 5 mM (Kenez et al., 2014). The receptor HCAR2 is part of a feedback mechanism down-regulating lipolysis (Gille et al., 2008). The time-dependent changes of the *HCAR2* mRNA as well as the HCAR2 protein abundance in SCAT may indicate a lipolytic regulation at the level of the receptor quantity, rather than on the concentration of its endogenous ligand BHBA during times when the respective BHBA concentrations are low. The HCAR2 protein expression was higher in SCAT than in RPAT on d 1, 21 and 100 after parturition. This points to a different relevance of the receptors in SCAT and RPAT and supports the notion of a different lipolytic capacity of SCAT and RPAT during the observed time.

TNFR1

The receptor TNFR1 has been shown to be necessary for TNF-α stimulated lipolysis and the inhibition of insulin stimulated glucose uptake in adipocytes (Sethi et al., 2000). The constant *TNFR1* mRNA abundance in SCAT and the time-dependent changes in RPAT further point to a tissue specific lipolytic regulation as already discussed. The receptor TNFR1, a strong activator of $NF_{\kappa B}$ mediates the pro-inflammatory activities of TNF-α (Puimege et al., 2014). In view of inflammatory pathways being involved in the homeorhetic adaptations to lactation (Farney et al., 2013), our results indicate that TNFR1 might play a role during this time in RPAT in which more *TNFR1* mRNA was detected. The constant *TNFR1* mRNA abundance in SCAT is in line with recently published data on *TNF-α* mRNA abundance, where no changes related to parturition and lactation but AT depot dependent differences were found (Friedrichs et al., 2014). Based on the results of that study on *TNF-α* data and our data on *TNFR1*, local regulation of insulin sensitivity may take place.

Relationship between the considered factors herein

The correlation studies showed no significant relation between serum apelin and glucose concentrations. However, a positive correlation was found for apelin and *TNFR1* in SCAT but not in RPAT. This might indicate a tissue-specific effect of apelin on insulin sensitivity and supports the notion of a tissue-specific regulation of insulin sensitivity (Saremi et al., 2014). A positive correlation between *HCAR1* and *HCAR2* was detected in both AT depots. This finding might indicate a co-regulation in the context of controlling lipolysis. The receptor *TNFR1* was positively correlated with *HCAR1* and *HCAR2* in both examined AT depots. Together with a higher *TNFR1* mRNA abundance at d 42 a.p. and d 21 p.p. in RPAT, this also supports the hypothesis of a differential lipolytic regulation in SCAT and RPAT. No correlation was found between serum lactate concentrations and *HCAR1* mRNA abundance. Thus the ligand most likely doesn´t regulate the receptor mRNA expression. Ahmed et al. (2010) postulated that lactate released from adipocytes acts in an auto- and paracrine manner to induce anti-lipolytic effects when plasma glucose levels are increased. This might explain the lacking correlation between *HCAR1* and serum lactate concentrations herein and indicates that the local lactate concentrations might be more relevant than the systemic ones. In our study, *HCAR2* mRNA in both SCAT and RPAT was positively correlated with serum glucose. This suggests a functional link between *HCAR2* and serum glucose concentrations to save energy in forms of triglycerides in dairy cows when glucose concentrations are elevated, a link that is also supported by in vivo glucose infusions resulting in increased lipogenesis (Al-Trad et al., 2009, Carra et al., 2013). The negative correlation with serum BHBA concentrations might indicate a negative feedback loop that down-regulates HCAR2 when BHBA concentrations are high in order to ensure lipolysis in dairy cows.

CONCLUSIONS

The presented results indicate a relationship between HCAR1, HCAR2 and TNFR1 on the level of transcription and point to a regulatory role in lipolysis during the transition period and in early lactation. Furthermore, we provide the first insight into the longitudinal regulation of *HCAR1* mRNA. A serum apelin profile for the corresponding period was recorded. Further studies are required to elucidate the functional roles of apelin and its receptor in glucose metabolism.

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Table 1. Nutrient contents of concentrates and roughage

Nutrient (g/kg DM)	Concentrate		Roughage	
	prepartum[a]	postpartum[b]	Corn	Grass
Crude Ash	42	47	36	94
Crude Protein	230	228	120	136
Ether Extract	39	39	29	32
ADF	45	44	222	310
NDF	163	152	461	506

[a]concentrate to roughage ratio: Low concentrate group 30:70, High concentrate group 60:40
[b]concentrate portion of 30% for all cows, increased to 50% within 16 days for the low concentrate group and within 24 days for the high concentrate group.

Table 2. Sequences of the primers and real-time polymerase chain reaction conditions used for the quantification of the target genes

Gene	Forward primer sequence (5´-3´) Reverse primer sequence (5´-3´)	Acc. no.[1]	bp	Con. (nM)[2]	mean Cq[3]	Annealing (s/°C)[4]	Elongation (s/°C)[5]	Efficiency
HCAR1[6]	TGCCCTTTCGGACAGACTAC CCAAAGGACACAGACAATGC	NM_001145234.1	218	200	31.8	60/60	60	96.6
HCAR2[7]	GGACAGCGGGCATCATCTC CCAGCGGAAGGCATCACAG	XM_010823378.1	140	200	30.4	30/61	30	92.4
TNFR1[8]	TGCCACTGGTGCTTCCAGCTC GCCCTGGACCCGGACAGTCAT	NM_174674.2	198	200	29.1	60/60	60	98.2

[1]NCBI Accession Number
[2]Concentrations for each primer
[3]Mean quantification cycle
[4]Initial denaturation for 10 min at 95°C; denaturation for 30 s at 95°C
[5]Elongation at 72°C
[6]Hydroxycarboxylic acid receptor 1
[7]Hydroxycarboxylic acid receptor 2, (Hosseini et al., 2012)
[8]Tumor necrosis factor-alpha receptor 1

Table 3. Coefficients of correlation (Pearson) for assessing relationships between the mRNA abundances of *HCAR1*, *HCAR2*, and *TNFR1* as well as the serum concentrations of apelin, resistin, glucose, NEFA, BHBA and lactate in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) of pluriparous dairy cows ($P < 0.05$)

			SCAT			RPAT		
	Apelin	Resistin	*HCAR1*	*HCAR2*	*TNFR1*	*HCAR1*	*HCAR2*	*TNFR1*
HCAR1				0.507	0.666		0.603	0.467
HCAR2					0.549			0.587
TNFR1								
Apelin		NS	0.372	NS	0.315	NS	NS	NS
Resistin			0.261			NS		
Glucose	NS	NS	NS	0.427	NS	NS	0.328	NS
NEFA	NS	0.661	NS	NS	NS	NS	NS	NS
BHBA	NS	NS	NS	-0.316	NS	NS	-0.237	NS
Lactate			NS			NS		

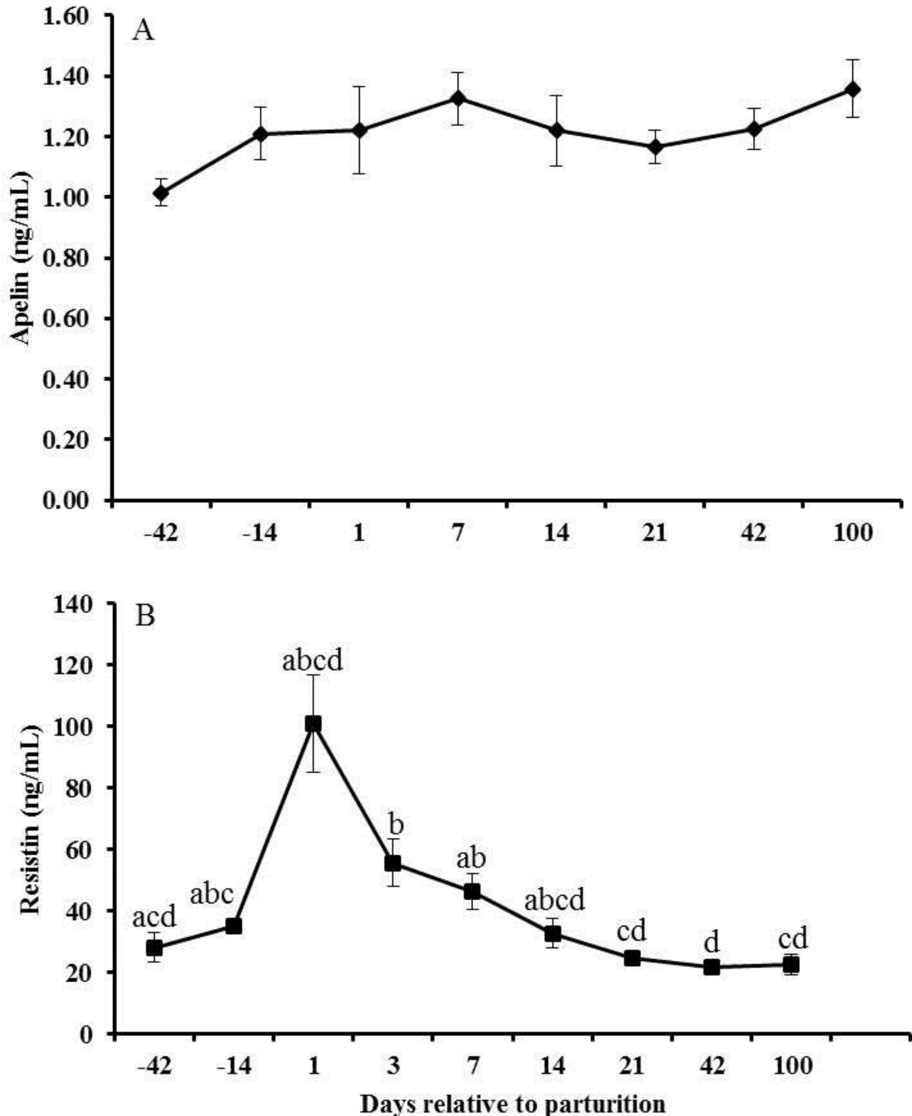


Figure 1. Time course of serum apelin (A) and resistin (B) concentrations from late pregnancy to early lactation of pluriparous dairy cows (means ± SEM). Different letters indicate significant differences between the individual sampling dates ($P < 0.05$).

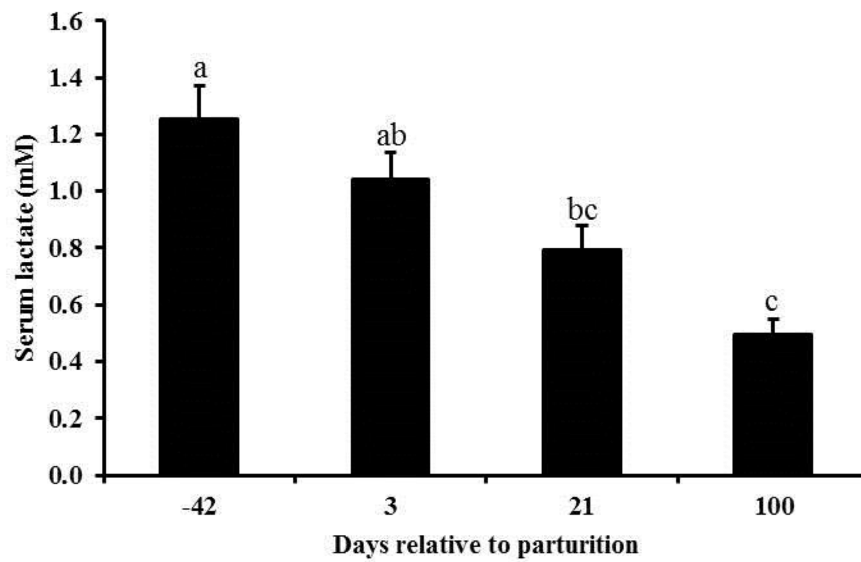


Figure 2. Serum L-lactate concentrations on day -42, 3, 21 and 100 relative to parturition in pluriparous dairy cows (means ± SEM). Different letters indicate significant differences between the individual sampling dates ($P < 0.05$).

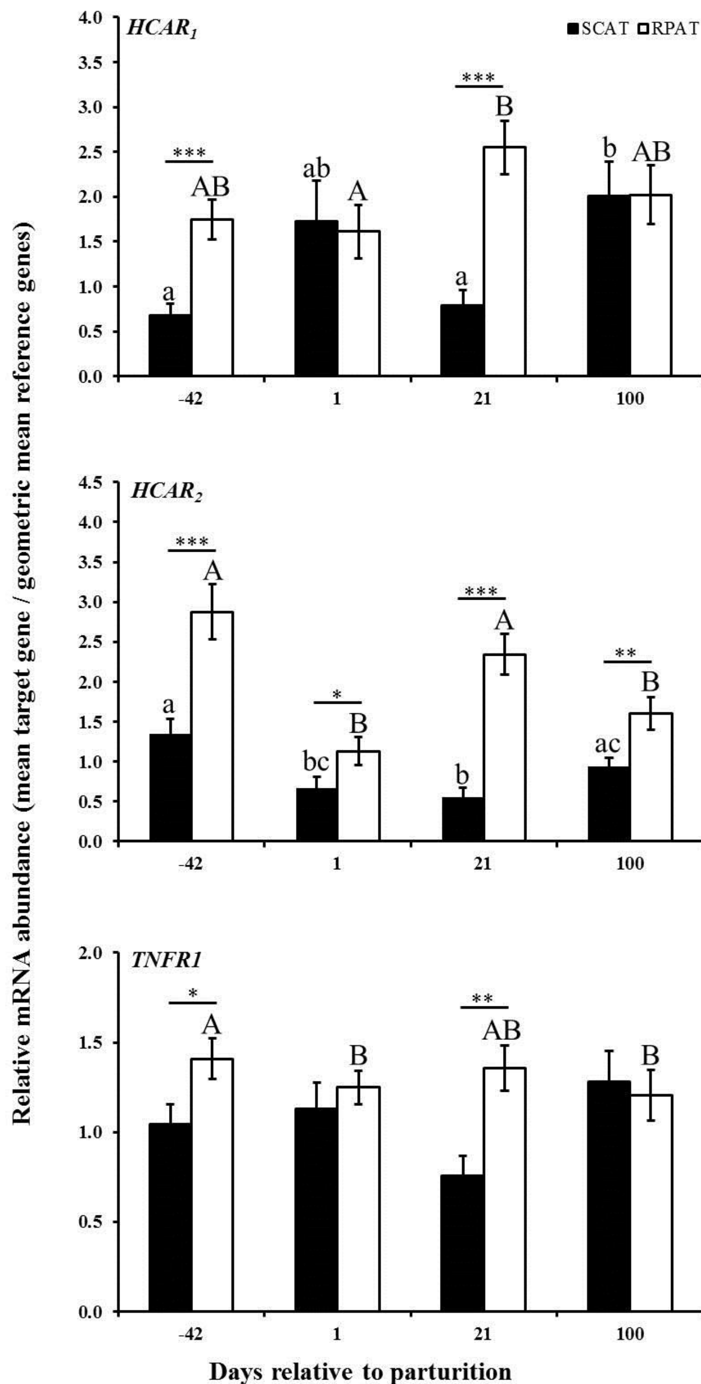


Figure 3. mRNA expression of hydroxycarboxylic acid receptor 1 *(HCAR1)*, hydroxycarboxylic acid receptor 2 *(HCAR2)* and tumor necrosis factor-alpha receptor 1 *(TNFR1)* on day -42, 1, 21 and 100 relative to parturition in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) of pluriparous dairy cows (means ± SEM). Different lower case characters indicate significant differences between the individual sampling dates in SCAT ($P < 0.05$), whereas different capital letters designate differences between the sampling dates in RPAT ($P < 0.05$). Asterisks mark a different mRNA abundance between SCAT and RPAT at the respective time-points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). For normalization lipoprotein receptor-related protein-10 *(LRP10)* and emerin *(EMD)* in SCAT, and LRP10, EMD, RNA polymerase II *(POLR2A)*, marvel domain containing 1 *(MARVELD1)* and eukaryotic translation initiation factor 3, subunit K *(EIF3K)* in RPAT were used as reference genes. For comparing SCAT and RPAT, LRP10, EMD, POLR2A, MARVELD, EIF3K and hippocalcin-like 1 (HPCAL) were used as reference genes.

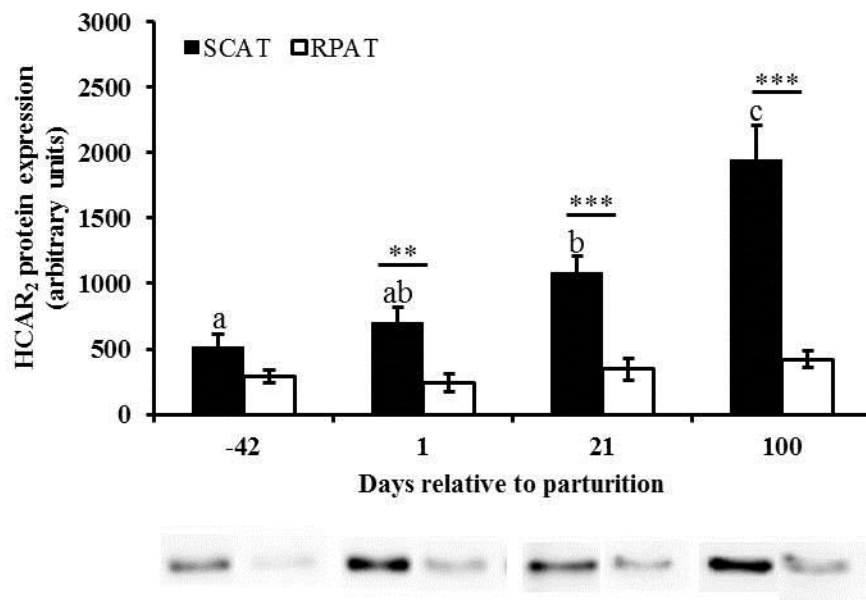


Figure 4. Protein expression of hydroxycarboxylic acid receptor (HCAR2) in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) on day -42, 1, 21 and 100 relative to parturition in pluriparous dairy cows (means ± SEM). Different letters indicate significant differences between the individual sampling dates in SCAT ($P < 0.05$). Asterisks mark a different protein expression between SCAT and RPAT at the respective time-points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Indian Ink staining was included as a loading and transfer control. Representative Immunoblots are shown below the respective bars.

Supplemental data:

Supplemental table 1. Effects of feeding diets with either a low (LC) or high (HC) amount of concentrate and with supplementing either 0 g (CON) or 24 g nicotinic acid (NA) per day on production and metabolic parameters of primiparous (PP) and pluriparous (PlP) cows during the dry period (LSMeans ± SE).

	Concentrate level (C)		Supplement (S)		Parity (P)		*P*-value		
	LC	HC	CON	NA	PP	PlP	C	S	P
Production data									
DMI (kg/d)	13.2 ± 0.3	15.1 ± 0.3	14.0 ± 0.3	14.3 ± 0.3	12.8 ± 0.3	15.5 ± 0.3	<0.001	0.391	< 0.001
Energy intake (MJ NEL)	91.7 ± 2.0	115.3 ± 2.0	102.4 ± 2.0	104.6 ± 2.0	93.7 ± 2.3	113.3 ± 1.8	<0.001	0.440	< 0.001
Energy balance (MJ NEL)	35.6 ± 1.9	58.9 ± 1.9	46.8 ± 1.8	47.8 ± 1.9	40.1 ± 2.1	54.5 ± 1.7	<0.001	0.716	< 0.001
BCS	3.5 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	0.256	0.084	0.826
Metabolic parameters									
NEFA (mM)	0.30 ± 0.02	0.34 ± 0.01	0.30 ± 0.01	0.33 ± 0.01	0.32 ± 0.02	0.31 ± 0.01	0.076	0.170	0.627
BHBA (mM)	0.58 ± 0.02	0.54 ± 0.02	0.56 ± 0.02	0.56 ± 0.02	0.50 ± 0.02	0.62 ± 0.02	0.160	0.801	0.001
Glucose	73.0 ± 1.0	69.9 ± 1.0	71.7 ± 1.0	71.1 ± 1.0	72.9 ± 1.1	70.0 ± 0.9	0.036	0.688	0.050

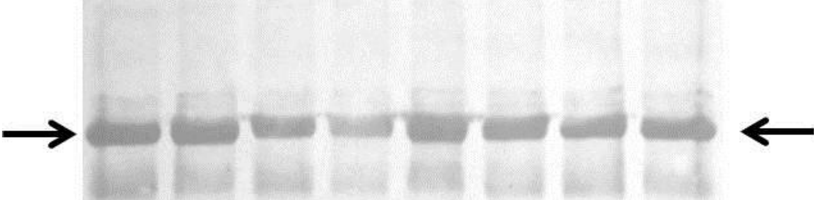
Table according to Tienken et al. (2015). The authors appreciate Taylor & Francis Ltd. (www.tandfonline.com) for the opportunity to use the table

Supplemental table 2. Effects of feeding prepartum diets with either a low (LC) or high (HC) amount of concentrate and with supplementing either 0 g (CON) or 24 g nicotinic acid (NA) per day on production, milk performance and metabolic parameters of primiparous (PP) and pluriparous (PlP) cows during lactation (LSMeans ± SE).

	Concentrate level (C)		Supplement (S)		Parity		*P*-value		
	LC	HC	CON	NA	PP	PlP	C	S	P
Production data									
DMI (kg/d)	17.0 ± 0.4	16.7 ± 0.3	16.7 ± 0.3	17.0 ± 0.4	15.3 ± 0.4	18.4 ± 0.3	0.470	0.490	< 0.001
Energy intake (MJ NEL)	124.9 ± 2.6	121.3 ± 2.5	122.0 ± 2.5	124.2 ± 2.6	112.4 ± 2.8	133.8 ± 2.3	0.320	0.520	< 0.001
Energy balance (MJ NEL)	-26.7 ± 3.1	-26.2 ± 3.0	-25.3 ± 2.9	-27.7 ± 3.1	-16.3 ± 3.4	-36.7 ± 2.7	0.913	0.555	< 0.001
BCS	3.0 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	0.901	0.921	0.272
Body weight (kg)	610 ± 9	613 ± 9	602 ± 9	622 ± 9	579 ± 9	644 ± 9	0.823	0.121	< 0.001
Milk performance data									
Milk yield (kg/d)	33.5 ± 1.1	32.9 ± 1.1	33.4 ± 1.1	33.1 ± 1.1	27.3 ± 1.2	39.1 ± 1.0	0.694	0.839	< 0.001
ECM yield (kg/d)	35.6 ± 1.3	34.1 ± 1.2	34.2 ± 1.2	35.6 ± 1.2	29.0 ± 1.4	40.7 ± 1.1	0.393	0.407	< 0.001
Fat (%)	4.58 ± 0.12	4.37 ± 0.12	4.31 ± 0.12	4.64 ± 0.12	4.45 ± 0.13	4.50 ± 0.11	0.234	0.054	0.739
Protein (kg/d)	3.38 ± 0.04	3.32 ± 0.04	3.33 ± 0.03	3.37 ± 0.04	3.41 ± 0.04	3.29 ± 0.03	0.240	0.505	0.024
Lactose (%)	4.85 ± 0.02	4.85 ± 0.02	4.84 ± 0.02	4.86 ± 0.02	4.88 ± 0.02	4.82 ± 0.02	0.902	0.343	0.053
Urea (mg/kg)	204 ± 6.4	198 ± 6.1	201 ± 5.9	201 ± 6.3	202 ± 6.8	200 ± 5.4	0.554	0.934	0.782
Metabolic parameters									
NEFA (mM)	0.40 ± 0.02	0.37 ± 0.02	0.37 ± 0.02	0.40 ± 0.02	0.36 ± 0.02	0.41 ± 0.02	0.191	0.218	0.090
BHBA (mM)	0.77 ± 0.05	0.76 ± 0.05	0.76 ± 0.05	0.77 ± 0.05	0.71 ± 0.05	0.82 ± 0.04	0.901	0.821	0.105
Glucose	53.6 ± 1.1	54.1 ± 1.0	53.1 ± 1.0	54.6 ± 1.1	55.1 ± 1.2	52.6 ± 0.9	0.705	0.328	0.104

Table according to Tienken et al. (2015). The authors appreciate Taylor & Francis Ltd. (www.tandfonline.com) for the opportunity to use the table

Supplemental figure 1. Exemplary membrane of HCAR2 showing the Indian Ink staining used for normalization (arrows point out the selected band used for calculations.)



4. Manuscript 2 (published in Journal of Dairy Science, 2016, 99: 1560-1570.)

Longitudinal changes in adipose tissue of dairy cows from late pregnancy to lactation, Part 2: The SIRT-PPARGC1A axis and its relationship with the adiponectin system

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ABSTRACT

The transition period in dairy cows is characterized by major changes in glucose and adipose tissue (AT) metabolism. The Sirtuin-1 (SIRT1) PPARγ co-activator 1α (PPARGC1A) axis might be related to the adiponectin (ADIPOQ) system to orchestrate the regulation of these processes. We aimed to assess the mRNA abundance of the aforementioned components in on visceral and one subcutaneous fat depot, together with the ADIPOQ concentrations in serum of dairy cows from late gestation to early lactation. In addition, the effect of two diets differing in energy density was tested. Twenty pluriparous German Holstein cows were all kept on the same silage-based diet until d 42 antepartum (a.p.). From then on until d 1 a.p., 10 animals each were assigned to either high-concentrate (HC, 60:40 concentrate:roughage) or

low concentrate diets (LC, 30:70). Both groups were further subdivided into a control and a niacin group, the latter receiving 24 g/d nicotinic acid from d -42 until d 24. From d 1 postpartum (p.p.) to d 24 p.p., the concentrate portion was increased from 30 to 50% for all cows. Biopsies of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) were taken at d -42, 1, 21 and 100 relative to parturition. Blood samples were drawn along with the biopsies as well as on d -21, -14,-7, -3, 1, 3, 7, 14, 21, 28, 35, 42, 63, 82 and 100 relative to calving. Quantification of target mRNAs was done using qPCR and serum ADIPOQ concentration was measured via ELISA. The feeding regimen did not affect the variables examined. Serum ADIPOQ concentrations decreased towards parturition, returned to pre-calving levels within 1 wk after parturition and remained on a constant level until the end of the experiment. The mRNA abundance of *SIRT1*, *PPARGC1A*, *NAMPT* and the ADIPOQ receptors 1 (*ADIPOR1*) and 2 (*ADIPOR2*) changed in SCAT and RPAT during the considered time-period. Comparing SCAT and RPAT, the mRNAs of *SIRT1*, *ADIPOR1* and *ADIPOR2* were more abundant in RPAT, whereas *PPARGC1A* and *NAMPT* were higher expressed in SCAT. The protein abundance of SIRT1 tended to increase from d -42 to d 21. At day 21 we detected more PPARGC1A protein in the LC group as compared to the HC group. The correlations observed point to a link between these factors and might hint to a functional role of the variables in the regulation of glucose metabolism. This study substantiates the existence of the SIRT1-PPARGC1A-axis and indicates a functional relationship between *SIRT1* and *ADIPOR1* in bovine AT.

Key words: dairy cow, transition period, Sirtuin-1, PPARGC1A, adiponectin

INTRODUCTION

Dairy cows undergo a plethora of metabolic changes during the transition from late gestation to lactation (Bauman and Currie, 1980) which includes among others the activation of the adrenergic system as most important trigger of lipolysis (McNamara and Hillers, 1986) During late pregnancy, dairy cows experience a period of insulin resistance which is, similar to other mammalian species like humans, maintained in early lactation (Bell and Bauman, 1997, Lain and Catalano, 2007). The adiponectin system is supposed to be part of the hormonal adaptations regulating insulin sensitivity (Giesy et al., 2012) and there are some indications that this system is possibly connected to the Sirtuin-1 (SIRT1) PPARγ co-activator 1α (PPARGC1A) axis (Iwabu et al., 2010). This network is likely involved in the orchestration of the complex metabolic adaptations during the transition period.

Sirtuin-1 belongs to a family of highly conserved NAD^+-dependent enzymes which deacetylate lysine residues on various proteins (Chaudhary and Pfluger, 2009). Depending on

the intracellular NAD^+/NADH ratio, these enzymes act as sensors to detect the cellular energy availability, i.e. a low energy level characterized by high NAD^+ and low NADH levels leads to an activation of SIRT1 (Imai et al., 2000, Chaudhary and Pfluger, 2009). This activation induces an increased lipolysis via a repression of PPARγ in differentiated adipocytes. Furthermore, SIRT1 was shown to be a modulator of adipogenesis (Picard et al., 2004). Sirtuin-1 mRNA was shown to be expressed in adipose tissue **(AT)** of bulls and Holstein male calves and reported to increase with the animals´ age (Ghinis-Hozumi et al., 2011, Liu et al., 2014).

One of the target proteins of SIRT1 is PPARGC1A. Activation of PPARGC1A via deacetylation induces gluconeogenic genes in liver whereas glycolytic genes are down regulated (Rodgers et al., 2005). The PPARGC1A protein was found to be expressed independent of the status of gestation or lactation in liver, kidney and muscle of beef cows (Wood et al., 2013). A regulatory element of SIRT1 function is nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD biosynthesis pathway in mammals. Activation of NAMPT leads to an elevation of the cellular NAD^+ level and subsequently activates SIRT1 (Revollo et al., 2004). Initially characterized as the human pre-B-cell colony-enhancing factor (PBEF) (Samal et al., 1994), NAMPT was also identified as an adipokine named visfatin (Fukuhara et al., 2005). Overexpression of visfatin in rodents activates PPARγ and ameliorates whole body insulin sensitivity (Sun et al., 2009).

In subcutaneous AT **(SCAT)** of Holstein dairy cows, visfatin mRNA abundance tended to decrease postpartum (p.p.) (Lemor et al., 2009). A further element involved in the regulation of SIRT1 activity is the adipokine adiponectin (ADIPOQ). Adiponectin is mainly expressed by adipocytes and exerts insulin sensitizing effects and inhibits lipolysis in AT (Yamauchi et al., 2001, Tschritter et al., 2003). In muscle cells of mice ADIPOQ was reported to activate SIRT1 by signaling through ADIPOQ receptor 1 (ADIPOR1) which subsequently lead to a decreased acetylation of PPARGC1A (Iwabu et al., 2010). Conversely, activation of SIRT1 was shown to decrease the secretion of ADIPOQ (Qiang et al., 2007). In Holstein dairy cows, serum ADIPOQ concentrations decrease towards parturition, reach a nadir at day 1 after calving and subsequently increase towards prepartum levels (Singh et al., 2014). Simultaneously, the *ADIPOR1* and *ADIPOR2* mRNA abundances were reported to decrease in subcutaneous AT during the transition period (Lemor et al., 2009).

The existence of the SIRT1-PPARGC1A axis was reported in adipose tissue of mice and is assumed to play a role in the regulation of insulin sensitivity (de las Heras et al., 2013). To our knowledge, so far nothing is known about this axis in AT of dairy cows. We assumed that

the axis' elements might be involved in the metabolic adaptation processes during the transition period in a tissue specific manner. Therefore the aim of the present study was to characterize the mRNA abundance of the aforementioned factors as well as PPARGC1A protein expression in SCAT and retroperitoneal AT **(RPAT)** of Holstein dairy cows during late pregnancy and early lactation and their relationship with the ADIPOQ system. We hypothesized that feeding different portions of concentrate during the prepartum period, resulting in differences in energy balance and a concomitant niacin supplementation might influence the bovine SIRT1-PPARGC1A axis in order to support the transition from late pregnancy to lactation.

MATERIALS & METHODS

Animals, Feeding and Sampling of Adipose tissue

Study design, feeding components, adipose tissue sample collection and processing are described in the companion paper by Weber et al. (2016). Table 1 shows the diet fed antepartum and p.p.

Blood Sampling and Analyses

Blood samples were drawn from the jugular vein on d -42, -21, -14, -7, -3, 1, 3, 7, 14, 21, 28, 35, 42, 63, 82 and 100 relative to calving. After clotting, blood was centrifuged at 2000 *g* for 15 min at 15°C and serum was stored at -80°C. Serum adiponectin concentrations were measured in duplicate using an in-house developed bovine specific ELISA as described in detail earlier (Mielenz et al., 2013). The measuring range as well as the linear range of the assay was 0.07-1.0 ng/mL. The intra- and inter-assay coefficients of variation were 7 and 11% respectively.

Western Blot Analysis

SCAT biopsy samples from all 20 cows at d -42 and d 21 as well as RPAT samples taken at d 21 were prepared for PPARGC1A Western blot analyses. For SIRT1, RPAT samples from the control cows at d -42, 1 and 21 as well as SCAT samples taken at d 21 were analyzed.

Tissue samples were homogenized in homogenization buffer containing 10 mM HEPES pH 7.4 and Complete protease inhibitor cocktail (one tablet per 10 mL, Roche, Mannheim, Germany) using the Precellys 24 system (Peqlab Biotechnologie GmbH, Erlangen, Germany). Homogenates were centrifuged twice (14000 x *g*, 4°C) to separate the fat layer. Aliquots of the supernatants were stored at -20°C until electrophoresis. The total protein concentration was measured using the Bradford assay (Roti Nanoquant K880, Carl Roth GmbH, Karlsruhe,

Germany). For PPARGC1A, the samples were diluted to 0.5 mg/mL with purified water and mixed with loading buffer (final concentrations: 0.1 M Tris HCL pH 6.8, 1% SDS, 0.0032 % bromophenol blue, 10% glycerol). The samples were loaded in duplicates (17.5 µg protein per lane) on 8% gels, separated by SDS-PAGE and transferred onto polyvinylidine difluoride membranes (PVDF, GE Healthcare Europe, Freiburg, Germany) with the use of the Trans-Blot Turbo transfer unit (Biorad Laboratories, Munich, Germany). Membranes were blocked in Tris-buffered saline solution-Tween (TBS-T) containing 10 % Roti-Block (Carl Roth GmbH) (Carl Roth GmbH) and 0.1 % Tween 20 (AppliChem GmbH, Darmstadt, Germany) for 1 h at room temperature. After blocking, a rabbit anti-PPARGC1A antibody (dilution 1:500, Merck Milipore, Merck KGaA, Darmstadt, Germany) was incubated in TBS-T containing 3% fat free milk powder (Carl Roth GmbH) and 0.1 % Tween 20 overnight at 4°C. This antibody was already successfully used by Wood et al. (2013) for assessing PPARGC1A protein in bovine liver and muscle tissue. After washing, membranes were incubated with a HRP conjugated corresponding secondary antibody (Mouse Anti-Rabbit IgG-HRP, SouthernBiotech, Birmingham, USA, dilution 1:25000) for 1 h at room temperature in TBS-T containing 10 % Roti-Block and 0.1 % Tween. Following another washing step, immunodetection was carried out using an enhanced chemiluminescence detection system (GE Healthcare). Imaging was performed with a VersaDoc MP4000 imaging system (Biorad) and the band intensities were densitometrically analyzed via the Image Lab software (Biorad). After immunodetection, Indian Ink staining was included as a loading and transfer control.

For SIRT1, the samples were diluted to 1 mg/mL and mixed with loading buffer and 0.2 M DTT. The samples were loaded in duplicates (20 µg protein per lane) on 10% gels and the SDS-PAGE and blotting was performed as described above. Membranes were blocked in TBS solution containing 5% fat-free milk powder (Carl Roth GmbH) and 0.1% Tween 20 (Applichem GmbH) for 1 h at room temperature and then incubated with primary antibodies against SIRT1 (ab13749, Abcam, Cambridge, UK) in 1:100 dilution overnight at 4°C. After washing, membranes were incubated with a HRP conjugated corresponding secondary antibody (Goat Anti-Rabbit IgG-HRP, SouthernBiotech, dilution 1:10000) for 1 h at room temperature. Detection of chemiluminescence and detection of bands carried using the same method as described above. Exemplary membranes showing the Indian Ink staining were included as supplemental figure 1 (PPARGC1A) and 2 (SIRT1).

Relative Quantification of mRNA

The preparation of the samples including RNA extraction and cDNA synthesis are described in detail in the companion paper by Weber et al. (2016). Relative quantification of the target

genes was performed with standard curves based on purified amplicons, except in case of *NAMPT* where serial dilutions of cDNA were used. As controls, a negative template control and a no reverse transcriptase control of the cDNA synthesis were carried along, together with a negative template control for each PCR run, containing 2 µl water instead of diluted template. The reactions were performed in Mx3000P systems (Stratagene, Amsterdam, the Netherlands and Agilent, Santa Clara, CA) with a total volume of 10 µL, including 2 µL cDNA (diluted 1:4), 1 µL primer mix, 2 µL water and 5 µL SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Nümbrecht, Germany). The sequences of the utilized primers and their respective conditions for quantitative PCR are provided in Table 2. Specificity of the polymerase chain products was confirmed for each primer by sequencing.

Reference gene stability

Selection, amplification and evaluation of the final reference genes were realized as described in the companion paper by Weber et al. (2016).

Statistical Analyses

Statistical analyses were performed using SPSS (version 22.0, SPSS Inc., Chicago, USA) on log-transformed mRNA data. No treatment effects were observed. Therefore data from the two feeding groups were merged for further analyses. The repeated comparison for the serum as well as the mRNA data between each time point was done using the mixed model procedure. The examined factors were considered as dependent variables; the level of concentrate and the niacin supplementation were incorporated as fixed effects, sampling day as repeated effect and their respective interactions were included into the model. As covariance structure autoregressive (first-order autoregressive structure with homogenous variances) followed by Bonferroni correction was used. The Student´s t-test was applied for assessing differences between SCAT and RPAT at each time point. Pearson correlation coefficient was calculated for assessing correlations. Data are expressed as means ± SEM. Statistical significance was set at $P < 0.05$ and P-values < 0.1 were defined as a trend.

RESULTS

Energy balance and mobilization of body fat

The strategy of feeding different portions of concentrate, supplemented with niacin, did not affect lipomobilization postpartum as indicated by the BCS as well as BHBA and NEFA concentrations. Also the energy balance was not affected by the feeding scheme postpartum (Tienken et al., 2015) (Supplemental table 1 and 2).

Adiponectin

The ADIPOQ concentrations in serum were not affected by treatment, but changes with time were observed. The concentrations decreased from d -42 reaching a nadir at d 1 after calving and increased to pre-calving levels within the first week after parturition. From d 7 onwards the ADIPOQ concentrations remained constant until the end of the experiment (Fig. 1).

SIRT1, PPARGC1A, NAMPT, ADIPOR1 and ADIPOR2 mRNA

No differences were observed between the feeding groups, thus merged data were analyzed for time-dependent effects.

The expression profiles of all five genes, except *ADIPOR2*, differed between SCAT and RPAT. For *SIRT1* mRNA abundance, time-dependent changes were observed in RPAT. *SIRT1* mRNA abundance decreased from d -42 until d 1 and then increased towards d 21. At d 100 *SIRT1* mRNA expression was lower than before parturition in RPAT (Fig. 2). For *PPARGC1A* time-dependent changes were restricted to SCAT, with greater mRNA abundance postpartum (Fig. 2). The *NAMPT* mRNA expression showed a very weak decrease after parturition on d 1 in SCAT and on day 100 in RPAT (Fig. 3). The mRNA abundance of *ADIPOR1* declined from d -42 to d 1 and d 21 after parturition and then increased back to the prepartum level from d 21 to 100 in SCAT. In RPAT the *ADIPOR1* mRNA abundance at all postpartum sampling dates was less than antepartum (Fig. 3). The mRNA expression of *ADIPOR2* was lower after parturition in SCAT and RPAT (Fig.3). Comparing SCAT and RPAT, *SIRT1* showed a higher mRNA abundance in RPAT on d -42 and d 21 (Fig. 2). For *PPARGC1Aα* (Fig. 2) and *NAMPT* (Fig. 3) higher mRNA expression was detected in SCAT. The mRNA abundance of *ADIPOR1* was higher in RPAT on d 21 (Fig. 3). The *ADIPOR2* mRNA expression was higher in RPAT postpartum (Fig. 3). The correlation coefficients between the variables assessed herein and NEFA, glucose, and BHBA (Tienken et al., 2015) are listed in Table 3. The highest positive correlation was found between *SIRT1* and *ADIPOR1* in both, SCAT (r = 0.720) and RPAT (r = 0.701). Negative correlations were strongest between NEFA and *visfatin* in RPAT (r = -0.401); in contrast this relationship was not significant in SCAT.

PPARGC1A protein

For PPARGC1A protein expression in SCAT a treatment effect was observed, i.e. the protein expression was higher in the LC group as compared to the HC group at d 21 after parturition. No differences in PPARGC1A protein expression were observed at day 21 between SCAT and RPAT (Fig. 4).

SIRT1 protein

The SIRT1 protein expression showed no changes related to treatment, thus data were pooled for further analysis. In RPAT, a trend for time-dependent changes of SIRT1 protein expression was detected. No differences were observed when comparing SIRT1 protein expression between SCAT and RPAT at d 21 after calving (Fig. 5).

DISCUSSION

We did not observe any treatment effect for the analyzed factors. This might be associated with the inability to induce differences in the energy balance p.p. by the prepartal feeding scheme we used. The BCS as well as lipolysis indicated by NEFA and BHBA concentrations in blood did not differ between the feeding groups (Tienken et al., 2015).

The serum ADIPOQ concentrations measured herein in pluriparous cows confirm recently published results (Singh et al., 2014) obtained with the same bovine-specific ELISA. The authors likewise reported a negative correlation between NEFA and adiponectin. Our results are also in accordance with earlier findings (Giesy et al., 2012) and support the authors´ hypothesis that the reduced ADIPOQ serum concentrations around parturition contribute to the homeorhetic adaptations during the respective time period and promote insulin resistance.

The decreased *ADIPOR1* and *ADIPOR2* mRNA abundance after parturition in SCAT and RPAT substantiates earlier findings in SCAT (Lemor et al., 2009, Saremi et al., 2014) and supports the authors´ assumption of a link to the reduced insulin sensitivity characteristically observed during that time. Adiponectin receptor mRNA expression was shown to be regulated by insulin in AT of mice (Tsuchida et al., 2004), as well as by growth hormone and prolactin in human (Nilsson et al., 2005) and mouse AT (Fasshauer et al., 2004). These hormones are known to change during the transition period (Accorsi et al., 2005). Thus the decreased *ADIPOR1* and *ADIPOR2* mRNA abundance might be the result of a complex regulatory system. High molecular weight ADIPOQ, the main form in dairy cattle (Giesy et al., 2012), was shown to possess a higher affinity to ADIPOR2 than ADIPOR1 (Kadowaki et al., 2006) and is associated with the improvement of insulin sensitivity (Pajvani et al., 2003). The higher *ADIPOR2* mRNA abundance and the higher correlation between the ADIPOQ receptors in RPAT thus point to a tissue-specific regulation of insulin sensitivity. The strongest correlation in this study was observed between *SIRT1* and *ADIPOR1* in SCAT and RPAT. Binding of ADIPOQ to ADIPOR1 was shown to regulate SIRT1 and AMP-activated protein kinase (AMPK) in muscle of mice (Iwabu et al., 2010). Since AMPK was discussed as an anti-lipolytic factor involved in the fine-tuning of lipolysis in cows (Locher et al., 2012), this

function of AMPK might partly be mediated via ADIPOR1 and SIRT1 in bovine AT, at least during early lactation. The observation, that ADIPOQ inhibits lipolysis via an AMPK-dependent pathway (Wedellova et al., 2011) might support this notion. It was further reported, at least in pancreas, that ADIPOQ inhibits apoptosis via ADIPOR1 and the AMPK SIRT1 pathway (Huang et al., 2014). Since the apoptotic rate does not change during lactation in SCAT of primiparous cows (Haussler et al., 2013), the down-regulation of the adiponectin system after parturition and the strong correlation between *ADIPOR1* and *SIRT1* might inhibit adipogenesis in order to ensure lipolysis. This might be supported by the fact, that SIRT1 attenuates adipogenesis (Picard et al., 2004). In this context, the observed correlation between *ADIPOR1* and *NAMPT*, as well as *SIRT1* and *NAMPT* might display a depot specific regulatory mechanism. For *SIRT1* and *NAMPT* a negative correlation was found only in SCAT. During SIRT1-dependent deacetylation processes, NAD is converted to NAM which inhibits SIRT1 activity (Bitterman et al., 2002). A high amount of NAM would thus lead to the inhibition of SIRT1, while activating NAMPT´s NAD synthetic function. The lacking relationship in RPAT might support our notion of ADIPOR1 and SIRT1 being involved in fat cell turnover, since in RPAT less apoptosis was reported compared to SCAT (Haussler et al., 2013). Song et al. (2013) reported a correlation between *SIRT1* and *ADIPOQ* mRNA abundance in SCAT and RPAT of women, with a higher correlation in SCAT. The authors related this finding to a possible relationship between SIRT1 and ADIPOQ gene transcription as discussed earlier (Qiao and Shao, 2006). The correlation observed herein indicates a similar relationship between SIRT1 and ADIPOR1 in bovine adipose tissue.

The mRNAs of *ADIPOR1* and *SIRT1* were positively correlated with the anti-lipolytic hydroxycarboxylic acid receptors 1 (*HCAR1*) and 2 (*HCAR2*) (companion paper) in both SCAT and RPAT, with *ADIPOR1* showing higher correlations in SCAT (Supplemental table 3). The adipokine ADIPOQ was reported to decrease lipolysis in mouse adipocytes (Qiao et al., 2011), and SIRT1 was reported to stimulate lipolysis by integrating β-adrenergic signaling, lipolysis and oxidative metabolism as important link (Picard et al., 2004, Khan et al., 2015). The β-adrenergic signaling pathway in controlling lipolysis is well known in dairy cows (Khan et al., 2013) with the beta-2 adrenergic receptors as being suggested as the most important subtype (Sumner and McNamara, 2007; Sumner and McNamara, 2007). During negative energy balance lipogenesis decreases and lipolysis increases (McNamara and Hillers, 1989). Since ADIPOQ showed an increase from d 1 to d 21 p.p. and for SIRT1 a trend for a time-effect was detected, these variables might be co-regulated in the context of controlling

lipolysis in dairy cows. Further theses variables might display a fine-tuning mechanism beside the main regulatory beta-adrenergic system.

Higher correlations between *ADIPOR1* and *HCAR1* as well as *HCAR2* in SCAT might hint to a higher lipolytic capacity of the retroperitoneal AT as discussed earlier (Locher et al., 2011). The catalytic activity of SIRT1 was recently shown to be important for the maintenance of insulin sensitivity in mice (Caron et al., 2014). The observed correlation in both AT depots might indicate a comparable role in the regulation of insulin sensitivity in cows; the observed trend for a time-dependent change of SIRT1 protein expression in RPAT together with the increasing serum ADIPOQ concentrations might indicate an improvement of insulin sensitivity. In murine hepatocytes resistin, a regulator of insulin resistance was reported to decrease SIRT1 mRNA abundance (Yu et al., 2013). This substantiates a relationship between SIRT1 and glucose metabolism, since we found increasing serum resistin concentrations towards parturition (companion paper).

During SIRT1-dependent deacetylation processes, NAD is converted to NAM, leading to the inhibition of the enzyme (Bitterman et al., 2002). To maintain SIRT1 activity, NAM has to be reconverted to NAD. The rate-limiting enzyme in this process is NAMPT/visfatin (Revollo et al., 2004). The decreased *NAMPT* mRNA expression in SCAT might lead to a reduced SIRT1 activity which in turn might contribute to the regulation of insulin sensitivity. The *NAMPT* mRNA abundance in subcutaneous AT detected herein was lower on d 100 p.p. as compared to d 42 a.p. These results are in accordance with observations from Lemor et al. (2009), who reported a trend towards a decreasing *NAMPT/visfatin* mRNA abundance postpartum. In contrast to this, we could not confirm the observed negative correlations between *NAMPT* and NEFA and *NAMPT* and BHBA. In our study we likewise detected a negative correlation between *NAMPT* and NEFA albeit only in RPAT and not in SCAT. The decreasing *NAMPT* mRNA abundance after parturition in RPAT might thus be a tissue dependent mechanism to decrease insulin-stimulated lipogenesis and to increase lipolysis. This might be supported by results obtained by von Soosten et al. (2011), who reported a higher mobilization of RPAT after parturition as compared to SCAT in primiparous German Holstein cows of rather low body weight and milk yield, as well as by Locher et al. (2011), who found a higher HSL-expression and phosphorylation in RPAT of pluriparous German Holstein cows, suggesting a higher lipolytic potential for this depot. In addition to its functions as an adipokine improving insulin sensitivity and biosynthetic enzyme, NAMPT/ visfatin was also characterized as a pro-inflammatory cytokine (Moschen et al., 2007). Since pro-inflammatory pathways are involved

in the homeorhetic adaptations to lactation (Farney et al., 2013), and the higher mRNA in RPAT, NAMPT/ visfatin might play a role in this AT depot during this time.

The PPARGC1A protein expression in SCAT was higher in the LC group as compared to the HC group at d 21. Since PPARGC1A is an important mediator of mitochondrial biogenesis (Liang and Ward, 2006), the increased protein expression in the LC group might hint to an increasing number of mitochondria. This might ameliorate the tense metabolic situation, compensating the more negative energy balance in the prepartum period.

CONCLUSION

The presented data substantiate the existence of the SIRT1-PPARGC1A axis in bovine adipose tissues. Further, a strong relationship between ADIPOR1 and SIRT1 was established at the level of transcription. The results indicate a relationship between the considered factors and point to a regulatory role in glucose metabolism during the transition period.

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Table 1. Diet composition antepartum vs. postpartum with different portions of concentrate vs. roughage in the prepartum period

Nutrient (g/kg DM)	Concentrate		Roughage	
	prepartum[1]	postpartum[2]	Corn	Grass
Crude Ash	42	47	36	94
Crude Protein	230	228	120	136
Ether Extract	39	39	29	32
ADF	45	44	222	310
NDF	163	152	461	506

[1]concentrate to roughage ratio: Low concentrate group 30:70, High concentrate group 60:40
[2]concentrate portion of 30% for all cows, increased to 50% within 16 days for the low concentrate group and within 24 days for the high concentrate group.

Table 2. Sequences of the primers and real-time polymerase chain reaction conditions used for the quantification of the target genes

Gene	Forward primer sequence (5´-3´) Reverse primer sequence (5´-3´)	Acc. no.[1]	bp	Con. (nM)[2]	meanCq [3]	Annealing (s/°C)[4]	Elongation (s/°C)[5]	Efficiency
SIRT1[6]	GGGGTTTCTGTTTCTTGTG ATGGTCTTGGGTCTTTTCTG	NM_001192980.1	139	600	29.3	59/60	60	89.9
PPARGC1A[7]	GGGGCAAATACACTCTTCCA TGGTCTTGTCTGCTTTGTCG	NM_1777945.3	185	200	32.2	60/60	60	94.5
NAMPT[8]	TGGCCACCGACTCATACAAG TCGGTCTTCTTTTCACGGCA	NM_001244141.1	97	600	27.1	59/60	60	94.2
ADIPOR1[9]	GCTGAAGTGAGAGGAAGAGTC GAGGGAATGGAGTTTATTGCC	NM_001034055.1	118	800	26.7	61/35	30	100.3
ADIPOR2[10]	GGCAACATCTGGACACATC CTGGAGACCCCTTCTGAG	NM_001040499.2	200	400	24.5	60/45	30	94.3

[1]NCBI Accession Number
[2]Concentrations for each primer
[3]Mean quantification cycle
[4]Initial denaturation for 10 min at 95°C; denaturation for 30 s at 95°C
[5]Elongation at 72°C
[6]Sirtuin-1
[7]PPARγ co-activator 1α
[8](Lemor et al., 2009)
[9]Adiponectin receptor 1, (Lemor et al., 2009)
[10]Adiponectin receptor 2, (Lemor et al., 2009)

Table 3. Coefficients of correlation (Pearson) between the serum concentrations of ADIPOQ, Glucose, NEFA and BHBA and the mRNA abundances of SIRT1, PPARGC1A, Visfatin, ADIPOR1 and ADIPOR2 in SCAT and RPAT of pluriparous dairy cows ($P < 0.05$).

		SCAT					RPAT				
	ADIPOQ	*ADIPOR1*	*ADIPOR2*	*SIRT1*	*PPARGC1A*	*NAMPT*	*ADIPOR1*	*ADIPOR2*	*SIRT1*	*PPARGC1A*	*NAMPT*
ADIPOQ							NS	NS	NS	NS	NS
ADIPOR1	NS										
ADIPOR2	NS	0.284					0.511				
SIRT1	NS	0.720	NS				0.701	0.357			
PPARGC1A	NS	NS	-0.288	NS			NS	NS	0.328		
NAMPT	NS	-0.388	0.274	-0.661	NS		0.372	0.462	NS	0.246	
Glucose	NS	0.413	0.366	NS	-0.275	NS	0.422	0.263	0.254	NS	0.257
NEFA	-0.338	-0.254	NS	NS	NS	NS	-0.286	-0.256	NS	NS	-0.401
BHB	NS	-0.294	NS	-0.243	NS	NS	NS	NS	NS	NS	NS

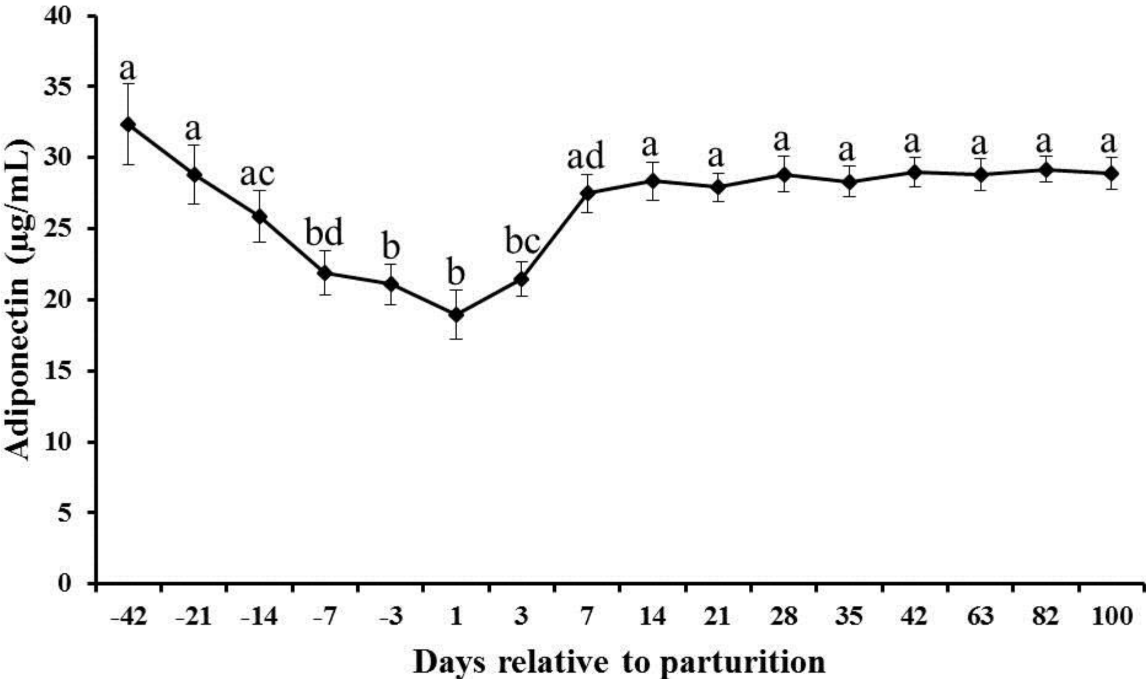


Figure 1. Time course of the adiponectin concentration in serum during late gestation and early lactation of pluriparous dairy cows (means ± SEM). Different letters indicate significant differences between the individual sampling dates ($P < 0.05$).

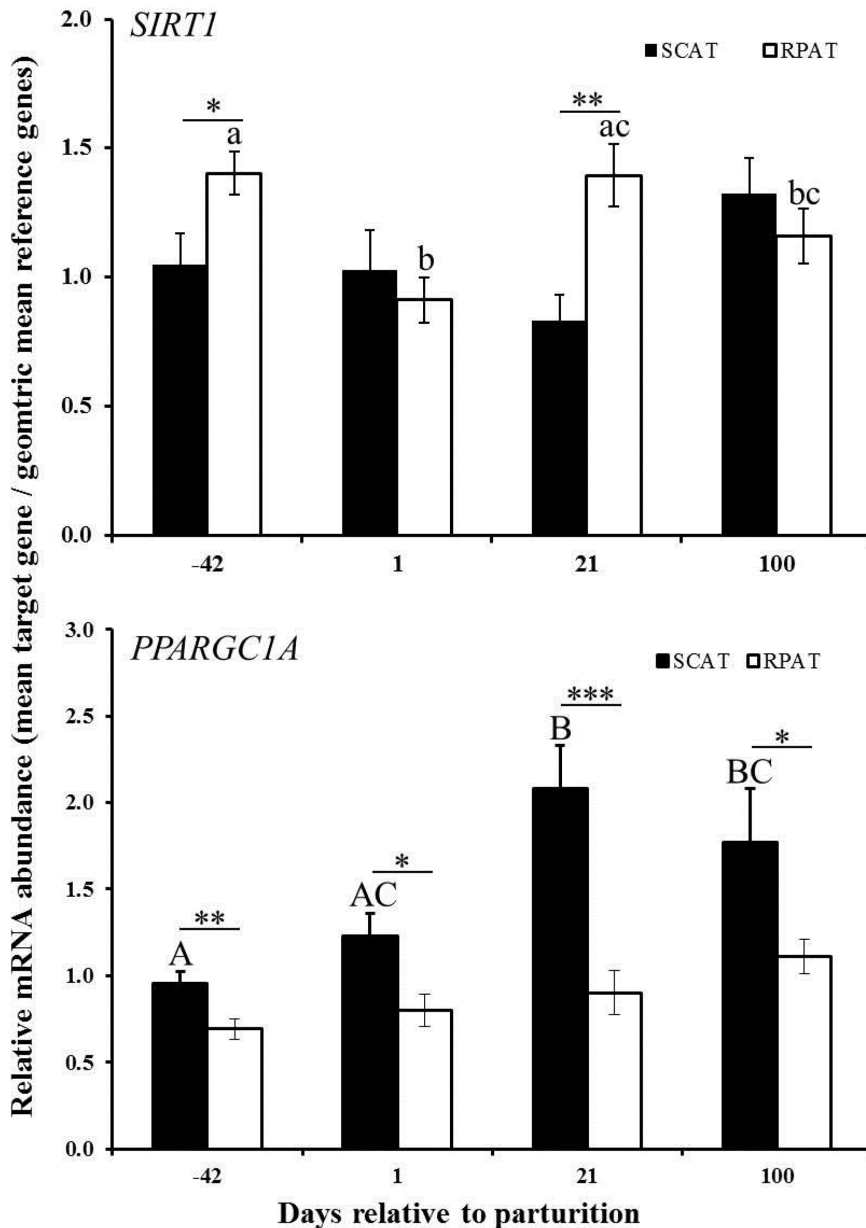


Figure 2. mRNA abundance of *SIRT1* and *PPARGC1A* on day -42, 1, 21 and 100 relative to parturition in SCAT and RPAT in pluriparous dairy cows (means ± SEM). Different capital letters indicate significant differences between the individual sampling dates in SCAT ($P < 0.05$), whereas different lower case characters designate differences between the sampling dates in RPAT ($P < 0.05$). Asterisks designate differences between SCAT and RPAT at the respective time-points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). For normalization lipoprotein receptor-related protein-10 (*LRP10*) and emerin (*EMD*) in SCAT, and *LRP10*, *EMD*, RNA polymerase II (*POLR2A*), marvel domain containing 1 (*MARVELD1*) and eukaryotic translation initiation factor 3, subunit K (*EIF3K*) in RPAT were used as reference genes. For comparing SCAT and RPAT, LRP10, EMD, POLR2A, MARVELD, EIF3K and hippocalcin-like 1 (HPCAL) were used as reference genes.

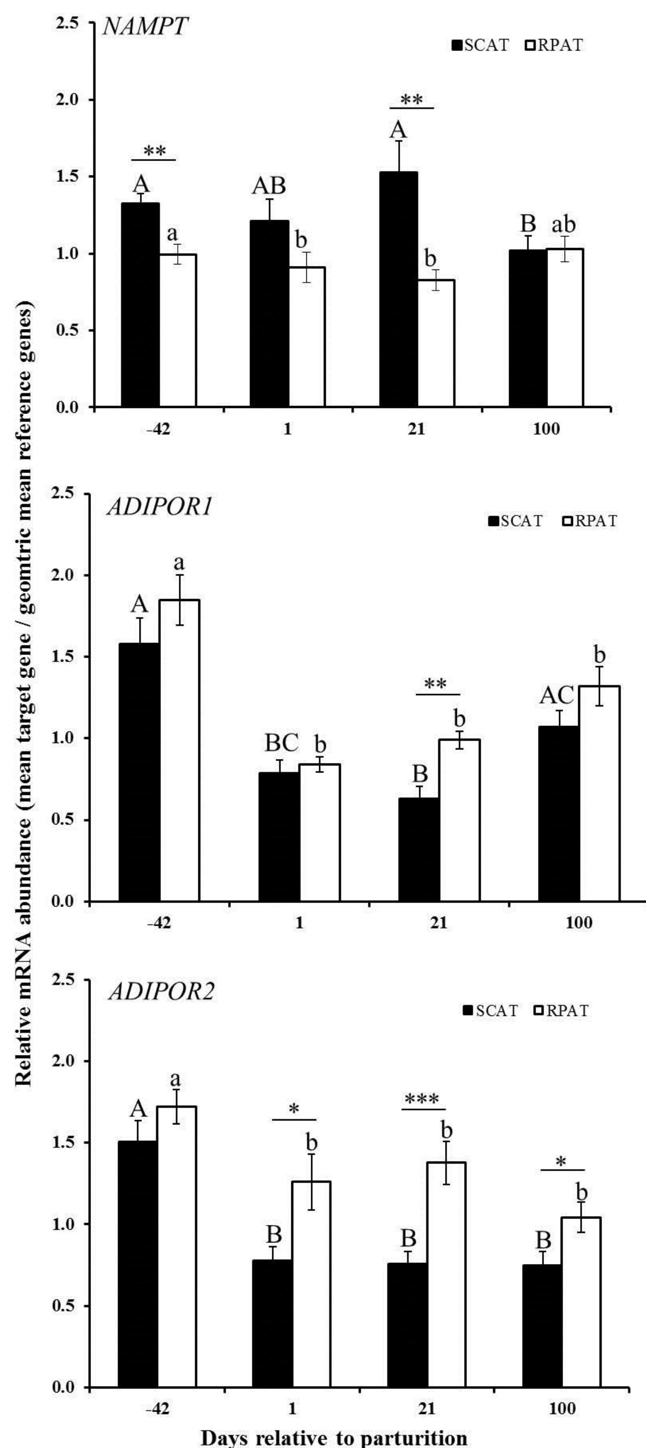


Figure 3. mRNA abundance of *NAMPT*, *ADIPOR1* and *ADIPOR2* on day -42, 1, 21 and 100 relative to parturition in SCAT and RPAT in pluriparous dairy cows (means ± SEM). Different capital letters indicate significant differences between the individual sampling dates in SCAT ($P < 0.05$), whereas different lower case characters designate differences between the sampling dates in RPAT ($P < 0.05$). Asterisks mark a different mRNA abundance between SCAT and RPAT at the respective time-points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). For normalization lipoprotein receptor-related protein-10 (*LRP10*) and emerin (*EMD*) in SCAT, and LRP10, EMD, RNA polymerase II (*POLR2A*), marvel domain containing 1 (*MARVELD1*) and eukaryotic translation initiation factor 3, subunit K (*EIF3K*) in RPAT were used as reference genes. For comparing SCAT and RPAT, LRP10, EMD, POLR2A, MARVELD, EIF3K and hippocalcin-like 1 (HPCAL) were used as reference genes.

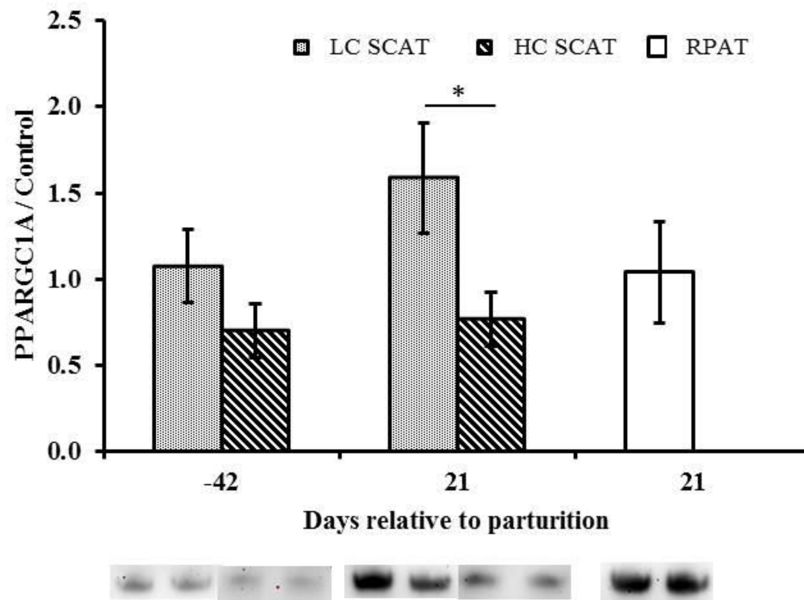


Figure 4. Protein expression of PPARGC1A in SCAT on days -42 and 21 and in RPAT on day 21 relative to parturition in pluriparous dairy cows (means ± SEM). Asterisk marks a different protein expression between the individual feeding groups (*P < 0.05). Indian Ink staining was included as a loading and transfer control. Representative Immunoblots are shown below the respective bars.

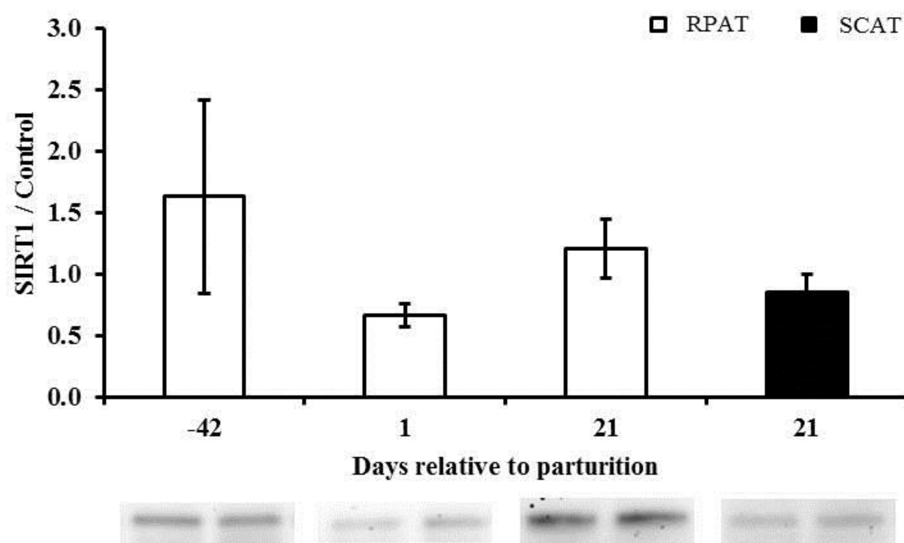


Figure 5. Protein expression of SIRT1 in RPAT on days -42, 1 and 21 and in SCAT on day 21 relative to parturition in pluriparous dairy cows (means ± SEM). There was a trend for a time effect in SCAT ($P < 0.1$). Indian Ink staining was included as a loading and transfer control. Representative Immunoblots are shown below the respective bars.

Supplemental data:

Supplemental table 1. Effects of feeding diets with either a low (LC) or high (HC) amount of concentrate and with supplementing either 0 g (CON) or 24 g nicotinic acid (NA) per day on production and metabolic parameters of primiparous (PP) and pluriparous (PlP) cows during the dry period (LSMeans ± SE).

	Concentrate level (C)		Supplement (S)		Parity (P)		*P*-value		
	LC	HC	CON	NA	PP	PlP	C	S	P
Production data									
DMI (kg/d)	13.2 ± 0.3	15.1 ± 0.3	14.0 ± 0.3	14.3 ± 0.3	12.8 ± 0.3	15.5 ± 0.3	<0.001	0.391	< 0.001
Energy intake (MJ NEL)	91.7 ± 2.0	115.3 ± 2.0	102.4 ± 2.0	104.6 ± 2.0	93.7 ± 2.3	113.3 ± 1.8	<0.001	0.440	< 0.001
Energy balance (MJ NEL)	35.6 ± 1.9	58.9 ± 1.9	46.8 ± 1.8	47.8 ± 1.9	40.1 ± 2.1	54.5 ± 1.7	<0.001	0.716	< 0.001
BCS	3.5 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	0.256	0.084	0.826
Metabolic parameters									
NEFA (mM)	0.30 ± 0.02	0.34 ± 0.01	0.30 ± 0.01	0.33 ± 0.01	0.32 ± 0.02	0.31 ± 0.01	0.076	0.170	0.627
BHBA (mM)	0.58 ± 0.02	0.54 ± 0.02	0.56 ± 0.02	0.56 ± 0.02	0.50 ± 0.02	0.62 ± 0.02	0.160	0.801	0.001
Glucose	73.0 ± 1.0	69.9 ± 1.0	71.7 ± 1.0	71.1 ± 1.0	72.9 ± 1.1	70.0 ± 0.9	0.036	0.688	0.050

Table according to Tienken et al. (2015). The authors appreciate Taylor & Francis Ltd. (www.tandfonline.com) for the opportunity to use the table.

Supplemental table 2. Effects of feeding prepartum diets with either a low (LC) or high (HC) amount of concentrate and with supplementing either 0 g (CON) or 24 g nicotinic acid (NA) per day on production, milk performance and metabolic parameters of primiparous (PP) and pluriparous (PlP) cows during lactation (LSMeans ± SE).

	Concentrate level (C)		Supplement (S)		Parity (P)		*P*-value		
	LC	HC	CON	NA	PP	PlP	C	S	P
Production data									
DMI (kg/d)	17.0 ± 0.4	16.7 ± 0.3	16.7 ± 0.3	17.0 ± 0.4	15.3 ± 0.4	18.4 ± 0.3	0.470	0.490	< 0.001
Energy intake (MJ NEL)	124.9 ± 2.6	121.3 ± 2.5	122.0 ± 2.5	124.2 ± 2.6	112.4 ± 2.8	133.8 ± 2.3	0.320	0.520	< 0.001
Energy balance (MJ NEL)	-26.7 ± 3.1	-26.2 ± 3.0	-25.3 ± 2.9	-27.7 ± 3.1	-16.3 ± 3.4	-36.7 ± 2.7	0.913	0.555	< 0.001
BCS	3.0 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	0.901	0.921	0.272
Body weight (kg)	610 ± 9	613 ± 9	602 ± 9	622 ± 9	579 ± 9	644 ± 9	0.823	0.121	< 0.001
Milk performance data									
Milk yield (kg/d)	33.5 ± 1.1	32.9 ± 1.1	33.4 ± 1.1	33.1 ± 1.1	27.3 ± 1.2	39.1 ± 1.0	0.694	0.839	< 0.001
ECM yield (kg/d)	35.6 ± 1.3	34.1 ± 1.2	34.2 ± 1.2	35.6 ± 1.2	29.0 ± 1.4	40.7 ± 1.1	0.393	0.407	< 0.001
Fat (%)	4.58 ± 0.12	4.37 ± 0.12	4.31 ± 0.12	4.64 ± 0.12	4.45 ± 0.13	4.50 ± 0.11	0.234	0.054	0.739
Protein (kg/d)	3.38 ± 0.04	3.32 ± 0.04	3.33 ± 0.03	3.37 ± 0.04	3.41 ± 0.04	3.29 ± 0.03	0.240	0.505	0.024
Lactose (%)	4.85 ± 0.02	4.85 ± 0.02	4.84 ± 0.02	4.86 ± 0.02	4.88 ± 0.02	4.82 ± 0.02	0.902	0.343	0.053
Urea (mg/kg)	204 ± 6.4	198 ± 6.1	201 ± 5.9	201 ± 6.3	202 ± 6.8	200 ± 5.4	0.554	0.934	0.782
Metabolic parameters									
NEFA (mM)	0.40 ± 0.02	0.37 ± 0.02	0.37 ± 0.02	0.40 ± 0.02	0.36 ± 0.02	0.41 ± 0.02	0.191	0.218	0.090
BHBA (mM)	0.77 ± 0.05	0.76 ± 0.05	0.76 ± 0.05	0.77 ± 0.05	0.71 ± 0.05	0.82 ± 0.04	0.901	0.821	0.105
Glucose	53.6 ± 1.1	54.1 ± 1.0	53.1 ± 1.0	54.6 ± 1.1	55.1 ± 1.2	52.6 ± 0.9	0.705	0.328	0.104

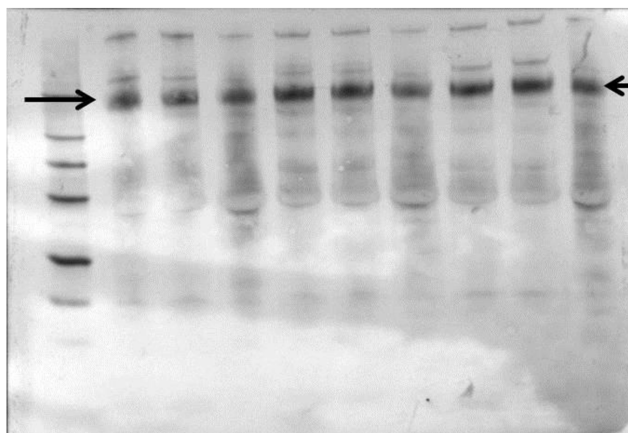
Table according to Tienken et al. (2015). The authors appreciate Taylor & Francis Ltd. (www.tandfonline.com) for the opportunity to use the table.

Supplemental table 3. Coefficients of correlation (Pearson) for assessing relationships between the mRNA abundances of *SIRT1* and *ADIPOR1* with *HCAR1* and *HCAR2* in SCAT and RPAT of pluriparous dairy cows ($P < 0.05$).

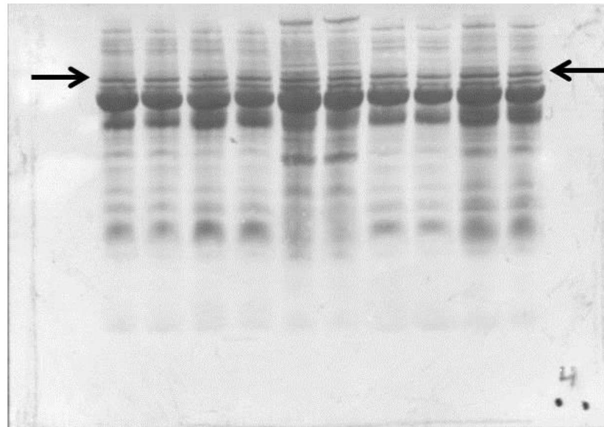
	SCAT		RPAT	
	ADIPOR1	*SIRT1*	*ADIPOR1*	*SIRT1*
HCAR1	0.477	0.741	0.354	0.665
HCAR2	0.744	0.716	0.581	0.749

Data on *HCAR1*, *HCAR2 is* presented in the companion paper (Weber et al., 2016)

Supplemental figure 1. Exemplary membrane of PPARGC1A showing the Indian Ink staining used for normalization (arrows point out the selected band used for calculations.)



Supplemental figure 2. Exemplary membrane of SIRT1 showing the Indian Ink staining used for normalization (arrows point out the selected band used for calculations.)



5. General discussion and future research perspectives

On the basis of the present study, the importance of several adipokines and related factors for the metabolic adaptations during the transition period of dairy cows was demonstrated. A more detailed knowledge about these metabolic processes is vital to promote a successful transition, resulting in an improved health status and an increased profitability of the cows, combining the interests of consumers and farmers. For farmers this is of special interest regarding the current situation on the milk market. Since April 2015, the European milk quota system has expired and enables the dairy farms to determine the production volume without European restrictions. In view of increasing milk quantities, the metabolic pressure increases and thereby the risk for transition diseases like ketosis, mastitis and abomasal displacement. Further, the understanding of the transition period is of importance regarding animal welfare. A successful transition might extend the productive life of the dairy cows, which decreased significantly over the last decades. This fundamental research provides insight into the physiological processes during the transition period as well as a basis for future research projects.

The adipokine apelin was reported to be involved in a variety of metabolic processes, e.g. in the regulation of glucose metabolism (Dray et al., 2008). In the present study, no changes in total serum apelin concentration related to treatment and time were observed, thus apelin was suggested as not being suitable to predict insulin sensitivity during the transition period. In human AT, TNF-α was shown to increase apelin expression (Daviaud et al., 2006). In AT of dairy cows, TNF-α mRNA was reported to remain constant during late gestation and early lactation (Friedrichs et al., 2014, Saremi et al., 2014). This might explain the fairly constant serum apelin concentrations observed herein. Apelin-dependent effects might further be mediated via the expression of its receptor APJ. Depending on the cell type, APJ is coupled to different G-proteins, resulting in different intracellular signaling pathways (Chaves-Almagro et al., 2015). Tissue specific changes in the APJ expression level might trigger the individual signaling pathways without changes in serum apelin concentrations. Thus APJ should be considered for prospective research projects.

As already discussed herein, possible effects of apelin might be mediated via the different apelin peptides, rather than via changing total serum apelin concentrations. In a recent review, a tissue specific expression of the endopeptidases involved in the cleavage of the different apelin peptides was suggested (Chaves-Almagro et al., 2015). This further supports the assumption that specific apelin peptides might play a role during the transition period.

In human epithelial cells the apelin-13 peptide was shown to play a role in angiogenesis (Kunduzova et al., 2008). Regarding this function, apelin might be of importance in primiparous cows, i.e. in the developmental process of the mammary gland during the first gestation and lactation.

As apelin was also shown to enhance mitochondrial biogenesis via increasing PPARGC1A expression and a weak correlation was observed between apelin and PPARGC1A in RPAT in the present study, apelin might partly be involved in the higher metabolic activity of RPAT (von Soosten et al., 2011) at least during the periparturient period in dairy cows. In addition, apelin might be important for the offspring. In an earlier study, apelin was found at high concentrations in bovine colostrum and was also detectable in commercially processed milk (Bouchard et al., 1993). Since administration of apelin in rats was reported to increase drinking behavior in a dose-dependent manner (Taheri et al., 2002), and apelin serum concentrations were shown to be increased during water deprivation in goats (Sato et al., 2012), in colostrum it might affect the calves' drinking pattern. Changes in apelin concentration during the transition period might probably be focused on milk and mammary gland and should be taken into consideration for future research. These assumptions show that the results provided herein do not indicate a functional role of apelin in glucose metabolism but introduce a wide range of possible regulatory functions which make apelin a promising research topic.

The observed correlation between resistin and serum NEFA supports the thesis of Reverchon et al. (2014), that resistin is likely involved in the regulation of lipolysis around calving. However, a bovine receptor for resistin has yet to be defined. The adenylyl cyclase-associated protein 1 (CAP1), which was identified as a receptor for resistin leading to inflammation in humans (Lee et al., 2014) might also be a promising candidate in the bovine, since inflammatory pathways are involved in the homeorhetic adaptations to lactation (Farney et al., 2013). Further, binding of resistin to CAP1 was reported to activate adenylyl cyclase (Lee et al., 2014). In adipocytes this might lead to increasing lipolysis and might be the mechanism explaining the correlation between serum NEFA and resistin.

For the receptor HCA_1, no correlations were observed with the serum lactate concentrations. Further, the measured lactate concentrations might only induce a minor receptor activation. During the fermentation of carbohydrates, lactate is produced in the rumen (Waldo and Schultz, 1956). Since lactate is only a minor intermediate in the rumen metabolism and is used for propionate formation (Counotte and Prins, 1981), the existence of a ligand other than

lactate was suggested. In humans, the phenolic acid 3,5-dihydroxybenzoic acid (3,5-DHBA) was identified as a specific ligand for HCA_1 (Liu et al., 2012). The authors suggested that the 3,5-DHBA concentration absorbed from food may provide additional activation of HCA_1 next to lactate. Since polyphenolic acids are rich e.g. in fruits and vegetables, grass and maize silage should be tested for the presence of 3,5-DHBA. If this specific phenolic acid is also present in dairy feed, it might occur in the rumen. Thus it might present a possible anti-lipolytic mechanism in the dairy cow and might point to a higher relevance of HCA_1 in the mesenteric or omental adipose tissue surrounding the intestine. These AT depots drain into the portal vein (Rebuffe-Scrive et al., 1989), thus a higher anti-lipolytic capacity of HCA_1 might be a safeguard mechanism to protect the liver against a high influx of fatty acids and reduce the development of fatty liver syndrome during the transition period. An alternative feeding strategy to decrease lipolysis might thus be an increasing amount of polyphenols in the ratio. Grape pomace was recently characterized as a suitable dietary source to increase the amount of polyphenols for ruminant feeding (Winkler et al., 2015). The affinity of the grape polyphenols to HCA_1 should be considered in future research studies, since a higher polyphenolic content might decrease lipolysis via HCA_1, reduce the risk for developing fatty liver and might thus improve the health status of periparturient dairy cows.

In this study, the existence of the bovine SIRT1-PPARGC1A axis and its relationship with the adiponectin system was substantiated. The highest correlation was observed between the mRNA abundance of SIRT1 and AdipoR1 in both SCAT and RPAT. We hypothesized there might be a relationship between these variables and the enzyme AMPK. Recently, visfatin was shown to stimulate glucose uptake via an AMPK-dependent pathway in skeletal muscle cells (Lee et al., 2015). Since visfatin is important for the regulation of SIRT1 function (Revollo et al., 2004), and the SIRT1-PPARGC1A axis was supposed to play a role in the regulation of glucose metabolism (de las Heras et al., 2013) this might support our notion of a functional relationship between the SIRT1-PPARGC1A axis and the AMPK in the dairy cow. To verify this idea, future research on the PPARGC1A axis and its role during the transition period might include the AMPK and should confirm the mRNA results of AdipoR1 and AdipoR2 observed herein on the level of protein expression. Further the enzyme activity of SIRT1 and the activity of PPARGC1A should be examined, to verify the functional relationship between these variables.

In the present experiment, the effects of a diet differing in the concentrate to roughage ratio and a concomitant nicotinic acid supplementation on variables potentially involved in the

periparturient glucose and lipid metabolism should be examined. However, the applied feeding regimen did not affect post partum AT mobilization as indicated by serum NEFA and BHBA concentrations. Further, no differences in the post partum energy balance were reported (Tienken et al., 2015) and consequently no treatment-dependent effects on the variables examined herein, excluding PPARGC1A, were observed.

The animals in this study received 24 g of a nicotinic acid supplement per day. Lipid lowering effects of nicotinic acid have already been observed at daily doses ranging from 3 g to 12 g per day (Dufva et al., 1983). Thus the selected niacin amount should have been suitable to exert an anti-lipolytic effect.

In dairy cows, only NAM was detected in serum after a NA supplementation given at an oral dose of 6 g per day (Niehoff et al., 2009). Thus the authors hypothesized, that a conversion of NA to NAM as observed in rodents might also take place in the bovine. This is supported by the fact, that NAM is the main transport form of niacin in the blood (Henderson, 1983). Kenez et al. (2014) showed that only NA was capable to induce an anti-lipolytic effect in bovine AT *in vitro*. In humans HCA_2 was reported to possess only a low affinity to NAM, i.e. NAM was ~ 1000 fold less capable to activate the receptor than NA (Wood et al., 2013). The possible conversion of NA to NAM might be the reason that serum NA was consistently under the limit of detection (Tienken et al., data not shown). Thus the NA concentrations reaching the AT might have been too low to induce an anti-lipolytic effect and to influence the observed variables herein. Although we did not observe any niacin effects in AT, effects on other tissues, e.g. muscle, cannot be excluded. In mice, pharmacological niacin doses were shown to improve the carnitine status in muscle tissue, which is important for the mitochondrial fatty acid utilization (Couturier et al., 2014). In sheep, the authors reported a niacin induced transition of muscle fibers from a glycolytic to an oxidative type and estimated this finding as particularly useful for periparturient dairy cows (Khan et al., 2013). In the present experiment, niacin might have induced a higher muscular capacity for using fatty acids, resulting in an improved energy status. For reasons of animal welfare, we passed on taking additional biopsies of muscle tissue. Based on these current results, a positive effect of a niacin supplementation on musculature cannot be excluded and muscle tissue should be considered for future research.

Besides lipid-lowering effects, some studies reported an increasing number of ruminal protozoa after daily supplementation of 6 g niacin (Erickson et al., 1990, Doreau and Ottou, 1996, Aschemann et al., 2012). The utilization of starch by entodinia supports a stable ruminal fermentation and further provides additional microbial protein to the cow (Erickson et

al., 1990). The increased protein provision might result in a better energy supply which might lead to a decreasing lipolysis debranching the cow's metabolism. Thus, a positive effect of the niacin supplementation cannot be completely excluded.
As already mentioned, the applied feeding regimen (low concentrate vs. high concentrate) did not affect AT mobilization post partum. According to a review from 2010 (Grummer et al.), influencing the energy status of early lactating cows through nutritional management strategies might be difficult. Therefore, the management of the dry period was discussed as an alternative approach to improve the energy status of transition dairy cows (Grummer, 2007, Grummer et al., 2010), a time frame typically considered as a period of recovery between two lactations (Beever, 2006). Shortening or eliminating this period may possibly improve the energetic situation of the periparturient dairy cow and consequently enhance reproductive efficiency. Cows undergoing no dry period did not enter a NEB and a shortening of the respective period resulted in a greater conception rate (Grummer et al., 2010). However, to consider animal welfare, the elongation of the operating period should also be taken into account. Further research might consider the duration of the dry period, in order to achieve a successful transition of the dairy cow.

From the current results, different mRNA expression patterns between the two adipose tissues examined were observed. To confirm these results, future studies should determine the protein expression of the presented variables exclusively examined on the level of transcription. Since potential effects of a niacin supplementation on the metabolic status might not be restricted to adipose tissue, effects on the dairy cows' metabolism cannot be excluded and future research should include e.g. muscle tissue as a promising target for potential niacin induced effects.

6. Summary

The transition from late gestation to early lactation in dairy cows is characterized by major changes in energy balance and of the metabolic and endocrine status. The animals have to mobilize body reserves, mainly in form of fat, to cover their energy demands and homeorhetic adaptations are necessary to ensure the nutrient drain towards the mammary gland. The understanding of adipose tissue (AT) metabolism is of special interest, since it plays a role in regulating metabolism via secreting messenger molecules, i.e. adipokines. Adipose tissue depots of different locations, i.e. visceral and subcutaneous AT, are known to differ in their structural and functional characteristics and may thus respond in divergent ways to the physiological changes during the transition period. Several feeding strategies exist to support the successful transition of dairy cows. Nicotinic acid is a known anti-lipolytic agent, which is used to release the metabolic pressure during the transition period. Albeit the effect on plasma NEFA is well known, potential effects on adipokines, receptors and enzymes in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) linked with lipid and glucose metabolism were not known. Therefor the present study aimed 1) to investigate effects of two different amounts of dietary concentrate and a subsequent niacin supplementation on different variables related to lipid and glucose metabolism, 2) to compare the mRNA expression of the target genes between SCAT and RPAT and 3) to discover possible relationships between the target genes and adipokines.

For the present experiment 20 pluriparous German Holstein cows were studied from d 42 prepartum until d 100 post partum. The animals were allocated to two different feeding groups, receiving either a high (concentrate to roughage ratio 60:40) or a low portion (concentrate to roughage ratio 30:70) of dietary concentrate before parturition. Both groups were further subdivided into a niacin group receiving 24 g nicotinic acid per day until d 24 post partum, and a control group. After parturition, a concentrate portion of 30% was fed to all cows and increased to 50% during 16 days for the low concentrate group and during 24 day for the animals of the high concentrate group. Biopsy samples from subcutaneous tail head and retroperitoneal adipose tissue were collected at d -42, 1, 21 and 100 relative to parturition. Blood samples were drawn along with the biopsies as well as on d -21, -14, -7, -3, 3, 7, 14, 28, 35, 42, 63 and 82 relative to calving.

Within **manuscript 1**, we aimed to characterize the serum concentration of the adipokines apelin and resistin as well as their relationship to receptors linked with lipolysis, i.e. the lactate receptor HCA_1, the niacin receptor HCA_2 and TNFR1, as well as to test the effects of diets differing in the concentrate to roughage ratio. The serum apelin concentrations remained

fairly constant during the observed time, thus it seems not be a suitable predictor for insulin sensitivity during the transition period. Resistin concentrations increased towards parturition and returned to pre-calving levels within one week post partum. Due to the high correlation with plasma NEFA, resistin seems to be involved in the lipolytic regulation around parturition. The different HCA_1 mRNA abundance in SCAT and RPAT might hint to a tissue specific lipolytic regulation. Results for HCA_2 and its endogenous ligand BHBA point to a relevant regulatory role only in ketonemic conditions. Different expression patterns in SCAT and RPAT further supports the notion of a depot-specific lipolytic capacity. The expression pattern of TNFR1 also substantiates this hypothesis. The observed positive correlations suggest a functional link between *HCA_2* and serum glucose concentrations to save energy in forms of triglycerides in dairy cows when glucose concentrations are elevated. The negative correlation with serum BHBA concentrations might indicate a negative feedback loop that down-regulates HCA_2 when BHBA concentrations are high in order to ensure lipolysis in the transition dairy cow.

Within **manuscript 2**, we aimed to characterize the functional interaction between the NAD-dependent enzyme SIRT1, its target PPARγ co-activator 1α (PPARGC1A) target, nicotinamide phosphoribosyltransferase (Nampt) and its relationship with the adiponectin system. The differential mRNA expression pattern of the AdipoQ receptors between SCAT and RPAT points to a tissue-specific regulation of insulin sensitivity. The strongest correlation was observed between SIRT1 and AdipoR1. Since AdipoR1 was shown to regulate SIRT1 and AMP-activated protein kinase (AMPK), the anti-lipolytic effect of AMPK in cows might partly be mediated by AdipoR1 and SIRT1. In addition, SIRT1 seems to be involved in the regulation of insulin sensitivity. The correlations indicate a relationship between the examined variables, substantiates the existence of the bovine SIRT1-PPARGC1a axis and further point to a regulatory role in glucose metabolism during the transition period.

The results of these studies provide expression profiles of genes, that indicate depot-specific regulatory functions of AT in glucose and lipid metabolism in periparturient dairy cows. These functions should further be substantiated on the level on protein expression.

7. Zusammenfassung

Die Übergangsphase von der späten Trächtigkeit zur frühen Laktation ist bei Milchkühen durch ausgeprägte Veränderungen der Energiebilanz, sowie des metabolischen und endokrinen Status gekennzeichnet. Zur Deckung des Energiebedarfs erfolgt die Mobilisierung körpereigner Reserven, hauptsächlich in Form von Fett, da der Energieaustrag über die Milch durch die nur langsam ansteigende Futteraufnahme noch nicht ausgeglichen werden kann. Zusätzlich erfolgen hormonelle und metabolische Anpassungsreaktionen, um den Nährstofffluss zur Milchdrüse zu gewährleisten. Das Fettgewebe steht hierbei besonders im Focus, da es durch die Sekretion von Signalmolekülen, den sogenannten Adipokinen, eine Rolle in der Regulation des Stoffwechsels spielt. Fettgewebe unterschiedlicher Lokalisation, subkutanes und viszerales Fettgewebe, unterscheiden sich hinsichtlich ihrer strukturellen und regulatorischen Merkmale. Dies könnte zu unterschiedlichen Reaktionen auf die physiologischen Veränderungen während der Transitphase führen. Der Einsatz unterschiedlicher Fütterungsstrategien dient der Unterstützung einer erfolgreiche Passage der Kuh durch diese Phase. Nikotinsäure wird als anti-lipolytisch wirksames Supplement eingesetzt, um den Stoffwechsel zu entlasten. Während der Effekt auf den NEFA Plasmaspiegel geläufig ist, sind mögliche Effekte auf Adipokine, Rezeptoren und Enzyme im subkutanen (SCAT) und retroperitonealen Fettgewebe (RPAT), die in Beziehung zum Fett- und Glukosestoffwechsel stehen noch weitgehend unbekannt. Aufgrund dessen zielte die vorliegende Arbeit darauf ab, 1) die Effekte unterschiedlicher Kraftfutteranteile sowie eine begleitende Niacinsupplementation auf Variablen des Fett- und Glukosestoffwechsels zu charakterisieren, 2) die mRNA Expression der Zielgene zwischen RPAT und SCAT zu vergleichen, sowie 3) Zusammenhänge zwischen den einzelnen Variablen zu ermitteln.

Das experimentelle Design der vorliegenden Arbeit umfasste 20 pluripare Kühe der Rasse Friesisch Holstein im Zeitraum zwischen Tag 42 vor der errechneten Kalbung bis Tag 100 nach der Kalbung. Die Tiere wurden in zwei Fütterungsgruppen aufgeteilt: eine Hochkonzentrat-Gruppe (HC) mit einem Kraftfutter : Raufutter Verhältnis von 60:40, sowie eine Niedrig-Konzentrat Gruppe (LC) mit einem Kraftfutter : Raufutter Verhältnis von 30:70. Beide Gruppen wurden weiterhin in eine Niacingruppe, sowie eine Kontrollgruppe unterteilt. Die Tiere der Niacingruppe erhielten bis zum Tag 24 nach der Kalbung 24 g Nikotinsäure pro Tag. Nach der Kalbung erhielten alle Tiere einen Kraftfutteranteil von 30%. Dieser wurde für die LC-Gruppe innerhalb von 16 Tagen und für die Tiere der HC-Gruppe innerhalb von 24 Tagen auf 50% erhöht und bis zum Ende der Studie gefüttert. An Tag -42, 1, 21 und 100 wurden mittels Biopsie Proben zweier Fettdepots entnommen: subkutanes Fettgewebe vom

Schwanzansatz sowie retroperitoneales Fettgewebe. Blutproben wurden zeitgleich mit den Biopsien, sowie an den Tagen -21, -14, -7, -3, 3, 7, 14, 28, 35, 42, 63 und 82 relativ zur Kalbung entnommen.

In **Manuskript 1** erfolgte die Charakterisierung des Serumprofils der Adipokine Apelin und Resistin sowie deren Beziehung zu verschiedenen mit der Lipolyse verbundenen Rezeptoren (der Laktatrezeptor HCA_1, der Nikotinsäure-bindende Rezeptor HCA_2 und TNFR1). Zusätzlich wurde der Effekt der unterschiedlichen Kraftfutteranteile in der täglichen Futterration sowie der Niacingabe untersucht. Die Serum Apelinkonzentration blieb weitgehend unverändert und eignet sich somit nicht als Indikator für die Beurteilung der Insulinsensistivität. Die Resistinkonzentration stieg zur Kalbung hin an und fiel innerhalb der ersten Woche nach der Kalbung wieder auf das vorgeburtliche Niveau. Aufgrund der hohen Korrelation zwischen Resistin und den NEFA-Werten, könnte Resistin eine Rolle bei der Regulation der Lipolyse im Zeitraum um die Geburt zu spielen. Die Unterschiede der HCA_2 mRNA-Expression zwischen SCAT und RPAT deuten auf eine depot-spezifische Regulation der Lipolyse hin. Unter Berücksichtigung der gemessenen Laktatwerte, die unterhalb der für eine Rezeptoraktivierung benötigten Konzentration liegen, lässt sich über die Existenz eines weiteren HCA_1-Liganden bei Milchkühen spekulieren. Die mRNA-Expression des HCA_2 sowie die gemessenen Konzentrationen seinen endogenen Liganden β-Hydroxybutyrat (BHBA), weisen auf eine regulatorische Rolle in ketotischen Zuständen hin. Die Unterschiede zwischen SCAT und RPAT sowie die Ergebnisse für TNFR1 unterstützen zudem die These einer depot-spezifischen Regulation der Lipolyse. Die ermittelten Korrelationen deuten auf einen funktionellen Link zwischen Glukose und HCA_2 hin, der dazu führt dass Energie in Form von Triglyceriden eingespart wird, wenn Glukose als Energiequelle zur Verfügung steht. Die negative Korrelation zwischen HCA_2 und BHB weist auf eine Rückkopplungschleife hin, die bei hohen BHBA-Konzentrationen zu einer verminderten HCA_2 mRNA-Expression führt um so die Lipolyse in der Transitphase zu gewährleisten.

In **Manuskript 2** wurde die funktionelle Interaktion zwischen dem NAD-abhängigen Enzym Sirtuin 1 (SIRT1), dem PPARγ co-activator 1α (PPARGC1A), der Nicotinamidphosphoribosyltransferase (Nampt) sowie das Verhältnis dieser Variablen zum Adiponektin (AdipoQ)-System charakterisiert. Das depot-spezifische mRNA-Expressionsmuster der beiden AdipoQ–Rezeptoren (AdipoR1 und AdipoR2) deutet auf eine Fettdepot-spezifische Regulation der Insulinsensitivität hin. Die stärkste Korrelation bestand zwischen AdipoR1 und SIRT1. Da AdipoR1 bereits als Regulator von SIRT1 sowie der AMP-aktivierten Kinase (AMPK) charakterisiert wurde, könnte dies über SIRT1 den anti-

lipolytischen Effekt der AMPK vermitteln. SIRT1 scheint zudem an der Regulation der Insulinsensitivität beteiligt zu sein. Die Korrelationen weisen auf einen funktionellen Zusammenhang zwischen den Variablen hin, belegen die Existenz der SIRT1-PPARGC1A Achse und deuten auf eine funktionelle Rolle im Glukosestoffwechsel hin.

Die vorliegende Arbeit stellt die Expressionsprofile der Zielgene zur Verfügung, die auf eine regulatorische Funktion der Variablen im peripartalen Glucosestoffwechsel von Milchkühen hinweisen. Zur Bestätigung der Funktionen sollten künftig die Proteinexpressionen charakterisiert werden.

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Abstracts in conferences

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