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Characterization of adiponectin at different physiological states in cattle based on an in-house developed immunological assay for bovine adiponectin

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Dedicated to my family

Abstract

Adipose tissue (AT), through secretion of adipokines, plays a central role in regulating metabolism. Adiponectin is one of the most abundant adipokines and is linked with several physiological mechanisms such as insulin sensitivity and inflammation. The aim of this dissertation was to characterize the effect of stage of lactation and of supplementation with conjugated linoleic acids (CLA) on blood adiponectin in dairy cows. In addition, adiponectin concentrations in different AT depots and the effect of lactational and dietary induced negative energy balance (NEB) on blood and milk adiponectin were studied in dairy cows. Adiponectin concentrations of blood were studied using serum samples obtained from multiparous (MP) and primiparous (PP) cows receiving either CLA or a control fat supplement from d -21 to d 252 relative to calving, and serum as well as AT samples [3 subcutaneous (sc): tail-head, sternum and withers and 3 visceral (vc): mesenterial, omental and retroperitoneal] from PP cows slaughtered at d 1, 42 and 105 of lactation. Effects of lactational and dietary induced NEB on plasma adiponectin were investigated in MP cows from d -21 to d 182 relative to calving with feed restriction for 3 weeks beginning at around 100 days of lactation. Blood adiponectin was decreased from d 21 ante partum, reached a nadir at calving and increased during the post partum period. CLA supplementation reduced circulating adiponectin post partum in both MP and PP cows and, as indicated by a surrogate marker of insulin sensitivity (RQUICKI) also resulted in decreased insulin sensitivity. The decline in blood adiponectin around parturition may result from reduced adiponectin protein expression in all fat depots. vcAT contained more adiponectin than scAT suggesting a relatively higher impact of vcAT on adiponectin blood concentrations. However, retroperitoneal AT had the lowest adiponectin content compared to the other fat depots and thus seems to play an unique role in lipid mobilization in dairy cows. NEB due to feed restriction about 100 days of lactation caused a decline in adiponectin secretion through milk but did not affect its plasma concentrations. In conclusion, the major changes in blood adiponectin occurred around parturition; dietary CLA supplementation reduced circulating adiponectin. Differing amounts of adiponectin per AT depot indicate differential contributions to circulating adiponectin. The present dissertation serves as a basis for further studies elucidating the role and regulation of adiponectin and other adipokines in various pathophysiological conditions in cattle.

Abstrakt

Fettgewebe spielt über die Sekretion von Adipokinen eine zentrale Rolle in der Regulation des Stoffwechsels. Adiponektin ist eines der am häufigsten vorkommenden Adipokine und ist mit verschiedenen physiologischen Prozessen wie Insulinsensitivität und Entzündungsreaktionen verbunden. Ziel der vorliegenden Dissertation war es, die Effekte des Laktationstadiums sowie einer Supplementation mit konjugierten Linolsäuren (CLA) auf den Blutspiegel von Adiponektin bei Milchkühen zu charakterisieren. Des Weiteren wurde die Adiponektinkonzentration in verschiedenen Fettdepots von Milchkühen ermittelt, sowie die Effekte von sowohl laktationsbedingter als auch fütterungsinduzierter negativer Energiebilanz (NEB) auf die Adiponektinkonzentration im Blut und in der Milch untersucht. Die Bestimmung der Adiponektinkonzentration erfolgte im Blut multiparer (MP) und primiparer (PP) Kühe, welche entweder mit CLA oder einem Kontrollfett gefüttert wurden im Zeitraum von Tag -21 bis Tag 252 relativ zur Kalbung. In einem weiteren Versuch wurde Adiponektin in Serum, und Fettgewebe [3 subkutane (sc) Depots: Schwanzansatz, Sternum und Widerrist, und 3 viszerale (vc) Depots: mesenterial, omental und retroperitoneal] bei PP Kühen gemessen, die an Tag 1, 42 und 105 während der Laktation geschlachtet wurden. Die Effekte einer laktationsbedingten sowie einer fütterungsinduzierten NEB auf die Plasmadiponektinkonzentration wurden in MP Kühen zwischen Tag 21 vor der Kalbung bis Tag 185 der Laktation untersucht. Die restriktive Fütterung erfolgte über einen Zeitraum von drei Wochen, beginnend an Tag 100 der Laktation. Die Adiponektinkonzentration im Blut sank von Tag 21 ante partum, erreichte ihren niedrigsten Wert zum Zeitpunkt der Kalbung und stieg im postpartalen Zeitraum wieder an. Die CLA-Supplementation führte sowohl bei MP als auch bei PP Kühen zu einer Reduktion der zirkulierenden Adiponektinkonzentration post partum und weißt zudem über einen Marker für Insulinsensitivität (RQUICKI) auf eine verminderte Insulinsensitivität hin. Der Abfall der Adiponektinkonzentration im Blut um den Zeitraum der Geburt könnte durch eine verringerte Adiponektin-Proteinexpression in den Fettdepots induziert sein. Das viszerale Fettgewebe enthielt mehr Adiponektin als das subkutane Fettgewebe, was einen relativ größeren Einfluss der viszeralen Depots auf die Blutadiponektinkonzentration vermuten lässt. Das retroperitoneale Fettgewebe wies aber, verglichen mit den anderen Depots, den geringsten Adiponektingehalt auf und scheint deswegen eine besondere Rolle in der Lipidmobilisation von Milchkühen zu spielen. Die fütterungsbedingte NEB im Zeitraum um Tag 100 der Laktation führte zu einer Abnahme der Adiponektinsekretion durch die Milch, beeinflusste jedoch nicht die Plasmakonzentrationen. Zusammenfassend zeigte sich, dass die größten Veränderungen der Adiponektinblutkonzentration im Zeitraum um die Geburt auftraten und das zirkulierende Adiponektin durch die CLA-Supplementation reduziert wurde. Die unterschiedlichen Gehalte der einzelnen Fettdepots weisen auf unterschiedliche Beiträge der Gewebe zur zirkulierenden Adiponektinkonzentration hin. Die vorliegende Dissertation stellt eine Basis für weiterführende Studien dar, um die Rolle und Regulation von Adiponektin sowie anderer Adipokine in verschiedenen pathophysiologischen Zuständen von Rindern zu klären.

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List of abbreviations

Approx.	approximately
AT	adipose tissue
AdipoQ	adiponectin
AdipoR1	adiponectin receptor R1
AdipoR2	adiponectin receptor R2
ANOVA	analysis of variance
a.p.	ante partum
B_0	absorbance of the maximum binding well
B/B_0 (%)	percent maximum binding
BCS	body condition score
BFT	back fat thickness
BHBA	ß-hydroxybutyrate
BW	body weight
c-9, *t*-11	*cis*-9, *trans*-11
CLA	conjugated linoleic acid
CON	control
CP	crude protein
d	day
DIM	day(s) in milk
DM	dry matter
EBW	empty body weight
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FA	fatty acids
FLI	Friedrich-Loeffler-Institute
GfE	German Society of Nutrition Physiology
GLM	general linear model
HF	Holstein-Friesian
Hp	haptoglobin
HSL	hormone sensitive lipase
IGF-1	insulin-like growth factor-1

IL	interleukin
IS	insulin sensitivity
IR	insulin resistance
LAVES	Lower Saxony State Office for Consumer Protection and Food Safety
LPL	lipoprotein lipase
mAb	monoclonal antibodies
ME	metabolizable energy
MP	multiparous
NEB	negative energy balance
NEFA	nonesterified fatty acids
NF-κB	nuclear factor-kappa B
PP	primiparous
p.p.	post partum
PMR	partial mixed ration
PPAR	peroxisome proliferator-activated receptor
r	Pearson correlation coefficient
ρ	Spearman correlation coefficient
RQUICKI	revised quantitative insulin sensitivity check index
RT	room temperature
sc	subcutaneous
SCC	somatic cell count
SEM	standard error of the means
TAG	triacylglycerides
TNF-α	tumor necrosis factor-alpha
t-10, *c*-12	*trans*-10, *cis*-12
vc	visceral
VEGF	vascular endothelial growth factor
WAT	white adipose tissue

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1. Introduction

Milk production of dairy cows has increased steadily based on a combination of several factors such as improved management, better nutrition and intense genetic selection. Selection of dairy cows directed towards higher milk production is often associated with changes in the pattern of nutrient partitioning (Oltenacu and Broom, 2010), decline in immune responses (Hoeben et al., 1997) and reproductive performance (Dobson et al., 2007) as well as higher incidence of metabolic problems such as milk fever and ketosis (Fleischer et al., 2001). Most of the metabolic diseases in dairy cows occur within the first 2 wk of lactation (Goff and Horst, 1997) and may cause severe economic loss in terms of reduced milk production, impaired fertility, extra-expenses for treatment and prevention efforts, and increased culling rates. Special feeding practices might help to minimize such economical losses and to achieve better animal health around calving and lactation. For this purpose, the diet for early lactating cows should be formulated to achieve rapid balancing of energy losses induced by milk secretion, faster adaptation of rumen function to the lactation diet, and immunomodulation to enable adequate immune functions (Goff and Horst, 1997). Feed supplements such as conjugated linoleic acids (CLA) have been demonstrated to exert beneficial effects on the immune system in humans (Song et al., 2005) and in animals such as mice (Yamasaki et al., 2003a) and pigs (Moraes et al., 2012). Conjugated linoleic acids are also known to inhibit milk fat synthesis in dairy cows, thus reducing the energy needed for milk production (Baumgard et al., 2000, Peterson et al., 2002). Thus, dietary supplementation with CLA might be a strategy to minimize the magnitude and duration of negative energy balance (NEB) and to improve immunity in early lactating dairy cows.

1.1. Physiological role of adipose tissue during the transition period and in early lactation of dairy cows

The transition period in dairy cows is defined as the period between 3 wk pre partum until 3 wk post partum and is characterized by dramatic changes in the endocrine status and energy balance of the animal (Grummer, 1995). Energy balance is the difference between energy consumed and energy required (for maintenance, growth, production, activity and fetal growth) (Grummer and Rastani, 2003). During the pre calving transition period, the nutrient demand is increased to support the final phase of conceptus growth and lactogenesis. This is accompanied by a gradual decline in feed

intake, with the most dramatic decrease during the final wk before calving (Grummer, 1995). During the early post partum period, voluntary feed intake is not increasing as fast as the energy demand for increased milk production, possibly resulting from calving stress and change in nutrient density of the diet. Consequently, dairy cows enter a state of NEB. In order to accomplish the increased energy demand during this period, the homeorhetic drive necessitates mobilization of body reserves, in particular fat from adipose tissue (AT). The rate and extent of AT mobilization depend on several factors such as body condition at the time of calving, parity, milk production and composition of diet (Komaragiri et al., 1998). Thus, AT plays a central regulatory role during the period of increased energy demand such as late pregnancy and early lactation in dairy cows.

1.1.1. Adipose tissue

Adipose tissue is a highly specialized loose connective tissue composed of adipocytes, collagen fibers and other cells summarized as the stromal vascular fraction such as preadipocytes, endothelial cells, fibroblasts, immune cells, blood vessels and nerves. Over the past one and half decades, numerous studies aimed to understand the role of AT in homeostatic and metabolic regulation (Deng and Scherer, 2010, Rajala and Scherer, 2003, Rosen and Spiegelman, 2006, Yamauchi et al., 2002). In consequence, our perception about AT has changed considerably. In addition to their ability to store or mobilize triglycerides, adipocytes synthesize and release a wide variety of bioactive molecules collectively termed as adipokines or adipocytokines. Examples of adipokines include adiponectin, leptin, resistin, visfatin, apelin, chemerin, omentin. Adipokines allow for the communication of AT with other organs of the body such as liver (Moschen et al., 2012), heart (Cherian et al., 2012), muscles (Zhou et al., 2007), brain (Bartness and Song, 2007), reproductive organs (Mitchell et al., 2005) as well as within the AT (Karastergiou and Mohamed-Ali, 2010). Adipose tissue is thus involved in the coordination of various biological processes including energy metabolism, neuroendocrine, cardiovascular, reproductive and immune functions through its secretions via autocrine, paracrine and endocrine mechanisms (Figure 1).

Mammals have two main types of AT depending on their cellular structure and functions i.e. brown AT and white AT. Brown AT is primarily present in newborns and hibernating mammals, and is involved in the process of heat generation. White AT is

the most abundant form of AT in adults and serves as a regulatory center for energy metabolism. Based on its anatomical locations, white AT is broadly classified into subcutaneous AT (scAT) and visceral AT (vcAT).

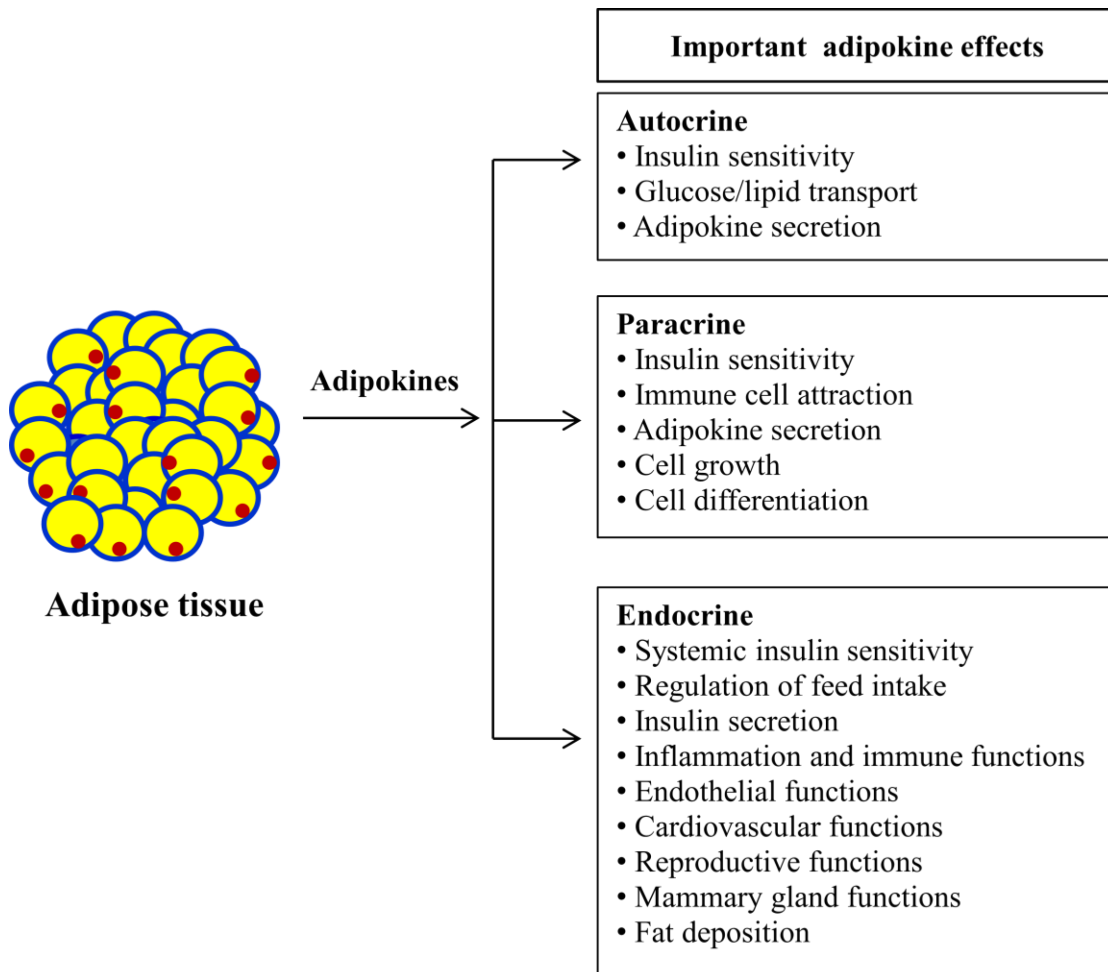

Figure 1. Physiological functions affected by adipokines (according to Blüher, 2012).

1.1.2. Subcutaneous and visceral AT

The scAT is located in the hypodermal layer of the skin, whereas the vcAT surrounds the inner organs in the abdominal cavity and mediastinum. Deep and superficial layers of scAT can be separated by the *Fascia superficialis* (Wajchenberg et al., 2002). Based on its locations, vcAT is divided into three major depots, i.e. mesenterial fat (more deeply buried around the intestines), omental fat (surround the intestines superficially), and retroperitoneal fat (near the kidneys, at the dorsal side of the abdominal cavity) (Wronska and Kmiec, 2012). Different AT depots (scAT and vcAT) display distinct

structural and functional properties; these may contribute to their differential role in physiological mechanisms (Table 1). The precise mechanism for the functional heterogeneity among fat depots is unclear.

Table 1. Differences in functional properties of subcutaneous adipose tissue (scAT) and visceral adipose tissue (vcAT) in humans, rats and dairy cows

Characteristics	scAT vs. vcAT	Reference
Responsiveness to caloric restriction	vcAT > scAT	Li et al., 2003[‡]
Catecholamine induced lipolysis	vcAT > scAT	Wajchenberg, 2000[‡]
Antilipolytic effects of insulin	scAT > vcAT	Wajchenberg, 2000[‡]
Insulin stimulated glucose uptake	vcAT > scAT	Virtanen et al., 2002[‡]
Metabolic activity and mitochondrial respiration	vcAT > scAT	Kraunsoe et al., 2010[‡]
Glucocorticoid and androgen receptors	vcAT > scAT	Björntorp, 1995[‡], Rebuffe-Scrive et al., 1985[‡]
Correlation with IS, diabetes type II, atherosclerosis and metabolic risk factors	vcAT > scAT	Cefalu et al., 1995[‡], Fox et al., 2007[‡], Kissebah, 1996[‡]
Expression of genes (HSL, ATGL) and of proteins (AMPKα1) related to lipolysis	RPAT > scAT	Locher et al., 2012*, Palou et al., 2009[†]
Expression of fatty-acid oxidation related genes (PPARα, CPT1)	scAT > RPAT	Palou et al., 2009[†]
Triglyceride turnover rate	RPAT > scAT	Palou et al., 2009[†]
Adipocyte size	RP AT > other vc (mesenterial, omental) and sc AT	Akter et al., 2011*

Reports in *dairy cows, †rats, ‡humans. AMPKα1: Adenosine monophosphate-activated protein kinase-α1; ATGL, adipose triglyceride lipase; CPT1; carnitine palmitoyltransferase-1; HSL: hormone sensitive lipase; IS: insulin sensitivity; PPARα/γ: Peroxisome proliferator-activated receptor-α/γ; RPAT: retroperitoneal adipose tissue.

1.2. Adipokines

Biologically active secretions of adipose tissue (adipokines) are diversified in terms of their protein structure and functions and are known to act at the local (autocrine/paracrine) and/or systemic (endocrine) level. They may be protein hormones (e.g. leptin, adiponectin, resistin, visfatin), cytokines (e.g. TNF-α, IL-6), chemokines (e.g. monocyte chemoattractant protein-1), proteins of the alternative complement system (e.g. adipsin, complement factor B), binding proteins (e.g. retinol binding protein), extracellular matrix proteins (e.g. monocyte chemotactic protein-1) or growth factors (e.g. nerve growth factor, vascular endothelial growth factor). Metabolic dysfunctions may partly result from an imbalance in the expression of pro- (e.g. leptin, resistin, TNF-α) and anti-inflammatory (e.g. adiponectin) adipokines; therefore, an adequate balance between different adipokines is crucial for determining homeostasis in the body (Ouchi et al., 2011). Adiponectin is one of the most important adipokines linked with several physiological mechanisms such as insulin sensitivity, immunity, reproduction, cardiovascular functions (Figure 2).

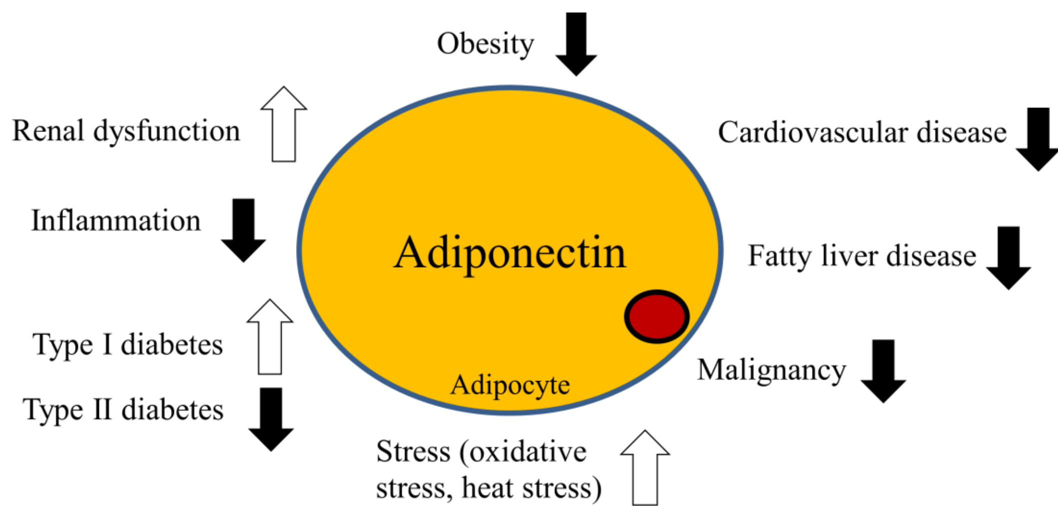

Figure 2. Patho-physiological states influencing circulating adiponectin concentration; ⇧, increase; ⬇, decrease (according to Scherer et al., 2006).

1.2.1. Adiponectin and adiponectin receptors

Adiponectin was independently characterized in the mid 1990s by four different research groups using distinct methods and was 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 a protein hormone expressed by differentiated adipocytes with circulating concentrations in the μg/mL range, thus accounting for approximately 0.01% of the total plasma protein (Arita et al., 1999). The adiponectin monomer is a 30 kDa polypeptide of 247 amino acids containing a N-terminal signal sequence, a variable domain, a collagen-like domain, and a C-terminal globular domain (Figure 3). It shares strong sequence homology with type VIII and X collagen and complement component C1q (Scherer et al., 1995). Post-translational modifications such as hydroxylation and glycosylation of the lysine residues within the collagenous domain are required for full activity of adiponectin (Wang et al., 2002). In contrast to several other adipokines, the plasma concentration of adiponectin is markedly reduced in visceral obesity (Goldstein and Scalia, 2004).

Adiponectin secretion from adipocytes is regulated by the endoplasmic reticulum (ER) proteins ERp44 and Ero1-Lalpha. Formation of the covalent bond between ERp44 and the thiol group of Cys39 on adiponectin molecule allows for the intracellular retention of adiponectin (thiol-mediated retention) while the disulfide bond formed between ERp44 and Ero1-L alpha releases adiponectin from ERp44 and allows its secretion from the adipocytes (Wang et al., 2007). Adiponectin is secreted from adipocytes as full-length adiponectin in three major isoforms i.e. low molecular weight (LMW), medium molecular weight (MMW) and high molecular weight (HMW) isoform (Figure 3) (Waki et al., 2003). The half life of MMW and HMW in mice is 4.5 and 9 h, respectively; neither MMW nor HMW are interconverted in circulation (Pajvani et al., 2003). Proteolytic cleavage of adiponectin molecules results in a product containing a globular head domain that still remains biologically active (Fruebis et al., 2001).

Three putative adiponectin receptors are identified so far, these are the adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2) and T-cadherin. Predominant expression of AdipoR1 and AdipoR2 has been observed in skeletal muscle and liver, respectively (Yamauchi et al., 2003). Adiponectin receptors have different affinities to the various adiponectin isoforms: AdipoR1 is a receptor for globular and full-length adiponectin isoforms whereas AdipoR2 has a higher affinity for full-length adiponectin isoforms than globular adiponectin (Kadowaki et al., 2006). Moreover, T-cadherin has

been identified as a receptor for the MMW and HMW adiponectin but not for the LMW or globular forms (Hug et al., 2004).

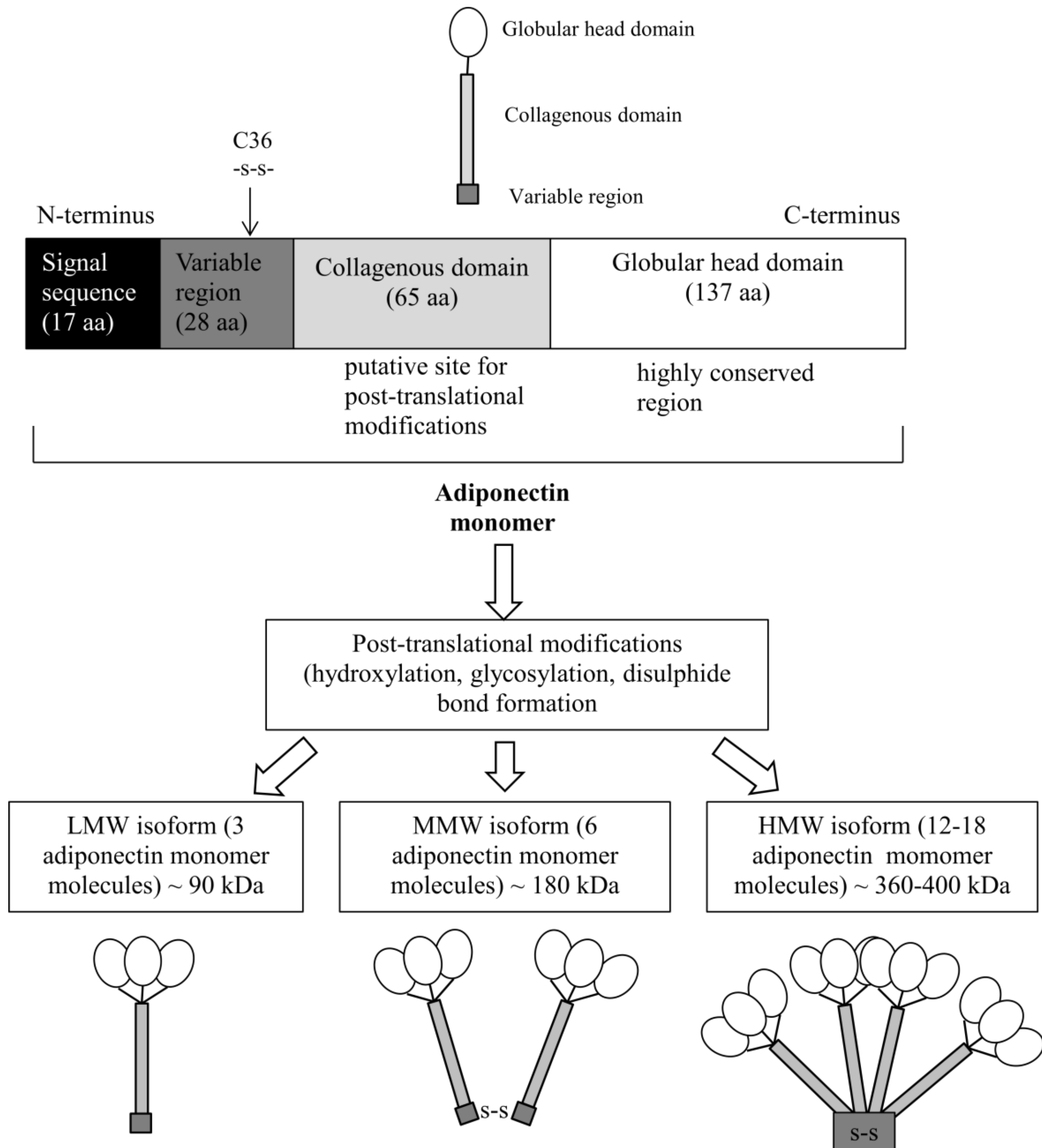

Figure 3. Structural features of adiponectin isofroms. N-terminus, amino-terminus; C-terminus, carboxy-terminus; aa, amino acid; LMW, low molecular weight; MMW, middle molecular weight, HMW, high molecular weight, S-S, disulfide bond (according to Berg et al., 2002; Waki et al., 2003 and Goldstein et al., 2009).

1.2.2. Adiponectin, insulin sensitivity and nutrient partitioning

Decreased insulin sensitivity (IS) is the situation in which normal insulin concentrations produce less biological response than expected. This might be due to changes in the sensitivity (the amount of hormones required for a desired response) and/or responsiveness (the maximal response to the hormone) of body tissues for insulin (Kahn, 1978). Early lactation in high yielding dairy cows is characterized by decreased IS in body tissues such as AT and muscle, thereby promoting mobilization of non-esterified fatty acids (NEFA), amino acids and sparing of glucose for increased nutrient demand of mammary gland for milk production (Bell, 1995).

The role of adiponectin as an insulin sensitizing adipokine was first identified in mice by three independent groups (Berg et al., 2001, Fruebis et al., 2001, Yamauchi et al., 2001). Plasma adiponectin decreases with the decrease of IS in rhesus monkeys (Hotta et al., 2001), in mice and in humans (Maeda et al., 2001). Moreover, positive associations of plasma adiponectin concentration with IS and inverse relationships with several components related to decline in IS, such as serum high-density lipoprotein cholesterol, triglycerides and diastolic blood pressure are observed in humans (Fernandez-Real et al., 2003, Matsubara et al., 2002). Adiponectin is known to stimulate glucose uptake and fatty acid oxidation by myocytes as well as to decrease gluconeogenesis in the liver thereby decreasing blood glucose concentrations (Yamauchi et al., 2002). The insulin sensitizing effect of adiponectin is mainly through inhibition of hepatic glucose production and increased fatty acid oxidation (Lihn et al., 2005). The metabolic and insulin sensitizing effects of adiponectin have not been fully explored in dairy cows.

Adiponectin inhibits lipolysis in humans and in mice (Qiao et al., 2011, Wedellova et al., 2011); therefore, lowered adiponectin concentrations facilitate the rate of lipolysis. The impact of adiponectin on IS and on metabolism of glucose and fatty acid contributes to nutrient partioning and thus may affect the nutrient availability in the mammary gland for milk production (Figure 4). Certain pathological conditions such as inflammation and endocrine hormones may affect the expression of adiponectin in AT. The regulation of adiponectin expression through other hormones and cytokines are summarized in Table 2.

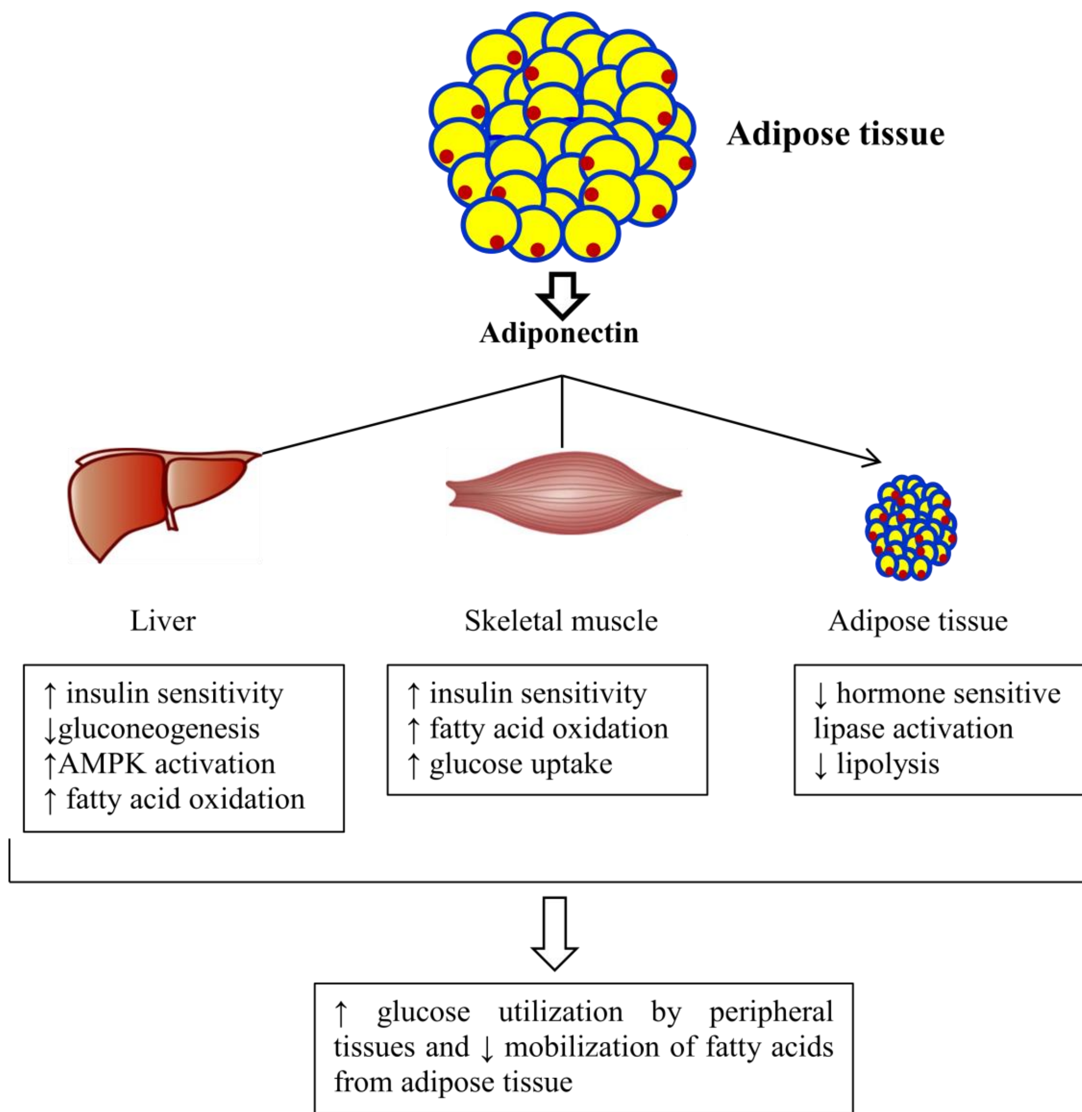

Figure 4. Insulin sensitizing and nutrient partitioning effects of adiponectin (according to Chandran et al., 2003).

Table 2. Influences of hormones and cytokines on adiponectin mRNA or protein expression in adipose tissue

Hormone/cytokine	Cell type	Effect	Reference
Insulin	epididymal primary adipocytes	↑ protein	Cong et al., 2007†
	omental adipocytes	↑ protein	Motoshima et al., 2002‡
	3T3L1 cells	↓ mRNA	Fasshauer et al., 2002
	perirenal and epididymal adipocytes	↓ mRNA	Sun et al., 2009*
Glucocorticoids	sc adipocytes	↓ protein	Degawa-Yamauchi et al., 2005‡
	3T3L1 cells	↓ mRNA	Fasshauer et al., 2002
Prolactin	sc abdominal adipocytes	↓ mRNA, protein	Nilsson et al., 2005‡
Growth hormone	sc abdominal adipocytes	↓ mRNA, protein	Nilsson et al., 2005‡
Testosterone	SGBS adipocytes	NE mRNA, protein	Horenburg et al., 2008‡
	3T3L1 cells	↓ mRNA, protein	Nishizawa et al., 2002# Li et al., 2011#
Progesterone	inguinal adipocytes	↓ mRNA (female) NE (male)	Stelmanska et al., 2012#
Estradiol	SGBS adipocytes	NE mRNA, protein	Horenburg et al., 2008‡
DHE	omental adipocytes	↑ mRNA	Hernandez-Morante et al., 2006‡
TNF-α	sc abdominal adipocytes	↓ mRNA	Bruun et al., 2003‡
	sc or omental adipocytes	↓ protein	Degawa-Yamauchi et al., 2005‡
	3T3L1 cells	↓ mRNA	Fasshauer et al., 2002; Ruan et al., 2002
IL-6	sc abdominal adipocytes	↓ mRNA	Bruun et al., 2003‡
C- reactive protein	3T3L1 cells	↓ mRNA	Yuan et al., 2007

Reports in *dairy cows, #mice, †rats, ‡humans; NE: no effect; sc: subcutaneous; DHE: dehydroepiandrosterone; TNF-α: tumor necrosis factor-α, IL-6: interleukin-6; SGBS: human preadipocyte cell line.

1.3. Conjugated linoleic acids (CLA)

The term conjugated linoleic acids (CLA) refers to a heterogeneous group of positional and geometric isomers of one of the omega-6 fatty acids, i.e. linoleic acid (*cis*-9, *cis*-12, octadecadienoic acid) (Figure 5). Based on the positioning of the double bond, 28 isomers of CLA are possible, however, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are the most common CLA isomers in the commercially available CLA preparations (Banni, 2002). *Cis*-9, *trans*-11 is the major CLA isomer in ruminant fat (~75–90% of total CLA) (Bauman et al., 2008). Dairy cows are able to synthesize the *cis*-9, *trans*-11 isomer endogenously by two different biosynthetic processes: First, as an intermediate product of biohydrogenation of linoleic acid by ruminal bacteria i.e. *Butyrivibrio fibrisolvens* (Kepler et al., 1966), and second, by desaturation of *trans*-11 $C_{18:1}$ (vaccenic acid), another intermediate in the biohydrogenation of unsaturated fatty acids, by the Δ^9-desaturase enzyme in body tissues such as AT and mammary gland (Bauman et al., 1999). Details about the metabolic functions of CLA and their role in adiponectin expression are given in following sub-sections.

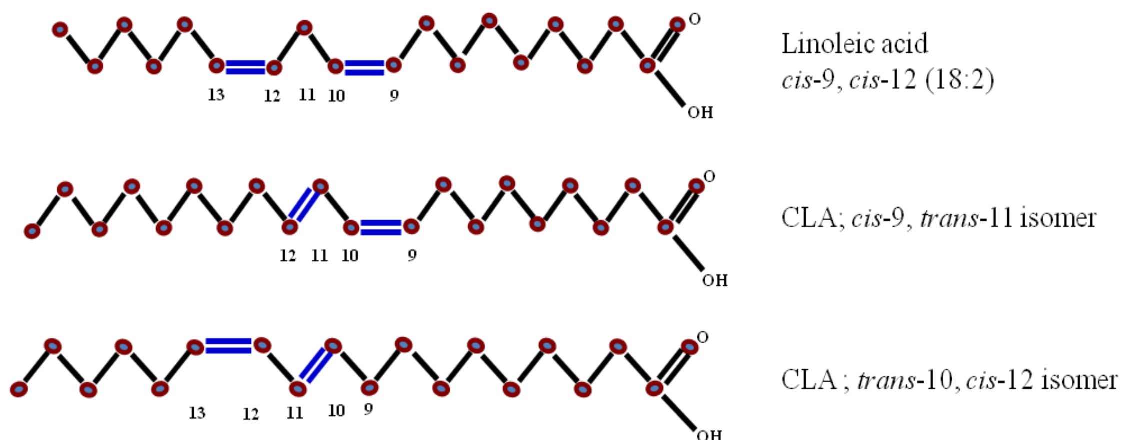

Figure 5. Linoleic acid and major conjugated linoleic acids (CLA) isomers (according to Wahle et al., 2004).

1.3.1. Effect of CLA on feed intake and energy balance

The ability of CLA to reduce food and energy intake in mice was suggested in some studies (Takahashi et al., 2002, West et al., 1998) but not in others (Ostrowska et al., 1999, Yamasaki et al., 2003b). It is reported that CLA supplementation increases energy expenditure in AKR/J mice (West et al., 2000) and thereby may affect the energy balance of the animal. Improvement in feed efficiency after CLA supplementation is also reported in pigs (Wiegand et al., 2001) and double-muscled Piemontese young bulls (Schiavon et al., 2010) but was not confirmed in cattle using Simmental heifers (Schlegel et al., 2012). Conflicting findings on the effects of CLA supplementation on dry matter intake and NEB have been reported in many studies on dairy cows (Table 3). The variation in the effects of CLA might be due to differences in the study design such as amount and type of CLA supplement fed (individual isomer or mixture of isomers), beginning and duration of supplementation and physiological condition of animals such as age and stage of lactation.

Table 3. Effects of CLA on dry matter intake (DMI) and post partum negative energy balance (NEB) in dairy cows

Variable	CLA Effect	Reference
DMI	inhibitory	Moallem et al., 2010; Pappritz et al., 2011; von Soosten et al., 2011
	stimulatory	Shingfield et al., 2004
	no effect	Perfield et al., 2002; Bernal-Santos et al., 2003; Odens et al., 2007; von Soosten et al., 2011
NEB	reduce	Shingfield et al., 2004; Odens et al., 2007
	aggravate	Pappritz et al., 2011; Hötger et al., 2013
	no effect	Bernal-Santos et al., 2003; Selberg et al., 2004; Moallem et al., 2010

1.3.2. Metabolic functions of CLA

Conjugated linoleic acids are known to affect body fat and energy metabolism in rodents and in humans (Smedman and Vessby, 2001, West et al., 1998). Results of an *in vitro* experiment on 3T3-L1 adipocytes suggest that the *trans*-10, *cis*-12 isomer affects fat metabolism by reducing lipoprotein lipase (LPL) activity, intracellular triglyceride

content and enhance glycerol release into the culture medium (Park et al., 1999). In addition, CLA induce apoptosis and increase fatty acid oxidation in 3T3-L1 preadipocytes (Evans et al., 2000, Evans et al., 2002). The t*rans*-10, *cis*-12 CLA isomer causes a change in body composition through reduction in body fat and increase in lean body mass in mice (Park et al., 1997). In cattle, the *trans*-10, *cis*-12 CLA decreased lipogenesis and expression of genes involved in milk lipid synthesis in the mammary gland and depressed the milk fat content (Baumgard et al., 2000, Baumgard et al., 2002a).

Lipoprotein lipase is a key enzyme in lipid metabolism. It catalyses the hydrolysis of the triacylglcerol component of chylomicrons and very low density lipoproteins, thereby providing NEFA for tissue utilization (Mead et al., 2002). Conjugated linoleic acids decrease LPL gene expression in mouse AT (Xu et al., 2003) and in the mammary gland of dairy cows (Baumgard et al., 2002a). Therefore, the effects of CLA on fat metabolism might be mediated through suppression of LPL expression in AT.

Several studies reported the ability of CLA to increase fatty acid oxidation in liver and AT (Evans et al., 2002, Martin et al., 2000, Ohnuki et al., 2001, West et al., 1998). This property of CLA might be partly responsible for the hypolipidemic effect of CLA in rodents (Sakono et al., 1999). An increased oxidation of [^{14}C] oleic acid is observed in 3T3-L1 preadipocytes treated with *trans*-10, *cis*-12 CLA for 6 d (Evans et al., 2002). *Trans-10*, *cis*-12 CLA increased AT and hepatic carnitine palmitoyltransferase activity, a rate-limiting enzyme for β-oxidation, in rats that consumed a diet containing 1% *trans*-10, *cis*-12 CLA for 6 wk (Martin et al., 2000). Therefore, it seems that *trans*-10, *cis*-12 is an isomer that contributes to most of the CLA effects on lipid metabolism.

Supplementation of CLA during early lactation in dairy cows leads to an increase in protein accretion and reduction in body mass (fat and protein) mobilization (von Soosten et al., 2012). Abomasal infusion of the *trans*-10, *cis*-12 CLA in dairy cows had no effect on blood variables such as glucose, insulin, NEFA, and leptin, whereas the lipolytic response to epinephrine was reduced (Baumgard et al., 2002b). Supplementation with a 1:1 mixture of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 reduced endogenous glucose production and decreased the expression of hepatic genes involved in this process i.e. cytosolic phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in dairy cows (Hötger et al., 2013). These results indicate that dietary CLA

supplementation might affect metabolic processes in dairy cows and alters the expression of hormones linked with the metabolism such as adiponectin.

1.3.3. Role of CLA in adiponectin expression

Inhibitory effects of CLA on adiponectin expression were observed in mice and in cell culture studies (Miller et al., 2008, Perez-Matute et al., 2007, Poirier et al., 2005). Treatment of 3T3-L1 cells with *trans*-10, *cis*-12 CLA is associated with a decline in cellular and secreted adiponectin content and impairment in the assembly of adiponectin isoforms (Miller et al., 2008). A recent study in dairy cows suggests that CLA supplementation causes a decrease in adiponectin mRNA expression in omental AT at 105 days of lactation (Saremi, 2013). However, information about the effect of CLA supplementation on circulating adiponectin concentrations in dairy cows was lacking. Based on the results of rodents and *in vitro* studies, the proposed mechanisms involved in the effect of CLA on adiponectin expression (Perez-Matute et al., 2007, Poirier et al., 2005) are presented in Figure 6.

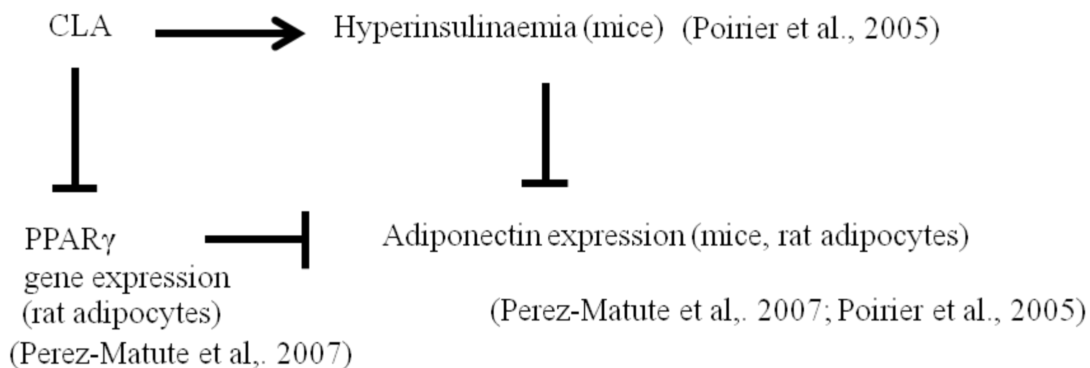

Figure 6. Mechanisms associated with the effect of CLA on adiponectin expression (→, stimulation; ⊣, inhibition).

1.4. Enzyme-linked immunosorbent assay (ELISA) for bovine adiponectin

Notwithstanding the well-developed knowledge about the biological role of adiponectin in humans and in laboratory animals, studies about the adiponectin in cattle have been

impeded due to the lack of reliable assays for bovine adiponectin. To overcome this shortcoming, an indirect competitive ELISA test was developed in-house for the measurement of adiponectin in bovine samples (Mielenz et al., 2013). Based on the principle of competition in antigen-antibody binding, the competitive ELISA is one of the formats of ELISA that can be used for quantitative measurements of analyte concentrations in a variety of samples with high sensitivity. For validating the in-house developed ELISA, several validation criteria such as intra-assay (reproducibility) and inter-assay (precision) coefficients of variation, spiking recovery, accuracy, specificity, sample stability, sensitivity or limit of detection and measuring range were assessed. Details of the ELISA validation, the assay protocol and the composition of buffers and solutions for the bovine adiponectin ELISA are presented in appendixes A, B and C, respectively.

1.4.1. Principle of the ELISA for bovine adiponectin

The adiponectin concentration in a sample is measured by recording the interference in an expected signal output. The extent of interference is based on the competition among sample adiponectin and coating adiponectin for primary antibody binding sites. More adiponectin in a sample results in binding of less peroxidase labelled secondary antibody in the well after washing and thus the signal obtained is weaker. Therefore, signal output is inversely proportional to the adiponectin concentration in the sample. The setup of the different components of the bovine adiponectin ELISA and a typical standard curve used for the calculation of adiponectin concentrations is presented in Figures 7 and 8, respectively.

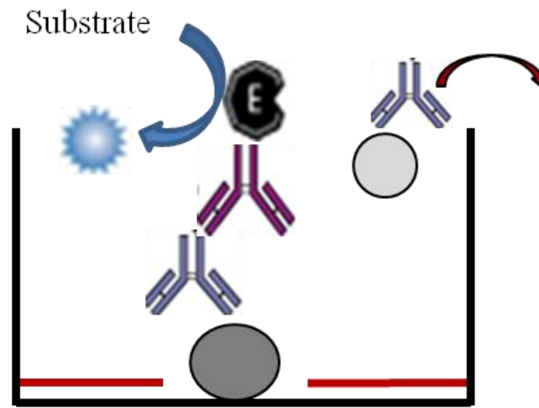

Figure 7. Setup of in-house developed indirect competitive bovine adiponectin ELISA. [dark grey circle], coating antigen (bovine serum adiponectin); [light grey circle], sample adiponectin; [antibody symbol] primary antibody; [antibody with E symbol], peroxidase conjugated secondary antibody.

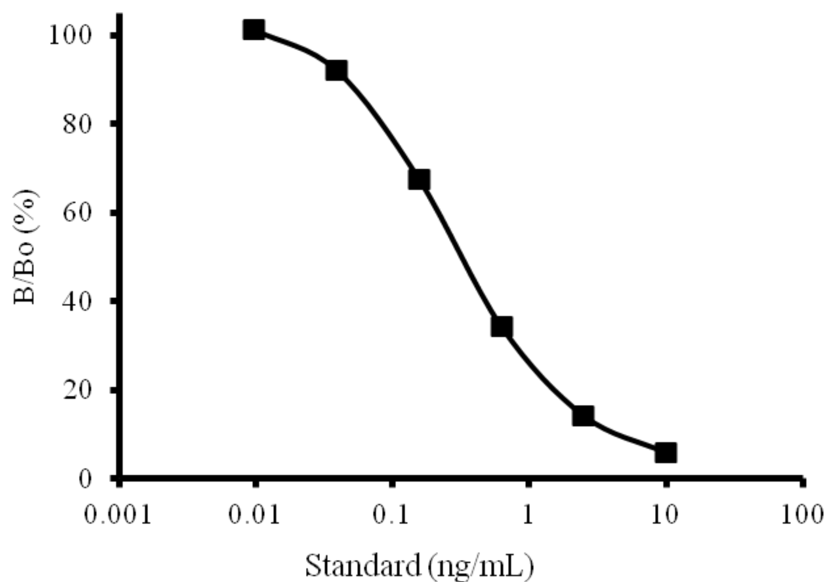

Figure 8. Typical standard curve of the ELISA for bovine adiponectin. B/B_0 (%) or percent of maximum binding is the ratio of absorbance of each well (B) and the absorbance of the maximum binding well (B_0) and multiplying by 100. The use of B/B_0 (%) on Y axis provides an advantage to compare results between assays performed on two different plates or two different days. This is because the absolute absorbance may differ from plate to plate or day to day however, the values of B/B_0 (%) should be reasonably consistent from one plate to the next.

1.5. Objectives

Adiponectin is one of the most abundant adipokines in circulation; however, information about its concentrations in blood and milk of dairy cows during different physiological conditions such as the periparturient period and lactation were lacking. In addition, potential differences between the different sc and vcAT depots in terms of adiponectin protein expression have not yet been assessed in dairy cattle. Furthermore, the variation in circulating and milk adiponectin concentrations after dietary CLA supplementation, during lactation or dietary induced NEB in ruminant species including cattle has not been studied previously. Therefore, the present research study has been designed to fill these gaps of knowledge with the following objectives:

1) To characterize adiponectin concentrations in blood and its tissue concentrations during different stages of lactation in dairy cattle by the in-house developed ELISA,
2) To evaluate the effect of CLA supplementation on blood and tissue adiponectin concentrations, and
3) To evaluate the effect of lactational and dietary induced negative energy balance on blood and milk adiponectin concentrations

2. Manuscript 1 (Published in General and Comparative Endocrinology, 2014, 198:13-21)

Supplementation with conjugated linoleic acids extends the adiponectin deficit during early lactation in dairy cows

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Abbreviated title: Adiponectin in CLA supplemented cows

Keywords: adiponectin, conjugated linoleic acids, dairy cow, lactation, parity

Abbreviations: ALR, adiponectin : leptin ratio; AT, adipose tissue; BCS, body condition score; CLA, conjugated linoleic acids; CON, control; DM, dry matter; HMW, high molecular weight; IS, insulin sensitivity; IR, insulin resistance; MMW, medium molecular weight; MP, multiparous; NEFA, nonesterified fatty acids; NE_L, net energy for lactation; PMR, partial mixed ration; PP, primiparous; RQUICKI, revised quantitative insulin sensitivity check index; sc, subcutaneous;

ABSTRACT

Decreasing insulin sensitivity (IS) in peripheral tissues allows for partitioning nutrients towards the mammary gland. In dairy cows, extensive lipid mobilization and continued insulin resistance (IR) are typical for early lactation. Adiponectin, an adipokine, promotes IS. Supplementation with conjugated linoleic acids (CLA) in rodents and humans reduces fat mass whereby IR and hyperinsulinemia may occur. In dairy cows, CLA reduce milk fat, whereas body fat, serum free fatty acids and leptin are not affected. We aimed to investigate the effects of CLA supplementation on serum and adipose tissue (AT) adiponectin concentrations in dairy cows during the lactation driven and parity modulated changes of metabolism. High yielding cows (n=33) were allocated on day 1 post partum to either 100 g/day of a CLA mixture or a control fat supplement (CON) until day 182 post partum. Blood and subcutaneous (sc) AT (AT) biopsy samples were collected until day 252 post partum to measure adiponectin. Serum adiponectin decreased from day 21 pre partum reaching a nadir at calving and thereafter increased gradually. The distribution of adiponectin molecular weight forms was neither affected by time, parity nor treatment. Cows receiving CLA had decreased serum adiponectin concentrations whereby primiparous cows responded about 4 weeks earlier than multiparous cows. The time course of adiponectin concentrations in sc AT (corrected for residual blood) was similar to serum concentrations, without differences between CLA and CON. CLA supplementation attenuated the post partum increase of circulating adiponectin thus acting towards prolongation of peripartal IR and drain of nutrients towards the mammary gland.

1. Introduction

The progression from pregnancy to lactation is characterized by comprehensive metabolic and endocrine changes. Fetal growth, lactogenesis and galactopoiesis require the targeted partitioning of nutrients towards the placenta and the mammary gland, a process that is accomplished by decreasing insulin sensitivity (IS) in peripheral tissues thus attenuating their uptake of glucose, amino acids and fatty acids and facilitating lipolysis in adipose tissue (AT) (Block et al., 2001). Considering the amount of nutrient output via milk, dairy cows present a biological extreme: in contrast to rodents and primates, in which insulin resistance (IR) develops during pregnancy and fades after parturition, dairy cows may maintain IR for several weeks and may excessively

mobilize body fat (Tamminga et al., 1997). Adipose tissue is a metabolically active tissue, communicating with other peripheral tissues and brain through secretion of bioactive molecules collectively termed as 'adipokines'. The circulating leptin concentrations reportedly change during pregnancy and around parturition (Block et al., 2001; Sattar et al., 1998) and are affected by parity in dairy cows (Wathes et al., 2007). Parity is considered as an important factor affecting metabolic and hormonal changes, since primiparous cows have not reached their adult body size and continue to grow during pregnancy and lactation thus showing significant differences in metabolism and lipolytic response when compared to later lactations (Theilgaard et al., 2002; Wathes et al., 2007).

Adiponectin is one of the most abundant adipokines in circulation (Hotta et al., 2001). Unlike other adipokines which increase with excess body fat mass, adiponectin is decreased in obese subjects (Kadowaki and Yamauchi, 2005). Adiponectin inhibits lipolysis in AT and decreases IR by stimulating fatty acid oxidation and reducing the triglyceride content in muscle and liver (Yamauchi et al., 2001). The low adiponectin expression in AT after onset of lactation, might contribute to the decrease in IS, which improves glucose supply for milk synthesis (Komatsu et al., 2007). Adiponectin is synthesized as a 30 kDa monomer and is subsequently assembled to various oligomers detectable as low molecular weight trimers, medium molecular weight (MMW) hexamers and high molecular weight (HMW) oligomers (Waki et al., 2003). The HMW isoform is the biologically active form of adiponectin since it is associated with activation of AMP activated protein kinase in muscle (Waki et al., 2003) and improvement in IS (Pajvani et al., 2003). Recently, the adiponectin : leptin ratio (ALR) was proposed as a more effective and reliable marker of IS (Inoue et al., 2005) and metabolic syndrome (Mirza et al., 2001) than adiponectin or leptin alone.

The term conjugated linoleic acids (CLA) refers to a mixture of positional and geometric isomers of octadecadienoic acids, a naturally occurring group of dienoic derivatives of linoleic acid. Supplements containing the two main isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 with a 50:50 ratio have been demonstrated to promote fat loss in rodents and obese or overweight humans and also to prevent body fat accumulation in mice (Park et al., 2007). Adverse side effects of using CLA mainly concern the induction of fatty liver and IR (Poirier et al., 2005; Riserus et al., 2002). In dairy cows, CLA supplementation mainly concerns the mammary gland by reducing milk fat

synthesis, whereas body fat and lactation induced lipolysis remain unaffected (Pappritz et al., 2011). In the present study, we hypothesized that for the homeorhetic adaptations to lactation, tissue and circulating adiponectin concentrations will be in support of peripartal IR and that the response to CLA will differ depending on stage of lactation and parity.

The objectives of the present study were (I) to characterize circulating adiponectin concentrations from late pregnancy to early lactation and then throughout lactation in dairy cows, (II) to evaluate potential effects of long term CLA supplementation and parity on circulating adiponectin and characterization of its molecular weight forms, (III) to compare the adiponectin serum concentration with ALR, (IV) to identify changes of the adiponectin concentrations in subcutaneous (sc) AT around parturition and early lactation, and (V) to characterize the relationship of insulin, IGF-1, nonesterified fatty acids (NEFA) concentrations in blood, body condition and systemic IS with adiponectin, leptin and ALR.

2. Materials and Methods

2.1. Animals and treatments

This study was conducted at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany. All animal experiments were approved by the Lower Saxony state office for consumer protection and food safety (LAVES, file no. 33.11.42502-04-071/07, Oldenburg, Germany). The experimental design has been described in detail elsewhere (Pappritz et al., 2011). Briefly, pregnant German Holstein Friesian cows either multiparous (MP, 2 to 4 preceding lactations, n = 22) or primiparous (PP, first pregnancy, n = 11) were studied from day (d) 21 pre partum until d 252 post partum. In period 1 (d 21 pre partum until the day of calving), all animals were housed in group pens according to their feeding group and had free access to water. The diet was a partial mixed ration (PMR) consisting of 63% silage and 37% concentrate (6.8 MJ NE_L/kg DM) on dry matter (DM) basis. The diets were formulated according to the recommendation of the Society of Nutrition Physiology ((GfE) Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie. Nr. 8. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder; Recommendations of energy and nutrient supply for dairy cows and breeding cattle). DLG-Verlag, Frankfurt am Main, Germany, 2001). On

d 1 post partum, the animals were randomly allocated to either the control group (CON; 11 MP cows and 6 PP cows) or the treatment group (CLA; 11 MP cows and 5 PP cows). In period 2 (from d 1 until d 182 post partum), the CLA animals received 100 g/d of a lipid encapsulated rumen-protected commercial CLA preparation (Lutrell® Pure, BASF SE, Ludwigshafen, Germany). The CLA supplement contained 12% each of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers of total fatty acid methyl esters. The animals consumed 7.6 g/d each of the *trans*-10, *cis*-12 and the *cis*-9, *trans*-11 CLA isomer (calculated, based on the analyzed proportions in the concentrate). The CON group received 100 g/d of a rumen-protected fat preparation (Silafat®, BASF SE) in which the CLA were substituted by stearic acid. During period 2, each cow of both groups received 4 kg/d additional concentrate (8.8 MJ NE_L/kg DM) containing the respective fat supplement. The detailed composition of the diet and the fatty acid profile of the fat supplements are provided elsewhere (Pappritz et al., 2011). To identify potential post supplementation effects, the animals were observed for further 12 weeks after the end of supplementation period (defined as period 3). The body condition score (BCS) of each animal was recorded at the blood sampling times according to a 5-point scale (1 = lean, 5 = fat) as previously described (Edmonson et al., 1989).

2.2. Blood sample collection, adipose tissue biopsies and preparation of tissue extracts

Blood samples from all animals were collected from the jugular vein on d -21, -14, -7, 1, 7, 14, 21, 35, 49, 70, 105, 140, 182, 189, 196, 210, 224, 238 and 252 relative to parturition. The plasma (heparin and EDTA) and serum samples were obtained following standard procedures and were stored at -80°C. Subcutaneous fat samples were obtained by biopsy as described previously (Saremi et al., 2012) from the tail head region at d -21, 1, 21, and 105 relative to parturition. Tissue samples were immediately snap frozen in liquid nitrogen, and then stored at -80°C. For determining the adiponectin concentrations in AT, the samples were homogenized in 2 volumes of homogenization buffer, i.e. 10 mM HEPES pH 7.4 with complete® protease inhibitor cocktail (Roche, Mannheim, Germany; 1 tablet/10 mL buffer) using a Precellys® 24 homogenizer in 2 mL tubes containing 1.4 mm zirconium oxide beads (Peqlab Biotechnologies GmbH, Erlangen, Germany). The homogenates obtained were centrifuged twice (14,000 × *g*, 10 min, 4°C) to separate the fat layer. The infranatants beneath were collected (without tissue debris) and stored at -80°C.

2.3. Adiponectin measurements in serum and adipose tissue

Serum samples and AT preparations were analyzed for adiponectin in duplicate using an in-house developed ELISA as described in detail earlier (Mielenz et al., 2013). Assay accuracy was confirmed by linearity and parallelism of diluted serum samples. The measuring range (consistent with the linear range) of the assay was 0.07-1.0 ng/mL and the limit of detection was 0.03 ng/mL. The intra- and interassay coefficients of variation (CVs) were 7 and 9%, respectively.

Correction for residual blood in the tissue extracts was done by comparing the transferrin (Tr) content of the tissue preparation to the Tr content of serum. Transferrin concentrations in each tissue preparation and the corresponding serum sample were determined by an ELISA specific for bovine Tr according to the manufacturer's directions (Bethyl Laboratories Inc., Montgomery, TX) with minor modifications. Briefly, microtiter plates (EIA plate 9018; Corning Costar, Cambridge, MA) were coated for 1 hour at 25°C with 1:100 diluted affinity purified anti-bovine transferrin antibody in carbonate buffer pH 9.6. Tris buffered saline (50 mM Tris, 0.14 M NaCl pH 8.0) with 0.05% Tween-20® was used for blocking (200 µL/well, 30 min, 25°C) and as a sample dilution buffer. After each incubation step, the plates were washed five times. Reference serum (250–3.9 ng/mL) and diluted samples were then applied in duplicate (100 µL/well). After 1 hour incubation at 25°C, 100 µL of 1: 50,000 diluted HRP detection antibodies were added to each well and incubated for 1 further hour. Subsequently, substrate solution (0.05 M citric acid, 0.055 M Na_2HPO_4, 0.05 M urea hydrogen peroxide, 0.02 % ProClin® 150 and 0.025% of tetramethylbenzidine) was added (150 µL/well) and incubated for 15 min at 25°C in the dark. The reaction was stopped with 50 µL 1 M oxalic acid, and the OD was determined at 450/630 nm with a microtiter plate reader (ELX800, BioTek Instruments Inc., Winooski, VT). The Tr concentrations in the AT preparation and in serum were calculated using the Gen5 2.0 software (BioTek). Accuracy was confirmed by linearity of diluted tissue extract and serum samples. The intra- and interassay CVs were 3.7 and 7.8%, respectively. Samples with Tr concentrations < 20 ng/mL were re-analyzed at lower dilution. For calculating the extent of tissue blood content, we used a normalization factor that was established as follows: the AT preparations were scored according to their visible hemoglobin content in 5 classes (0 to 5; 0 was for colorless samples and 5 for deep redish samples). The Tr content of all samples from class 0 was averaged and the mean value obtained was

considered as threshold (0.03 ± 0.00; n = 5), i.e. only values above this threshold were considered as being indicative for significant AT blood content. The normalization factor was then calculated by forming the ratio between the mean Tr values of all samples and the threshold value (0.10 / 0.03 = 3.3). Subsequently, the Tr concentration in each individual sample was divided by the normalization factor to obtain the actual Tr coming from the blood (normalized Tr concentration in tissue preparations). Further calculations for correction for residual blood were done as described elsewhere (de Boer et al., 2005). Briefly, the ratio of the Tr concentration in the AT preparation and in the corresponding serum sample was calculated; the amount of adiponectin attributable to tissue blood content was calculated by multiplying serum adiponectin (µg/mL) with this ratio and the resulting product was then subtracted from the total adiponectin measured in the AT preparation. Samples showing negative values after calculation for correction for residual blood were handled as missing values, since blood adiponectin concentrations were exceeding those in the tissue. The concentration of total protein in the AT preparations was determined by the Bradford assay (Roti-Nanoquant, Carl Roth GmbH, Karlsruhe, Germany). The blood-corrected adiponectin concentrations of the tissue preparations (ng/mL) were normalized and are presented as ng adiponectin/mg total protein and ng adiponectin/g wet tissue to take potential changes in tissue protein content into account.

To characterize the molecular weight forms of circulating adiponectin, serum samples from d 1 and d 105 of lactation (each from PP and MP cows of CON and CLA group) were exemplarily examined by Western blot analysis under nonreducing, nonheat-denaturing conditions. All samples were diluted with ultrapure water to achieve an approximately equal concentration of adiponectin according to preceding ELISA analysis (0.75 ng adiponectin/lane). The diluted samples were mixed with sample buffer (final concentration: 0.064 M Tris HCl pH 6.8, 1% SDS, 0.01% bromophenol blue, 10% glycerol). Before loading on 8% SDS gels, samples were centrifuged for 5 min at 10,000 × *g*, 4°C. Proteins separated by SDS-PAGE were transferred onto a polyvinylidene diflouride membrane (PVDF) (GE Healthcare Europe, Freiburg, Germany) using tank blotting with the Criterion Blotter System (Bio-Rad Laboratories, Munich, Germany). After 5 min of incubation in 15% acetic acid, 10% ethanol and further washing with 50% methanol (2 × 1 min), the membranes were dried for 3 min at 60°C. The first antibody, same as in the ELISA (Mielenz et al., 2013), was used (0.25

μg/mL) and incubated for 30 min under shaking. After washing (3 × 5 min), a 1:100,000 dilution of the HRP conjugated secondary antibody (goat-anti-rabbit antibody; SouthernBiotech, Birmigham, AL) was incubated for further 30 min. After washing (3 × 5 min), the immune complex was detected with an enhanced chemiluminescence detection system (GE Healthcare) using CL-XPosure film (Thermo Scientific, Munich, Germany). The molecular weight of the developed bands was assessed by comparing with a prestained molecular weight marker [Protein Marker VI (10-245) Applichem, Darmstadt, Germany].

2.4. Other blood variables

Plasma insulin was analyzed using a commercially available double antibody radioimmunoassay (DSL-1600, Diagnostic Systems Laboratories Inc., Webster, TX). The intra- and interassay CVs were 6.3% and 8.8%, respectively. Plasma IGF-1 was measured by 2-site-immunoradiometric assay (IRMA, DSL-5600 Active IGF-I-IRMA; Diagnostic Systems Laboratories Inc.). The intra- and interassay CVs were between 3.4% and 8.2%, respectively. Serum leptin concentrations were measured by a competitive ELISA (Sauerwein et al., 2004) and the intra- and interassay CVs were 3.8% and 9.3%, respectively. Plasma NEFA and glucose were estimated by enzymatic analysis using commercial kits (NEFA HR [2] R1+ R2 Set, Wako Chemicals GmbH, Neuss, Germany; Hexokinase Fluid 5 + 1, MTI Diagnostics GmbH, Idstein, Germany) and results are described elsewhere (Pappritz et al., 2011).

The 'Revised Quantitative Insulin Sensitivity Check Index' (RQUICKI) provides good and linear correlations with estimates of IS such as homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) in human populations (Rabasa-Lhoret et al., 2003). It was also suggested as a measure of IS in ruminants (Holtenius and Holtenius, 2007). Estimation of RQUICKI was done according to Perseghin et al. (Perseghin et al., 2001), i.e. RQUICKI = 1 / [log(Glucose, mg/dL) + log(Insulin, μU/mL) + log(NEFA, mmol/L)], in which a low RQUICKI index indicates decreased insulin sensitivity. The serum leptin concentrations and mRNA abundance of adiponectin in the sc AT biopsies were quantified earlier (Saremi et al., 2014) and were used to evaluate potential correlations.

2.5. Statistical analyses

All statistical analyses were performed using SPSS (version 19.0, SPSS Inc., Chicago, IL). Data were tested for normal distribution using the Shapiro-Wilk test. Homogeneity of variances was tested using the Levene's test. The mixed model procedure was used each for serum adiponectin concentrations or ALR as a dependent variable. Treatment (CON or CLA) and parity (MP or PP) were considered as fixed factor, sampling days as repeated effects, and their respective interactions were included into the model. The covariance structure autoregressive (first-order autoregressive structure with homogenous variances) followed by Bonferroni correction were used for serum adiponectin and ALR data. Differences at each time point between CON and CLA groups were compared for MP and PP cows by Student's *t*-test or Mann-Whitney *U* test. For the tissue adiponectin concentrations, treatment was not significant and thus data were merged and compared across times using the Friedman test. Pearson`s correlations (2-tailed) were calculated between adiponectin, leptin, ALR and all other blood variables; in addition, data of BCS and RQUICKI were also used to calculate correlations. All data are presented as arithmetic means ± SEM, significance was declared for P-values < 0.05 and a trend was noted when $0.05 \leq P \leq 0.10$.

3. Results

3.1. Adiponectin serum concentrations: Time course, parity and CLA effects

Time dependent changes of serum adiponectin concentrations during the entire experimental period for both treatment and parity groups are shown in Figure 1. Serum adiponectin decreased from d 21 before calving to parturition, reached a nadir at the time of calving in all groups, and increased gradually thereafter (Figure 1). Treatment effects with decreased serum adiponectin concentrations in the CLA groups were observed in both PP and MP cows. The CLA effect was detected earlier in PP cows (d 21 post partum) than in MP cows (d 49 post partum) and persisted until d 140 post partum in both PP and MP cows (Figure 1). Serum adiponectin concentrations in PP cows were 1.4-fold higher at d 21 post partum compared to d 21 pre partum ($P < 0.05$).

After the end of the supplementation period no carry-over effect of CLA was observed in PP cows, however, the MP cows treated previously with CLA tended to lower adiponectin serum concentrations on d 189 ($P = 0.051$), d 238 ($P = 0.054$) and d 252 ($P = 0.091$) as compared to MP CON cows. Considering the parity effect, serum adiponectin concentrations tended to be higher in MP cows than in PP cows of both the

CON (1.12-fold) and the CLA (1.15-fold) group (*P* = 0.055 and *P* = 0.079, respectively). The *P* values for the different factors and their interaction for serum adiponectin during the different experimental periods are summarized in supplemental Table 1.

3.2. Adiponectin to Leptin Ratio (ALR)

The time dependent changes of ALR are shown in Figure 1. No treatment effect was observed in the corresponding serum leptin concentrations (provided in supplemental Figure 2). Considering the entire experimental period, no CLA effect on the ALR of PP cows was observed, whereas CLA-treated MP cows tended to have lower ALR ($P = 0.071$) than CON MP cows. However, when limited to period 2, the ALR of the CLA group was about 1.5-times lower compared to the CON group ($P < 0.05$) in MP cows. In PP cows, no treatment effect was observed after the end of supplementation, whereas MP CON cows had 1.5- and 1.4-times higher ALR values on 189 and 238 d post partum, respectively ($P < 0.05$).

No parity effect on ALR was observed in the CLA cows, whereas in the CON group, MP cows had 1.3-fold higher ALR than PP cows ($P < 0.05$). The *P* values for different factors and their interactions for ALR during different experimental periods are provided in supplemental Table 2.

3.3. Molecular weight distribution of serum adiponectin in primiparous and multiparous cows treated with or without CLA

Using SDS-PAGE and Western immunoblotting without reducing and heat-denaturing, adiponectin was detected as HMW oligomers of approximately 300 kDa and smaller medium molecular forms (MMW) as exemplary shown in Figure 2. Based on this and other blots, the MW distribution was apparently neither affected by parity nor by CLA treatment.

3.4. Adiponectin concentrations in adipose tissue

The portion of blood derived adiponectin from total adiponectin measured in the tissue preparations was 47.3 ± 1.4%. The adiponectin content in the biopsy preparations attributable to blood was 1.32 ± 0.08 µg/mL, ranging from 0.19-5.7 µg/mL. The corrected adiponectin concentrations measured in sc AT were related both to mg of total

protein and to g of wet tissue and are presented in Figure 3 A and B. No treatment or parity effect was found on tissue adiponectin concentrations except higher concentrations in CLA group than CON group on d 1 (0.7 ± 0.1 vs. 0.3 ± 0.1 µg/g wet tissue; P = 0.004) and lower values in PP cows than in MP cows on d 21 pre partum (51.7 ± 15.1 vs. 63.0 ± 6.3 ng/mg protein; P = 0.023 and 0.3 ± 0.1vs. 0.7 ± 0.1 µg/g wet tissue; P = 0.019). No further parity or treatment effect was found for the tissue adiponectin concentration at any other time point.

3.5. Correlations of adiponectin, leptin and ALR with other variables

Serum and sc AT adiponectin concentrations were positively correlated both on the basis of µg/mg protein basis (r = 0.333; P < 0.001) and a µg/g tissue basis (r = 0.391; P < 0.001). Neither the tissue adiponectin protein concentrations, nor the serum concentrations were correlated with the sc AT adiponectin mRNA abundance.

Time dependent changes in blood concentration of glucose, NEFA, insulin, RQUICKI, leptin, and IGF-1 are shown in supplemental Figure 1 and 2. The correlations of serum adiponectin, leptin and ALR with BCS, NEFA, RQUICKI, insulin and IGF-1 are presented in Table 1. Irrespective of treatment, serum adiponectin in MP and PP animals was positively correlated with RQUICKI [(MP, r = 0.232; P = 0.003) (PP, r = 0.135; P = 0.008)] and negatively correlated with BCS [(MP, r = -0.421; P < 0.001) (PP, r = -0.303; P < 0.001)] and NEFA [(MP, r = -0.255; P < 0.001) (PP, r = -0.281; P < 0.001)].

4. Discussion

4.1. Circulating adiponectin and ARL during late pregnancy and throughout lactation

For studying the importance of adiponectin for the metabolic adaptation to the energetic needs of lactation, dairy cows provide a unique model due to their high milk yield, the extent and duration of negative energy balance at the onset of lactation with concomitantly maintained IR. Investigations about adiponectin in cattle have been impeded by the lack of valid assays; using semiquantitative Western immunoblotting, the peripartal changes recently characterized (Giesy et al., 2012; Mielenz et al., 2013) were herein confirmed using an ELISA developed for bovine adiponectin (Mielenz et al., 2013) and further longitudinal data about the entire lactation period with newly established pregnancies were provided. The circulating adiponectin concentrations

decreased from d 21 pre partum, reached a nadir at the time of parturition and thereafter increased gradually. In humans, plasma adiponectin levels are reportedly lower at the immediate post partum period than during pregnancy (Mazaki-Tovi et al., 2007). Similarly, blood adiponectin was demonstrated to slightly decline as pregnancy advances, reaching lowest concentrations after delivery and one month thereafter (Asai-Sato et al., 2006). Changes in circulating adiponectin levels during pregnancy and lactation can result from alterations in its expression, secretion, and/or clearance. Hyperinsulinemic conditions, such as pregnancy, are associated with decreased adiponectin receptors (Kadowaki and Yamauchi, 2005), thereby reduced adiponectin sensitivity might be compensated by an increase in adiponectin levels (Mazaki-Tovi et al., 2007).

In rodents, adiponectin increases glucose uptake in skeletal muscle and reduces hepatic glucose production by suppressing the expression of molecules involved in gluconeogenesis (Yamauchi et al., 2002). Similarly in humans, plasma adiponectin is negatively associated with both basal- and insulin-suppressed endogenous glucose production as well as with insulin-stimulated whole-body glucose uptake (Stefan et al., 2003). Due to ruminal fermentation of carbohydrates from feed, cows depend almost entirely on gluconeogenesis, with propionate from microbial fermentation being the main substrate (Aschenbach et al., 2011). Gluconeogenesis is of particular importance during lactation to ensure an adequate glucose supply of the mammary gland to synthesize lactose, the key osmotic regulator of milk volume (Reynolds et al., 1988). Adiponectin suppresses gluconeogenesis (Zhou et al., 2005). Therefore, the reduction in adiponectin concentrations around parturition was suggested as a physiological mechanism to improve gluconeogenesis and increase glucose supply to the mammary gland for milk production (Saremi et al., 2014). In view of the loss of body condition (from 3 weeks before calving until 3 weeks thereafter, BCS dropped from 3.5 ± 0.07 and 3.3 ± 0.06 to 3.1 ± 0.05 and 3.1 ± 0.06 in MP and PP cows, respectively) with high rates of lipolysis as indicated by rapidly increasing NEFA concentrations, an increase rather than a decrease of adiponectin might have been expected since circulating adiponectin is commonly increasing in obese patients after diet induced weight loss (Oberhauser et al., 2012). The physiological prioritization of lactation, which is particularly emphasized in dairy cows genetically selected for high milk yields, might thus uncouple the relation between body fat and adiponectin in favor of maintaining IR.

In addition, a shift in the ratio between visceral and subcutaneous fat depots towards more visceral fat might play a role, since circulating adipokine levels are decreasing with increasing central adiposity (Arita et al., 1999); however, the portion of the different fat depots cannot be adequately quantified in living large animals.

Late pregnancy, both in humans and animals, is characterized by low-grade inflammatory conditions in AT (de Castro et al., 2011; Zhang et al., 2011) with increased production of pro-inflammatory cytokines like IL-6 and TNF-α (Radaelli et al., 2006). These cytokines and also monocyte chemoattractant protein-1 suppress adiponectin synthesis and secretion by adipocytes (Chazenbalk et al., 2010). In addition, the hormonal changes related to parturition, i.e. cortisol (Goff and Horst, 1997), estradiol-17 β, prolactin (Bell, 1995), insulin (Oda et al., 1989), and growth hormone (Bell, 1995; Oda et al., 1989) are likely to affect (together with the aforementioned cytokines) the circulating adiponectin concentrations. Adiponectin has recently been identified as mediator of the metabolic effects of fibroblast growth factor-21 (FGF21) in terms of glucose homeostasis and IS in mice (Lin et al., 2013). However, when comparing the pattern of the serum concentrations of FGF21 during the periparturient period reported for in dairy cows to the one of the adiponectin concentrations, the peak of FGF21 at the day of calving (Schoenberg et al., 2011) coincides with a nadir of adiponectin (Giesy et al., 2012; Mielenz et al., 2013 and present study). For the main FGF receptors concerned (FGFR1c, FGFR2c) and the co-receptor protein ß-Klotho, the mRNA expression in sc AT remained unchanged when comparing 40 d pre partum to d 7 of lactation (Schoenberg et al., 2011), but corresponding data for the day of parturition are not available. It thus remains open whether the phenomenon of low adiponectin during high FGF21 concentrations indicates an uncoupling of FGF21 and adiponectin during this specific metabolic and hormonal situation, or whether it is a general difference related to the species investigated, their sex and their physiological status.

Serum adiponectin concentrations were affected by parity; MP cows tended to have higher adiponectin concentrations than PP cows. Parity may affect blood hormones and metabolites, since PP animals are still growing and nutrients are directed both to fetal and maternal growth (Coffey et al., 2006). Recently, ALR was suggested as a more effective marker for IS (Inoue et al., 2005) and metabolic syndrome (Mirza et al., 2001) than adiponectin or leptin alone. In another human study, adiponectin was considered as superior to leptin or ALR for identifying metabolic changes (Mojiminiyi et al., 2007). In

this study, ALR followed a similar pattern as adiponectin around parturition. Based on the higher frequency of significant correlations between adiponectin and insulin, NEFA, and RQUICKI, as compared to ALR, we postulate that adiponectin alone is a more meaningful marker of metabolic changes around parturition in dairy cows.

4.2. CLA induced changes in circulating adiponectin and ALR

The transition from late pregnancy to lactation comprises extensive physiological and metabolic adaptation to accomplish the nutrient demand for milk production. Based on their milk fat reducing effects, CLA have been suggested to alleviate the metabolic stress that dairy cows undergo at the onset of lactation by decreasing the milk energy output. The milk fat reducing effect was confirmed for the animals studied herein (Pappritz et al., 2011). Adiponectin is involved in regulation of glucose, fatty acid and energy metabolism (Yamauchi et al., 2001). In the present study, we demonstrated that the increase of circulating adiponectin post partum was mitigated in PP and MP dairy cows by supplementation with CLA. Contradicting results have been published regarding the effect of conjugated linoleic acid (CLA) on adiponectin in monogastrics both *in vitro* and *in vivo*, whereas ruminant species including cattle have not been studied previously. When a mixture of the *cis*-9, *trans*-10 and the *trans*-10, *cis*-12 CLA was used as in our present study, decreasing (Poirier et al., 2005) as well as increasing (Martins et al., 2010) effects on adiponectin are reported. In addition, a lack of CLA effects on circulating adiponectin is documented in human patients (Joseph et al., 2011; Norris et al., 2009). In view of the emerging notion that adiponectin is a downstream effector of FGF21, recent reports that the *trans*-10, *cis*-12 CLA isomer induces hepatic expression of FGF21 through PPAR-α in mice (Yu et al., 2012), would rather point to an CLA-induced increase of adiponectin. However, the adiponectin response to CLA seems complex and probably depends on the basal (patho-) physiological condition, in particular body fat portion, the dosage used and the effective tissue concentrations of the relevant CLA isomers and the concentration of other hormones such as insulin (Perez-Matute et al., 2007).

Several authors have demonstrated that CLA reduce body fat mass in mice and humans (DeLany et al., 1999; Wang and Jones, 2004), mainly through the *trans*-10, *cis*-12 CLA isomer (Wang and Jones, 2004). Hyperinsulinemia triggered by dietary CLA was suggested as a reason for decreased adiponectin plasma levels in mice (Poirier et

al., 2005). Indeed, the CLA effects on systemic IS in our study resulted mainly from increased insulin concentrations whereas NEFA and glucose, the other variables used to calculate the RQUICKI, remained largely unchanged.

After withdrawal of CLA, a trend for a carry-over effect of CLA was observed in MP but not in PP cows. However, the metabolic and endocrine characteristics recorded herein offer no conclusive explanation for this divergent reaction in animals of different parity.

We investigated the CLA effect on the distribution of circulating adiponectin MW forms in PP and MP cows. We observed that adiponectin circulates as HMW and MMW oligomers, these molecular weight forms were previously established as the primary forms of circulating adiponectin in cattle (Giesy et al., 2012; Raffelsieper et al., 2012; Suzuki et al., 2007). The exclusive presence of HMW and absence of MMW adiponectin isoforms in bovine serum reported recently (Giesy et al., 2012), might be due to a different antibody, gel system (6% vs. 8% SDS-PAGE gel) and/or differences in the sample preparation and blotting conditions. No CLA or parity effect on the MW distribution pattern of circulating adiponectin during d 1 and d 105 of lactation was observed. In contrast, results from a study in mice and in adipocyte cell culture suggest that using exclusively the *trans*-10, *cis*-12 CLA isomer impaired the assembly of MMW forms and reduced the cellular as well as the secreted MMW adiponectin levels (Miller et al., 2008).

4.3. Adipose tissue adiponectin concentration

Adiponectin is produced mainly in AT and circulates in blood at high concentrations (Maeda et al., 1996). Its secretion is assumed to follow synthesis without significant accumulation within the tissue (Hotta et al., 2001). Blood contained in the biopsy samples may thus bias the actual tissue concentrations. We therefore used the Tr concentration in tissue extracts and serum as a marker for the extent of blood contamination in each individual sample. About 20% of total adiponectin concentrations measured in the tissue preparation were attributable to blood adiponectin. The variation of blood content observed in the tissue samples stresses the importance of correcting adiponectin concentrations from AT for the respective blood adiponectin content, at least for biopsy samples. However, the correction for residual blood we did might still result in negative values (in this study 3 out of 126 samples which were omitted in the

statistical analyses). In addition, the consideration of tissue blood content does not allow for differentiating the degree of angiogenesis in different AT or different sections within a AT depot. Moreover, assessing the MW distribution of adiponectin in tissue preparations would be compromised and we thus renounced on doing so.

The lowest (blood corrected) tissue adiponectin concentrations were observed at the time of parturition. This is in agreement with a previous study showing that adiponectin secretion from sc AT (expressed as per tissue weight) is reduced in obesity and IR in humans (Hoffstedt et al., 2004). In addition, the lowest expression at the time around calving was also confirmed in terms of mRNA (Saremi et al., 2014), albeit the serum and sc AT adiponectin concentrations were not correlated with adiponectin mRNA abundance in sc AT in the current study. No correlation between plasma and mRNA was also found in sc AT of mice (Combs et al., 2003), Rhesus monkeys (Hotta et al., 2001) and humans (Garaulet et al., 2004). When comparing late pregnant (d 40 pre partum) versus early lactating cows (d 7 post partum), the decline of adiponectin plasma concentrations was not mirrored in adiponectin mRNA abundance which remained constant in sc AT (Giesy et al., 2012). Based on the similar time course of serum and sc AT adiponectin protein concentrations with the correlation established between serum and tissue, sc AT may contribute to declining adiponectin concentrations around parturition. However, further studies need to address the expression of adiponectin mRNA and protein in different sc and visceral AT depots and their relationship with blood adiponectin concentration.

4.4. Correlations of adiponectin, leptin and ALR with measures of body condition, blood metabolites and metabolic hormones

When all animals were considered irrespective of treatment and parity, adiponectin was negatively associated with BCS but not with insulin. We observed significant inverse associations between circulating adiponectin and plasma NEFA and IGF-1 concentrations, which is in agreement with earlier human studies (Lavoie et al., 2009; Morcov et al., 2012). The ALR was unable to provide any additional information through correlations as compared to adiponectin alone, except a negative relationship with IGF-1 for the pooled data.

5. Conclusions

In summary, circulating adiponectin concentrations gradually decreased pre partum with lowest concentrations at the time of parturition, and increased thereafter. Long term dietary CLA supplementation reduced circulating adiponectin concentrations in both PP and MP cows. Concerning parity, there was a trend for lower adiponectin concentrations in PP than in MP cows. In addition, a carry-over effect of CLA after cessation of the supplementation was exclusively observed in MP cows. There was no apparent effect of treatment, parity or day of lactation on the molecular weight distribution of the protein. The lowest adiponectin concentrations in sc AT were observed at the time of parturition and increased during early lactation. No relationship between circulating or tissue adiponectin concentrations with adiponectin mRNA abundance in sc AT was found. Circulating adiponectin was positively correlated with RQUICKI indicating that reduced adiponectin around calving promotes IR and thus facilitates nutrient partitioning towards the mammary gland. The precise mechanisms that change the circulating adiponectin concentrations around parturition, and whether CLA interferes locally with other hormones and cytokines produced by AT, to alter expression and secretion of adiponectin remains to be determined.

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Table 1. Pearson correlation coefficients of adiponectin (µg/mL), leptin (ng/mL) and adiponectin : leptin ratio (ALR) with blood variables [presented for control (CON) and conjugated linoleic acid (CLA) groups during period of supplementation i.e. from d 1 to d 182 of lactation (Period 2) and for pooled data (Period 1 + 2 + 3) of multiparous (MP) and primiparous (PP) cows as well as for merged data (irrespective of parity and treatment)

			Period 2			Pooled (Period 1 + 2 + 3)		
Variable	Parity	Group	Adiponectin	Leptin	ALR	Adiponectin	Leptin	ALR
BCS	MP	CON	-0.408**			-0.392**		-0.310**
		CLA	-0.389**		-0.396**	-0.409**	0.373**	-0.460**
	PP	CON	-0.440**		-0.398**	-0.414**	0.329**	-0.546**
		CLA				-0.306**		-0.291*
NEFA, mmol/L	MP	CON	-0.394**			-0.294**		
		CLA	-0.422**			-0.239**	-0.189*	
	PP	CON	-0.511**				0.295*	
		CLA	-0.549**		-0.411*	-0.276*		0.287*
Insulin, µU/mL	MP	CON	0.335**		0.295*			
		CLA	0.324**			0.235**	0.275**	
	PP	CON						
		CLA						
IGF-1, ng/mL	MP	CON						-0.284**
		CLA					0.492**	-0.326**
	PP	CON		0.366*			0.354*	-0.417**
		CLA				-0.315*		-0.324**
RQUICKI	MP	CON	0.272*					
		CLA						
	PP	CON						
		CLA	0.485**		0.370*	0.360**		0.355**
Merged data (irrespective of parity and treatment)								
BCS			-0.388**		-0.305**	-0.403**	0.212**	-0.423**
NEFA, mmol/L			-0.326**		-0.174*	-0.221**	-0.133*	
Insulin, µU/mL								
IGF-1, ng/mL						-0.208**	0.323**	-0.360**
RQUICKI			0.244**			0.126*		

Reported correlations are limited to $P \leq .01$; BCS = Body Condition Score; NEFA = Non-esterified fatty acids; IGF-1 = Insulin like growth factor-1; RQUICKI = Revised Quantitative Insulin Sensitivity Check Index; $^{*}P \leq .01$; $^{**}P \leq .001$

Supplemental Table 1. *P* values for fixed factors and their interactions using linear mixed model for serum adiponectin during different experiment periods.

Effects		Factors	Period 2	Period 3	Period 2 + 3	Period 1 + 2 + 3
Treatment	CON	Time	<0.001	ns	<0.001	<0.001
		Parity	ns	T (0.076)	ns	T (0.055)
		Time × Parity	T (0.057)	ns	ns	ns
	CLA	Time	<0.001	0.024	<0.001	<0.001
		Parity	0.07	ns	ns	T (0.079)
		Time × Parity	ns	ns	ns	ns
Parity	MP	Time	<0.001	ns	<0.001	<0.001
		Treatment	T (0.066)	0.034	0.019	0.022
		Time × Treatment	0.001	ns	0.017	0.023
	PP	Time	<0.001	ns	<0.001	<0.001
		Treatment	0.003	ns	0.042	ns
		Time × Treatment	ns	ns	ns	ns
Pooled		Time	<0.001	ns	<0.001	<0.001
		Treatment	0.002	ns	0.005	0.003
		Parity	T (0.064)	ns	0.034	0.009

Period 1 = before parturition (d -21 until parturition), Period 2 = period of supplementation (d 1 to d 182 of lactation), Period 3 = period after supplementation (d 183 of lactation until end of experiment). Pooled = merged data of multiparous and primiparous cows of control and treatment groups; MP (multiparous cows), PP (primiparous cows), CON (control group), CLA (treatment group), T (Trend), ns (not significant). In period 1, a significant parity effect was observed ($P = 0.004$).

Supplemental Table 2. *P* values for fixed factors and their interactions using linear mixed model for serum adiponectin to leptin ratio during different experiment periods.

Effects		Factors	Period 2	Period 3	Period 2 + 3	Period 1 + 2 + 3
Treatment	CON	Time	<0.001	ns	<0.001	<0.001
		Parity	ns	0.016	0.01	0.006
		Time × Parity	ns	ns	ns	ns
	CLA	Time	0.032	ns	0.048	0.004
		Parity	ns	ns	ns	ns
		Time × Parity	ns	ns	ns	ns
Parity	MP	Time	<0.001	ns	<0.001	<0.001
		Treatment	ns	T (0.057)	T (0.075)	T (0.071)
		Time × Treatment	0.020	ns	T (0.084)	ns
	PP	Time	<0.001	0.047	0.001	<0.001
		Treatment	0.011	ns	ns	ns
		Treatment × Time	ns	ns	ns	ns
Pooled		Time	<0.001	ns	<0.001	<0.001
		Treatment	T (0.081)	ns	T (0.086)	T (0.084)
		Parity	0.036	0.017	0.005	0.003

Period 1 = before parturition (d -21 until parturition), Period 2 = period of supplementation (d 1 to d 182 of lactation), Period 3 = period after supplementation (d 183 of lactation until end of experiment). Pooled = merged data of multiparous and primiparous cows of control and treatment groups; MP (multiparous cows), PP (primiparous cows), CON (control group), CLA (treatment group), T (Trend), ns (not significant). In period 1, no parity effect was observed.

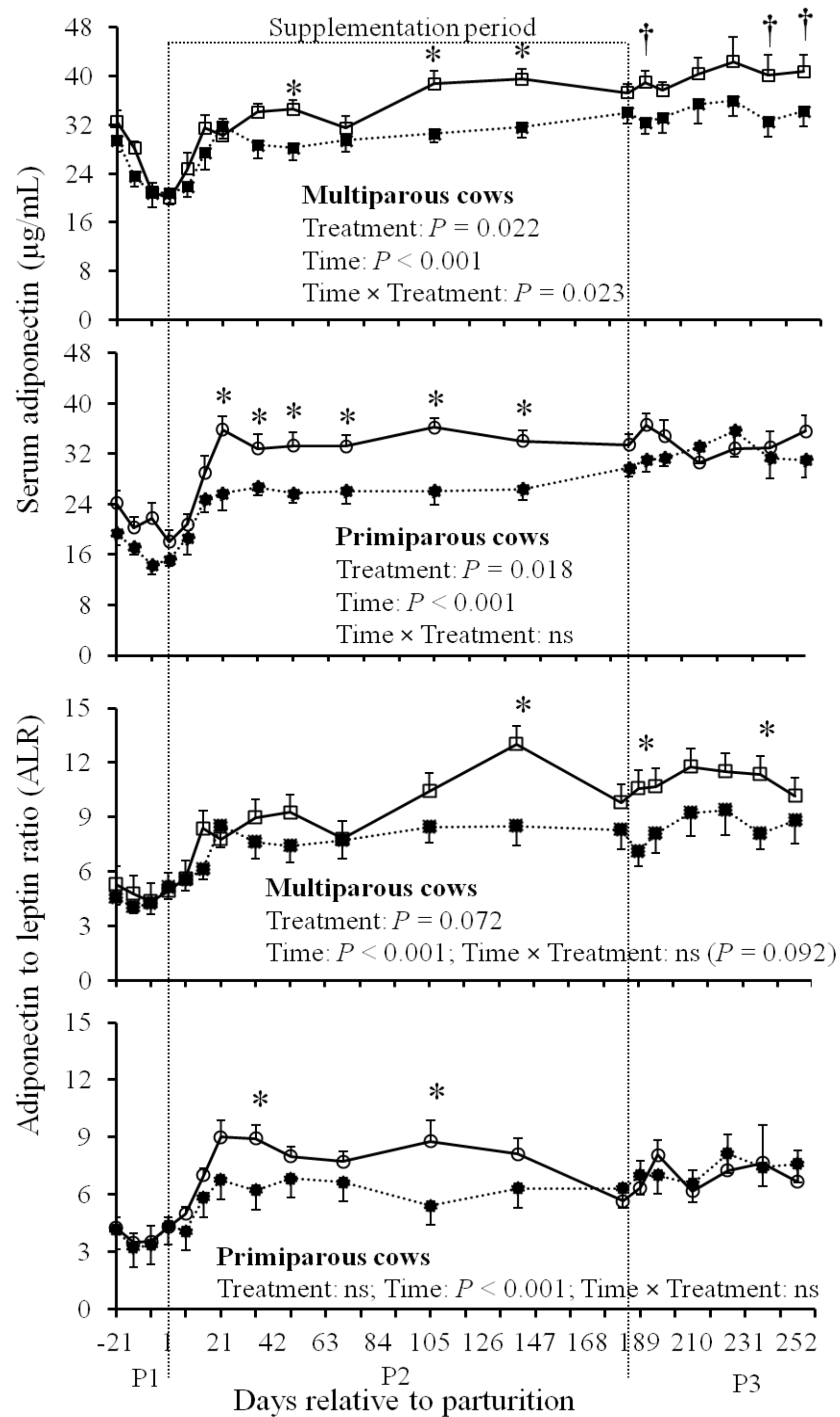


Figure 1. Serum adiponectin concentrations (means ± SEM) and serum adiponectin leptin ratio (ALR) in multiparous (MP) and primiparous (PP) cows receiving 100 g/d of either conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) or a control fat supplement (CON, Silafat®, BASF SE) from d 1 to d 182 post partum. The statistical results included in the graph comprise the entire experimental period (d -21 to d 252 post partum) considering time and treatment as factors. * indicate differences ($P < .05$) and † indicate trends for comparison between CON and CLA groups at the individual time points. A detailed statistical evaluation of the different periods and the effect of parity are provided in Tables 1 and 2. CON: straight lines, MP cows (□), n = 11, PP cows (○) n = 6; CLA: dashed lines, MP cows (■) n = 11, PP cows (●) n = 5; ns = not significant. P1 = experimental period 1 (d -21 until calving), P2 = experimental period 2 (d 1 until 182 post partum) during which the CON or CLA supplement was fed; this period is also indicated by the dotted vertical lines, P3 = experimental period 3 (after withdrawal of the supplements, d 183 post partum until end of experiment).

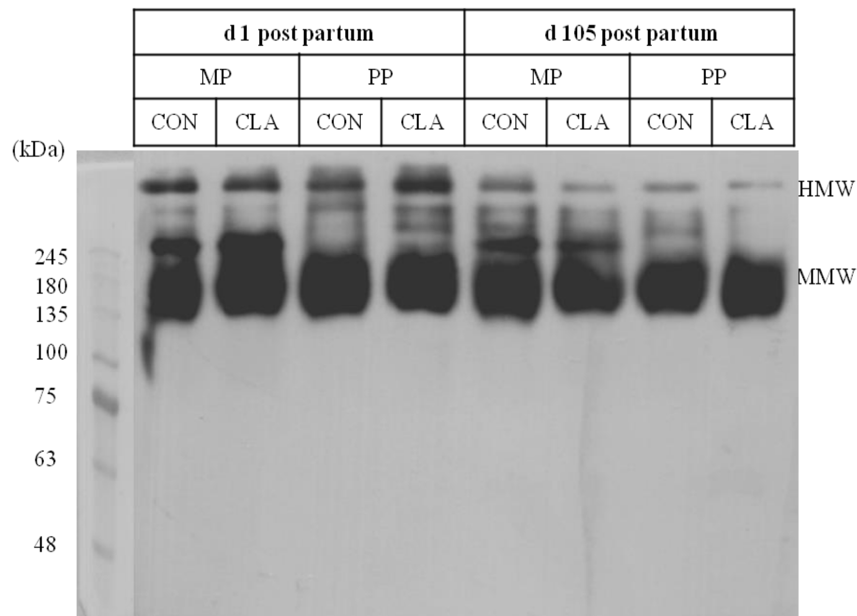


Figure 2. Exemplary Western blot of adiponectin multimeric isoforms [high molecular weight (HMW) and middle molecular weight (MMW)] under nonreducing, nonheat-denaturing conditions with sera from multiparous (MP) or primiparous (PP) cows receiving a control fat (CON, Silafat®, BASF SE, Ludwigshafen, Germany) or conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE) on d 1 and d 105 post partum.

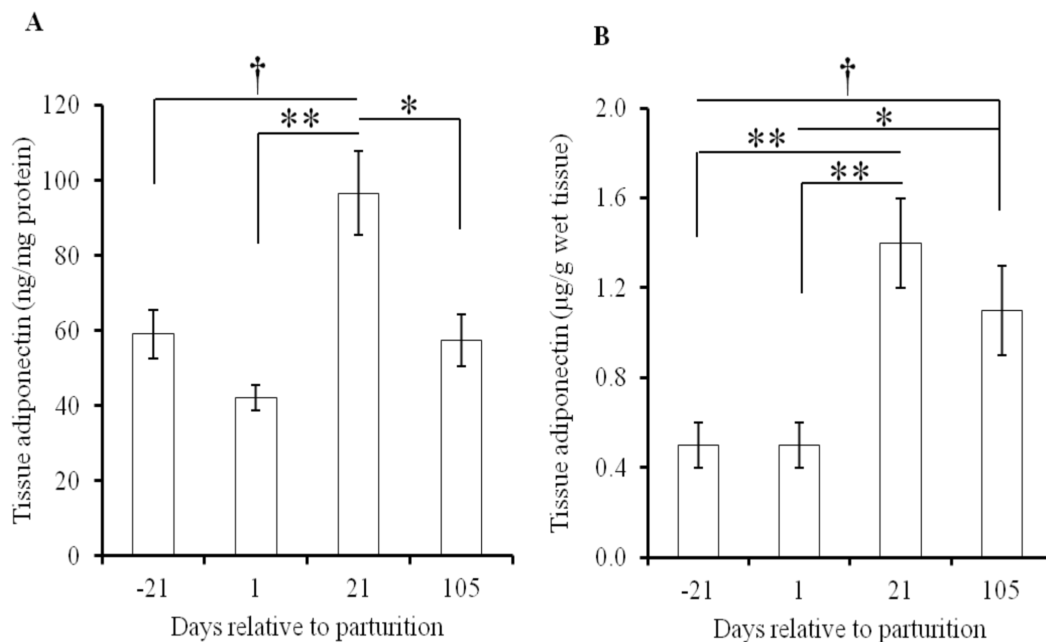
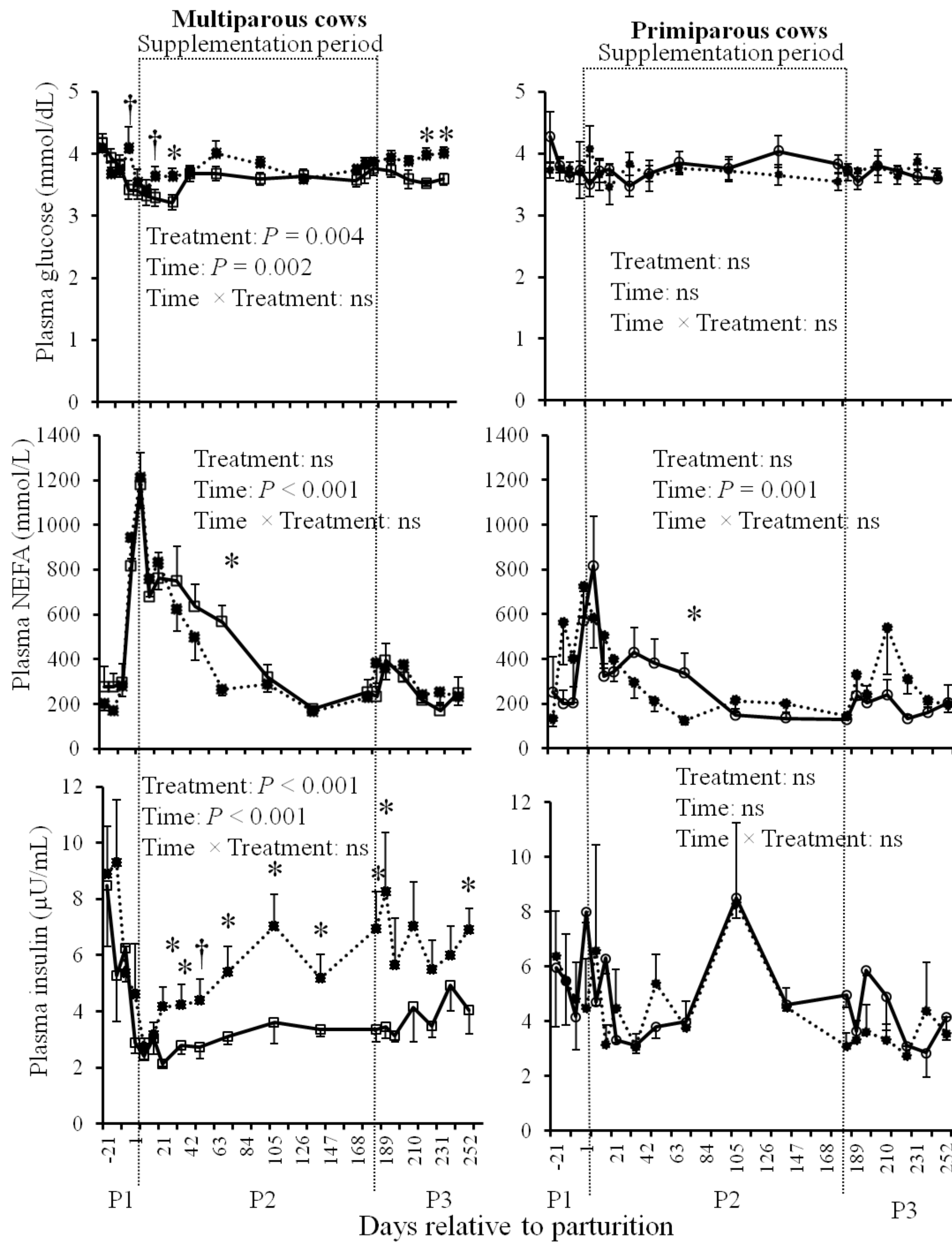
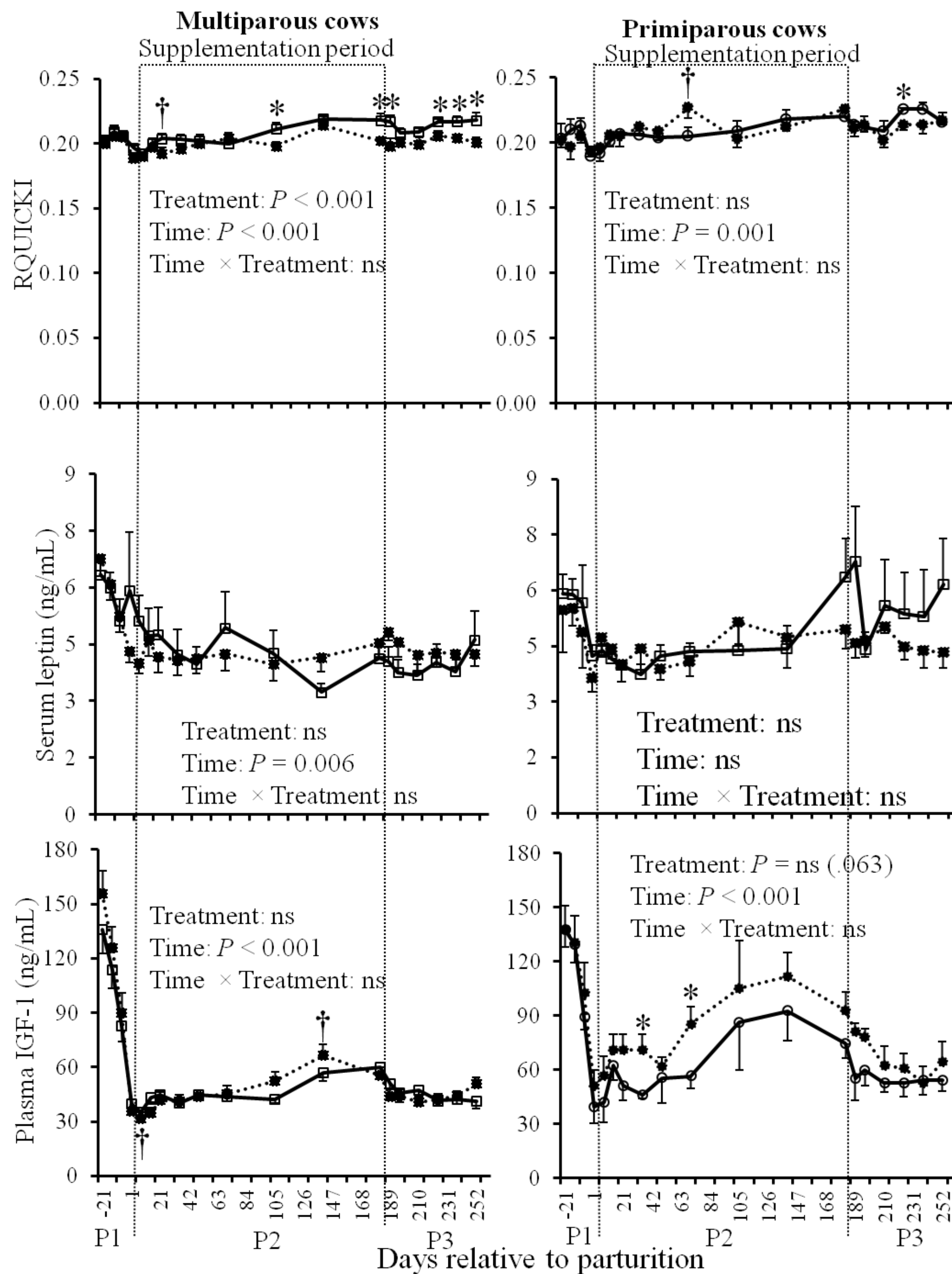


Figure 3. Adiponectin concentrations (means ± SEM) in adipose tissue **(A)** ng/mg total protein and **(B)** ng/g wet tissue basis corrected for blood-derived adiponectin (n = 33). * or ** indicate differences at $P < 0.05$ or $P < 0.001$, respectively and † indicates trend ($P < 0.1$). Data were merged for analysis since no treatment or parity effect was observed (with the exception of a treatment effect on d 21 for the ng/mg total protein values).



Supplemental Figure 1. Circulating concentrations of glucose, NEFA and insulin (means ± SEM) in multiparous (MP) and primiparous (PP) cows receiving 100 g/d of either conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) or a control fat supplement (CON, Silafat®, BASF SE) from d 1 to d 182 postpartum. The statistical results included in the graph comprise the entire experimental period (d -21 to d 252 postpartum) considering time, treatment and parity as factors. * indicate differences (P < .05) and † indicate trends for comparison between CON and CLA groups at the individual time points. Significant parity effect (P < .001) was observed for NEFA with lower values in PP cows. CON: straight lines, MP cows (□), n = 11, PP cows (○) n = 6; CLA: dashed lines, MP cows (■) n = 11, PP cows (●) n = 5; ns = not significant. P1 = experimental period 1 (from d -21 until calving), P2 = experimental period 2 (from d 1 until 182 postpartum) during which the CON or CLA supplement was fed; this period is also indicated by the dotted vertical lines, P3 = experimental period 3 (after withdrawal of the supplements, d 183 postpartum until end of experiment).



Supplemental Figure 2. Revised Quantitative Insulin Sensitivity Check Index (RQUICKI), circulating concentrations of leptin and IGF-1 (means ± SEM) in multiparous (MP) and primiparous (PP) cows receiving 100 g/d of either conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) or a control fat supplement (CON, Silafat®, BASF SE) from d 1 to d 182 postpartum. The statistical results included in the graph comprise the entire experimental period (d -21 to d 252 postpartum) considering time, treatment and parity as factors. * indicate differences ($P < .05$) and † indicate trends for comparison between CON and CLA groups at the individual time points. Significant parity effect ($P < .01$) was observed for RQUICKI and IGF-1 with lower values in MP cows. CON: straight lines, MP cows (□), n = 11, PP cows (○) n = 6; CLA: dashed lines, MP cows (■) n = 11, PP cows (●) n = 5; ns = not significant. P1 = experimental period 1 (from d -21 until calving), P2 = experimental period 2 (from d 1 until 182 postpartum) during which the CON or CLA supplement was fed; this period is also indicated by the dotted vertical lines, P3 = experimental period 3 (after withdrawal of the supplements, d 183 postpartum until end of experiment).

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Lactation driven dynamics of adiponectin supply from different fat depots to circulation in cows

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ABSTRACT

Adipose tissue (AT) depots are heterogeneous in terms of morphology and adipocyte metabolism. Adiponectin, one of the most abundant adipokines, is known for its insulin sensitising effects and its role in glucose and lipid metabolism. Very little is known about the presence of adiponectin protein in visceral and subcutaneous AT depots. We assessed serum adiponectin and adiponectin protein concentrations and the molecular weight forms in visceral (mesenterial, omental and retroperitoneal) and subcutaneous (sternum, tail-head and withers) AT of primiparous dairy cows during early lactation. Primiparous German Holstein cows (n = 25) were divided into a control (CON) and a

conjugated linoleic acid (CLA) group. From day 1 of lactation until slaughter, CLA cows were fed 100 g of a CLA supplement/d (about 6% of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers each), whereas the CON cows received 100 g of a fatty acid mixture/d instead of CLA. Blood samples from all animals were collected from 3 week before calving until slaughter on day 1 (n = 5, CON cows), 42 (n = 5 each of CON and CLA cows) and 105 (n = 5 each of CON and CLA cows) of lactation when samples from different AT depots were obtained. Adiponectin was measured in serum and tissue by ELISA. In all AT depots adiponectin concentrations were lowest on day 1 compared to day 42 and day 105, and circulating adiponectin reached a nadir around parturition. Retroperitoneal AT had the lowest adiponectin concentrations, however, when taking total depot mass into consideration, the portion of circulating adiponectin was higher in visceral than subcutaneous AT. Serum adiponectin was positively correlated with adiponectin protein concentrations but not with the mRNA abundance in all fat depots. The CLA supplementation did not affect adiponectin concentrations in AT depots. Furthermore, inverse associations between circulating adiponectin and measures of body condition (empty body weight, back fat thickness and visceral AT mass) were observed. In all AT depots at each time, adiponectin was present as high (about 300 kDa) and medium (about 150 kDa) molecular weight complexes similar to that of the blood serum. These data suggest differential contribution of AT depots to circulating adiponectin.

Keywords: adipose tissue depots, adiponectin, dairy cow, lactation

1. Introduction

Adipose tissue (AT) is considered as an active endocrine organ synthesising and secreting a series of bioactive molecules collectively termed as adipokines. Adiponectin (AdipoQ), one of the most abundant circulating adipokines is primarily expressed by adipocytes and exerts mainly insulin sensitising effects [1]. Synthesised as a 30 kDa monomer, AdipoQ is assembled to various oligomeric forms via disulfide bonds through an amino-terminal cysteine and may occur in circulation as low molecular weight (LMW; trimer), medium molecular weight (MMW; hexamer) and high molecular weight (HMW; congregated multimers) oligomers [2]. Among all molecular weight isoforms, the HMW isoform is considered as the most biologically active, being associated with improving insulin sensitivity (IS) [3] and activation of AMP activated

protein kinase in muscle [2]. Regulation of adiponectin isoform secretion is regulated at the level of AT [3] and the spontaneous inter-conversion of hexamers (MMW) and HMW do not occur in circulation [4]. Besides its role in regulation of glucose and lipid metabolism, pro- and anti-apoptotic functions of AdipoQ have also been demonstrated (reviewed by [5]). Therefore, AdipoQ might affect the rate of apoptosis also in AT through autocrine or paracrine mechanisms.

The AT from different anatomical locations differ in fatty acid metabolism [6]. Visceral AT (vcAT) mass is more strongly correlated with IS [7], increased risk for developing diabetes II and atherosclerosis [8], than subcutaneous AT (scAT). Regardless of weight loss interventions such as caloric restriction, pharmacological therapy, or exercise, individuals preferentially lose vcAT mass [9], thus suggesting that vc adipocytes have a higher lipolytic capacity. Moreover, in rodents and humans, removal of sc fat did not improve IS, supporting the hypothesis that vcAT may be metabolically more active [10,11]. Site dependent differences in the functional activities of vc (retroperitoneal AT) and sc AT depots have also been shown for dairy cows [12,13]. When studying the role of AT in processes of homeorhetic regulation in which metabolism of all body tissues is dynamically adapted to support a physiological state, such as pregnancy or lactation, the anatomical location of AT requires careful consideration. Most mammals undergo a period of negative energy balance during early lactation, however, in dairy cows selected for high milk yields both the extent and the duration of negative energy balance exceed the ones in other mammalian species [14-16]. In early lactation, the priority of the mammary gland for glucose uptake is accomplished by insulin-independent glucose transporters in the mammary gland together with a decrease of IS in other peripheral tissues [17]. Concomitant with the peripartal decrease in IS, the circulating AdipoQ concentrations were recently demonstrated to decline using semiquantitative Western immunoblot methods [18,19]. Recent human studies suggest that by 24 h after delivery there is a decline in HMW and MMW isoforms which correspond with approximately an 80% reduction of serum AdipoQ concentrations [20].

Based on the heterogeneity of the metabolic activity in different AT depots and the relationship of AdipoQ with IS and lipolysis, we hypothesised that the expression of the AdipoQ protein in sc and vc AT depots will change during early lactation. Dietary supplementation with conjugated linoleic acids (CLA) affect lipid metabolism and is

known to cause decline in milk fat content in dairy cows [21,22]. Therefore, we aimed to assess the AdipoQ protein concentrations and the distribution of its different isomeric forms (LMW, MMW, and HMW) in 3 different sc and 3 different vc AT depots at 3 time points during the first 105 days of lactation in primiparous dairy cows with or without CLA supplementation. Assuming that not only the AdipoQ protein expression by each individual AT will change but also the mass of the individual depot, we took the respective fat masses into consideration to estimate the portion of circulating AdipoQ present in different fat depots. In addition, we tested for correlations between AT AdipoQ, serum AdipoQ, adipocyte size, adipocyte apoptotic rate, several blood metabolites, and hormones including a surrogate marker of IS [Revised Quantitative Insulin Sensitivity Check Index (RQUICKI)] and variables characterising body fat such as body condition score (BCS), back fat thickness (BFT), live weight and empty body weight (EBW).

2. Materials and methods

2.1. Experimental animals and treatments

In compliance with the European Union Guidelines concerning the protection of experimental animals and with approval by the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany (File Number 33.11.42502-04-071/07), the present experiment was conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute, Braunschweig, Germany. The experimental design is described in detail elsewhere [23]. In brief, primiparous lactating German Holstein-Friesian cows (n = 25) with an average age at parturition of 23 ± 0.2 months were included in the study 3 wk before the expected date of calving and were slaughtered on d 1, 42 and 105 of lactation. For this purpose, the animals were kept in a free-stall barn, with free access to water. Before parturition, all cows received a similar diet consisting of 60% corn silage and 40% grass silage on a dry matter (DM) basis (6.7 MJ of NE_L/kg DM) ad libitum and 2 kg of concentrate/d (6.7 MJ of NE_L/ kg DM). During lactation, the partial mixed ration (PMR) fed comprised 38% corn silage, 25% grass silage (DM basis, 7.5 MJ of NE_L/kg DM) and 37% PMR-concentrate (13.5 MJ of NE_L/ kg DM). In addition, each animal was provided with concentrate by a computerized concentrate feeding station (3.5 kg/d on a DM basis). Feed ingredients of the concentrates are presented in Table 1.

Beginning at the day of calving, the animals were randomly assigned to receive either a supplement containing CLA (100 g/d Lutrell® Pure, BASF SE, Ludwigshafen, Germany; group CLA, n = 10) or a control (CON) fat supplement in which CLA was replaced by stearic acid (Silafat®; BASF SE, 100 g/d; group CON, n = 15) until experimental slaughter. The supplements were provided with the concentrate; the CLA group consumed 6.0 g/d of the *trans*-10, *cis*-12 CLA isomer and 5.7 g/d of the *cis*-9, *trans*-11 CLA isomer (calculated based on the analysed proportion in concentrates) [23]. Five animals of the CON group were slaughtered on d 1 and thereafter five animals of both groups were slaughtered each at d 42 and d 105 of lactation.

2.2. Blood and tissue sample collection

Blood samples were collected from the jugular vein on d -21, -14, -7, -3, 1 (n = 25), 7, 14, 21, 28, 42 (n = 20) and 105 (n = 10) relative to parturition. Serum samples were obtained by clotting followed by centrifugation (3000 × *g*, 10 min, 4°C) and stored at -80°C until analysed. Body weight was automatically recorded when leaving the milking parlor. Body condition was scored using a scale of 1 to 5 (1 = thin, 5 = obese) [24] and BFT was determined by ultrasound immediately before slaughter. After the morning milking on the respective slaughter days (d 1, 42 or 105), the animals were transported to the experimental slaughter facilities, weighed, stunned with a captive bolt, and exsanguinated. Samples from different AT depots were collected, rapidly frozen in liquid nitrogen and stored at -80°C until analysed. Body parts were weighed as they were dissected from the animal. The EBW comprises of weights of all body parts except digesta and content of urinary and gall bladder [23]. Mesenteric, omental and retroperitoneal fat depots were dissected and weighed during the slaughter process. Subcutaneous fat which include fat from different sites such as sternum, tail-head and withers was dissected manually from the skin and body with a knife and weighed.

2.3. Preparation of tissue samples for AdipoQ analysis

After thawing the tissue samples on ice, they were homogenised in 2 vol of homogenisation buffer [10 mM HEPES pH 7.4 with complete® protease inhibitor cocktail (one tablet/10 mL buffer, Roche, Mannheim, Germany)] using the Precellys® 24 homogeniser (Peqlab Biotechnologies GmbH, Erlangen, Germany). Homogenates were centrifuged twice (14,000 × *g*, 10 min, 4°C) to separate the fat layer. The

infranatant (without fat and tissue debris) were collected and stored at -20°C until analysis.

2.4. Sample analyses

Serum samples were analysed for AdipoQ in duplicate using an in-house developed ELISA as described in detail earlier [19]. Prior to the AdipoQ measurements in tissue preparations, parallelism was established and assay accuracy was determined by linearity of serial samples dilutions (Fig. 1). The intra- and inter-assay coefficients of variation were 7.0% and 11.0%, respectively. The total protein concentration of the tissue preparations was determined using the Bradford Assay [Roti-Nanoquant (K880), Carl Roth GmbH, Karlsruhe, Germany]. The AdipoQ concentrations of tissue preparations (ng/mL) were normalised to total protein concentration or wet tissue weight and are presented as ng AdipoQ/mg total protein or ng AdipoQ/g wet tissue. The amount of AdipoQ (µg) present in individual AT depots was estimated by multiplying the AdipoQ in each g of tissue with the mass of the respective depot. The values were further used to estimate the portion of circulating AdipoQ present in individual AT depots.

To characterise the different molecular weight (MW) forms of AdipoQ in tissue preparations in comparison with serum, AT samples from d 1, 42 and 105 of lactation were exemplarily examined by Western blot analysis under non reducing, non heat-denaturing conditions. Based on preceding ELISA results, the samples were diluted with ultrapure water to obtain approximately the same amount of AdipoQ in each lane (0.75 ng AdipoQ per lane). The diluted samples were mixed with sample buffer (0.064 M Tris HCl pH 6.8, 1% SDS, 0.01% bromophenol blue, 10% glycerol). Before loading on 8% SDS gels, samples were centrifuged for 5 min at 10,000 × *g*, 4°C. Proteins separated by SDS-PAGE were transferred onto a polyvinylidene diflouride membrane (PVDF; GE Healthcare Europe, Freiburg, Germany) using tank blotting (Criterion Blotter System, Bio-Rad Laboratories, Munich, Germany). After 1 h of blocking the membrane in Tris-buffered saline-Tween (TBS-T) + 10% Roti®-Block (Carl Roth, Karlsruhe, Germany), the first antibody (polyclonal rabbit antiserum generated against bovine adiponectin; 0.25 µg/mL), i.e. the same as used in the ELISA [19], was incubated for 60 min under shaking at room temperature. After 4 washing steps (5 min each), the HRP conjugated secondary antibody (goat-anti-rabbit antibody; 1:100,000, Southern Biotech, Birmingham, AL, USA) was incubated for another 60 min at room

temperature. Finally, the membrane was again washed (4 × 5 min, TBS-T) and the immune complex was then detected with an enhanced chemiluminescence detection system (GE Healthcare) using CL-XPosure film (Thermo Scientific, Munich, Germany). The MW of the bands obtained were assessed by comparison with the bands of the MW marker [Biotinylated Protein Ladder, #7727, Cell Signaling Technology, Danvers, MA, USA].

As a measure of IS, RQUICKI was calculated according to the following equation [25]: RQUICKI = 1 / [log (Glucose, mg/dL) + log (Insulin, µU/mL) + log (NEFA, mmol/L)]. The data for body weight, BCS, BFT, AT depot masses, adipocyte cell size from 100 adipocytes per section, portion of apoptotic cells, plasma IGF-1, plasma NEFA and AdipoQ mRNA abundance in the different fat depots were presented earlier [26-29] and were used for the correlation analysis in the present study.

2.5. Statistical analyses

The serum and tissue AdipoQ concentrations were not affected by CLA supplementation, therefore merged data from both groups were used for statistical analyses. Data for all variables were first tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variances with the Levene's test. Comparison for serum AdipoQ concentration and RQUICKI between each time points was done using mixed model procedure (general linear model). Sampling days was considered as repeated effect. The covariance structure autoregressive (first-order autoregressive structure with homogenous variances) followed by Bonferroni correction was used. Mann-Whitney *U* test was used to analyse the differences in AT depots for AdipoQ concentrations, amount of AdipoQ, portion of circulating AdipoQ present and time dependent changes in the mass. Correlation coefficients between variables were calculated using Pearson correlation analysis. Backward stepwise multivariate linear regression analyses were conducted to identify significant independent predictors of serum AdipoQ concentration. The variables which were related to serum AdipoQ ($r \geq 0.55$) were included in the models. Some of the variables such as AT AdipoQ concentration and tissue mass were highly correlated to each other therefore, to avoid multicollinearity, they were entered separately into regression models. Serum AdipoQ was regarded as dependent variable and AT measures such as tissue AdipoQ concentration, adipocyte cell size, AT depot mass as well as total body fat mass were

analysed as independent variables. For correlation and regression analyses the variables were log transformed, wherever necessary, to satisfy the statistical assumptions of normal distribution for these tests. All statistical analyses were carried out using SPSS for Windows version 19.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as means ± S.E.M., P-values < 0.05 were considered significant and a trend was defined at $P \leq 0.10$.

3. Results

3.1. Serum AdipoQ and RQUICKI profiles from late pregnancy to early lactation

The time dependent changes in serum AdipoQ concentrations and RQUICKI are shown in Fig. 2. Neither serum AdipoQ concentrations nor RQUICKI were affected by CLA supplementation ($P > 0.01$). The serum AdipoQ concentrations decreased from d 21 pre partum, reaching a nadir at 3 d pre partum (23% lower concentration compared to d 21 pre partum). Thereafter, the concentration increased by about 2-fold until d 14 postpartum ($P < 0.001$) and then remained unchanged. The RQUICKI values decreased from d 7 pre partum and reached the lowest level at the time of parturition (about 24% lower than d 7). After calving RQUICKI values gradually increased and reached pre partum levels within the first wk of lactation.

3.2. Tissue AdipoQ concentrations

Being not affected by CLA supplementation ($P > 0.01$), the tissue AdipoQ concentrations (ng/mg protein and ng/g wet tissue) in sc and vcAT depots are presented in Fig. 3 irrespective of the treatment. Tissue AdipoQ concentrations in all AT depots were lowest on d 1 compared to d 42 and d 105. Retroperitoneal AT had about 1.6-fold lower AdipoQ concentrations than the mesenterial, omental, tail-head and withers fat depots ($P < 0.01$). When normalised to wet tissue weights, lowest AdipoQ concentrations were observed in retroperitoneal AT compared to all other fat depots at all time points.

3.3. Total amount of AdipoQ in AT depots

The total amount of AdipoQ (µg) contained in each individual fat depot was calculated using the respective depot concentration and the depot mass during different days of lactation and is presented as Fig. 4 A. The corresponding change in fat mass

(kg) is provided in Fig. 4 B. Significant declines in fat depot mass on d 42 and d 105 compared to d 1 were limited to omental and retroperitoneal AT, whereas the depot mass of mesenterial and sc AT remained unchanged (Fig. 4 B). Concentrations of AdipoQ were lowest in retroperitoneal AT, and were accompanied by decreased adipocyte size and depot mass during early lactation. However, when changes in depot mass were calculated as a portion of total fat mass (%), a significant decline (1.3-fold, $P < 0.05$) was observed only in retroperitoneal AT. With the exception of retroperitoneal AT, the amount of AdipoQ in all vc and sc AT depots was significantly lower on d 1 compared to d 42 and d 105 (Fig. 4 A). In the retroperitoneal depot, the amount of AdipoQ on d 1 and d 42 was about 2.5-fold lower compared to d 105 ($P < 0.01$). Omental AT contained the highest and retroperitoneal AT contained the lowest amounts of AdipoQ at all time points. Irrespective of time, the AdipoQ amount was highest in omental AT (1709 ± 331.6 µg), whereas retroperitoneal AT (488 ± 81.4 µg) had 2.1, 3.5 and 2.5 fold lower amounts ($P < 0.001$) than mesenterial, omental and sc AT depots, respectively.

3.4. Estimated portion of circulating AdipoQ in individual fat depots

The total plasma volume was calculated considering 36.9 mL/kg body weight, given for dairy cows of 2 to 5 years of age [30]. Considering this, the amount of AdipoQ (µg) in mesenterial, omental, retroperitoneal, and sc AT corresponded to about 0.19 %, 0.28 %, 0.09 % and 0.21% of the amount of AdipoQ (µg) in circulation, respectively. When taking together all vc depots, the portion of AdipoQ contained in the vc depots was consistently higher than in the sc depot (7-fold on d 1, $P = 0.002$; 2.2-fold on d 42, $P = 0.003$ and 3-fold on d 105, $P < 0.001$) (Fig. 5). The AdipoQ present in all fat depots considered in this study (vc and sc AT) comprised 0.78% of the total AdipoQ present in the circulation.

3.5. Correlation analyses

The relationships of serum AdipoQ concentration with tissue AdipoQ, AT mass, total tissue AdipoQ, adipocyte size and AdipoQ mRNA of each fat depot as well as with measures of body condition such as total fat mass, BCS, BFT, live and empty body weight are presented in Table 2. Relationships of adipocyte size with tissue AdipoQ concentration or RQUICKI are presented in Table 3. Correlation coefficients between

tissue AdipoQ and plasma concentrations of IGF-1 and NEFA are presented in Table 4. The portion of apoptotic cells (%) in sternum AT was positively correlated with tissue AdipoQ [ng/mg protein (r = 0.421, *P* = 0.036) and ng/g tissue (r = 0.400, *P* = 0.048)]. This relationship was not observed in any other fat depot. No significant correlation was found between serum AdipoQ and the portion of apoptotic cells in any of the fat depots. Among all measures of body condition, only BFT was associated with the AT AdipoQ concentration of all depots except sternum (Table 5). An inverse relationship was observed between live body weight (kg) with AT AdipoQ concentrations (ng/mg protein) in mesenterial AT (r = -0.421, *P* = 0.036), retroperitoneal AT (r = -0.456, *P* = 0.022) and sc AT from withers (r = -0.449, *P* = 0.024). Significant relationships between depot mass (kg) and tissue AdipoQ concentration were limited to retroperitoneal AT (r = -0.456, *P* = 0.022 and r = -0.608, *P* = 0.001 for ng/mg protein and for ng/g tissue, respectively). The tissue AdipoQ concentrations and RQUICKI were not correlated, however, RQUICKI was correlated with the amount of AdipoQ in retroperitoneal AT (r = 0.411, *P* = 0.046), but not in other fat depots.

3.6. Multivariate regression analysis

Retroperitoneal and tail-head AdipoQ but not the adipocyte sizes were identified as independent predictor for the circulating AdipoQ concentrations (Table 6).

3.7. Molecular weight distribution of AdipoQ in adipose tissue depots

Using SDS-PAGE and Western immunoblotting without reducing and heat-denaturing conditions, AdipoQ in tissue preparations and in serum samples was detected as HMW oligomers of approximately 300 kDa and MMW of approximately 150 kDa as exemplarily shown in Fig. 6 (A, B, C). The molecular weight distribution pattern was similar in serum and all AT depots.

4. Discussion

Adipose tissue and circulating AdipoQ concentrations were measured in this study using an in-house developed and validated competitive ELISA. In view of the serum concentrations exceeding by far the ones in AT, we estimated the amount of residual blood in the tissue samples using the transferrin concentration in the tissue preparations and in serum to be able to correct for blood derived adiponectin. However, in contrast to AT samples obtained through biopsies, the blood content in AT samples

collected after exsanguination at slaughter was negligible i.e. less than 1% vs about 5% for biopsy samples (unpublished observation). Serum AdipoQ decreased around parturition confirming earlier studies in which semiquantitative Western immunoblotting was used [18,19] and our previous results using the aforementioned ELISA [19]. The underlying mechanisms for the decline in circulating AdipoQ concentrations around parturition are not entirely known. Our present results indicate that the concentration of AdipoQ was significantly lower in all fat depots at the time of parturition and increased during early lactation irrespective of the anatomical site. Therefore, decreasing circulating AdipoQ around parturition may be the collective outcome of significant reduction of AdipoQ protein expression in all fat depots. The hormonal changes associated with parturition are probably related with the changes in circulating AdipoQ as AdipoQ expression and secretion is reportedly suppressed by prolactin, growth hormone, glucocorticoids and TNF-α *in vitro* [31-33]. Secretion of blood hormones via colostrum and milk is a convenient means of biological clearance from the maternal circulation [34]. The rate of secretion might be affected by the integrity of the blood-milk barrier. In dairy cows, the permeability of mammary gland tight junctions is increased during late pregnancy [35] and is closed following parturition [36]. This might result into higher secretion of blood AdipoQ into the colostrum as reported for certain other blood hormones such as progesterone, estrogen and prolactin [37] and consequently contribute to the low blood AdipoQ concentrations around calving.

Adiponectin is known for its insulin sensitizing [38] and lipolysis inhibiting effects [39]. In rodents, AdipoQ increases glucose uptake and fatty acid oxidation in skeletal muscle as well as reduces hepatic gluconeogenesis [40]. Therefore, lower AdipoQ concentrations in circulation around parturition might contribute to increased lipolysis, increased glucose production in liver and decreased IS of peripheral organs and thus help in nutrient availability to the mammary gland for enhanced milk production after calving.

We observed nearly similar characteristic patterns of the time dependent changes in serum AdipoQ concentrations and RQUICKI around parturition and found a significant weak positive correlation between these variables during the post-calving period suggesting an *in vivo* effect of AdipoQ on IS. The RQUICKI and adipocyte cell size in all fat depots were negatively correlated and this points to adipocyte size as an

important determinant of whole body IS. This is in line with earlier *in vitro* studies suggesting that AT of obese subjects, with enlarged cells, is less insulin responsive [41] and that stimulation with insulin increases the amount of glucose transporter (GLUT4) in the plasma membrane of small fat cells but not of large fat cells [42].

Certain fatty acids such as linoleic acid, palmitic acid and eicosapentaenoic acid are known to reduce AdipoQ gene expression in 3T3-L1 adipocytes [43]. In addition, inhibitory effects of CLA on AdipoQ expression has been observed in mice and in cell culture studies [44,45]. In the present study, no effect of dietary CLA supplementation on AT or circulating AdipoQ concentrations was observed. Based on the determination of fat content and the fatty acid composition of different fat depots in the same animals, the transfer of supplemented CLA isomers into the AT depots was only marginal [46]. Therefore, we assume that the absence of CLA effects on tissue and circulating AdipoQ might be due to the insufficient dose of supplemented CLA to these animals.

Rate of synthesis, secretion and clearance are the main determinants for circulating concentrations of metabolites and hormones. Adiponectin has relatively long half life (2.5 to 9 h) and is dependent on its molecular weight form with greater half life of HMW isomer compared to the MMW isomer (9 vs 4.5 h) [4,47]. Between different AT depots AdipoQ secretion vary substantially [48]. After treatment with the insulin sensitizer thiazolidinedione, Motoshima et al. [48] showed that increased circulating AdipoQ levels derived from the omental rather than the scAT depot. Moreover, rate of AdipoQ secretion by adipocytes from scAT seems to play only a minor role in variation of its blood concentration [47]. Thus, the secretion rate alone may not provide an optimal estimate of circulating AdipoQ concentrations. The variables such as tissue AdipoQ concentration and the changes in the mass of fat depots during the period of energy demand such as early lactation may thus give an idea about relative competence of AT depots for the variation in circulating AdipoQ concentrations.

Studies on AT in dairy cows are mainly limited to sc fat, since the vc fat depots in the alive animal can neither be quantified due to the lack of appropriate methods for large animals such as magnetic resonance imaging, nor routinely be biopsied for tissue analyses. Therefore data about metabolic and endocrine differences between vc and sc AT depots are largely lacking. To determine the predominant site of AdipoQ secretion, we measured the AdipoQ concentration in various fat depots at different periods of lactation and further analysed their relationship with serum AdipoQ concentrations and

with measures of AT depot and body condition. Retroperitoneal AT had both the lowest AdipoQ concentration and the lowest total amount of AdipoQ. In addition, when calculated as a proportion of EBW (%), a decline is observed only in retroperitoneal AT in the same animals [23]. Therefore, the retroperitoneal AT seems unique as compared to the other fat depot in lipid mobilisation during early lactation. *In vitro* studies on human and mouse adipocytes suggest an inhibitory effect of AdipoQ on lipolysis [39,49], therefore, the lower concentration of AdipoQ in retroperitoneal AT might contribute to the higher lipolytic rate in this depot. The physiological significance of depot specific variations in lipolysis is unknown, however, it has been reported that in contrast to the retroperitoneal AT, which drains into the systemic circulation, other vcAT depots release free fatty acids (FFA) directly to the liver via the portal vein [50]. Increased delivery of FFA to the liver impairs the ability of insulin to suppress hepatic gluconeogenesis; in addition, increased systemic FFA concentrations from retroperitoneal AT may inhibit insulin mediated glucose uptake in skeletal muscle [51]. Therefore, a higher rate of lipolysis in retroperitoneal and/or other vcAT depots might have positive effects on glucose homeostasis in early lactation by partitioning nutrients towards the mammary gland.

In the current study, serum AdipoQ was negatively correlated with the mass of each individual vcAT depot (mesenterial, omental and retroperitoneal AT) as well as with the total vcAT mass, however, no relation was observed with scAT mass. In line with our findings, studies in humans reported that vcAT is negatively associated with circulating AdipoQ, whereas scAT has no significant impact [52,53]. However, this relationship might be different in other physiological situations since Frederiksen *et al.* [54] found that scAT rather than vcAT was negatively associated with AdipoQ in healthy young men.

As shown by multiple regression analysis, the amount of AdipoQ in omental AT, tail-head AdipoQ concentration, retroperitoneal tissue mass and its AdipoQ concentration were observed as significant independent predictors for circulating AdipoQ concentration. This is in partial agreement with other studies reporting that AdipoQ concentrations are inversely correlated with both vcAT and scAT, however, only vcAT was identified as independent determinant of AdipoQ levels [52,55]. Therefore, decreased circulating AdipoQ concentrations seems closer related to increased vcAT rather than scAT [55].

Considering both, the variation in protein expression and change in tissue mass, fat depots contribute differently to the circulating concentrations of AdipoQ. Again, the importance of the retroperitoneal depot for energy homeostasis through lipolysis during the period of high energy demand is supported.

An inverse relationship of serum AdipoQ concentration with adipocyte size in retroperitoneal AT and in sc fat from withers was observed, whereas adipocyte sizes of other fat depots were not significantly related. In agreement with these findings, an increase in adipocyte sizes was reported to reduce AdipoQ mRNA expression in parametrial AT [56]. Adipocyte size is an important determinant for the secretion of adipokines and reduced AdipoQ expression in large adipocytes *in vivo* may be due to down regulatory effect of cytokines such as TNF-α, IL-6, or IL-8, being released by surrounding cells such as preadipocytes and macrophages [57]. Recently, we reported that retroperitoneal adipocyte sizes were mostly larger than the adipocytes from other sites, independent of time of lactation [26], therefore, the lower AdipoQ concentration in retroperitoneal AT might be due to relatively large size of their adipocytes. Adiponectin is known to affect apoptosis both *in vivo* and *in vitro*. However, controversial reports exist whether AdipoQ might have pro- or anti-apoptotic effects, depending on the source and the tissue sensitivity of AdipoQ as well as on the time and concentration of the administration [5]. Although the relationship of tissue AdipoQ with the number of apoptotic cells (%) was limited to the sternum AT in the present study, the positive correlation suggests pro-apoptotic functions of AdipoQ in bovine AT. We observed a negative relationship of serum AdipoQ with measures of body condition such as live weight, EBW, BCS and BFT which is in concordance with studies in mice and in humans suggesting that AdipoQ gene expression and plasma levels are significantly reduced in obesity [38]. Abundance of AdipoQ mRNA in different fat depots of the same animals are presented earlier [28]. In our study, the AdipoQ mRNA abundance was not correlated with serum or tissue AdipoQ concentrations similar to earlier studies in humans and animals [58,59]. Factors such as translational regulation, post-transcriptional modification and rate of secretion or degradation might be more important in regulating circulating AdipoQ concentrations than the gene transcription in adipocytes alone.

Under non reducing and non heat-denaturing conditions, almost all of the AdipoQ in AT and serum was present as complexes of hexamers (about 150 kDa), and

higher MW forms (about 300 kDa). The HMW and MMW forms were previously established as the primary forms of circulating AdipoQ in cattle [60], thus confirming the results. The exclusive presence of a HMW form and absence of MMW AdipoQ in bovine serum reported recently [18], might be due to a different antibody, gel system (6% vs 8% SDS-PAGE gel) and/or differences in sample preparation and blotting conditions. For the AdipoQ isoforms in AT, our results are in partial agreement with the finding of Phillips *et al.* [61], who reported that under non reducing conditions almost all of the AdipoQ in human adipocytes is present as complexes consistent with higher MW forms, hexamers and trimers. The similar MW distribution pattern of AdipoQ in tissue preparation and serum in our study indicate little or no interchange between AdipoQ molecular forms in circulation, as reported earlier [62].

In summary, we report that fat depots contained different concentrations and amounts of AdipoQ during early lactation thus implying differential contribution to the circulating AdipoQ concentrations in primiparous dairy cows. In general, vcAT contained more AdipoQ than scAT suggesting a higher impact of vcAT to circulating concentrations. Decreased circulating AdipoQ concentrations reflect the decline of tissue AdipoQ concentrations around parturition which may promote lipolysis and energy supply for the milk production in dairy cows.

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Table 1. Pearson's correlation coefficient (r) between log serum AdipoQ (µg/mL) and AT variables in dairy cows.

AT depot	Variables	log serum AdipoQ	
		r	*P*
vcAT depot			
Mesenterial AT	log tissue AdipoQ, ng/mg protein	**0.541**	**0.006**
	log total tissue AdipoQ, µg	**0.430**	**0.036**
	Tissue mass, kg	**-0.425**	**0.038**
	Adipocyte size, μm^2	-0.253	0.232
	Tissue AdipoQ mRNA	0.275	0.205
Omental AT	log tissue AdipoQ, ng/mg protein	**0.661**	**<0.001**
	log total tissue AdipoQ, µg	**0.572**	**0.003**
	Tissue mass, kg	-0.400	0.053
	Adipocyte size, μm^2	-0.278	0.189
	Tissue AdipoQ mRNA	-0.077	0.726
Retroperitoneal AT	log tissue AdipoQ, ng/mg protein	**0.573**	**0.003**
	log total tissue AdipoQ, µg	0.366	0.079
	Tissue mass, kg	**-0.616**	**0.001**
	Adipocyte size, μm^2	**-0.551**	**0.005**
	Tissue AdipoQ mRNA	0.216	0.311
scAT depot			
Sternum AT	log tissue AdipoQ, ng/mg protein	**0.642**	**0.001**
	Adipocyte size, μm^2	-0.371	0.074
	Tissue AdipoQ mRNA	-0.220	0.301
Tail-head AT	log tissue AdipoQ, ng/mg protein	**0.635**	**0.001**
	Adipocyte size, μm^2	-0.306	0.146
	Tissue AdipoQ mRNA	0.167	0.446
Withers AT	log tissue AdipoQ, ng/mg protein	**0.754**	**<0.001**
	Adipocyte size, μm^2	**-0.529**	**0.008**
	Tissue AdipoQ mRNA	-0.124	0.581
Other body measures			
Total vcAT mass, kg		**-0.513**	**0.010**
Total scAT mass, kg		-0.208	0.328
Total fat mass, kg (vcAT + scAT)		**-0.475**	**0.019**
Live body weight, kg		-0.377	0.069
EBW, kg		**-0.427**	**0.038**
BCS		-0.361	0.083
BFT (cm)		**-0.550**	**0.005**

Abbreviations: AT = adipose tissue, vc = visceral, sc = subcutaneous, Total vcAT = mesenterial AT + omental AT + retroperitoneal AT, EBW = empty body weight, BCS = body condition score, BFT = back fat thickness. Boldface indicates significant associations.

Table 2. Relationships (Pearson's correlation coefficients, r) of adipocyte sizes (μm^2)[a] with log tissue AdipoQ concentrations (ng/g tissue) and RQUICKI in dairy cows.

AT depot	log tissue AdipoQ		RQUICKI	
	r	*P*	r	*P*
Adipocyte size (vcAT depot)				
Mesenterial AT	-0.166	0.427	**-0.442**	**0.031**
Omental AT	**-0.421**	**0.036**	**-0.423**	**0.039**
Retroperitoneal AT	**-0.620**	**0.001**	**-0.564**	**0.004**
Adipocyte size (scAT depot)				
Sternum AT	-0.343	0.093	**-0.621**	**0.001**
Tail-head AT	-0.142	0.499	-0.351	0.093
Withers AT	**-0.645**	**0.001**	**-0.411**	**0.046**

Abbreviations: AT = adipose tissue, vc = visceral, sc = subcutaneous, RQUICKI = Revised Quantitative Insulin Sensitivity Check Index.
[a] Data have been published earlier [26].
Boldface indicates significant associations.

Table 3. Pearson's correlation coefficient (r) between log tissue AdipoQ and log plasma IGF-1 (ng/mL)[a] and log NEFA (µEq/L)[b] concentration in dairy cows.

AT depot	Variables	Plasma IGF-1		Plasma NEFA	
		r	*P*	r	*P*
vcAT					
Mesenterial AT	log tissue AdipoQ, ng/mg protein	0.218	0.295	-0.153	0.467
	log tissue AdipoQ, ng/g tissue	**0.570**	**0.003**	**-0.502**	**0.010**
	log total tissue AdipoQ, µg	**0.548**	**0.005**	**-0.478**	**0.016**
Omental AT	log tissue AdipoQ, ng/mg protein	0.314	0.126	-0.289	0.161
	log tissue AdipoQ, ng/g tissue	0.324	0.115	-0.346	0.090
	log total tissue AdipoQ, µg	0.204	0.328	-0.231	0.266
Retroperitoneal AT	log tissue AdipoQ, ng/mg protein	0.112	0.593	0.304	0.140
	log tissue AdipoQ, ng/g tissue	**0.413**	**0.040**	**-0.524**	**0.007**
	log total tissue AdipoQ, µg	0.284	0.168	**-0.534**	**0.006**
scAT					
Sternum AT	log tissue AdipoQ, ng/mg protein	**0.463**	**0.020**	-0.375	0.065
	log tissue AdipoQ, ng/g tissue	**0.472**	**0.017**	0.379	0.062
Tail-head AT	log tissue AdipoQ, ng/mg protein	0.301	0.143	-0.257	0.215
	log tissue AdipoQ, ng/g tissue	**0.453**	**0.023**	-0.355	0.002
Withers AT	log tissue AdipoQ, ng/mg protein	0.327	0.111	-0.268	0.196
	log tissue AdipoQ, ng/g tissue	0.330	0.107	-0.248	0.232

Abbreviations: AT = adipose tissue, vc = visceral, sc = subcutaneous.
[a] and [b] Data have been published earlier [27] and [23], respectively.
Boldface indicates significant associations.

Table 4. Pearson's correlation coefficient (r) between log tissue AdipoQ concentration (ng/g tissue) and measures of body condition in dairy cows.

log tissue	BW (kg)[a]		BFT (cm)[a]		BCS[a]		EBW (kg)[a]	
AdipoQ	r	*P*	r	*P*	r	*P*	r	*P*
vcAT								
Mesenterial AT	-0.272	0.189	**-0.461**	**0.020**	-0.243	0.243	**-0.442**	**0.027**
Omental AT	-0.315	0.125	**-0.521**	**0.008**	**-0.438**	**0.029**	-0.339	0.098
Retroperitoneal AT	-0.357	0.080	**-0.508**	**0.010**	-0.295	0.152	-0.391	0.053
scAT								
Sternum AT	-0.214	0.304	-0.204	0.329	-0.103	0.626	-0.276	0.182
Tail-head AT	-0.175	0.401	**-0.527**	**0.007**	-0.366	0.072	-0.319	0.121
Withers AT	**-0.521**	**0.008**	**-0.443**	**0.027**	-0.316	0.124	**-0.502**	**0.010**

Abbreviations: AT = adipose tissue, BW = body weight, BFT = back fat thickness, BCS = body condition score, EBW = empty body weight.
[a] Data have been published earlier [23].
Boldface indicates significant associations.

Table 5. Multiple linear regression analyses of the relationship between adipose tissue (AT) depot measures with serum AdipoQ concentration. Parameters identified as significant independent predictors for serum adiponectin in dairy cows are presented.

Predictor variable	Standardized beta coefficient	*P*	Adjusted R^2
Model 1			0.654
log retroperitoneal AdipoQ, ng/g tissue	0.650	<0.001	
log tail-head AdipoQ, ng/g tissue	0.366	0.009	
Model 2			0.608
Retroperitoneal tissue mass, kg	-0.564	<0.001	
log omental total AdipoQ, µg	0.515	0.001	

Note: For model 1 AdipoQ concentrations (ng/mg tissue) in each individual AT (mesenterial, omental, retroperitoneal, sternum, tail-head, withers AT), whereas for model 2 RP tissue mass (kg), RP adipocyte size (μm^2), wither adipocyte size (μm^2), log omental total AdipoQ (µg) were included due to their strong relationship with serum AdipoQ ($r \geq 0.55$).

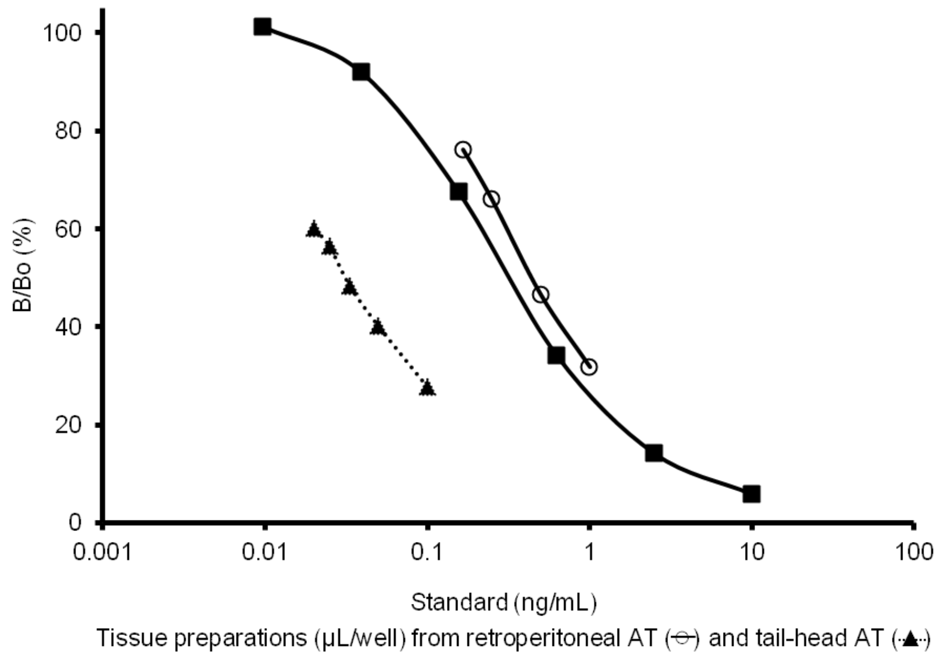

Fig. 1. Representative serial dilution curve for tissue preparations of visceral (retroperitoneal) and subcutaneous (tail-head) AT depots, demonstrating parallelism with the standard curve. B/Bo = Percent of maximum binding.

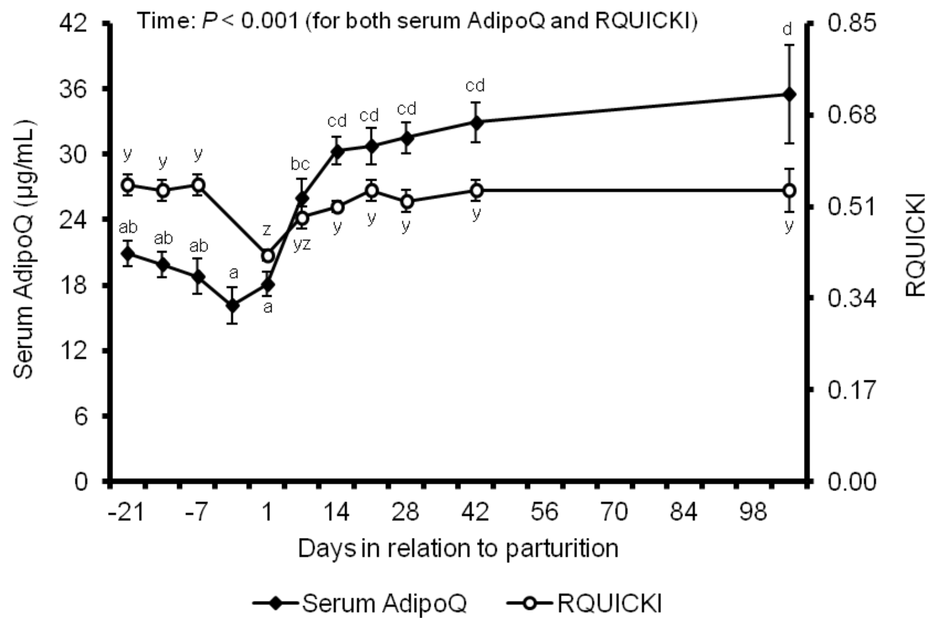

Fig. 2. Time dependent changes in serum adiponectin (AdipoQ) concentrations (µg/ml) and Revised Quantitative Insulin Sensitivity Check Index (RQUICKI). Different letters (a – d for serum AdipoQ; y and z for RQUICKI) indicate significant differences ($P < 0.05$; general linear mixed model) between the means at different time points. Data are presented as means ± S.E.M. In post partum period, serum AdipoQ was significantly correlated with RQUICKI ($r = 0.236$, $P = 0.007$).

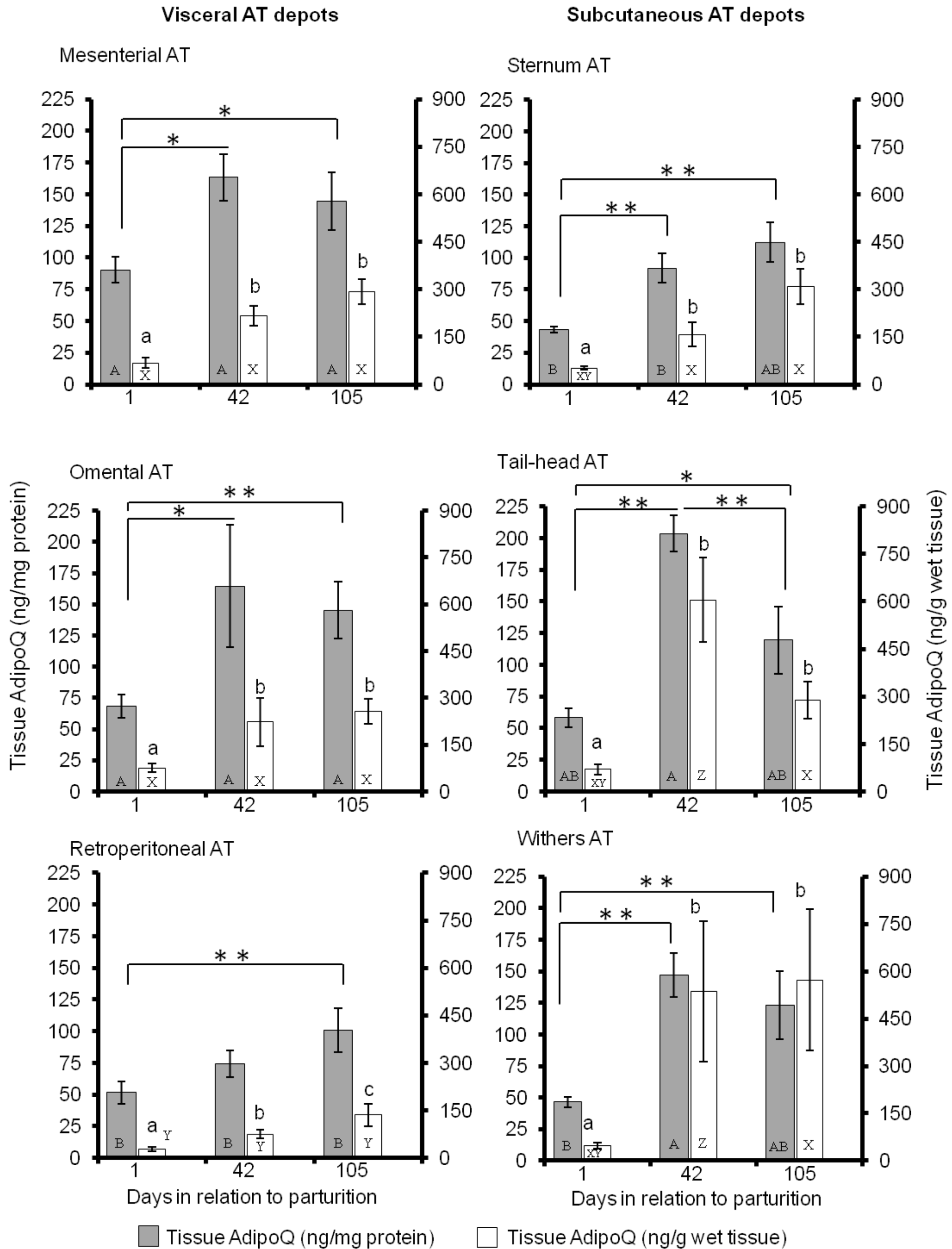

Fig. 3. Tissue adiponectin (AdipoQ) concentration (ng/mg total protein and ng/g wet tissue) in visceral and subcutaneous adipose tissue (AT) depots in different lactation periods i.e. d 1, 42 and 105 after parturition. Significant differences in AdipoQ concentrations within tissue are indicated by asterisks (**P* < 0.05 and ***P* < 0.01; Mann-Whitney *U* test) for ng/mg total protein or different small letters (a – c, *P* < 0.05) for ng/g wet tissue. Capital letters within the bars indicate significant differences in AdipoQ concentrations between different AT depots at d 1, 42 and 105 (A, B, C for ng/mg total protein and X, Y, Z for ng/g wet tissue; *P* < 0.05; Mann-Whitney *U* test). Data are presented as means ± S.E.M.

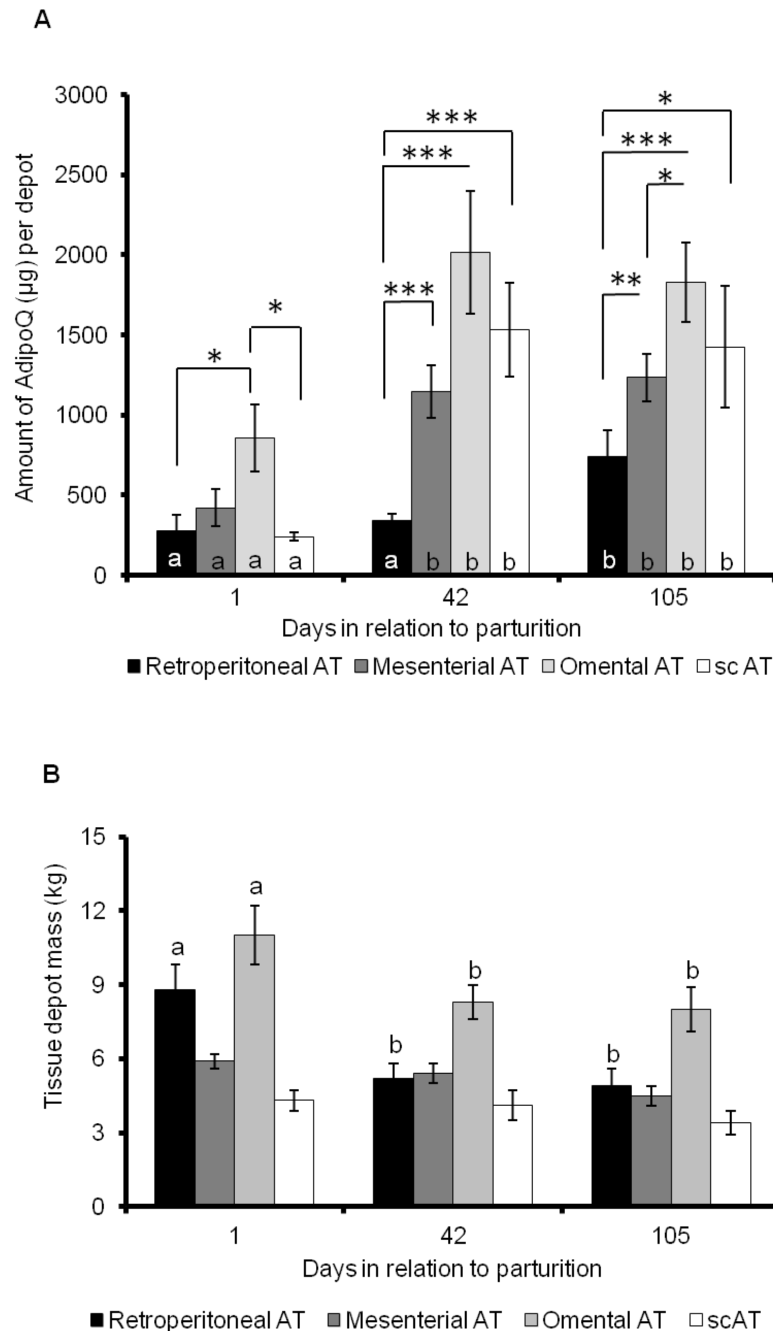

Fig. 4. (A) Amount of adiponectin (AdipoQ, µg) in different visceral (retroperitoneal, mesenterial, omental) and subcutaneous (sc) adipose tissue (AT) depots in different lactation periods i.e. d 1, 42 and 105 after parturition. Asterisks indicate significant differences on day of observation (*$P < 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$; Mann-Whitney *U* test). Different letters within the bars (a, b) designate significant difference between days within depot ($P < 0.05$; Mann-Whitney *U* test); **(B)** Time dependent changes in absolute depot mass (kg) of visceral (mesenterial, omental and retroperitoneal) and subcutaneous (sc) (pooled for sternum, tail-head and withers fat) adipose tissue (AT) depots in different lactation periods i.e. d 1, 42 and 105 after parturition. Different letters (a, b) designate significant difference between days within depot ($P < 0.05$, Mann-Whitney *U* test). Data are presented as means ± S.E.M.

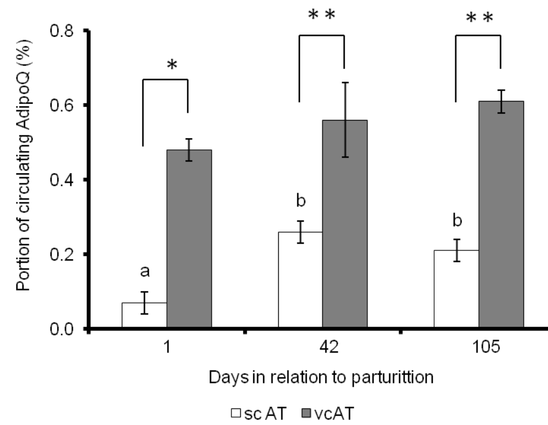

Fig. 5. Portion (%) of circulating adiponectin (AdipoQ) present in visceral (vc) (pooled for mesenterial, omental and retroperitoneal fat depot) and subcutaneous (sc) (pooled for sternum, tail-head and withers fat depot) adipose tissue (AT) depots in different lactation periods i.e. d 1, 42 and 105 after parturition. Estimated amount of AdipoQ in circulation (mg) on d 1, 42 and 105 was 358.2 ± 51.2, 591.8 ± 48.4 and 665.5 ±72.3, respectively. Asterisks indicate significant differences (*$P < 0.05$, **$P \leq 0.01$; Mann-Whitney *U* test). Different letters within the bars (a, b) designate significant difference between days within depot ($P < 0.05$, Mann-Whitney *U* test). Data are presented as means ± S.E.M.

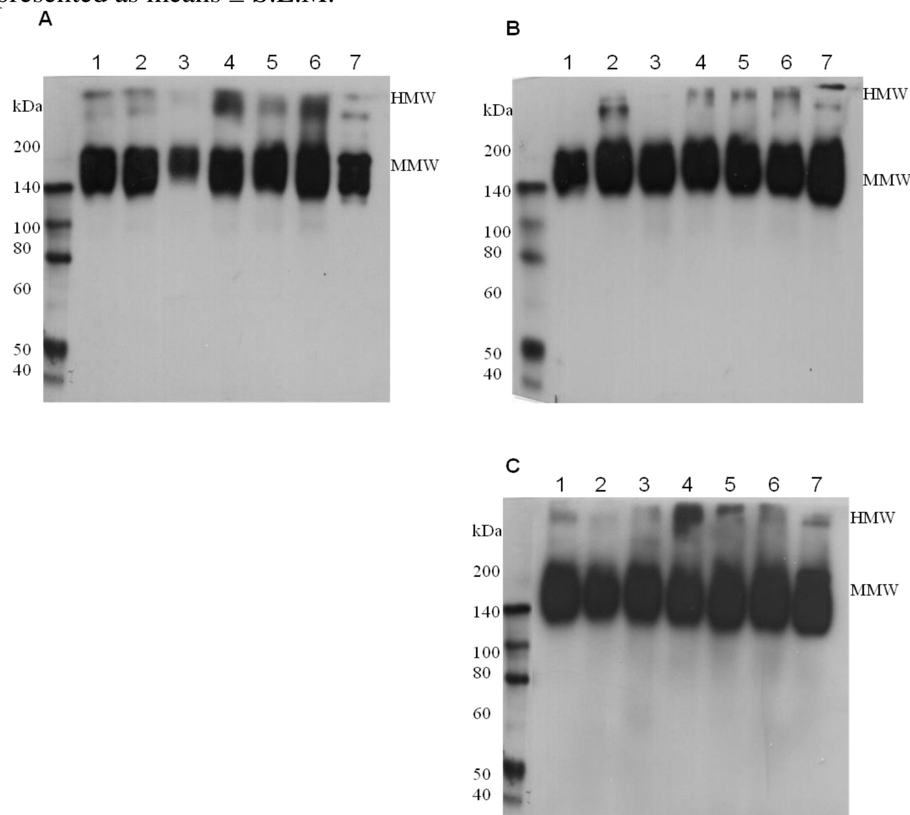

Fig. 6. Exemplary Western blots of AdipoQ multimeric isoforms (HMW, high molecular weight; MMW, middle molecular weight) under non reducing and non heat-denaturing conditions in adipose tissue (AT) extracts and serum at d 1 **(A)**, d 42 **(B)** and d 105 **(C)** of lactation. *Lane1*, mesenterial AT extract; *Lane 2*, omental AT extract; *Lane 3*, retroperitoneal AT extract; *Lane 4*, wither AT extract; *Lane 5*, tail-head AT extract; *Lane 6*, sternum AT extract; *Lane 7*, serum. The band corresponds to the low molecular weight (LMW) isoform (at the level of approximately 70 kDa; [2]) and was not observed in tissue extract or serum at any time point.

4. Manuscript 3 (Accepted; Journal of Dairy Science; doi.org/10.3168/jds.2013-7598)

Short communication: **Circulating and milk adiponectin change differently during energy deficiency at different stages of lactation in dairy cows**

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ABSTRACT

Adiponectin, one of the most abundant adipokines in circulation, is known for its role in regulation of body metabolism. The aim of this study was to evaluate the effects of a negative energy balance (NEB) at 2 stages of lactation (lactational NEB at the onset of lactation and an induced NEB by feed restriction near 100 d of lactation) on circulating adiponectin concentrations. We also investigated the effect of feed restriction on adiponectin concentrations in milk and the relationships of blood and milk adiponectin with selected plasma or milk variables and with measures of body condition. Plasma adiponectin was measured in 50 multiparous Holstein dairy cows throughout 3 experimental periods, i.e. period 1 = 3 wk ante partum up to 12 wk post partum, 2 = 3 wk of feed restriction started at around 100 days in milk with a control (n

= 25) and feed restricted group (50% of energy requirements; n = 25), and 3 = subsequent realimentation period for 8 wk. Milk adiponectin was investigated among twenty one multiparous cows at wk 2, wk 12 of period 1 and wk 2 of period 2. Adiponectin concentrations in plasma and skim milk were measured using an in-house ELISA specific for bovine adiponectin. Major changes in circulating adiponectin concentrations were observed during the periparturient period whereas energy deficiency during established lactation at around 100 days in milk and subsequent refeeding did not affect plasma adiponectin. At the second wk of feed restriction, adiponectin secretion via milk was reduced (mg/d; $P < 0.01$) and a trend for lower adiponectin concentrations in milk was observed (µg/mL; $P = 0.087$). Mean adiponectin concentrations in milk (0.61 ± 0.03 µg/mL) were about 52-fold lower ($P < 0.001$) than the mean plasma adiponectin concentrations (32.1 ± 1.0 µg/mL). The share of adiponectin from blood that is secreted via milk per day was estimated as 2.7%. In view of the nearly similar extent of NEB in both periods of energy deficiency, decreasing adiponectin concentrations seem important for accomplishing the adaptation to the rapidly increasing metabolic rates in early lactation whereas the lipolytic reaction towards feed restriction-induced NEB during established lactation seems to occur largely independent of changes in circulating adiponectin.

Key words: adiponectin, milk, energy deficiency, dairy cow

Short Communication

With the onset of lactation in high producing dairy cows, milk synthesis leads to an approximately 4-fold increase in total energy requirements compared to pregnant and non lactating state (Block et al., 2001), which is often accompanied by insufficient voluntary feed intake. The period of negative energy balance (**NEB**) is associated with changes of several metabolic key hormones such as growth hormone (**GH**), IGF-1 and the responsiveness of peripheral tissues for these hormones (Rhoads et al., 2007). The period of energy demand such as early lactation in dairy cattle is characterized by an increase in lipolysis and a decrease in lipogenesis (McNamara and Hillers, 1986). During lipolysis, fatty acids in the form of non-esterified fatty acids (**NEFA**) are released from adipose tissue (**AT**) and serve as a major energy substrate for body tissues.

Adipose tissue is not only an energy storage organ, but also synthesizes and secretes a wide range of metabolically active hormones, proteins and other bioactive molecules, collectively termed as adipokines. Changes in metabolic activities of AT during the period of NEB may affect the expression of adipokines and vice versa e.g. energy deficiency in periparturient cows reduce leptin gene expression and its plasma concentrations (Block et al., 2001).

Produced mainly by AT, adiponectin one of the most abundant adipokines in circulation is known to be involved in regulation of glucose and fatty acid metabolism (Yamauchi et al., 2002). Studies about the concentration of adiponectin in bovine body fluids have been impeded by the lack of inexpensive, straightforward and reliable assays for bovine adiponectin. Using semi quantitative Western immune blotting the circulating adiponectin concentrations in dairy cows were recently characterized and were shown to be lowest during the periparturient period (Giesy et al., 2012, Mielenz et al., 2013). Using an ELISA system we recently developed specifically for bovine adiponectin, this time course was confirmed (Mielenz et al., 2013) and supports that adiponectin participates in coordinating the metabolic changes during the period of NEB in early lactation.

In rats, short-term (48 h) dietary fasting (no feed) decreased and refeeding increased adiponectin gene expression in perirenal AT (Zhang et al., 2002) as well as in subcutaneous and epididymal AT (Bertile and Raclot, 2004). However, the influence of feed restriction or complete feed deprivation on circulating adiponectin remains obscure since previous rodent studies have reported contradictory findings with unchanged (after 48 h of fasting) (Zhang et al., 2002), decreased (after 8 h of fasting and remain constant thereafter until 48 h) (Gui et al., 2003), or increased (after feed restriction of 60% of the baseline intake) (Combs et al., 2003) adiponectin blood concentrations.

Changes in milk concentration of hormones and metabolites might reflect variations in their blood concentrations. Earlier reports suggest that the induced NEB by feed restriction in dairy cows alters milk yield and its composition such as fat and protein percentage (Gross et al., 2011a) or BHBA and citrate concentration (Nielsen et al., 2003). The objectives of the present study were to investigate the effect of lactational versus feed restriction induced NEB on plasma and milk adiponectin concentrations and their relationship with circulating concentrations of metabolic hormones such as leptin, GH, IGF-1, insulin, triiodothyronine, thyroxin; variables

linked to the fat metabolism such as NEFA, BHBA, total cholesterol, triglyceride and other variables such as urea, Revised Quantitative Insulin Sensitivity Check Index (**RQUICKI**), DMI, BW, BCS, back fat thickness (**BFT**), longissimus dorsi muscle diameter (**MD**) and energy balance.

The animal trial was conducted at the Agricultural Experimental Unit Hirschau of the Technical University Munich, Germany, and was approved by the state department for animal welfare affairs. The animal experiment and feeding regimen was described in detail previously (Gross et al., 2011a). Briefly, the study included 50 multiparous Holstein dairy cows (3.2 ± 0.2 lactations, mean ± SEM) from wk 3 ante partum (**a.p.**) to approximately wk 26 post partum (**p.p.**). Period 1 was from wk 3 a.p. till wk 12 p.p., where all animals were treated as one group. The restriction phase (period 2) started at 98 ± 7 DIM and lasted for 3 wk. In this period animals were allocated equally to either a control group (**C**, n = 25) or a restriction group (**R**, n = 25). The wk before the start of the feed restriction was considered as wk 0, when all animals were treated as one group. Calculations for dietary requirement of energy and protein followed the recommendations of the Society of Nutrition Physiology (GfE, 2001). At the start of period 2, an energy deficiency of approximately 50% of cow's requirements was induced by feeding R cows a diet with a lower energy content (6.24 vs 6.53 MJ of NE_L/kg of DM) compared to the C cows and decreased amount of concentrate (0.4 kg DM/d; whereas C cows received concentrate according to the individual requirements for milk production). After 3 wk of feed restriction, period 3 (8 wk duration) started during which R cows were (re)fed to the level of the C cows (realimentation period).

Blood samples (n = 50) were collected weekly via jugular vein puncture in K_3EDTA-coated (18 mL) evacuated blood collection tubes (Greiner, Frickenhausen, Germany). Plasma samples were obtained by centrifugation (2,000 × *g*, 15 min) and stored at -20 °C until analyzed. Cows were milked twice daily at 0500 and 1500 h and daily milk yield was recorded electronically. Milk samples (n = 21) at 2 consecutive milkings were collected at wk 2, wk 12 (period 1) and wk 2 of the energy restriction period (period 2) for adiponectin analysis. Milk samples were vortexed properly to ensure sample uniformity before skimmed by centrifugation (3,000 × *g*, 30 min, 4 °C). Skim milk was harvested between the supernatant (fat layer) and the pellet (debris and cells) and stored at -20 °C until analysis.

Plasma and skim milk samples were analyzed for adiponectin using an in-house competitive ELISA as described in detail earlier (Mielenz et al., 2013). Before measurement of milk adiponectin, assay accuracy was confirmed by linearity and parallelism of diluted milk samples. The measuring range of the assay was 0.07-1.00 ng/mL and the limit of detection was 0.03 ng/mL. The intra- and inter-assay coefficients of variations for skim milk were 5% and 12%, respectively.

Enzymatic kits from bioMérieux SA, Geneva, Switzerland were used for measurements of total cholesterol (no. 61219), triglycerides (no. 61236) and urea (no. 61974) in plasma. The profiles of other variables such as NEFA, BHBA, DMI, BW, EB, BCS, BFT, MD, milk yield (Gross et al., 2011a); and RQUICKI, insulin, IGF-1, GH, leptin, triiodothyronine, thyroxin (Gross et al., 2011b) have been published elsewhere. In this paper the values were used to calculate the potential association with plasma and milk adiponectin concentrations.

Adiponectin secretion (mg/d) via milk was calculated by multiplying the milk adiponectin concentration (mg/L) with the milk yield (L/d). In addition, assuming that all milk adiponectin is derived from the circulation, the following calculations were done to estimate the portion of circulating adiponectin (%) secreted via milk per day:

- Total blood volume of a cow (mL) = Body weight (kg) × 57 (Hansard et al., 1953)
- Plasma volume (mL) = Blood volume (mL) × 0.658 (Jones et al., 1956)
- Amount of adiponectin present in circulation (mg) = Plasma adiponectin concentration (mg/mL) × plasma volume (mL)
- Amount of adiponectin secreted via milk (mg) = Milk adiponectin concentration (mg/L) × milk yield (L)

Portion of blood adiponectin secreted via milk (%)

$$= \frac{\text{Amount of adiponectin secreted via milk (mg)}}{\text{Amount of adiponectin present in circulation (mg)}} \times 100$$

All statistical analyses were performed using SPSS (version 19.0, SPSS Inc., Chicago, IL). Data were tested for normal distribution using the Shapiro-Wilk test and homogeneity of variances was tested using Levene's test. One-way repeated measures ANOVA followed by Bonferroni post hoc test was conducted for multiple comparisons of plasma adiponectin concentrations at different time points during period 1. During

period 2 and 3, plasma adiponectin concentrations between C and R groups were compared with the linear mixed model procedure followed by Bonferroni correction. The model included individual animal as a repeated subject, and wk, treatment and their interaction as fixed effects. Milk adiponectin concentrations, adiponectin secretion and portion of plasma adiponectin secreted per d in period 1 and period 2 were compared by student's *t*-test. Pearson correlation coefficient was calculated for statistical analysis of relationship among variables. All data are presented as means ± SEM, significance was declared for P-values < 0.05 and a trend was noted when $P \leq 0.10$.

Time dependent changes in plasma adiponectin concentrations during different experimental periods are shown in Figure 1. Plasma adiponectin decreased from wk 3 a.p. and reached lowest concentrations at the time of parturition; thereafter the concentration increased gradually and approached a.p. values (3 wk a.p.) within 3 wk of lactation. The time and treatment dependent changes in milk adiponectin concentration and daily milk adiponectin secretion are presented in Figure 2.

When normalized to the amount of milk protein content, the mean milk adiponectin concentration was 18.5 ± 0.9 ng/mg milk protein. Irrespective of time and treatment, milk adiponectin represented about 0.002% of total milk protein. The mean adiponectin concentration in milk (0.6 ± 0.03 μg/mL) was about 52-fold lower than the mean plasma adiponectin concentration (32.1 ± 1.0 μg/mL). Considering all time points (wk 2, wk 12 of period 1 and wk 2 of period 2), 2.7% of circulating adiponectin were secreted through milk per day. The portions of circulating adiponectin secreted through milk in different periods are presented in Figure 3. Neither the plasma nor the milk adiponectin concentration was correlated with the milk yield. No significant association of plasma and milk adiponectin with plasma leptin and GH was observed. The coefficients of correlation between plasma and milk adiponectin concentrations and adiponectin secretion with plasma, milk and other variables are presented in Table 1 and Table 2.

The transition from late gestation to lactation is a period of comprehensive physiological and metabolic adaptations to accomplish the nutrient demand of the mammary gland for milk production. Adiponectin is known to be involved in regulating glucose and fatty acid metabolism (Yamauchi et al., 2002), therefore, adiponectin concentrations are likely to change during this period of NEB. We observed decreased plasma adiponectin concentrations from 3 wk a.p. until parturition and subsequently

gradually increased concentrations rebounding over the next 4 wk of lactation. Similar characteristic patterns of peripartal changes of circulating adiponectin were recently characterized in dairy cows using semiquantitative Western immunoblotting (Giesy et al., 2012, Mielenz et al., 2013) and the ELISA used herein (Mielenz et al., 2013). Ruminants largely depend on gluconeogenesis for their glucose requirements, therefore, efficient gluconeogenesis in dairy cows is the major pathway for maintaining adequate glucose supply for the mammary gland (Reynolds et al., 1988). Adiponectin increases glucose uptake and lactate production in skeletal muscle and reduces the expression of molecules involved in gluconeogenesis in liver (Yamauchi et al., 2002), thus, suppresses hepatic glucose production (Zhou et al., 2005). Furthermore, adiponectin is known to improve insulin sensitivity (Yamauchi et al., 2001). Together with decreasing insulin sensitivity in peripheral tissue, decreasing adiponectin concentrations may support partitioning of glucose towards mammary gland. Moreover, decreasing adiponectin concentrations might increase hepatic gluconeogenesis to improve glucose supply to the mammary gland for milk production. The precise functional importance of the periparturient reduction of circulating adiponectin in dairy cows remains to be characterized.

We did not observe significant effects of feed restriction and subsequent realimentation on plasma adiponectin concentrations even though the extent of NEB was lower than that observed at the time of initiation of lactation (Gross et al., 2011a). The unchanged plasma adiponectin in R cows compared to the C cows indicates that its circulating concentration is not affected by energy balance. The finding of unchanged adiponectin concentrations during feed restriction agrees with results of energy restriction (4 d) study in human (Imbeault et al., 2004) or total feed deprivation studies in rodents (48 h fasting) (Combs et al., 2003, Kmiec et al., 2005) and humans (48-72 h fasting) (Gavrila et al., 2003, Merl et al., 2005). However, another report suggests that adiponectin concentration is decreased in mice after 8 h of fasting (Gui et al., 2003). The discrepancy in these results might be due to age, physiological state, extent and duration of fasting and/or species differences.

From wk 2 to wk 12 p.p., milk adiponectin concentrations and adiponectin secretion declined 2-fold and 1.8-fold, respectively. In contrast, earlier human studies reported either unchanged (Bronsky et al., 2011) or increased milk adiponectin concentrations during the first 180 days after birth (Ozarda et al., 2012). The milk yield

of the animals in the present study was similar at wk 2 and wk 12 (Gross et al., 2011a) therefore, the observed decline in adiponectin secretion in period 1 resulted from decreased milk adiponectin concentration. In period 2, we observed a trend towards reduced adiponectin concentrations in milk in the R group compared to the C group. In addition, milk yield was significantly reduced in the R group during this period (Gross et al., 2011a) therefore, the reduction of both milk adiponectin concentration and milk yield lead to decreased adiponectin secretion in R cows.

Rate of synthesis and clearance determine the blood concentration of hormones and metabolites. Adiponectin secretion rate from human adipocytes play only a minor role in variation of its blood concentration (Hoffstedt et al., 2004). Moreover, liver mediated clearance rate of adiponectin in mice is relatively stable (half-life ~ 75 min) and did not affect by 24 h fasting (Halberg et al., 2009). Therefore, decline in adiponectin secretion through milk during the period of feed restriction might provide an additional mechanism to prevent significant fall in its plasma concentration during this period. Compared to the concentration of leptin in milk that are around 6 ng/mL (Pinotti and Rosi, 2006), the mean milk adiponectin concentration observed herein (616.4 ± 32.6 ng/mL) were about 100-fold higher, suggesting that similar to the blood concentrations, adiponectin in milk is present in much higher concentration then other adipokines.

In humans and in animals such as horses, adiponectin mRNA expression and serum concentrations are decreased with an increased portion of body fat (Kearns et al., 2006, Kern et al., 2003). In dairy cows, an increase in fat mass could be expected with improvement in the body condition measures such as BW, BCS, and BFT. This might explain the observed negative correlations of plasma adiponectin with measures of body condition (BW, BCS, BFT, MD) in the present study. The positive correlation between plasma adiponectin and insulin concentrations might indicate that the adiponectin secretion by adipocytes is stimulated by insulin (Blümer et al., 2008). Moreover, inhibitory effects of adiponectin on lipolysis (Qiao et al., 2011) and/or stimulatory effects on fatty acid oxidation in liver and muscles (Yamauchi et al., 2002) could explain the inverse association between adiponectin and NEFA observed in the current study. Adiponectin might increase DMI via stimulating the hypothalamic AMP-activated protein kinase as reported in mice (Kubota et al., 2007). However, the precise role of adiponectin in regulation of feed intake in dairy cows warrants further

investigation. The observation that plasma adiponectin and leptin were not correlated in the present study supports the notion that they independently regulate biological functions in the body such as insulin sensitivity (Koebnick et al., 2008).

To summarize, our data showed that the most prominent changes in plasma adiponectin occur around parturition. Feed restriction and subsequent realimentation did not affect its circulating concentrations; therefore, we assume that energy balance is affecting plasma adiponectin only in case of the initial adaptations to the rapidly increasing metabolic rates in early lactation. Feed restriction resulted in reduced adiponectin secretion via milk and a tended to decrease milk adiponectin concentrations. The putative biological significance of milk adiponectin and its relationship with other milk hormones and metabolites still remains to be characterized.

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Table 1. Pearson correlation coefficients between plasma adiponectin (µg/mL) and plasma, milk and other variables (n = 50)

Variables	Period 1			Period 2		Period 3	Entire duration
	Before calving	After calving	Entire period 1	C Group	R Group		
Plasma variables							
NEFA (mmol/L)§		-0.225***	-0.148***	0.227*			-0.190***
BHBA (mmol/L)§	-0.315***						
Insulin (µU/mL)‡		0.219***	0.208***	0.365***			0.226***
Milk variables#§							
Milk adiponectin (ng/mL)			-0.360*	0.542†			
Milk fat (%)			-0.290†		-0.777**		-0.283*
Milk protein (%)			-0.366*				-0.355**
Other variables							
RQUICKI‡		0.111†					
DMI (kg/d)§		0.313***	0.351***		0.277*		0.185***
BW (kg)§	-0.17*		-0.185***		-0.258*		-0.152***
BCS§	-0.395***	-0.357***	-0.402***		-0.299**	-0.313***	-0.374***
BFT (mm)§	-0.149†	-0.284***	-0.277***	-0.263*	-0.248*	-0.301***	-0.253***
EB (MJ of NE_L/d)§		0.253***		0.208**	0.389***		

Period 1 = 3 wk before calving until wk 12 of lactation; Period 2 = period of energy restriction (wk 15 until wk 17 of lactation); Period 3 = period of realimentation (wk 18 until wk 25 of lactation); Before calving = 3 wk before calving until calving; After calving = calving until wk 12 of lactation; Entire duration = 3 wk before calving until end of experiment

C = control; R = energy restriction; RQUICKI = revised quantitative insulin sensitivity check index; TG = triglyceride; DMI = dry matter intake; BW = body weight; EB = energy balance; BCS = body condition score; BFT = back fat thickness

#All data at wk 2, 12 of period 1 and wk 2 of period 2 (n = 21) were used for analysis

Correlation coefficients > 0.1 with significant and trend associations are presented. †$P \leq 0.10$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

Data have been presented earlier §Gross et al. (2011a); ‡Gross et al. (2011b)

Table 2. Pearson correlation coefficients of milk adiponectin concentration (µg/mL) and milk adiponectin secretion via milk (mg/d) with milk, plasma and other variables

Variables	Milk Adiponectin	Adiponectin secretion
Milk variables[§]		
Milk yield (kg)		0.478***
Milk fat (%)	0.466***	0.421***
Milk protein (%)	0.322**	
Plasma and other variables		
Leptin (ng/mL)[‡]	-0.399**	-0.282*
NEFA (mmol/L)[§]	0.613***	0.683***
BHBA (mmol/L)[§]	0.327*	0.404**
Glucose (mmol/L)[§]	-0.493***	-0.470***
RQUICKI[‡]	-0.431***	-0.462***
BW (kg)[§]		0.422***
BCS[§]	0.283*	0.397***
BFT (mm)[§]		0.343***
EB (MJ of NE_L/d)[§]	-0.304**	-0.354**

Data at wk 2, wk 12 of period 1 and wk 2 of period 2 were used for analysis (n = 21)
Correlation coefficients > 0.1 with significant associations are presented. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$
RQUICKI = revised quantitative insulin sensitivity check index; BW = body weight; BCS = body condition score; BFT = back fat thickness; EB = energy balance
Data have been presented earlier §Gross et al. (2011a); ‡Gross et al. (2011b)

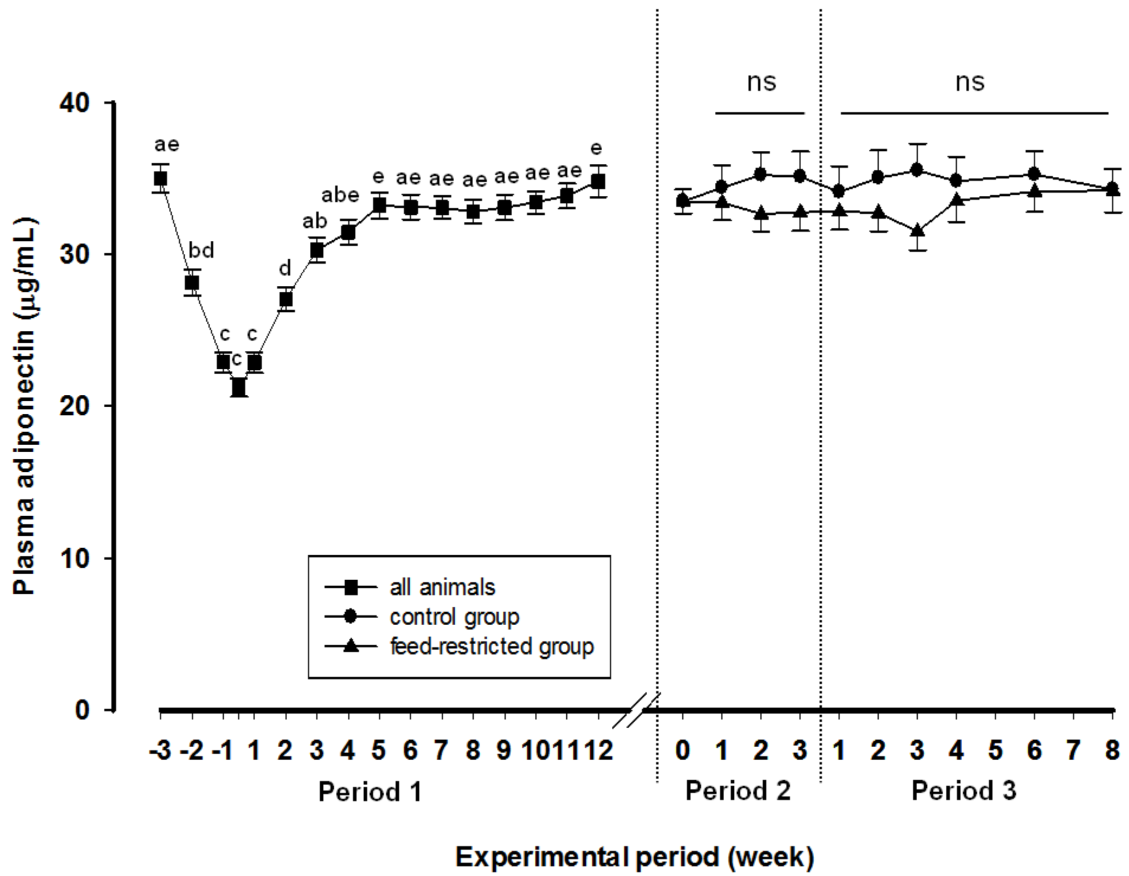

Figure 1. Plasma adiponectin concentration (µg/mL) in cows (n = 50) during the experimental period 1 (from wk 3 ante partum up to wk 12 post partum), period 2 (3 wk of feed restriction, started at about 100 DIM), and period 3 (8 wk of realimentation). Data are given as mean ± SEM. Significant differences ($P < 0.05$) between the time points during period 1 are presented as lower case (one-way repeated measure ANOVA followed by Bonferroni post hoc test). ns indicate no significant differences (linear mixed model) between the control (n = 25) and feed-restricted (n = 25) groups.

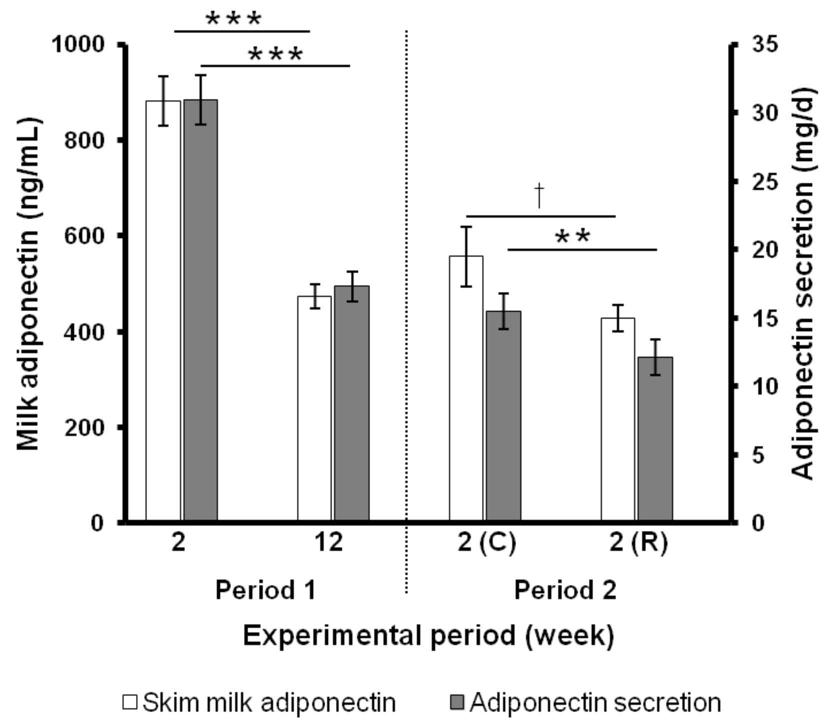

Figure 2. Skim milk adiponectin concentration (ng/mL) and adiponectin secretion via milk (mg/d) at wk 2 and wk 12 of period 1 (n = 21) and control (C, n = 11) and feed-restricted (R, n = 10) groups at wk 2 of period 2. Data are given as mean ± SEM. Significant differences are presented as *** $P < 0.001$; ** $P < 0.01$ and trend as † $P < 0.10$ (Student's *t*-test).

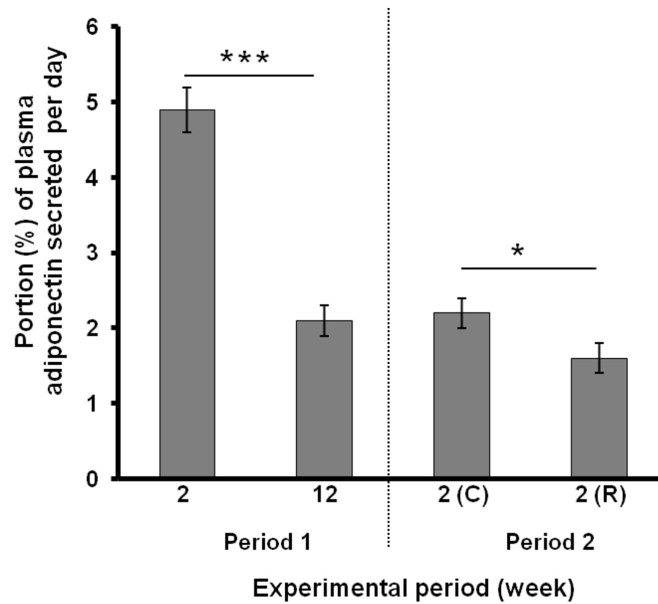

Figure 3. Portion of plasma adiponectin (%) secreted via milk per d at wk 2 and wk 12 of period 1 (n = 21) and control (C, n = 11) and feed-restricted (R, n = 10) groups at wk 2 of period 2. Data are given as mean ± SEM. Significant differences are presented as *** $P < 0.001$; * $P < 0.05$ (Student's *t*-test).

5. General discussion and future research prospectives

Circulating adiponectin profile during periparturient period and lactation in dairy cows

The present study was carried out to characterize circulating adiponectin concentrations around parturition and during almost the entire lactation (d -21 to d 252 relative to calving) in primiparous (PP) and multiparous (MP) cows using an in-house developed ELISA specific for bovine adiponectin (Mielenz et al., 2013). Most of the changes in circulating adiponectin were observed around calving with a characteristic pattern of progressive decline from d 21 pre partum, lowest concentrations at the time of calving and thereafter a gradual increase. In humans, the blood adiponectin concentration is slightly declining as pregnancy advances, reaching the lowest concentrations after delivery (Asai-Sato et al., 2006). The precise underlying mechanisms for the decline in adiponectin concentrations around parturition are not known so far. However, endocrine changes around parturition might affect this variation since *in vitro* adiponectin expression and secretion is suppressed by hormones associated with calving such as prolactin, growth hormone and glucocorticoids (Fasshauer et al., 2002, Nilsson et al., 2005).

Effect of dietary CLA supplementation on circulating adiponectin

In the present thesis a fat supplement containing 12% each of the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 CLA isomers was shown to reduce the post partum circulating adiponectin concentrations in both PP and MP dairy cows. Decreasing (Poirier et al., 2005) as well as increasing (Martins et al., 2010) effects of CLA on adiponectin expression are reported in rodents. *In vivo* and *in vitro* studies suggest that PPARγ ligands such as thiazolidinediones and rosiglitazone increase expression and plasma concentrations of adiponectin (Iwaki et al., 2003, Maeda et al., 2001, Motoshima et al., 2002). Incubation of cells such as primary rat adipocytes, human preadipocytes or 3T3-L1 cells with the *trans*-10, *cis*-12 CLA isomer decrease PPARγ gene expression (Granlund et al., 2003, Kang et al., 2003, Perez-Matute et al., 2007). Therefore, it is suggested that the inhibitory effect of CLA on adiponectin expression is probably mediated through the suppression of PPARγ (Perez-Matute et al., 2007).

Adiponectin concentrations in adipose tissue depots

Our results indicate that irrespective of the anatomical site, the adiponectin concentration was significantly lower in all sc (tail-head, sternum and withers) and vc (mesenterial, omental and retroperitoneal) fat depots at the time of parturition compared to early lactation. This change is likely to be the determinant for the lowest adiponectin concentration in blood at the time of calving. Visceral AT contained higher amounts of adiponectin than scAT, proposing a relatively higher contribution of vcAT on blood adiponectin concentrations. Similarly, adiponectin expression is significantly higher in vcAT than in scAT in lean rats suggesting that vc fat is the predominant contributor to plasma adiponectin (Altomonte et al., 2003). However, retroperitoneal AT, a type of vcAT depot, contained the lowest concentration and lowest total amount of adiponectin compared to the other vc and sc fat depots in the present study. A significant reduction in adipocyte size during early lactation was observed only in retroperitoneal AT (Akter et al., 2011) and when calculated as a portion of empty body weight (%), a decline was observed only in this depot (von Soosten et al., 2011). Protein expression of hormone-sensitive lipase (rate-limiting enzyme in the lipolytic pathway) and the extent of its phosphorylation were higher in retroperitoneal AT compared to the sc AT of dairy cows during early lactation (Locher et al., 2011), indicating a higher lipolytic activity in this depot. Based on these features of the retroperitoneal AT, it seems that in dairy cows, retroperitoneal AT plays an unique role and participates more actively in lipid mobilization compared to other fat depots.

Relationship of adiponectin with insulin sensitivity

In the present research we observed nearly similar patterns of time dependent periparturient changes in serum adiponectin and IS (estimated by the Revised Quantitative Insulin Sensitivity Check Index). In addition, a positive correlation between these variables during the post parturient period was observed, suggesting an *in vivo* effect of adiponectin on IS in dairy cows. Decreasing blood adiponectin concentrations around parturition thus might result in decreased IS of peripheral tissues (Yamauchi et al., 2001), decreased glucose uptake by skeletal muscles and increased hepatic gluconeogenesis (Yamauchi et al., 2002). Therefore, lower blood adiponectin

concentration around calving might improve supply of glucose to the mammary gland for enhanced milk production during early lactation.

Negative energy balance and adiponectin concentration

No effect of induced NEB caused by feed restriction during established lactation (about 100 DIM) and subsequent realimentation was observed on plasma adiponectin concentration, even though the extent of NEB due to feed restriction (-62.7 ± 2.0 MJ of NE_L/d) was higher compared to the immediate post partum period (-46.1 ± 3.4 MJ of NE_L/d) (Gross et al., 2011). However, after calving blood adiponectin concentrations and energy balance were at their lowest values, increased gradually during early lactation and were positively correlated during post partum period. The differential effects of NEB on circulating adiponectin concentrations at the 2 stages of lactation show that decline in blood adiponectin concentrations around parturition in dairy cows are not only due to the energy deficiency, but mainly due to the specific metabolic regulations during this period.

Future research prospectives

Precise mechanisms that regulate changes in the circulating adiponectin around parturition and its physiological importance in cattle remain to be characterized. The putative biological significance of milk adiponectin in the growth, development and health status of the offspring remain to be identified. Identification of blood adiponectin as a diagnostic marker for the periparturient metabolic disorders may represent important consideration in future studies.

6. Summary

The transition from late pregnancy to lactation in dairy cows is characterized by marked changes in energy balance, and the metabolic and endocrine status of the animal. Adipose tissue (AT) plays an important role in regulating metabolism and other body functions such as immunity and reproduction through its secretion of adipokines. Adipose tissue depots at different anatomical locations [subcutaneous (sc) and visceral (vc) AT] are distinct in their structural and functional properties; therefore, they may respond in divergent ways to physiological changes such as the onset of lactation. Adiponectin is one of the most abundant adipokines and is linked with several physiological functions such as insulin sensitivity, immunity and inflammation. Conjugated linoleic acids (CLA) might affect adiponectin expression and secretion, as known from cell culture and rodent studies. In dairy cows, CLA feed supplements are used to decrease milk fat thus potentially alleviating the output of energy with milk during the first weeks of lactation when voluntary feed intake is mostly not covering the needs for maintenance and milk production; albeit the effect on mammary lipogenesis is well characterized, potential effects on body fat, in particular adiponectin secretion, were not known. Therefore, the experiments conducted herein aimed 1) to characterize the adiponectin concentrations in blood and different sc and vc AT depots from dairy cows during different stages of lactation, 2) to test the effect of CLA supplementation on blood and tissue adiponectin concentrations, and 3) to evaluate the effect of lactational versus dietary induced negative energy balance (NEB) on blood and milk adiponectin concentrations.

In two different CLA trials, German Holstein cows were allocated to either a CLA group (100 g/d of a fat supplement containing 12% each of the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 isomer) or a control group (100 g/d of a control fat in which CLA was substituted by stearic acid). In trial 1, both multiparous (MP, n = 22) and primiparous (PP, n = 11) cows received CLA (11 MP, 5 PP) or the control fat (11 MP, 6 PP) from d 1 to d 182 of lactation; blood samples as well as biopsy samples from tail-head scAT were collected from MP and PP cows from d -21 to d 252 relative to calving. In trial 2, PP cows (n = 25) were accordingly treated and AT samples from different locations [3 vcAT depots (omental, mesenterial, and retroperitoneal) and 3 scAT depots (sternum, tail-head, and withers)] and blood samples were collected at slaughter on days 1 (n = 5), 42 (n = 10, 5 per group) and 105 (n = 10, 5 per group) of lactation. In a third

trial, the effects of NEB at 2 stages in lactation (lactational, "natural" NEB at the onset of lactation versus feed restriction-induced NEB in established lactation at ~ 100 d in milk) on circulating and milk adiponectin concentration were studied. The experiment included MP Holstein dairy cows (n = 50) and the entire duration was divided into 3 periods, i.e. period 1 = 3 wk ante partum until 12 wk post partum, period 2 = 3 wk of feed restriction starting at around 100 days in milk with a control (n = 25) and a feed restricted group (70% of energy requirements; n = 25), and 3 = realimentation period for 8 wk. Plasma adiponectin was measured at different time points throughout the experiment. Milk adiponectin was investigated in a subset of 21 cows at wk 2, wk 12 of period 1 and wk 2 of period 2. Adiponectin concentrations in samples collected in all experiments were measured using an in-house developed ELISA that is the first assay which allows for valid quantification of bovine adiponectin suitable for large sample series.

We herein demonstrated that major changes in blood adiponectin concentration appear during the periparturient period with a gradual decline from d 21 ante partum to lowest levels at the time of calving and subsequent post partum gradual increase. Supplementation with a 1:1 mixture of *cis*-9, *trans*-10 and *trans*-10, *cis*-12 CLA caused a reduction in post partum circulating adiponectin in both PP and MP dairy cows. A carry over effect of CLA beyond the time of supplementation was limited to MP cows. Nearly similar patterns of periparturient changes in serum adiponectin and IS (estimated by the Revised Quantitative Insulin Sensitivity Check Index) were observed. This observation and the result of positive associations between these variables during the post parturient period suggest *in vivo* effects of adiponectin on IS in dairy cows.

Irrespective of the anatomical site, adiponectin concentrations in all fat depots were lowest at the time of parturition and increased during early lactation. The decline in circulating adiponectin around parturition thus likely results from reduced expression of the adiponectin protein in all fat depots. The finding that vcAT contained more adiponectin than scAT suggests a relatively higher contribution of vcAT to the blood concentrations. However, retroperitoneal AT as one of the vc depots contained the lowest adiponectin concentration and also, when related to depot mass, the lowest total amount of adiponectin compared to the other fat depots. No effect of induced NEB due to feed restriction and subsequent re-feeding was observed on plasma adiponectin concentrations, suggesting that circulating adiponectin is not affected by energy balance

during established lactation near 100 d of lactation. Besides providing information about longitudinal changes of blood adiponectin concentrations and the effect of CLA, the present thesis contributes to improving the knowledge about differential expression of adiponectin in various AT depots and changes in blood concentrations in dairy cows. Moreover, the dissertation serves as a basis for further studies exploring the role of adiponectin in various pathophysiological conditions in cattle and, potentially also in other livestock species. In future, the specific mechanisms for regulating the periparturient changes in circulating adiponectin concentrations and its physiological importance remain to be elucidated.

7. Zusammenfassung

Die Übergangsphase von der späten Trächtigkeit zur frühen Laktation ist bei Milchkühen von ausgeprägten Veränderungen der Energiebilanz sowie des metabolischen und endokrinen Status gekennzeichnet. Das Fettgewebe spielt durch die Sekretion von Adipokinen eine wichtige Rolle in der Regulation des Stoffwechsels und anderer Körperfunktionen wie beispielsweise Immunität und Reproduktion. Fettgewebsdepots unterschiedlicher anatomischer Lokalisation [subkutan (sc) und viszeral (vc)] verfügen über unterschiedliche strukturelle und funktionelle Eigenschaften. Dies könnte dazu führen, dass sie auf verschiedene Weise auf physiologische Veränderungen wie das Einsetzen der Laktation reagieren. Adiponektin ist eines der am häufigsten vorkommenden Adipokine und ist mit verschiedenen physiologischen Funktionen wie Insulinsensitivität, Immunität und Entzündungsreaktionen verbunden. Konjugierte Linolsäuren (CLA) könnten, wie bereits aus Zellkulturstudien und Studien mit Nagetieren bekannt, die Adiponektinexpression beeinflussen. Bei Milchkühen werden CLA-Supplemente zur Senkung des Milchfettgehaltes verwendet. Dies dient der Verringerung des Energieverlusts über die Milch in den ersten Wochen der Laktation, während derer die Futteraufnahme meist nicht ausreicht, um den Energiebedarf für die Grunderhaltung und die Milchproduktion zu decken. Obgleich die CLA-Effekte auf die Lipogenese der Milchdrüse bereits gründlich aufgezeigt wurden, sind mögliche Einflüsse auf das Körperfett, insbesondere auf die Adiponektinsekretion noch unbekannt. Demzufolge zielen die hier durchgeführten Experimente darauf ab, 1) die Adiponektinkonzentrationen in Blut und verschiedenen sc und vc Fettdepots von Milchkühen in unterschiedlichen Laktationsstadien zu charakterisieren, 2) die Effekte einer CLA-Supplementation auf die Adiponektinkonzentration in Serum und Milch zu testen und 3) die Effekte einer laktationsbedingten sowie einer ernährungsinduzierten negativen Energiebilanz (NEB) auf Blut- und Milchadiponektinkonzentrationen zu beurteilen.

In zwei Versuchen wurden Holstein Kühe in eine CLA-Gruppe (100 g Fettsupplement pro Tag mit je 10% *cis*-9, *trans*-11 und *trans*-10, *cis*-12 Isomer) oder eine Kontrollgruppe (100 g Fettsupplement pro Tag, Ersatz der CLA durch Stearinsäure) eingeteilt. In Versuch 1 erhielten sowohl multipare (MP, n = 22), als auch primipare (PP, n = 22) Kühe von Tag 1 bis Tag 182 der Laktation entweder CLA (11 MP, 5 PP)

oder das Kontrollfett (1 MP, 6 PP). Blutproben sowie Biopsien subkutanen Fettgewebes vom Schwanzansatz wurden von allen Kühen im Zeitraum von Tag -21 bis 252 relativ zur Kalbung entnommen. In Versuch 2 erfolgte eine entsprechende Fütterung von 25 PP Kühen und nach den Schlachtungen an Tag 1 (n = 5) , 42 und 105 (jeweils 5 Tiere pro Gruppe) der Laktation wurden Proben unterschiedlicher Fettdepots [3 vc Depots (omental, mesenterisch und retroperitoneal) und 3 sc Depots (Schwanzansatz, Widerrist und Sternum)], sowie Blutproben entnommen. In einem dritten Versuch wurden die Effekte einer NEB in zwei Stadien der Laktation (die natürliche NEB zu Beginn der Lakation sowie eine durch eine restriktive Fütterung hervorgerufene NEB um Tag 100 während der Laktation) auf die zirkulierenden Adiponektinkonzentrationen sowie die Konzentrationen in der Milch untersucht. Der Versuch umfasste 50 Holstein Kühe und der gesamte Versuchszeitraum wurde in 3 Perioden eingeteilt: Periode 1 von drei Wochen ante partum bis zwölf Wochen post partum, Periode 2 mit drei Wochen restriktiver Fütterung begann an Tag 100 der Laktation mit einer Kontrollgruppe (n = 25) und einer Restriktionsgruppe (70 % des Energiebedarfs; n = 25), gefolgt von Periode 3, einer Realimentationsphase über einen Zeitraum von acht Wochen. Die Bestimmung der Plasmaadiponektinkonzentration erfolgte an verschiedenen Zeitpunkten während des Versuchs. Die Messung der Adiponektinkonzentrationen in den Proben aller Versuche erfolgte mit einem hauseigenen ELISA, der als erster Assay eine valide Quantifizierung von bovinem Adiponektin in einem umfangreichen Probenset ermöglicht.

Wir konnten aufzeigen, dass die wesentlichsten Veränderungen der Blutadiponektin-konzentration während der peripartalen Phase auftreten, mit einer sukzessiven Verringerung ausgehend von Tag 21 ante partum hin zu einem Tiefstwert zum Zeitpunkt der Kalbung und einem anschließenden graduellen Anstieg post partum. Die Supplementation mit einer 1:1 Mischung von *cis*-9, *trans*-10 und *trans*-10, *cis*-12 CLA führte zu einer Verringerung des nachgeburtlich zirkulierenden Adiponektins sowohl in PP als auch in MP Kühen. Ein carry-over Effekt des CLA über den Supplementationszeitraum hinaus beschränkte sich auf die MP Kühe. Es wurden annähernd dieselben Muster der peripartalen Veränderungen von Serumadiponektin und IS (geschätzt anhand des bereinigten Quantitativen Insulin Sensitivitäts Check Index) ermittelt. Diese Beobachtung sowie das Ergebnis der positiven Assoziation der

Variablen während der postpartalen Periode lässt *in vivo* Effekte von Adiponektin auf die IS von Milchkühen vermuten.

Unabhängig von der anatomischen Lokalisation zeigten alle Fettgewebsdepots die geringsten Adiponektinkonzentrationen zum Zeitpunkt der Kalbung, gefolgt von einem Anstieg während der frühen Laktation. Die Verringerung der zirkulierenden Adiponektinkonzentration um den Geburtszeitpunkt resultiert daher wahrscheinlich aus der reduzierten Proteinexpression in allen Fettdepots. Der höhere Adiponektingehalt im vc Fettgewebe deutet darauf hin, dass dieses Gewebe einen vergleichsweise höheren Beitrag zu den Blutkonzentrationen leistet als das sc Gewebe. Das retroperitoneale Fettgewebe als eines der viszeralen Depots enthielt aber die geringste Adiponektinkonzentration, sowie die geringste Gesamtadiponektinmenge bezogen auf die Depotmasse. Die durch eine restriktive Fütterung hervorgerufene NEB sowie die anschließende Realimentation hatten keinen Einfluss auf die Plasmadiponektinkonzentrationen im Zeitraum um Tag 100 der Laktation. Dies lässt vermuten, dass die zirkulierenden Adiponektinkonzentrationen während der Laktation keiner Beeinflussung durch die Energiebilanz unterliegen. Neben der Bereitstellung von Informationen über die longitudinalen Veränderungen der Adiponektinkonzentration im Blut und der Effekte von CLA, trägt die vorliegende Arbeit zu einem verbesserten Wissen über die unterschiedliche Adiponektinexpression in verschiedenen Fettgeweben sowie über Veränderungen der Blutadiponektinkonzentrationen bei. Darüber hinaus dient diese Dissertation als Grundlage für zukünftige Studien, die die Rolle von Adiponektin in unterschiedlichen pathophysiologischen Zuständen beim Rind oder anderen Spezies untersuchen. Die spezifischen Regulationsmechanismen der peripartalen Veränderungen der zirkulierenden Adiponektinkonzentrationen sowie deren physiologische Bedeutung bleiben weiterhin zu klären.

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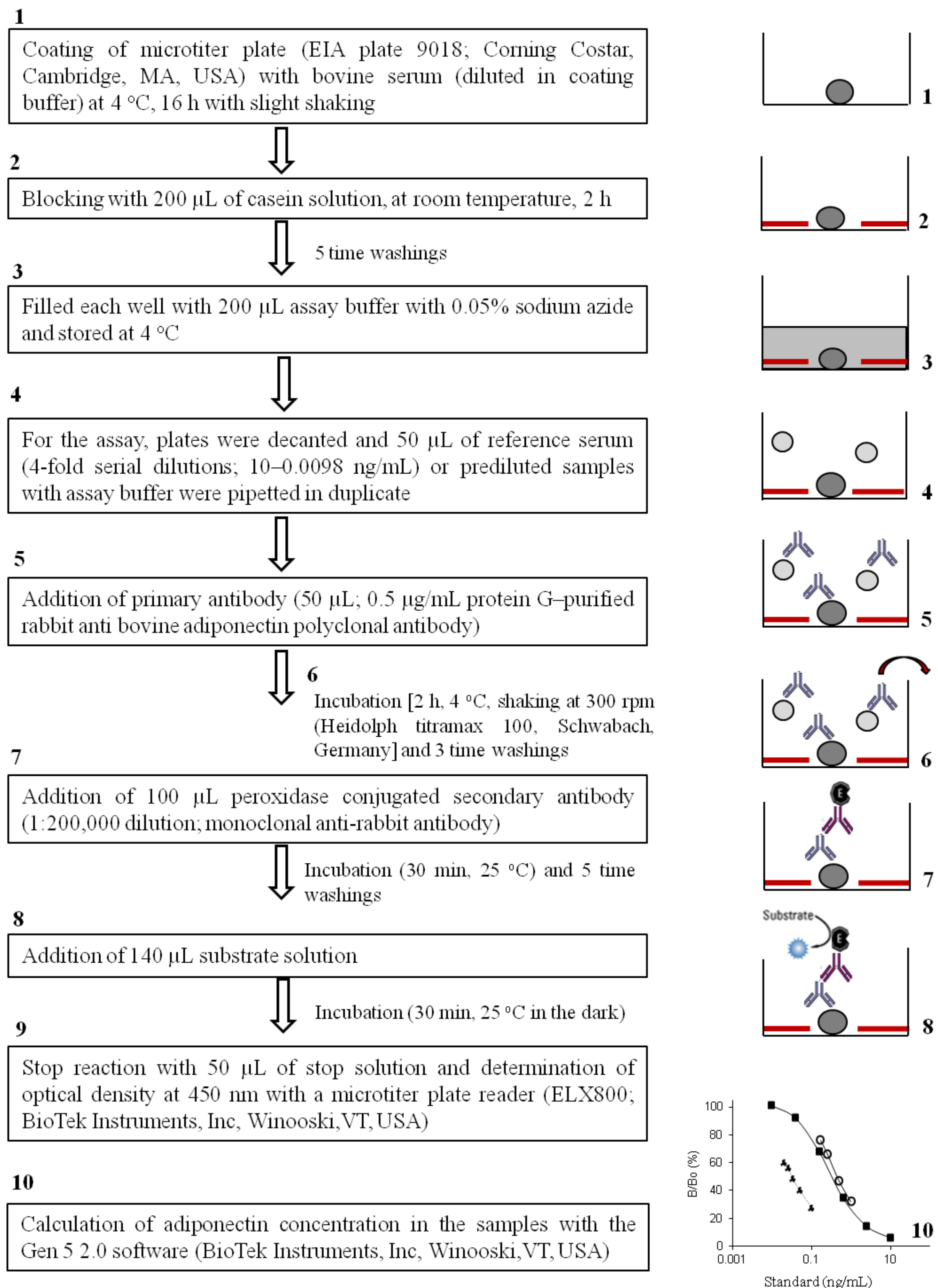
9. Appendixes

Appendix A: Validation of in-house developed ELISA for bovine adiponectin

Criterion	Validation approach
Intra-assay (reproducibility) and inter-assay (precision) coefficients of variation	Determined by repeated measurements (n = 7) of 2 different serum samples at 2 different dilutions (n = 4) within single assay and in different assays (n = 7). The mean intra- and inter-assay coefficients of variation were 7% and 11%, respectively.
Spiking recovery	Assessed by calculate the ratio of expected vs measured adiponectin concentrations. Two different serum samples with differing adiponectin concentrations (10.2 and 28.8 µg/mL) were mixed at 11 different ratios and were measured. The mean recovery was 112% ± 8.23%. In addition, the mean recovery of 3 different concentrations of purified adiponectin spiked into a serum sample in 4 different dilutions was 119% ± 8.02%.
Accuracy	Measured by demonstrating linearity and parallelism of serial sample dilutions and the standard curve. Dilutional linearity was also tested for various other bovine samples such as saliva, urine, follicular fluid, seminal fluid, preparations from different scAT and vcAT depots, cell culture supernatant of differentiated bovine preadipocytes and of mouse 3T3-L1 cells, bovine whole milk, skim milk and milk serum as well as blood serum from different species such as sheep, goat, camel, mouse, pig, horse, roe deer, human, chicken, and goose. Dilution linearity was not observed in samples from species other than cattle.
Specificity	Determined by testing the cross-reactivity with bovine albumin (Roth), calf skin collagen (Sigma-Aldrich), and the structurally homologous human complement factor C1q (GenWay Biotech, San Diego, CA, USA). Negligible cross-reactivity (i.e. <0.0001%) was observed for all 3 proteins.

Sample stability	Similar concentrations were observed in bovine serum samples after freeze/thaw cycles (12 times, -20 ^{o}C, CV < 15%), thus suggesting no effect of repeated freeze/thaw cycles on adiponectin measurements.
Sensitivity or limit of detection	Defined by the least detectable dose i.e. the lowest concentration of adiponectin that could be distinguished from a zero blank by replicated determination (n = 10) (mean value + 3 SDs). Sensitivity of the assay was 0.03 ng/mL.
Measuring range	Estimated as the concentration range in which the standard curve was approximately linear. Measuring range for the assay was 0.07 – 1.0 ng/mL.

Appendix B: Steps followed in the ELISA for bovine adiponectin



Note: composition of buffers and solution used in ELISA is given in appendix C.

Appendix C: Chemicals, buffers and solutions used in the ELISA for bovine adiponectin

Chemicals, buffers and solutions*	Composition	Firm
Protease inhibitor Complete™	1 tablet in 2 mL H_2O	Roche
Coating buffer (pH 9.6)	50 mM $NaHCO_3$	Roth
	200 µL/L Protease inhibitor	
	20 mL/L Proclin 150®	Supelco
Casein solution (pH 7.2)	0.05 M NaOH	Applichem
	1.5 mM EDTA (Disodium salt dihydrate)	Roth
	2.5% Casein	Sigma
	200 µL/L Protease inhibitor	
	20 mL/L Proclin 150®	
Assay buffer (pH 7.3)	0.12 M NaCl	Roth
	0.02 M Na_2HPO_4	AppliChem
	0.01 M EDTA	Roth
	0.005% Chlorhexidine	Sigma
	0.1% Gelatine (Gelatine Hydrolysate)	Sigma
	0.05% Tween®20	AppliChem
	0.002% Phenol red	Sigma
	200 µL/L Protease inhibitor	
	20 mL/L Proclin 150®	
Substrate buffer (pH 4.05)	0.05 M Citric acid	AppliChem
	0.055 M Na_2HPO_4	
	0.05% Urea peroxide	Sigma
	20 mL/L Proclin 150®	
TMB-solution	12.5 mg TMB (3, 3, 5, 5-Tetramethylbenzidine)	AppliChem
	1 mL DMSO (Dimethyl sulfoxide)	AppliChem
Substrate solution	18 mL substrate buffer	
	360 µL TMB-solution	
Stop solution	1 M Oxalic acid	Roth
Washing buffer (10-fold; pH 6.8)#	1.36 M NaCl	
	81 mM Na_2HPO_4	
	27 mM KCl	Roth
	15 mM KH_2PO_4	Roth
	20 mL/L Proclin 150®	
	55 g/L Tween®20	

* Ultrapure water was used for preparing all buffers and solutions.
# For preparing working washing solution: 10 mL of washing buffer/ L ultrapure water

10. Acknowledgments

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11. Publications derived from this doctoral thesis and related works

11.1. Papers and manuscripts

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

(2) **Singh, S. P.,** S. Häussler, J. F. L. Heinz, S. H. Akter, B. Saremi, U. Müller, J. Rehage, S. Dänicke, M. Mielenz and H. Sauerwein. 2014. Lactation driven dynamics of adiponectin supply from different fat depots to circulation in cows. Domest. Anim. Endocrin. (in press; DOI: 10.1016/j.domaniend.2013.12.001).

(3) **Singh, S. P.,** S. Häussler, J. J. Gross, R. M. Bruckmaier, and H. Sauerwein. 2014. Short communication: Circulating and milk adiponectin change differently during energy deficiency at different stages of lactation in dairy cows. J. Dairy Sci. (accepted; doi.org/10.3168/jds.2013-7598).

(4) Mielenz, M., B. Mielenz, **S. P. Singh,** C. Kopp, J. Heinz, S. Häussler, and H. Sauerwein. 2013. Development, validation, and pilot application of a semiquantitative Western blot analysis and an ELISA for bovine adiponectin. Domest. Anim. Endocrin. 44: 121–130.

(5) Weber, C., C. Hametner, A. Tuchscherer, B. Losand, E. Kanitz, W. Otten, **S. P. Singh**, R. M. Bruckmaier, F. Becker, W. Kanitz, and H. M. Hammon. 2013. Variation in fat mobilization during early lactation in high yielding dairy cows affect feed intake, body condition as well as glucose and lipid metabolism. J. Dairy Sci. 96:165–180.

(6) Heinz, J. F. L., **S. P. Singh,** U. Janowitz, M. Hoelker, D. Tesfaye, K. Schellander, and H. Sauerwein. Characterization of adiponectin concentrations and molecular weight forms in bovine body fluids related to reproduction (submitted).

(7) Kopp, C., **S. P. Singh**, P. Regenhard, H. Sauerwein and M. Mielenz. *Trans*-cinnamic acid increases adiponectin and the phosphorylation of AMP-activated protein kinase via G-protein coupled receptor 109A in 3T3-L1 adipocyte (submitted).

11.2. Abstracts in conferences

(1) Singh S. P., S. Häußler, J. Gross, R. M. Bruckmaier, and H. Sauerwein. 2013. Circulating adiponectin concentrations in dairy cows during a negative energy balance in early lactation and during an energy-restriction at 100 days in milk (*oral presentation*). Proceedings of the 67th annual meeting of the GfE (Society of Nutrition Physiology) Conference, 19–21.03.13, University of Göttingen, Göttingen, Germany, Page 83, ISBN 978-3-7690-4106-4.

(2) Singh S. P., B. Mielenz, M. Mielenz, S. Dänicke, J. Rehage, S. Häussler and H. Sauerwein. 2013. Towards characterizing the usefulness of serum adiponectin concentrations to estimate the risk for metabolic diseases in dairy cows (*poster presentation*). Proceedings of the 16th International Symposium of the World Association of Veterinary Laboratory Diagnosticians (WAVLD), 05–08.06.2013, Berlin, Germany, Page, ISBN.

(3) Singh S. P., J. Heinz, S. Dänicke, S. Häussler, and H. Sauerwein. 2013. Identification of adiponectin in bovine milk and characterization of its concentrations during early lactation (*poster presentation*). Book of Abstracts of the 15th International Conference on Production Diseases in farm animals (ICPD), 24–28.06.2013, Swedish University of Agricultural Sciences, Uppsala, Sweden, Page 129, ISBN 978-91-576-9150-7.

(4) Singh S. P., H. Sauerwein, M. Steyer, T. Ettle, M. Rodehutscord, and S. Häussler. 2013. Relationships of leptin and adiponectin serum concentrations with measures of body condition in Simmental cows (*poster presentation*). Book of Abstracts of the 15th International Conference on Production Diseases in farm animals (ICPD), 24–28.06.2013, Swedish University of Agricultural Sciences, Uppsala, Sweden, Page 139, ISBN 978-91-576-9150-7.

(5) Singh S. P., S. Häussler, S. Dänicke, M. Mielenz, and H. Sauerwein. 2013. Characterization of serum adiponectin during lactation in dairy cows supplemented with conjugated linoleic acids (*oral presentation*). Joint Annual Meeting of American Dairy Science Association (ADSA)–American Society of Animal Science (ASAS), Indianapolis, Indiana, USA, 08–12.07.2013. J. Dairy Sci. Vol. 96 (E-Suppl. 1): 208–209.

(6) Singh S. P., S. Häussler, D. Tesfaye, M. Hölker, K. Schellander and H. Sauerwein. 2013. Characterization of follicular fluid adiponectin and its

relationship with blood adiponectin during estrous cycle in cattle (*poster presentation*). Joint Annual Meeting of American Dairy Science Association (ADSA)–American Society of Animal Science (ASAS), Indianapolis, Indiana, USA, 08–12.07.2013. J. Dairy Sci. Vol. 96 (E-Suppl. 1): 247.

(7) **Singh S. P.,** S. Häussler, O. Wellnitz, R. M. Bruckmaier and H. Sauerwein. 2013. Influence of intramammary lipopolysaccharide challenge on milk and plasma adiponectin in dairy cows (*poster presentation*). Joint Annual Meeting of American Dairy Science Association (ADSA)–American Society of Animal Science (ASAS), Indianapolis, Indiana, USA, 08–12.07.2013. J. Dairy Sci. Vol. 96 (E-Suppl. 1): 572.

(8) **Singh S. P.,** S. Häussler, J. J. Gross, R. M. Bruckmaier and H. Sauerwein. 2013. Adiponectin concentrations in cows' milk during induced negative energy balance (*poster presentation*). Joint Annual Meeting of American Dairy Science Association (ADSA)–American Society of Animal Science (ASAS), Indianapolis, Indiana, USA, 08–12.07.2013. J. Dairy Sci. Vol. 96 (E-Suppl. 1): 233.

(9) Häussler, S., **S. P. Singh,** L. Laubenthal, L. Locher, J. Winkler, U. Meyer, S. Dänicke and H. Sauerwein. 2013. Impact of increased oxidative stress through excessive accumulation of adipose tissue on circulating adiponectin concentrations in dairy cows (*poster presentation*). Joint Annual Meeting of American Dairy Science Association (ADSA)–American Society of Animal Science (ASAS), Indianapolis, Indiana, USA, 08–12.07.2013. J. Dairy Sci. Vol. 96 (E-Suppl. 1): 114.

(10) Kopp C., **S. P. Singh,** H. Sauerwein and M. Mielenz. 2013. Niacin increases adiponectin secretion in differentiated bovine preadipocytes *in vitro* via G-protein coupled receptor 109A (*poster presentation*). Joint Annual Meeting of American Dairy Science Association (ADSA)–American Society of Animal Science (ASAS), Indianapolis, Indiana, USA, 08–12.07.2013. J. Dairy Sci. Vol. 96 (E-Suppl. 1): 121.