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# Histomorphological and immunohistochemical characterization of different fat depots of dairy cows during early lactation

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***To my parents and my family***

**Histomorphological and immunohistochemical characterization of different fat depots of dairy cows during early lactation**

Early lactation is attended by massive fat mobilization, decreased insulin sensitivity and immunosuppression. The aim of this dissertation was to study the effects of lactation and conjugated linoleic acid (CLA) on adipocyte size and on the invasion of phagocytic immune cells in different fat depots of dairy cows during the first 105 days in milk (DIM). The present study focused on two aspects: First, mean adipocyte areas ($\mu m^2$) of different subcutaneous (SC) and visceral (VC) fat depots were assessed to investigate the effects of CLA on adipocyte size, with regards to the changes during early lactation. Second, adipose tissue (AT) sections were immunohistochemically stained to characterize different fat depots of early-lactating dairy cows in terms of phagocytic immune cell present. In addition, the changes in the portion of phagocytic immune cells in AT of early-lactating cows were compared with non-pregnant, over-conditioned heifers. The timely changes of adipocyte size were non-uniform in different SC and VC fat depots: retroperitoneal adipocyte sizes were significantly smaller at 105 DIM compared to 1 DIM, whereas SC adipocytes remained unchanged. When comparing the control and CLA group, adipocyte sizes were smaller both in SC and in VC fat depots to different extent in CLA supplemented cows. Immunohistochemical analyses of different fat depots revealed a low incidence of phagocytic immune cell infiltration in early-lactating cows. The average portion of macrophages in a few positive AT samples was slightly lower in SC versus VC depots. No significant alterations in this infiltration phenomenon, with regards to DIM and CLA supplementation were observed during early lactation. Moreover, increased accumulation of phagocytic immune cells in the SC fat of non-pregnant, over-conditioned heifers might be related to large adipocytes, secreting higher amounts of chemoattractant adipokines. In conclusion, dietary CLA supplements have site-specific effects on adipocyte size of different fat depots in dairy cows. CLA-induced decreases in adipocyte size indicate lipolytic and/or antilipogenic effects of CLA on AT. Moreover, early-lactating cows are not ‘obese’ and may thus lack significant infiltration of phagocytic cells into AT and therefore, these immune cells might have no major role in the immunologic and metabolic adaptations during early lactation. The results are of general importance to Animal Science and provide a general basis for understanding CLA effects on body fat depots in dairy cattle.

**Histomorphologische und immunhistochemische Charakterisierung verschiedener Fettdepots von Milchkühen während der frühen Laktation**

Während der Fühlaktation kommt es zu einer massiven Fettmobilisierung, verringerter Insulinsensitivität und Immunsuppression. Das Ziel dieser Dissertation war es, den Einfluss konjugierter Linolsäuren (CLA) auf die Adipozytengröße ($\mu m^2$) sowie die Einwanderung phagozytierender Immunzellen in subkutane (SC) und viszerale (VC) Fettdepots bei Milchkühen während der ersten 105 Laktationstage zu untersuchen. Zusätzlich zum immunhistochemischen Nachweis der Immunzellen bei frühlaktierenden Kühen, wurde die Anzahl phagozytierenden Zellen im Fettgewebe von nicht tragenden, überkonditionierten Färsen untersucht und verglichen. Die Adipozytengröße variierten in den verschiedenen SC und VC Fettdepots: An Tag 105 der Laktation war die Adipozytengröße im retroperitonealen Fett signifikant geringer, als an Tag 1 der Laktation, wobei die Adipozyten im SC Fett unverändert blieben. Beim Vergleich zwischen Kontroll- und CLA-Supplementationsgruppe waren die Adipozyten sowohl in den SC als auch in den VC Fettdepots der CLA Tiere kleiner. Die immunhistochemischen Färbungen zeigten eine geringfügige Infiltration phaygozytierender Immunzellen bei frühlaktierenden Kühen in allen untersuchten Fettdepots. In den wenigen positiven Fettproben war die Anzahl von Makrophagen in SC niedriger als in VC Fettdepots. Bezüglich Laktationsverlauf und CLA Supplementation konnten keine signifikanten Veränderungen festgestellt werden. Eine erhöhte Anreicherung phagozytierender Immunzellen im SC Fett von nicht tragenden überkonditionierten Färsen könnte mit der Anzahl großer Adipozyten im Zusammenhang stehen, da diese mehr Chemokine freisetzen. Die CLA-Supplementation zeigt somit gewebespezifische Effekte auf die Adipozytengröße von Milchkühen, die auf lipolytische oder antilipogene Effekte der CLA im Fettgewebe zurückgeführt werden. Zudem sind frühlaktierende Kühe nicht „übergewichtig“ und weisen daher wahrscheinlich eine geringere Infiltration phagozytierender Zellen ins Fettgewebe auf, weshalb diese Immunzellen offenbar keine große Rolle in der immunologischen und metabolischen Adaptation während der frühen Laktation spielen. Die Ergebnisse sind von allgemeiner Bedeutung für die Nutztierwissenschaften und liefern grundlegende Erkenntnisse für das Verständnis von CLA-Effekten auf das Körperfett bei Milchkühen.

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**List of abbreviations**

| | |
|---|---|
| ADD1/SREBP-1 | Adipocyte determination and differentiation factor-1 |
| ADF | Acid detergent fiber |
| Approx. | Approximately |
| AT | Adipose tissue |
| ATM | Adipose tissue macrophages |
| BCS | Body condition score |
| BHBA | ß-hydroxybutyrate |
| BW | Body weight |
| °C | Degrees centigrade |
| *c* | *Cis* |
| CAMs | Cellular adhesion molecules |
| C/EBP | CCAAT/enhancer binding protein |
| *Cis*-9, *trans*-11 | *c*9,*t*11 |
| CLA | Conjugated linoleic acid |
| cm | Centimeter |
| CON | Control |
| CP | Crude protein |
| d | Day |
| DIM | Day(s) in milk |
| DM | Dry matter |
| e.g. | For example |
| EBW | Empty body weight |
| EC | Endothelial cells |
| EDTA | Ethylenediaminetetraacetic acid |
| et al. | And others |
| FA | Fatty acids |
| Fig. | Figure |
| FLI | Friedrich-Loeffler-Institute |
| g | Gram |
| GLM | General linear model |
| GPR109 | G-protein-coupled receptor109 |

| | |
|---|---|
| h | Hour |
| HF | German Holstein-Friesian cows |
| HSL | Hormone sensitive lipase |
| i.e. | That is |
| IGF-1 | Insulin-like growth factor-1 |
| IL | Interleukin |
| kg | Kilogram |
| L | Liter |
| LANUV-NRW | North Rhine-Westphalia State Agency for Nature, Environment, and Consumer Protection |
| LAVES | Lower Saxony State Office for Consumer Protection and Food Safety |
| LPL | Lipoprotein lipase |
| mAb | Monoclonal antibodies |
| MCP-1 | Monocyte chemoattractant protein-1 |
| ME | Metabolizable energy |
| MIF | Macrophage migratory inhibitory factor |
| min | Minute |
| MIP-1α | Macrophage inflammatory protein-1 alpha |
| MJ | Megajoule |
| ml | Milliliter |
| mmol | Millimole |
| mRNA | Messenger ribonucleic acid |
| MT | Metallothionein |
| n | Number of samples |
| NDF | Neutral detergent fiber |
| NEB | Negative energy balance |
| NEFA | Nonesterified fatty acids |
| $NE_L$ | Net energy for lactation |
| $NE_M$ | Net energy for maintenance |
| NF-κB | Nuclear factor-kappa B |
| ng | Nanogram |
| NGF | Nerve growth factor |
| *P* | Probability |

| | |
|---|---|
| PAI-1 | Plasminogen activator inhibitor-1 |
| PG | Prostaglandin |
| PMR | Partial mixed ration |
| PPAR | Peroxisome proliferator-activated receptor |
| PUFA | Polyunsaturated fatty acids |
| r | Correlation coefficient |
| RANTES | Regulated upon activation, normally T-expressed, and presumably secreted |
| RP | German Red Pied steers |
| RT | Room temperature |
| RXR | Retinoic X receptors |
| SAA | Serum amyloid A |
| SC | Subcutaneous |
| SDF-1α | Stromal cell-derived factor-1 alpha |
| SEM | Standard error of the means |
| SM | Simmental heifers |
| SVF | Stromal-vascular fraction |
| *t* | *Trans* |
| TAG | Triacylglycerides |
| TF | Tissue factor |
| TGF-ß | Transforming growth factor-beta |
| TNF-α | Tumor necrosis factor-alpha |
| *Trans*-10, *cis*-12 | *t*10,*c*12 |
| µEq | Microequivalent |
| $\mu m^2$ | Square micrometer |
| VC | Visceral |
| VEGF | Vascular endothelial growth factor |
| vol/vol | Volume per volume |
| vs. | Versus |
| WAT | White adipose tissue |
| wt/vol | Weight per volume |
| yr | Year(s) |
| ZAG | Zinc-α2-glycoprotein |

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# 1 Introduction

## 1.1 Metabolic stress in dairy cows during early lactation

Most dairy cows, especially high-yielding breeds, are susceptible to metabolic disorders during early lactation. The cows in this period undergo a situation of compromised immune response which predispose to infectious diseases like mastitis, metritis, and laminitis (Heuer et al., 1999; Collard et al., 2000). Dairy cows presenting such disturbances in early lactation cause economic losses (Drackley, 1999). Moreover, metabolic disorders during early lactation are related to decreased fertility (Butler and Smith, 1989). Although most cows overcome this period without showing signs of clinical disease, the dairy industry loses thousands of cows each year for culling due to metabolic or infectious diseases, and thus encounters increasing replacement costs. Dry matter (DM) intake of dairy cows is low in early lactation, whereas energy demand for milk production is high, which leads to cows being in negative energy balance (NEB). Therefore, energy has to be mobilized from body reserves, mainly from the adipose tissue (AT) to support milk production and animal body maintenance. High-yielding dairy cows lose approx. 60% or more of their body fat in the first weeks after parturition (Knop and Cernescu, 2009). Extreme fat mobilization from AT results in increased circulating concentrations of nonesterified fatty acids (NEFA) and ß-hydroxybutyrate (BHBA) that may result in metabolic disorders like fatty liver and ketosis (Herdt, 2000).

## 1.2 Adipose tissue

### 1.2.1 Adipose tissue function

Adipose tissue is a highly specialized connective tissue dealing mainly with processes for storage and release of energy. According to its diverse biochemical and functional characteristics, it is classified into two distinct types: white AT (WAT) and brown AT. In the present study the focus lies on WAT, since it is the most abundant one and quantitatively plays the most important role in adults (Marra and Bertolani, 2009).

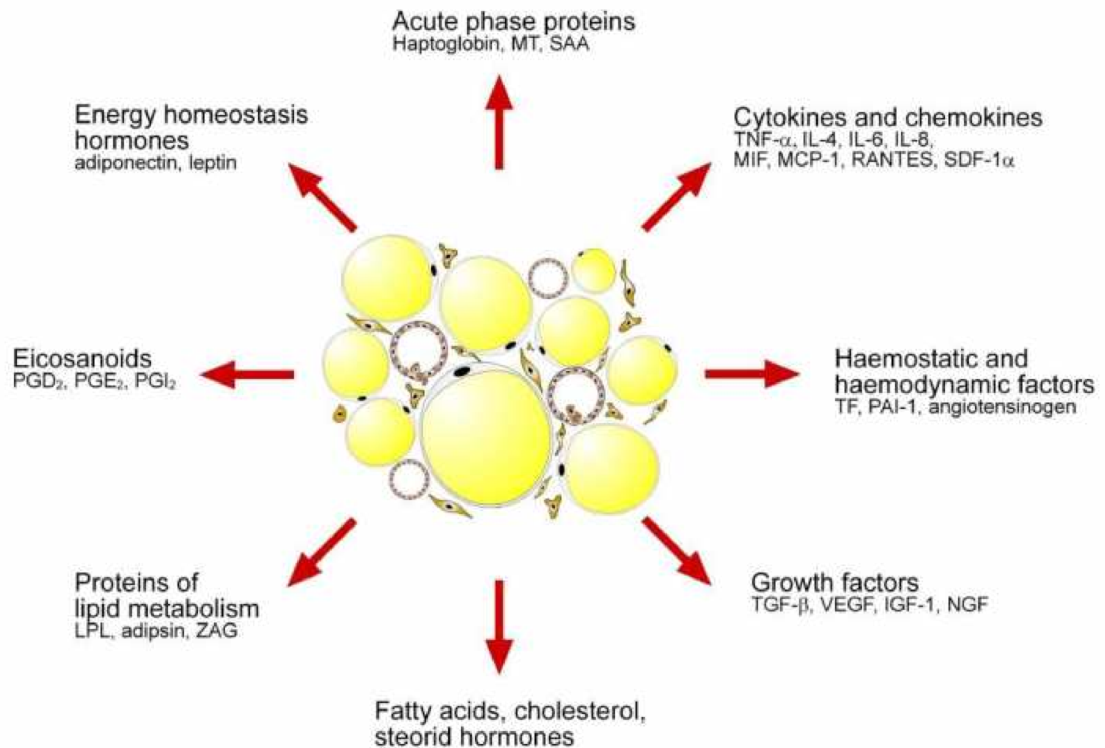


**Fig. 1: Schematic representation of the multiple adipose-derived factors.** Abbreviations: MT, metallothionein; SAA, serum amyloid A; TNF-α, tumor necrosis factor-alpha; IL, interleukin; MIF, macrophage migratory inhibitory factor; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation, normally T-expressed, and presumably secreted; SDF-1α, stromal cell-derived factor 1 alpha; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; TGF-ß, transforming growth factor-beta; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; LPL, lipoprotein lipase; ZAG, zinc-α2-glycoprotein; PG, prostaglandin (modified from Bing and Trayhurn, 2009; Skurk et al., 2009).

White AT stores and releases energy in the form of triacylglycerides (TAG) and plays an important role in the regulation and maintenance of energy homeostasis. During periods of fuel surplus, glucose and fatty acids (FA) are taken up by adipocytes and converted into TAG for storage. The term lipogenesis encompasses the processes of FA and subsequent TAG synthesis. During periods of fuel deprivation, the TAG are broken down in the adipocytes into FA and glycerol and then released into the circulation; this process is termed lipolysis (Boschmann, 2001). Two key enzymes: lipoprotein lipase (LPL; located extracellularly) and hormone sensitive lipase (HSL; located intracellularly) are involved in the regulation of lipogenesis and lipolysis, respectively.

White AT also serves as a cushion for various organs and is necessary for mechanical and thermal insulation (Klaus, 2001).

Since the discovery of leptin in 1994, it has been recognized that adipocytes produce and secrete a wide range of molecules, collectively termed as adipokines (Fig. 1). Thus, WAT has been accepted as an important endocrine and secretory organ (Ahima, 2006). The adipokines are involved in a wide range of physiological and metabolic processes including appetite regulation, glucose metabolism, insulin sensitivity, lipid metabolism, inflammation, and immune functions (Frühbeck, 2008). Numerous proinflammatory, antiinflammatory, immunomodulating proteins and peptides (in excess of 100) that belong to the cytokine, chemokine, complement, and growth factor families have been identified in adipocytes, which points to a role of WAT in the innate immune system (Pattrick et al., 2009). At the same time, WAT expresses receptors for most of these factors, which warrant an extensive crosstalk at a local and systemic level in response to specific external stimuli or metabolic changes (Frühbeck, 2008). Moreover, WAT undergoes macroscopic changes, such as hypertrophy, stiffness, neovascularization and changes of cellular composition, such as monocytic infiltration, alterations of adipocyte size, and changes in the ratio of preadipocytes to mature adipocytes (plasticity of the WAT) and is directly involved in inflammatory processes (Schäffler and Schölmerich, 2010).

### 1.2.2 Morphology of white adipose tissue

At the cellular level, WAT is a heterogeneous tissue containing mature adipocytes and the stromal-vascular fraction (SVF). Mature adipocytes represent about 50% of the total cell content of WAT (Hausman, 1985) and store the TAG which in turn constitute up to 85% of tissue weight (Trayhurn et al., 2006). The remaining SVF consists of many cell types, including preadipocytes, fibroblasts, pericytes, blood and endothelial cells, diverse precursor cells and immune cells, such as macrophages and T-lymphocytes. The multicellularity of WAT is involved in autocrine, paracrine, and endocrine processes and crosstalks (Hauner, 2005); however, their differential contribution to the total pool of adipokines is not well clarified (Weisberg et al., 2003; Fain et al., 2004; Antuna-Puente et al., 2008; Fain et al., 2008).

White AT is not a single homogeneous compartment; rather it displays depot and sex-specific metabolic properties and is subject to differential neural and endocrine regulation (Ross et al., 1996; Mack, 2011). White AT depots are distributed throughout the body and can be roughly categorized as subcutaneous (SC) and visceral (VC) depots (Shen et al., 2003). Some other non-visceral WAT depots include: inter-, intra-, and perimuscular AT (Frühbeck, 2008). Small quantities can also be found within and around many organs and blood vessels (Cinti, 2005). The SC depot comprises the fat layer found between the dermis and the aponeuroses and fasciae of the muscles (Frühbeck, 2008). Shen et al. (2003) proposed a classification for the VC depots, consisting of the AT distributed in three body cavities, such as intrathoracic (e.g., extra- and intrapericardial), intraabdominal (e.g., omental, mesenteric, and retroperitoneal), and intrapelvic (e.g., gonadal and urogenital).

There is mounting evidence that WAT is distinct both in cellular makeup and size, as well as vasculature content and lymphatic profusion (Wright-Piekarski, 2010). White AT depots are shown to have varying metabolic functions. When comparing the omental and SC depots in humans, the omental depot has a greater effect on overall metabolism (O'Rourke, 2009). It has been suggested that the effect may be due to any of the following: its vascular proximity to the liver (portal theory), by higher production of adipokines and cytokines (Wajchenberg et al., 2002), or by its method of expansion: hyperplasia vs. hypertrophy (Joe et al., 2009). The portal theory suggests that the proximity of VC depots to the liver, combined with its higher lipolytic rate compared with SC depots, leads to an increase in free FA being delivered to the liver, which in turn contributes to liver insulin resistance (Kabir et al., 2005).

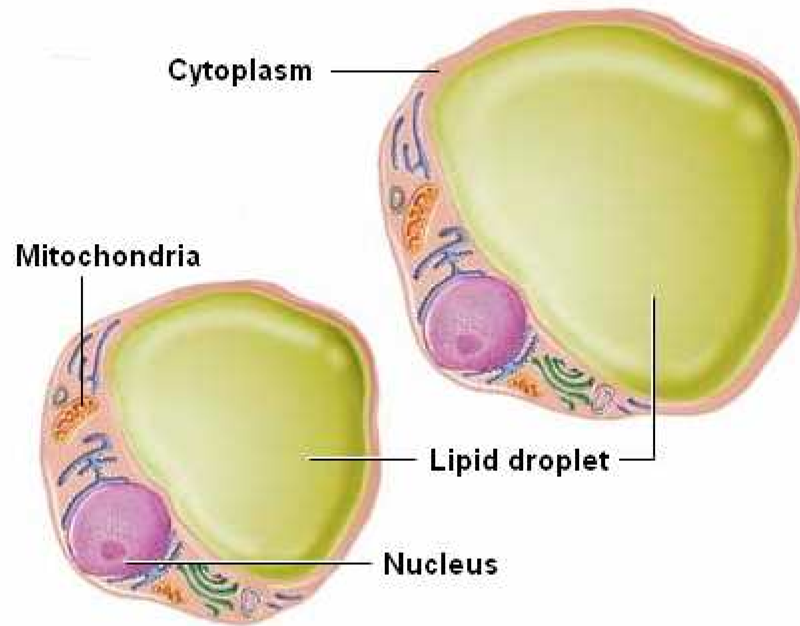


**Fig. 2:** **Morphology of typical mature adipocytes** (modified from www.Adamimages.com)

Within the WAT, unilocular adipocytes are individually held in place by delicate reticular fibers clustering in lobules bounded by fibrous septa. It is highly vascularized and innervated by the sympathetic (Cinti, 2001) and the parasympathetic (Kreier and Buijs, 2007) nervous system. Mature adipocytes contain a single, large lipid inclusion surrounded by a thin layer of cytoplasm. The lipid mass compresses the flattened nucleus to an eccentric position, producing a signet-ring appearance (Fig. 2). Mitochondria present in the thicker portion of the cytoplasmic rim, near the nucleus. The characteristic spherical form of adipocytes varies enormously in size, ranging from 20 to 200 µm in diameter in humans which is due to the ability of the cells to accumulate different amounts of TAG in a single vacuole (Frühbeck, 2008).

### 1.2.3 Growth of white adipose tissue

White AT growth requires continuous remodeling of the vascular network, mainly the microvasculature, which is then subject to neovascularization and/or remodeling of existing capillaries (Christiaens and Lijnen, 2009). In the past, it was believed that the number of adipocytes does not increase after birth. According to the report of Schling

and Löffler (2002), WAT can increase either due to hypertrophy of existing adipocytes or hyperplasia where the number of adipocytes is increased. The determining factors of AT mass in adults are not completely understood; however, increased lipid storage in fully differentiated mature adipocytes, resulting in hypertrophied adipocytes appears to be the most important (Bjorntorp, 1974; Hirsch and Batchelor, 1976). In contrast, hyperplasia and the determination of adipocyte number appear to occur during childhood (Knittle et al., 1979; Spalding et al., 2008). Moreover, the number of adipocyte remains constant in adulthood in lean and obese individuals, even after marked weight loss which had been stable for two years at the time of the measurements (Spalding et al., 2008). Nevertheless, if it comes to massive increases in body weight (BW) and adipocytes have reached a critical size, hyperplasia also appears to occur (Hirsch and Batchelor, 1976), which is achieved by inducing adipogenesis. Adipogenesis also takes place in normal weight individuals since WAT is a plastic organ with an annual turnover of adipocytes of approx. 10% (Spalding et al., 2008).

### 1.2.4 Adipogenesis

Adipogenesis refers to the process of development of fat cells from preadipocytes to mature adipocytes that are capable of metabolizing lipid (lipogenesis and lipolysis). The two main steps involved in adipogenesis are proliferation and differentiation of the progenitor fat cells (Fig. 3). The adipose cell lineage originates from multipotent mesenchymal stem cells that develop into adipoblasts. These adipoblasts gives rise to preadipocytes that proliferate to expand the cell population. Differentiation is the transition from undifferentiated fibroblast-like preadipocytes into mature round lipid-filled fat cells (Butterwith, 1994). Proliferating preadipocytes undergo growth arrest initiated through contact inhibition. Following growth arrest, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through the subsequent differentiation steps, leading to the progressive acquisition of the morphological and biochemical characteristics of mature adipocytes (Gregoire, 2001).

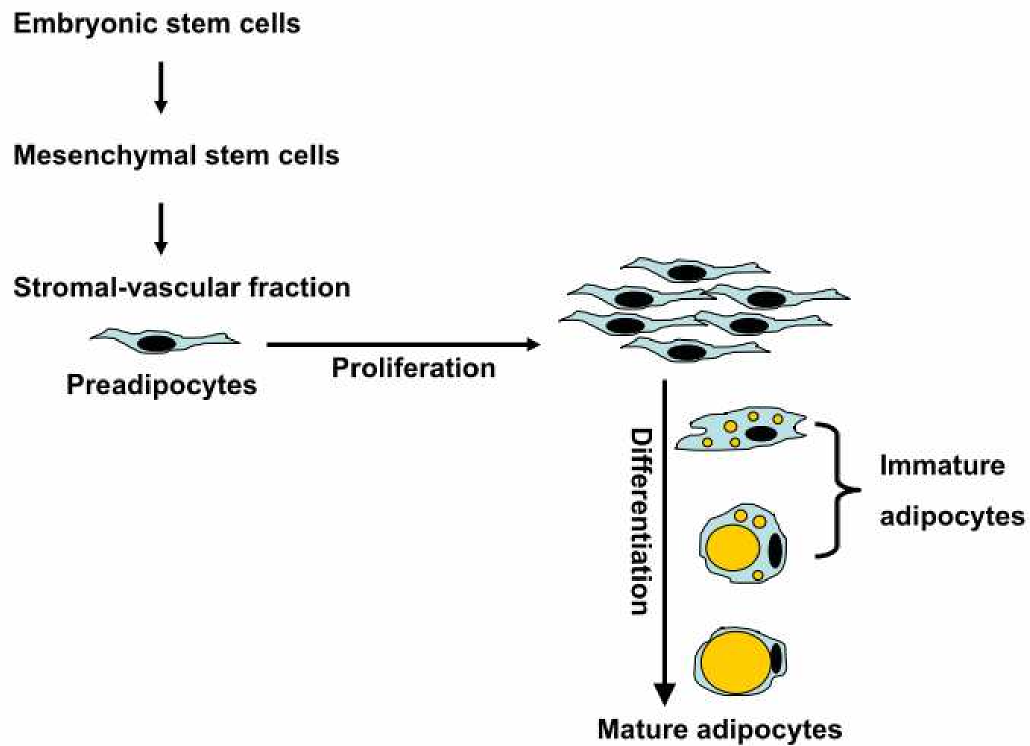


**Fig. 3:** **Schematic representation of the major processes of adipogenesis.** Deriving from mesenchymal stem cells, preadipocytes represent a cell group of the stromal-vascular fraction in the adipose tissue. After hormonal stimulation, preadipocytes proliferate (*in vitro* until confluency) before the process of differentiation can be induced. During differentiation, the cells collect lipid droplets until they become all lipid-filled mature adipocytes (modified from Gummersbach, 2008).

Adipogenesis is regulated by the hormonally induced coordinated expression and activation of two main groups of transcription factors, the CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR)-γ (Rosen et al., 2002). Some of the key features of adipogenic differentiation can be summarized as follows: At early differentiation, C/EBP-β and δ are expressed and increase the expression of PPAR-γ2. PPAR-γ2 heterodimerizes with retinoic X receptors (RXR) and activates C/EBP-α which exerts positive feedback on PPAR-γ2 until both transcription factors reach maximum levels as differentiation proceeds. Additionally, sterol responsive element binding protein-1, also called adipocyte determination and

differentiation factor-1 (ADD1/SREBP-1) can increase the transcriptional activity of PPAR-γ, while Forkhead box C2 can enhance the activity of C/EBP-α, PPAR-γ, and ADD1/SREBP-1 (Gregoire et al., 1998; Rosen and Spiegelman, 2000; Gregoire, 2001; Valet et al., 2002; Farmer, 2006).

### 1.2.5 Immune cell infiltration in white adipose tissue

Many cell types can infiltrate into WAT, including immune cells such as T-lymphocytes and macrophages with their number being increased in obese mice (Weisberg et al., 2003; Xu et al., 2003) and humans (Cancello et al., 2006; Curat et al., 2006). It has been suggested that especially the adipose tissue macrophages (ATM) induce local and systemic insulin resistance by promoting a proinflammatory micromilieu (Surmi and Hasty, 2008). As yet, it is neither clear what initiates the increased immune cell infiltration nor the sequence in which the different immune cell types appear in WAT. Severe hypertrophy of adipocytes, being mostly connected with FA flux, hypoxia, adipocyte cell death, increased leptin secretion, and endothelial dysfunction may be all contributing factors for immune cell infiltration into WAT (Surmi and Hasty, 2008).
There is evidence that recruitment of neutrophils occurs before monocyte infiltration into WAT (Elgazar-Carmon et al., 2008). However, the number of T-lymphocytes in WAT correlates with the waist circumference of type 2 diabetes patients. Additionally, it was shown in a mouse model of high-fat diet induced obesity that T-lymphocyte infiltration precedes the accumulation of macrophages in WAT, during the development of insulin resistance (Kintscher et al., 2008).
Monocytes infiltrate the tissue and differentiate to macrophages producing further cytokines such as TNF-α and IL-1 (Mosser, 2003), which are very potent proinflammatory activators of endothelial cells (Petzelbauer et al., 1993), adipocytes (Wang and Trayhurn, 2006), and other cell types. Additionally, an *in vitro* co-culture study of macrophages and adipocytes showed that the TNF-α, released from macrophages, interferes with adipocyte insulin signaling and induces lipolysis (Suganami et al., 2005). These mechanisms altogether may result in a positive feedback loop and are shown in Fig. 4.

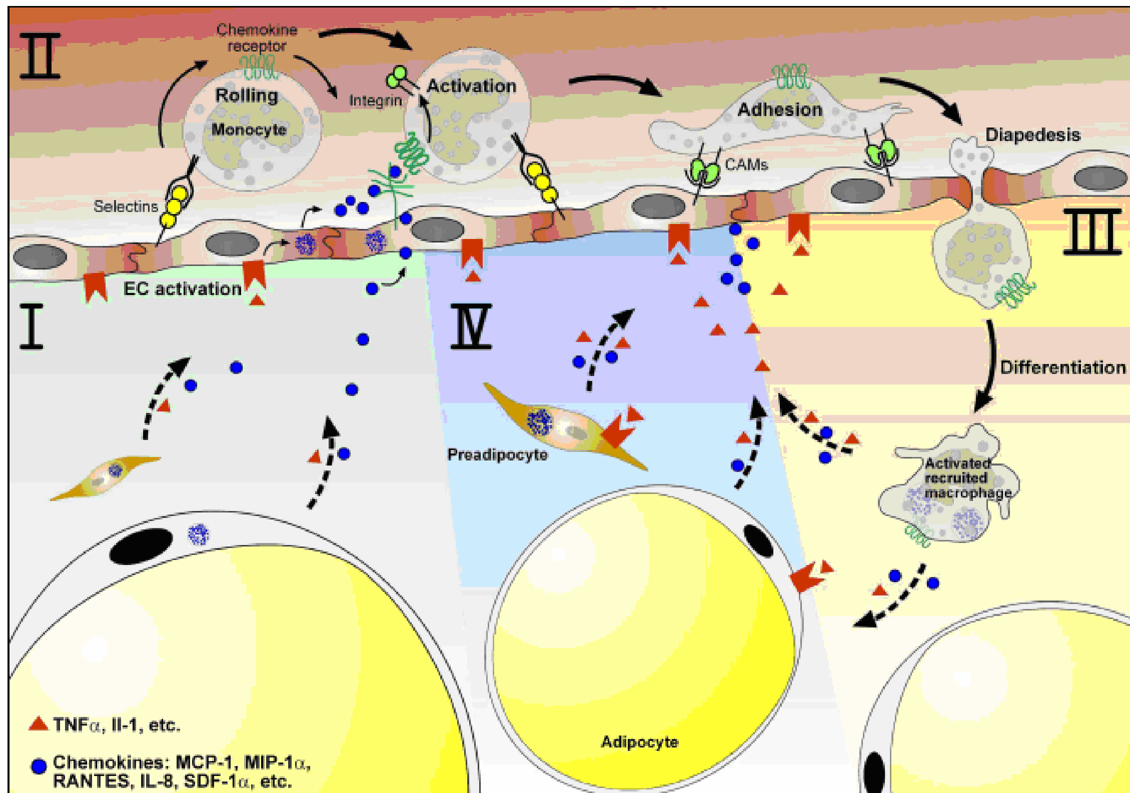


**Fig. 4:** **Monocyte infiltration into white adipose tissue.**
**I:** Some adipokines (e.g., TNF-α) are able to activate the endothelium. This is characterized by the expression of selectins and adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) and the release of chemokines from the endothelial cells. **II:** Activated monocytes and other immune cells adhere to the adhesion proteins on the endothelium via integrins which is followed by transmigration. **III:** After the diapedesis the monocytes differentiate into macrophages. Activated macrophages release large amounts of TNF-α and IL-1 and several chemokines. **IV:** The released cytokines from macrophages activate the endothelium itself and trigger the adipocytes and preadipocytes to change their secretion to a more inflammatory pattern. Abbreviations: CAMs, cellular adhesion molecules; EC, endothelial cells; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1 alpha; RANTES, regulated upon activation, normally T-expressed, and presumably secreted; SDF-1α, stromal cell-derived factor-1 alpha; TNFα, tumor necrosis factor-alpha (modified from Mack, 2011).

Monocyte recruitment may be initiated by an activated endothelium, adipocytes, preadipocytes, and other cells residing in WAT, producing chemoattractants such as MCP-1 and IL-8 (Fain et al., 2004; Fain and Madan, 2005). Macrophages can be distinguished in M1-classically activated and M2-alternatively activated subpopulations, as well as intermediate types having both M1 and M2 characteristics and are found in WAT (Lumeng et al., 2007; Zeyda et al., 2007; Bourlier et al., 2008). M1 macrophages are induced by inflammatory agents, have phagocytic activity, predominantly secrete proinflammatory cytokines and are important in the initiation of inflammation. M2 macrophages are induced by IL-4 and IL-13 and secrete predominantly antiinflammatory cytokines having a reparative and remodeling function by promoting the formation of new blood vessels and are important in terminating inflammatory process (Mosser, 2003; Bourlier et al., 2008). So far, most research on ATM infiltration has focused on their contribution to the development of obesity-associated pathologies through systemic effects in nonruminant species (Bouloumié et al., 2005; Schäffler and Schölmerich, 2010). In contrast to nonruminants, no report exists about immune cell infiltration into bovine AT.

## 1.3 Conjugated linoleic acid

### 1.3.1 Structure and origins

Conjugated linoleic acid (CLA) designates a group of unsaturated FA with two conjugated double bonds that are found naturally in milk and meat of ruminants. They were first discovered by Pariza and his group when investigating the carcinogenic components of grilled beef (Pariza and Hargraves, 1985). These modified FA are formed by the biological or industrial hydrogenation of linoleic acid (18:2, n-6), and were surprisingly found to have anticancer rather than procancer properties. The term CLA describes a group of positional and geometric isomers of linoleic acid, where one or both of the double bonds are either in the *cis* or the *trans* configuration and *trans*-posed to different positions along the acyl chain with the bonds separated by a simple carbon-carbon linkage rather than by the normal methylene group (-$CH_2$-).

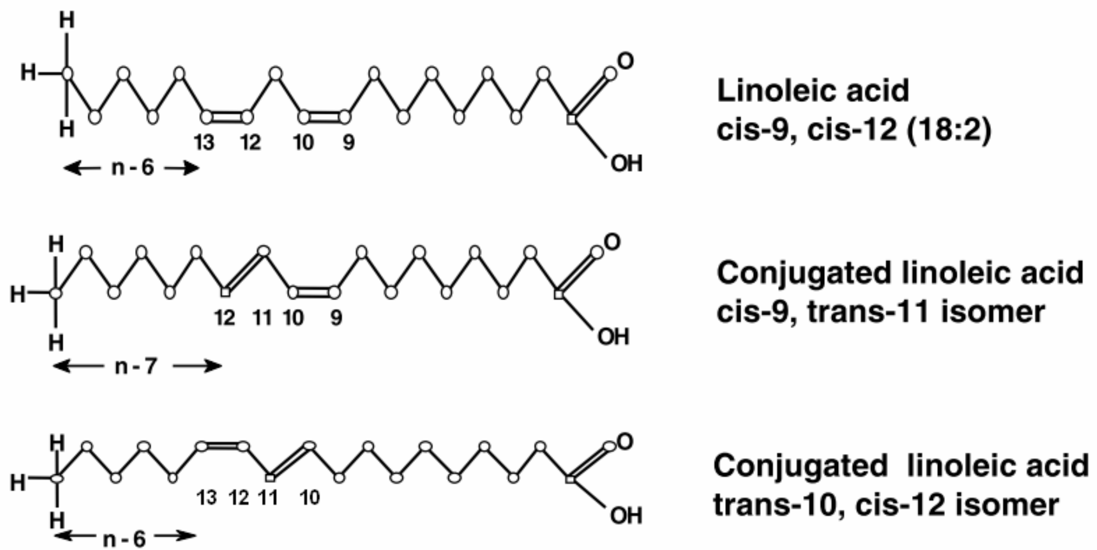


**Fig. 5: Structure of the linoleic acid and its two main conjugated derivatives**

The structure of the *cis*-9, *trans*-11 (*c*9,*t*11) and the *trans*-10, *cis*-12 (*t*10,*c*12) isomers of CLA, compared to their natural homologue linoleic acid are shown in Fig. 5. A number of *cis-cis*, *cis-trans*, *trans-cis* and *trans-trans* isomers with the double bonds at various locations along the acyl chain, from carbon-6 to carbon-15, have been identified by various chemical reductive, chromatographic, and spectroscopic techniques (Adlof, 2003; Christie, 2003; Dobson, 2003).

The major natural sources of CLA are the body tissues, predominantly the AT, of ruminants. Ruminant meat, particularly the fat associated with meat, is an important dietary source of CLA, contributing in the region of 25 to 30% of the total intake in Western populations (McGuire et al., 1999; Parodi, 2003). Milk, yogurt, cheese, and butter are also an excellent dietary source of CLA. The proportion of CLA ranges from 0.34 to 1.07% of the total fat in dairy products, and from 0.12 to 0.68% of the total fat in raw or processed beef products (Dhiman et al., 2005; Silveira et al., 2007; Mendis et al., 2008). The CLA content of food is dependent on several factors including the season and the animal’s breed, nutritional status, and age (Dhiman et al., 2005).

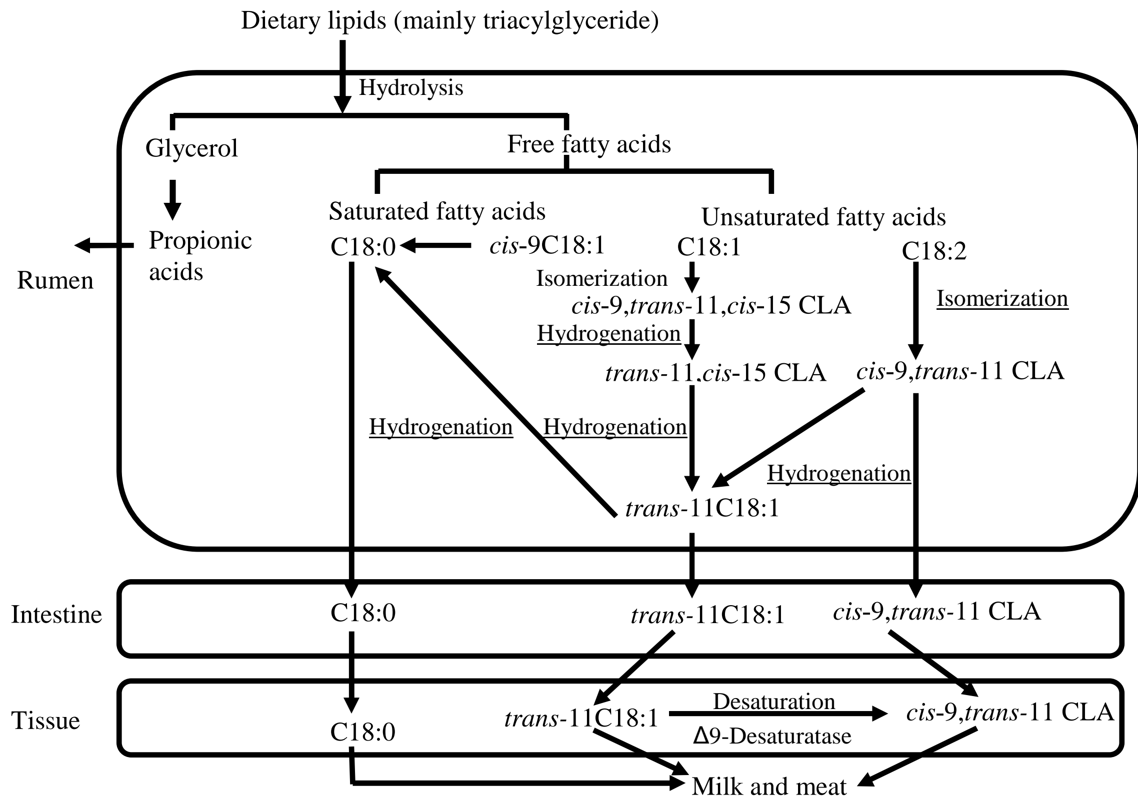


**Fig. 6:** **Biosynthesis of CLA and its incorporation into milk and meat of ruminants** (modified from Griinari and Bauman, 1999)

The rumen of ruminants is likened to a large anaerobic fermentation vat which contains microbes capable of biohydrogenating the ingested polyunsaturated FA (PUFA), derived largely from forage but also from other feed sources (e.g., added grain or fish oils). The first step in the biohydrogenation of dietary linoleic acid results in the formation of the *c*9,*t*11 isomer, due to isomerization and transposition of the delta-12 double bond. This is the most abundant natural isomer present in ruminant AT (over 90% of total CLA) and has been termed rumenic acid (McGuire et al., 1999; Parodi, 2003). Kepler and Tove (1967) extracted a linoleate isomerase from the rumen bacteria (*Butyrivibrio fibrisolvens*) which is responsible for the isomerization of linoleic acid into RA in the first step. Further hydrogenation of rumenic acid results in the production of *trans*-11-18:1 vaccenic acid, which is the major *trans*-monounsaturated FA present in the fats of ruminant food products (McGuire et al., 1999; Parodi, 2003). The pathways for the biosynthesis of CLA and its incorporation into milk and meat of ruminants are shown in Fig. 6.

### 1.3.2 Effective isomers and general health claims

Many CLA isomers exist and the biological properties can differ among CLA isomers. The predominant isomer in naturally occurring foods is the *c*9,*t*11, with minor but significant proportions of the *t*10,*c*12 (McGuire et al., 1999; Parodi, 2003). Although several other isoforms of CLA have been identified (i.e., *t*9,*t*11; *c*9,*c*11; *t*10,*t*11; and *c*10,*c*12), the *c*9,*t*11 and *t*10,*c*12 isomers appear to be the most biologically active (Wallace et al., 2007). The *c*9,*t*11 isomer comprises approx. 90% of CLA found in ruminant meats and dairy products and the *t*10,*c*12 isomer comprises the remaining 10%. This contrasts with commercial preparations of CLA where proportions of the two main isomers are usually equal, although the chemical method for synthesis will allow a variety of ratios for the two isomers in the final mixture (McGuire et al., 1999; Parodi, 2003).

The growing interest in CLA research over the past three decades attributed to identification of numerous beneficial health effects in various animal models and in cultures of various types of animal and human cells. Potential beneficial health effects of CLA include its anticarcinogenic, antiatherogenic, antiobesity, antidiabetogenic, and antiinflammatory properties. Dietary CLA also have reported to exert beneficial regulatory effects on immune function, lipid and eicosanoid metabolism, cytokine and immunoglobulin production, and can modulate the expression of a number of genes, either directly or through specific transcription factors involved in the many metabolic processes they affect (Nicolosi et al., 1997; Park et al., 1997; Cook and Pariza, 1998). Dietary CLA supplementation has been reported to change body composition by reducing fat to lean body mass ratio in several experimental animals including mice (Park et al., 1997; DeLany et al., 1999; Park et al., 1999), pigs (Ostrowska et al., 1999), rats and chickens (Pariza et al., 1996), also in obese humans (Blankson et al., 2000).

### 1.3.3 Spectrum of activity and mechanisms of action of CLA in monogastric species

Regarding the fat-decreasing effect, there is much evidence showing that CLA decreases preadipocyte differentiation in animal (Brodie et al., 1999; Kang et al., 2003) and human (Brown et al., 2003) preadipocytes. This finding is in contrast to another study that has found no significant effect of CLA on the expression of key transcription factors of adipogenesis (Choi et al., 2000). One of the mechanisms proposed to explain the effect of CLA on AT mass is via the PPAR. The PPAR are ligand-activated nuclear hormone receptors that control the expression of genes involved in cellular metabolism and differentiation (Spiegelman, 1998; Desvergne and Wahli, 1999). Both *c*9,*t*11 and *t*10,*c*12 are potent ligands of PPAR-α and -δ, with PPAR-α showing the highest affinity (Moya-Camarena et al., 1999). It has been suggested that increased β-oxidation and energy expenditure due to activation of PPAR-α may contribute to the antiadipogenic effect of the *t*10,*c*12 isomer (Sakono et al., 1999; Delany and West, 2000). Conversely, one study using PPARα- knock-out mice demonstrated that the effect of CLA on body composition is independent of PPARα activation (Peters et al., 2001). Brown et al. (2003) reported that the *t*10,*c*12, but not the *c*9,*t*11 isomer of CLA decreases glucose and FA uptake and oxidation. Moreover, the *t*10,*c*12 isomer decreases preadipocyte differentiation by decreasing PPAR-γ expression (Hargrave et al., 2002; Brown and McIntosh, 2003; Brown et al., 2003), whereas the *c*9,*t*11 isomer increases the expression of PPAR-γ.

Regarding the effects of CLA on adipokine secretion, the *c*9,*t*11 and *t*10,*c*12 isomers seem to have opposing effects. The *t*10,*c*12 isomer promotes ATM infiltration and decreases leptin and adiponectin secretion, which in turn contributes to insulin resistance and hyperinsulinemia in CLA fed animals and humans (Clément et al., 2002; Poirier et al., 2006; Pérez-Matute et al., 2007). CLA-induced shifts in the expression of these adipokines are therefore important both for metabolism and immune function. The *t*10,*c*12 isomer rapidly induces inflammatory factors, such as TNF-α and IL-6 gene expression in WAT (Ahn et al., 2006; Poirier et al., 2006). *In vitro* studies with 3T3-L1 and human adipocytes have also shown that the *t*10,*c*12 isomer directly stimulates the IL-6 secretion (Chung et al., 2005; Poirier et al., 2006). In contrast, the *c*9,*t*11 isomer improves insulin and glucose metabolism. Supplementation with *c*9,*t*11 isomer results

in low ATM infiltration with concomitant decrease in inflammatory cytokines, which may improve insulin sensitivity (Moloney et al., 2007).
In addition to the initial health benefits, CLA supplementation has also been reported to be associated with some deleterious effects both in mice and humans (Tsuboyama-Kasaoka et al., 2000; Riserus et al., 2004). Supplementation with CLA induced insulin resistance, stable hyperinsulinemia, and fatty liver (West et al., 1998; Delany et al., 1999; Tsuboyama-Kasaoka et al., 2000; Clément et al., 2002). These effects are entirely attributable to the *t*10,*c*12 isomer (Clément et al., 2002), but are also observed when using commercial CLA mixture (West et al., 1998). These effects might be the negative consequences of increased energy expenditure, fecal energy loss, apoptosis, FA oxidation, and lipolysis as well as decreased preadipocyte differentiation and lipogenesis (Wang and Jones, 2004).

### 1.3.4 Use of CLA in dairy cows

The use of CLA as a supplement to dairy cows was initially conceived to increase its content in milk for the benefit of consumer health. The findings of Loor and Herbein (1998) and Chouinard et al. (1999) with abomasal infusion studies demonstrated that CLA supplements decreased milk fat yield by inhibiting *de novo* lipogenesis in the mammary gland. Subsequent work in lactating dairy cows by Baumgard et al. (2000) demonstrated that the isomer involved in that effect was the *t*10,*c*12 isomer, albeit the *c*9,*t*11 isomer had less effect (Perfield II et al., 2007). These findings are also consistent with other studies in nursing women (Masters et al., 2002) and lactating rats (Ringseis et al., 2004). These results suggested a great potential for the use of CLA supplement as a tool to manipulate milk composition. The milk fat-decreasing effects of CLA have been also reported in other supplementation trials (Giesy et al., 2002; Perfield II et al., 2002; Bernal-Santos et al., 2003; von Soosten et al., 2011). Those studies were done with high-yielding cows and demonstrated decreased milk fat content but variable results regarding milk and protein yield. The high-yielding dairy cows are challenged to meet its nutritional requirements, particularly in early lactation. Thus, inhibition of milk fat excretion may have favored positive responses in milk and milk protein production (Mackle et al., 2003; de Veth et al., 2006; Kay et al., 2007). This could be beneficial for saving energy to counteract the

physiological NEB in early-lactating dairy cows. However, CLA supplementation seems to leave body fat mostly unaffected in dairy cows because body condition score (BCS), NEFA, and plasma leptin concentrations are not different in CLA supplemented cows versus control group (Baumgard et al., 2002; Kay et al., 2006).
In contrast to the studies in monogastric species, no reports exist about body fat-decreasing effects of CLA in ruminants. Also reports about immune cell infiltration into bovine AT, in particular addressing the heterogeneity of the SC versus VC depots are lacking. Herein, we hypothesized that dietary supplementation of CLA has site-specific effects on adipocyte size of different fat depots in dairy cows during early lactation. It was also hypothesized that phagocytic immune cell infiltration in bovine AT is independent of immunologic and metabolic adaptations during early lactation. Therefore, the present research study has been designed with the following objectives:

1) To characterize different SC and VC fat depots of dairy cows in terms of adipocyte size during early lactation,

2) To investigate phagocytic immune cell infiltration in bovine AT,

3) To evaluate the effects of CLA on adipocyte size of different fat depots of dairy cows during the first 105 DIM, and

4) To investigate the potential effects of CLA on the invasion of phagocytic immune cells into WAT of dairy cows.

The goal of the aforementioned approaches was to improve the knowledge about the effects of long-term dietary CLA supplementation on body fat depots of dairy cows, with regards to the changes during early lactation. The results are of general importance to Animal Science and provide a general basis for the regulatory role of AT in ruminants.

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## Physiological and conjugated linoleic acid-induced changes of adipocyte size in different fat depots of dairy cows during early lactation

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## Abstract

The aim of this study was to investigate the effects of lactation and conjugated linoleic acid (CLA) supplementation on adipocyte sizes of subcutaneous (SC) and visceral (VC) fat depots in primiparous dairy cows during the first 105 days in milk (DIM). German Holstein-Friesian cows (n = 25) were divided into a control (CON) and a CLA group. From the first DIM until sample collection, CLA cows were fed 100 g of CLA supplement/d (about 6% of *c*9,*t*11 and *t*10,*c*12 isomers each), whereas the CON cows received 100 g/d of a fatty acid mixture instead of CLA. The CON cows (n = 5 each) were slaughtered at 1, 42, and 105 DIM, and the CLA cows (n = 5 each) were slaughtered at 42 and 105 DIM. Adipose tissues from three SC depots (tailhead, withers, and sternum) and from three VC depots (omental, mesenteric, and retroperitoneal) were sampled. Hematoxylin-eosin staining was done to measure adipocyte area ($\mu m^2$). Retroperitoneal adipocyte sizes were mostly larger than adipocytes from the other sites independent of lactation time and treatment. Significant changes related to duration of lactation were limited to retroperitoneal fat: adipocyte sizes were significantly smaller at 105 DIM than at 1 DIM in CON cows. Adipocyte sizes were decreased in SC from tailhead at 105 DIM and from sternum at 42 DIM in CLA versus CON cows, whereas for VC depots adipocyte sizes were decreased in mesenteric fat at 42 and 105 DIM, and in omental and retroperitoneal fat, at 105 DIM in CLA versus CON cows. Within the CLA group, adipocyte sizes were smaller in the SC from the tailhead at 105 DIM than at 42 DIM. Adipocyte sizes and depot weights were significantly correlated in SC depots (r = 0.795, $P < 0.01$) in the CLA group and in retroperitoneal fat both in the CON (r = 0.698, $P < 0.01$) and the CLA (r = 0.723, $P < 0.05$) group. In conclusion, CLA-induced decreases in adipocyte size indicate lipolytic or antilipogenic effects of CLA, or both effects, on adipose tissue in primiparous dairy cows.

Key words: adipocyte, conjugated linoleic acid, dairy cow, lactation

## Introduction

The most metabolically stressful period in dairy cows is the early lactation, when milk production abruptly rises and an enormous metabolic drain is imposed on the body. The energy requirements at this period exceed the available energy from feed intake, resulting in a condition of negative energy balance (NEB). High-yielding dairy cows are then susceptible to various metabolic disorders and compromised immune response and fertility (Butler and Smith, 1989; Mallard et al., 1998). To meet the nutritional demands of milk synthesis, dairy cows need to mobilize body reserves (Bauman and Elliot, 1983). When the decrease in body fat is refined (i.e., taking the share of different fat depots into consideration), the proportional change in different time intervals after calving is different when comparing the subcutaneous (SC) depots with different visceral (VC) depots (Butler-Hogg et al., 1985).

Conjugated linoleic acid (CLA) comprises a group of geometric and positional conjugated dienoic isomers produced during biological or industrial hydrogenation of linoleic acid (C18:2, n-6). *Cis*-9, *trans*-11 (*c*9,*t*11) is the predominant natural CLA isomer occurring exclusively in ruminant meat and milk; commercially available CLA preparations contain this isomer together with the *trans*-10, *cis*-12 (*t*10,*c*12) isomer in equimolar concentrations (Poirier et al., 2006). Conjugated linoleic acid has attracted considerable attention because of their antiobesity, anticarcinogenic, antiatherogenic, antidiabetogenic and immunomodulating properties in certain animal models (Belury, 2002; McLeod et al., 2004). Dietary CLA supplementation alters lipid metabolism and causes a reduction in milk fat secretion in lactating animals (Loor and Herbein, 1998; Ringseis et al., 2004). The *t*10,*c*12 isomer has been reported to be specific for the mammary gland and responsible for the decreased milk fat synthesis in lactating dairy cows (Baumgard et al., 2001), whereas the *c*9,*t*11 isomer had less effect (Perfield II et al., 2007). Nevertheless, the *t*10,*c*12 isomer causes a decreased body fat accretion in growing animals by decreasing *de novo* lipogenesis (Bauman et al., 2000). In addition, dietary CLA supplementation has been found to decrease body fat mass in several monogastric species such as in pigs (Ostrowska et al., 1999), mice (Poirier et al., 2005; Halade et al., 2010) and also in obese humans (Blankson et al., 2000). Studies with bovine preadipocyte culture suggest that the *t*10,*c*12 isomer inhibited differentiation of preadipocytes to mature adipocytes (House et al., 2005; Smith et al., 2009; Lengi and

Corl, 2010). Moreover, CLA-induced decrease in body fat mass in rats was due to a decrease in adipocyte size rather than adipocyte number (Azain et al., 2000). However, CLA supplementation seems to leave body fat unaffected because BCS and NEFA and leptin concentrations were not different from untreated controls (CON) at least when using relatively short treatment periods in dairy cows (Baumgard et al., 2002; Kay et al., 2006). To our knowledge, no report about body fat-decreasing effects of CLA in dairy cows exists, but more detailed histological studies of adipose tissues, in particular addressing the heterogeneity of SC versus VC fat depots are lacking. Therefore, we assessed the mean adipocyte areas ($\mu m^2$) of SC and VC to characterize 6 different fat depots in terms of adipocyte size and to investigate: 1) what physiological changes in adipocyte size of different fat depots occur in primiparous dairy cows during the first 105 DIM, and 2) whether and to what extent a rumen-protected CLA supplement changes the adipocyte size of different fat depots in dairy cows.

## Materials and methods

### *Animals, diets, and treatments*

The animal experimentation was done according to the European Union Guidelines and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, File number 33.11.42502-04-071/07), Oldenburg, Germany. The experimental design and main results with regard to performance and body composition are described elsewhere (von Soosten et al., 2010). In brief, German Holstein-Friesian cows (n = 25) with a mean BCS of 3.0 (scale = 1 to 5) were housed in a free-stall barn at the Experimental Station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany. The cows were fed according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). Water was provided ad libitum. Before parturition, cows received a diet consisting of a partial mixed ration (PMR, 60% corn silage and 40% grass silage, 6.7 MJ of $NE_L$/kg of DM) on a DM basis ad libitum and 2 kg of concentrate/d (6.7 MJ of $NE_L$/kg of DM). The feed ingredients and chemical composition of the postpartum diets fed from parturition until slaughter with the FA profiles of the fat supplements used are shown in Table 1.

The cows were randomly allocated to either the CON (n = 15) or the CLA (n = 10) group. From 1 DIM until sample collection, the CLA cows were fed 100 g of rumen-protected CLA (Lutrell pure; BASF, Ludwigshafen, Germany) per d, containing equal proportions of each of the *c*9,*t*11 and *t*10,*c*12 isomer. The pelleted CLA-concentrate contained about 6% each of the CLA isomers (calculated proportion in the CLA-concentrate; D. von Soosten, FLI, Braunschweig, Germany, personal communication). For the CON group, CLA were substituted by 100 g of an analogous fatty acid mixture (Silafat, BASF). The CON cows (n = 5 each) were slaughtered at 1, 42, and 105 DIM, whereas the CLA cows (n = 5 each) were slaughtered at 42 and 105 DIM. Energy balance was calculated according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001).

***Measurements and sample collection***

At slaughter, adipose tissue samples from SC and VC depots were immediately excised from the cows. Adipose tissues from three SC depots (tailhead, withers and sternum) and from three VC depots (omental, mesenteric and retroperitoneal) were sampled. The mesenteric fat was collected from the fat around the jejunum and the retroperitoneal fat was sampled from the pararenal fat, located superficial to the renal fascia. All adipose tissue depots were then dissected and weighed. Milk samples were taken twice per week. Blood was collected in Vacutainer tubes containing sodium heparin or potassium EDTA (Becton Dickinson Vacutainer Systems USA, Rutherford, NJ) on the day of slaughter by venipuncture before slaughtering. Blood samples were analyzed for plasma concentrations of NEFA and BHBA with commercial kits (NEFA-C, Wako Chemicals GmbH, Neuss and RANBUT, Randox Laboratories GmbH, Wülfrath, Germany). The empty BW (EBW) was calculated as sum of the weights of all body parts of the cows which were recorded during the slaughter process without claws cut off, ingesta, and contents of urinary and gall bladder.

### *Histomorphology*

Adipose tissue samples (n = 150) were fixed in 4% paraformaldehyde (Roth, Karlsruhe, Germany) overnight, dehydrated with an ascending graded series of isopropanol (Roth), cleared in Rotihistol (Roth), infiltrated with a mixture of Rotihistol and melted paraffin (50:50 mixture) at 60ºC and finally embedded into paraffin wax. Ten-micrometer-thick tissue sections were cut on a rotation microtome (SLEE, Mainz, Germany) cooled with Cryospray (Roth) and mounted on SuperFrost Plus slides (Menzel, Braunschweig, Germany). They were dried at 60°C for 2 h and subsequently at 37°C for overnight. After deparaffinizing in Rotihistol followed by rehydration with a descending graded series of isopropanol, finally the sections were stained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and 1% wt/vol eosin Y (AppliChem, Darmstadt) and mounted with Kaiser's glycerol gelatine (Merck). All stained tissue sections were studied by light microscopy by one person to avoid individual variation and samples were blind-analyzed to prevent any bias. From each stained section, five random images were taken at 100× magnification on a light microscope (Leica DMR, Leica Microsystems, Wetzlar, Germany) with a JVC digital color camera KY-F75U (Hachioji Plant of Victor Company, Tokyo, Japan) connected to a computer. Adipocyte areas of 100 adipocytes from five randomly selected fields per section were measured using DISKUS software (4th version; Hilgers, Koenigswinter, Germany) and then averaged. The unit of mean adipocyte area was square micrometer ($\mu m^2$).

### *Statistical analyses*

Data for all variables (EBW, depot weight, adipocyte area, energy balance, and plasma NEFA and BHBA concentrations) were tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variances with the Levene's test. Statistical analyses were made using general linear model (GLM) followed by a Bonferroni post-hoc test to compare the differences within the CON groups and within the fat depots of both CON and CLA groups. For GLM, significance was defined as $P < 0.05$ and trends for physiological responses were declared at $P < 0.10$. The Student's *t*-test was used to analyze the differences between the CON and the CLA group at 42 and 105 DIM as well as within the CLA group on the respective day. Therefore, the

threshold for statistical significance was defined as $P < 0.017$ after the Bonferroni α-correction for multiple comparisons (n = 3; $\alpha = 0.05/3 = 0.017$). Pearson correlation coefficients were used to examine the relations between adipocyte sizes and depot weights of different adipose tissues, and between adipocyte sizes and plasma NEFA and BHBA concentrations, respectively. All analyses were done using SPSS software, version 17.0 (SPSS, Inc., Chicago, Illinois). Values are given as means ± SEM.

## Results

The animals were in NEB at 1 DIM, whereas higher ($P < 0.001$) and positive EB values were reached in both groups by 42 and 105 DIM (Fig. 1). Empty BW was not different between sampling day or groups; EBW was numerically lower at 42 DIM ($P < 0.10$) than at 1 DIM in CON cows and lower than at 105 DIM in both CON and CLA cows (Table 2). Comparing the different fat depot weights from 1, 42, and 105 DIM, differences ($P < 0.05$) were limited to the retroperitoneal depot for which 52 and 63% of the mass recorded at 1 DIM were reached at 42 and 105 DIM, respectively (Table 2). For all other depots, numerical decreases, not reaching the level of significance, were observed from 1 to 42 DIM. From 42 to 105 DIM, numerical, but insignificant, increases were noticed for SC and omental fat, but not for mesenteric fat in the CON cows. In CLA cows, all fat depots were consistently lighter at 105 versus 42 DIM, but again, the differences did not reach the level of significance (Table 2).

### *Plasma NEFA and BHBA concentrations*

In CON cows, plasma NEFA concentrations were higher at 1 DIM than at 42 DIM (2.4-fold; $P = 0.01$) and at 105 DIM (2.6-fold; $P = 0.007$), respectively. No differences existed in CLA versus CON groups and also within the CLA group (Fig. 1). Concerning plasma BHBA concentrations, no differences were observed within the CON group, between the CON and the CLA group, or within the CLA group.

### *Histomorphological study*

Histomorphological examination revealed that the adipocytes were individually held in place by delicate reticular fibers clustering in lobules bounded by fibrous septa within the adipose tissue both in SC and VC depots. In SC depots, higher portions of connective tissue fibers were found than in VC depots (Fig. 2). Typical, unilocular, signet-ring shaped adipocytes and small multilocular adipocytes were found in all the fat depots examined. Small arteries and venules were present within the septa in all adipose tissues.

### *Adipocyte sizes of different fat depots in CON and CLA cows*

In CON cows, retroperitoneal adipocyte sizes were 1.4-fold larger ($P = 0.01$) than in SC depot from the sternum at 1 DIM (Table 3). Adipocyte sizes tended to be larger ($P < 0.10$) in retroperitoneal fat than that of SC depots from the tailhead and withers and of VC depot from mesenteric fat in CON cows at 1 DIM. The same trend ($P < 0.10$) for larger adipocyte size was also found in retroperitoneal fat when compared with SC depot from the withers in CON cows at 105 DIM. In CLA cows, retroperitoneal adipocyte sizes were 1.4-fold larger than those in SC depots from the withers ($P = 0.041$) and sternum ($P = 0.037$) at 42 DIM (Table 3). Representative pictures of an SC depot (from tailhead) and also of a VC depot (from retroperitoneal) are shown in Fig. 2 for each treatment and sampling day.

### *Influence of lactation time on adipocyte size during early lactation*

No effect of duration of lactation on adipocyte sizes of SC depots from the tailhead and sternum in CON cows was observed. Adipocyte sizes of SC depot from the withers in CON cows tended to be smaller ($P < 0.10$) at 105 DIM than at 1 DIM (Table 3). Conversely, for VC depots, significant changes related to the duration of lactation were limited to retroperitoneal fat. In CON cows, retroperitoneal adipocyte sizes were 1.2-fold smaller ($P < 0.05$) at 105 DIM than at 1 DIM (Table 3), and tended to be smaller ($P < 0.10$) at 42 DIM than at 1 DIM. Moreover, no effect of duration of lactation on adipocyte sizes of omental and mesenteric fat depots was observed.

***Effects of CLA on adipocyte size during early lactation***

The SC depot from the tailhead had 1.9-fold smaller (*P* = 0.001) adipocyte sizes in CLA cows than in CON cows at 105 DIM, whereas SC depot from the sternum had 1.3-fold smaller (*P* = 0.01) adipocyte sizes in CLA cows than in CON cows at 42 DIM (Table 3). For VC depots, the adipocyte size was decreased 1.5-fold (*P* = 0.011) in omental fat in CLA versus CON cows at 105 DIM. In mesenteric fat, adipocyte size was 1.5-fold smaller in CLA cows than in CON cows both at 42 DIM (*P* = 0.002) and 105 DIM (*P* = 0.008) each. Moreover, the adipocyte size of retroperitoneal fat was 1.5-fold smaller (*P* = 0.006) in CLA versus CON cows at 105 DIM (Table 3). Within the CLA group, adipocyte size of SC depot from the tailhead was decreased 1.4-fold (*P* = 0.005) from 42 to 105 DIM (Table 3). To assess the extent of CLA effects, percentage differences (%) between adipocyte sizes of different fat depots of CON and CLA groups were calculated; for both sampling days, the corresponding CON group was taken as 100%. Conjugated linoleic acid supplemented cows had 16, 22, and 24% numerical decreases in adipocyte sizes in SC depots from the tailhead, withers, and sternum, respectively, compared with the CON cows at 42 DIM, whereas 47, 33, and 29% numerical decreases in adipocyte sizes were observed in SC depots from the tailhead, withers and sternum, respectively, in CLA cows compared with the CON cows at 105 DIM. For VC depots, the CLA cows had 22, 32, and 16% numerical decreases in adipocyte sizes in omental, mesenteric, and retroperitoneal fat, respectively, compared with the CON cows at 42 DIM, whereas 32, 33, and 34% numerical decreases in adipocyte sizes were observed in omental, mesenteric, and retroperitoneal fat, respectively, in CLA cows compared with the CON cows at 105 DIM.

***Correlations between adipocytes sizes and depot weights of different adipose tissues***

We examined the correlation between adipocyte areas and depot weights of SC and different VC depots at 1, 42, and 105 DIM. For the entire SC depot, the correlation was calculated between the mean adipocyte areas of all 3 SC depots examined and the weight of the entire SC fat. In CON cows, a strong positive correlation was found in retroperitoneal fat depot (r = 0.698, *P* = 0.004, n = 15). In CLA cows, strong positive relationships were observed in the SC depot (r = 0.795, *P* = 0.006, n = 10) and in the

retroperitoneal depot (r = 0.723, $P$ = 0.018, n = 10). Very strong relationships were found in retroperitoneal depot both in CON (r = 0.905, $P$ = 0.035, n = 5) and CLA cows (r = 0.892, $P$ = 0.042, n = 5) at 105 DIM.

***Correlations between adipocytes sizes and plasma NEFA and BHBA concentrations***

We examined the correlation between adipocyte areas of different SC and VC depots and plasma NEFA and BHBA concentrations, respectively, at 1, 42, and 105 DIM. In CON cows, a strong positive correlation was found between retroperitoneal adipocyte sizes and plasma NEFA concentrations (r = 0.698, $P$ = 0.004, n = 15), whereas no significant relationships were detectable in any of the fat depots from the CLA cows (Table 4). A very strong inverse relationship was found between SC adipocyte sizes from withers and plasma NEFA in CON cows at 42 DIM (r = –0.990, $P$ = 0.001, n = 5). However, no significant relationships were found between adipocyte sizes and plasma BHBA concentrations in any of fat depots from the CON cows, whereas a strong positive correlation was found only in the SC depot from the sternum (r = 0.651, $P$ = 0.041, n = 10) in CLA cows. Very strong positive relationships were found in omental depot of CON cows (r = 0.879, $P$ = 0.05, n = 5) and in SC depots from the tailhead (r = 0.989, $P$ = 0.001, n = 5) and from the sternum (r = 0.880, $P$ = 0.049, n = 5) of CLA cows at 105 DIM (Table 4).

## Discussion

The experimental primiparous cows experienced NEB on first sampling day at 1 DIM, but were returned to positive EB on the day of second slaughter series i.e., 42 DIM irrespective of CLA treatment. This is in contrast to the duration of NEB in pluriparous cows (Bauman and Currie, 1980). However, the trend for lower EBW at 42 DIM than at 1 DIM might reflect the effects of the preceding NEB; conversely, numerically higher EBW at 105 DIM than at 42 DIM may indicate the effects of positive EB in dairy cows, irrespective of CLA treatment (Table 2; Fig. 1).

The size of adipocytes within an individual can vary considerably from depot to depot. It has been reported that the adipocytes from retroperitoneal fat have consistently larger adipocyte areas than the adipocytes from SC, omental and mesenteric fat depots in cows

(Pike and Roberts, 1984). In the present study, we found that the adipocytes from retroperitoneal fat had significantly larger adipocyte areas than that of SC from the sternum in CON cows at 1 DIM. A tendency of increasing adipocyte size of retroperitoneal fat compared to that of SC depots from the tailhead and withers and of VC depot from mesenteric fat in CON cows at 1 DIM was observed. This could be related to metabolic differences between adipocytes from various depots, as known from monogastric species (Arner, 1998). Indeed, gene expression profiles from SC and VC fat depots in cattle (Hishikawa et al., 2005) support the notion of characteristic biochemical differences in these depots.

***Physiological changes of adipocyte size during early lactation***

Bovine adipose tissue undergoes coordinated metabolic adaptations via a decrease in *de novo* lipogenesis and an increase in rates of lipolysis during early lactation (McNamara and Hillers, 1986). In the present study, we observed the physiological changes of adipocyte sizes of different fat depots during the first 105 DIM. Adipocytes from different regions have been reported to show a differential response during pregnancy and lactation in women, due to the condition of changing fat cell metabolism in different regions (Rebuffe-Scrive et al., 1985). Fat mobilization occurs in cows from late pregnancy through to midlactation, and the different depots respond to changing physiological states in different ways. Butler-Hogg et al. (1985) estimated from their dissection experiments that the rates of change on a g/d basis are highest from calving to peak-lactation (42 DIM) in all depots whereby the intermuscular and the SC depots are the main contributors. Until midlactation (154 DIM), the rate of change in SC fat was only about 1% of the rate of change observed during the preceding period, whereas omental and mesenteric fat were further mobilized at 13 and 28% of the preceding rate. Perirenal retroperitoneal fat was not further decreased after peak-lactation (Butler-Hogg et al., 1985). In our study, we found that the adipocyte size of retroperitoneal fat was 1.2-fold smaller in CON cows at 105 DIM than at 1 DIM, and also tended to be smaller at 42 DIM than at 1 DIM, suggesting that retroperitoneal fat was preferentially mobilized during peak-lactation. This finding is in agreement with the aforementioned study (Butler-Hogg et al., 1985). Moreover, the retroperitoneal fat depot weight was significantly decreased at 42 DIM and numerically decreased at 105 DIM compared

with 1 DIM, suggesting that the retroperitoneal fat depot reacted most sensitively to the advancement of lactation.

Although significant changes related to duration of lactation were limited to retroperitoneal fat, data in Table 3 showed numerically decreased adipocyte size up to 105 DIM in almost all of the fat depots examined. Active ongoing lipolysis, as evident from plasma NEFA concentrations, was thus, limited to the first sampling day at 1 DIM (Fig. 1). This rapid rise in plasma NEFA might be due to the stress of calving (Grummer, 1995). However, the time course of adipocyte decrease accompanying or following lipolysis or both, has not been characterized, at least to our knowledge. We thus speculate that the effects of lipolysis on adipocyte size were continued up to 105 DIM.

***CLA-induced changes of adipocyte size during early lactation***

The efficacy of the CLA supplementation in terms of milk fat decrease was confirmed in dairy cows during early lactation (D. von Soosten and S. Dänicke, FLI, Braunschweig, Germany, unpublished observations). We herein demonstrate for the first time CLA-induced changes of adipocyte size in dairy cows during early lactation. It has been reported that CLA decreased adipocyte size in rats, as to the significant reduction in mean adipocyte diameter (Sisk et al., 2001). The use of mean adipocyte diameter is an alternative method for estimating body fat and adipose tissue cellularity in lactating cows (Waltner et al., 1994).

The body fat-decreasing effects of CLA have been also reported in other studies in rats (Azain et al., 2000; Noto et al., 2007) and pigs (Corino et al., 2005). Those studies were limited in that the adipocyte size was measured in only one fat depot (i.e., SC). DeLany et al. (1999) showed that dietary CLA supplementation decreased body fat accumulation in VC depots in mice. Poulos et al. (2001) found that CLA increased the proportion of smaller adipocytes and decreased the proportion of larger adipocytes of retroperitoneal fat depot in rat pups. In the present study, we observed that dietary CLA supplementation decreased adipocyte sizes of different SC and VC depots to different extents in dairy cows during the first 105 DIM. Moreover, the extent of CLA-induced decreased in adipocyte sizes is consistently higher at 105 DIM than at 42 DIM both for SC and VC depots, as indicated by the percent decreases. Specific regional metabolic

differences that explain the differential regional response to CLA treatment are not well clarified. The regional differences could be explained by differences in accretion rates of different fat depots during the trial (Djian et al., 1983). It has also been reported that adipocytes in SC and VC depots show differences in basal metabolic properties, for example, in regulating volume and lipid composition as well as present differential gene expression profiles in humans (Montague et al., 1998) and cattle (Hishikawa et al., 2005). Within the CLA group, the adipocyte size significantly decreased only in SC depot from the tailhead, suggesting that the adipocyte size may not differ with the time of CLA supplementation.

***Mechanisms of action of CLA***

The mechanisms behind a possible fat-decreasing effect of CLA are currently not well clarified. Both *in vitro* and *in vivo* studies in monogastric species demonstrated that CLA decreases adipocyte cellularity by decreasing adipocyte proliferation (Brodie et al., 1999; Evans et al., 2000) or adipocyte size (Azain et al., 2000; Brown et al., 2001; Evans et al., 2000). The *t*10,*c*12 isomer has been associated with decreased body fat mass in mice, whereas no such changes in body composition have been observed using the *c*9,*t*11 isomer (Park et al., 1999b). The *t*10,*c*12 isomer also induces adipocyte apoptosis in mice (Tsuboyama-Kasaoka et al., 2000). In addition, this CLA isomer decreases preadipocyte differentiation by down-regulation of peroxisome proliferator-activated receptor gamma expression in humans (Brown and McIntosh, 2003). The CLA preparation used in the present study contained equal amounts of *c*9,*t*11 and *t*10,*c*12 isomers. The effects of CLA presented in this study could, therefore, result from either or both of these isomers.

The metabolic effects reported for CLA in adipose tissue suggest increased fat mobilization, as evidenced by increasing lipolysis and glycerol release, and decreased lipid deposition, as evidenced by decreasing activity of lipoprotein lipase (Park et al., 1997). These changes in lipid metabolism were exclusively associated with the *t*10,*c*12 isomer of CLA (Park et al., 1999b). The antilipogenic effect of CLA is accounted for by decreased lipid filling of adipocytes in CLA supplemented rats (Azain et al., 2000). Decreased rates of lipogenesis have also been suggested to be the mechanism for the decrease in body fat accretion in CLA supplemented humans (Brown et al., 2003),

growing pigs (Ostrowska et al., 1999) and mice (Tsuboyama-Kasaoka et al., 2000). Thus, the effects of CLA on adipogenesis and lipid metabolism in animals are isomer-, dose-, time-, and species-dependent (Evans et al., 2002). West et al. (1998) reported decreases in specific adipose tissue depots in male mice fed CLA ranging from 40 to 80%. Spalding et al. (2008) suggested that adipocyte number remains stable in adulthood in lean and obese individuals, although significant weight loss can result in a decrease in adipocyte volume; however, the decrease in body fat mass in response to CLA can be attributed to decreased adipocyte size rather than adipocyte number (Azain et al., 2000). This basis for the reduction in fat mass is consistent with metabolic changes (decreased lipid deposition and increased lipolysis) and has been reported previously (Park et al., 1997; Park et al., 1999a, b).

In contrast to the studies in monogastric species, whether, and to what extent, supplemental CLA dose changes the body fat in dairy cows is not yet been clarified. One mRNA expression study indicates that lipid synthesis is stimulated by short term CLA supplementation in adipose tissue of cows (Harvatine et al., 2009). Decreased rates of glucose utilization for lipid synthesis could be a mechanism by which CLA decrease body fat (Baumgard et al., 2002). However, in studies with shorter durations of CLA supplementation (up to 5 wk) than in our experiment, metabolic parameters and hormones such as NEFA, glucose, insulin and leptin as well as BCS were not indicating any systemic changes in lipid metabolism and energy homeostasis (Baumgard et al., 2002; de Veth et al., 2006; Kay et al., 2006). In our present study, both plasma NEFA and BHBA concentrations were not different between CON and CLA groups. Moreover, a very strong inverse relationship was detectable in SC depot from the withers in CON cows at 42 DIM, possibly indicating lipolysis during the physiological changes related to early lactation. Furthermore, both *in vitro* and *in vivo* bovine studies suggest that BHBA may play an important role in dairy cows for its feedback inhibitory effect on lipolysis (Metz et al., 1974). The receptor for BHBA [i.e., G-protein-coupled receptor109 (GPR109)] is predominantly expressed in adipose tissues of monogastrics (Soga et al., 2003) and dairy cows (Lemor et al., 2009). Taggart et al. (2005) also reported that BHBA inhibits adipocyte lipolysis in mice via PUMA-G (also known as GPR109). Based on the positive correlation between adipocyte size and plasma BHBA from our present study, the processes of lipolysis might be increased in adipose tissues in a depot-specific manner both in CON and CLA supplemented cows. We, thus,

speculate that lipolysis might be elevated during the physiological changes related to early lactation. In addition, the decreases in adipocyte size that we observed in CLA supplemented cows may indicate lipolytic or antilipogenic effects of CLA, or both effects, on adipose tissue in primiparous dairy cows.

## Conclusions

Significant physiological changes related to the duration of lactation were limited to retroperitoneal fat depot in CON cows during early lactation. Conjugated linoleic acid-induced decreases in adipocyte size in the present long-term study indicate that CLA does affect body fat in dairy cows similar to what is known from CLA supplemented monogastrics. The present study suggested, for the first time, that dietary supplementation of CLA has site-specific effects on adipocyte size of different fat depots in dairy cows. These results may contribute to an improved and comprehensive assessment of CLA effects on animal health in dairy cows.

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**Table 1: Feed ingredients and chemical composition of the postpartum concentrates and partial mixed ration (PMR)**

| Item[1] | Concentrate[2] CON | Concentrate[2] CLA | PMR[3] |
|---|---|---|---|
| Feed ingredients (%) | | | |
| Wheat grain | 39.5 | 39.5 | |
| Dried sugar beet pulp | 29 | 29 | |
| Rapeseed meal | 20 | 20 | |
| Soybean meal | 6.5 | 6.5 | |
| Vitamin/mineral premix[4] | 2 | 2 | |
| CON fat supplement | 2.5 | - | |
| CLA supplement | - | 2.5 | |
| Calcium carbonate | 0.5 | 0.5 | |
| Analyzed chemical profile | | | |
| DM (g/kg) | 873 | 871 | 445 |
| Nutrient (g/kg DM) | | | |
| Crude ash | 65 | 69 | 62 |
| CP | 182 | 180 | 124 |
| Ether extract | 50 | 44 | 28 |
| Crude fiber | 96 | 97 | 183 |
| ADF | 134 | 133 | 208 |
| NDF | 259 | 260 | 405 |
| Energy[5] (MJ/kg DM) | | | |
| ME | 13.9 | 13.7 | 11.9 |
| $NE_L$ | 8.9 | 8.7 | 7.5 |
| CLA[4] (g/kg DM) | | | |
| C18:2 *c*9,*t*11 | 0.0 | 1.6 | 0.0 |
| C18:2 *t*10,*c*12 | 0.0 | 1.7 | 0.0 |
| Fatty acid profiles of fat supplements | | | |
| | CON[6] | CLA[7] | |
| Fatty acid (% by wt of total fatty acids) | | | |
| Palmitic acid (C16:0) | 10.89 | 10.89 | |
| Stearic acid (C18:0) | 87.30 | 50.31 | |
| Oleic acid (C18:1 c9) | <0.01 | 10.66 | |
| CLA | | | |
| C18:2 *c*9,*t*11 | 0.06 | 11.99 | |
| C18:2 *t*10,*c*12 | 0.02 | 11.88 | |
| Other CLA | 0.15 | 0.95 | |
| Others | 1.58 | 3.32 | |

[1]*c = cis; t = trans.*

[2]CON = control; CLA = conjugated linoleic acid.

[3]Partial mixed ration containing 25% grass silage, 38% corn silage and 37% PMR-concentrate (13.5 MJ of ME /kg of DM) on DM basis.

[4]Per kilogram of mineral feed, contains: 140 g of Ca; 120 g of Na; 70 g of P; 40 g of Mg; 6 g of Zn; 5.4 g of Mn; 1 g of Cu; 100 mg of I; 40 mg of Se; 5 mg of Co; 1,000,000 IU of vitamin A; 100,000 IU of vitamin D3; and 1,500 mg of vitamin E.

[5]Calculation based on nutrient digestibility's measured in rumen-cannulated wethers (GfE, 1991).

[6]CON fat supplement.

[7]CLA supplement.

**Table 2: Empty BW (EBW; kg) and different fat depot weights (kg) in control (CON) and conjugated linoleic acid (CLA) supplemented dairy cows during the first 105 DIM**

| Item | DIM | | | |
|---|---|---|---|---|
| | 1 | 42 | 105 | *P*-value[1] |
| CON (n = 15), means ± SEM | | | | |
| EBW | 444 ± 10.5 | 395 ± 12.4 | 432 ± 11.9 | 0.052 |
| Depot weight | | | | |
| Mean Subcutaneous (SC) depot | 4.32 ± 0.36 | 3.29 ± 0.40 | 3.75 ± 0.71 | 0.481 |
| Visceral (VC) depots | | | | |
| Omental | 11.0 ± 1.04 | 7.58 ± 0.53 | 8.53 ± 1.21 | 0.110 |
| Mesenteric | 5.93 ± 0.31 | 4.97 ± 0.39 | 4.44 ± 0.46 | 0.094 |
| Retroperitoneal | 8.76 ± 0.87[a] | 4.58 ± 0.65[b] | 5.56 ± 1.01[ab] | **0.024** |
| CLA (n = 10), means ± SEM | | | | *P*-value[2] |
| EBW | | 399.3 ± 10.94 | 410.66 ± 18.60 | 0.650 |
| Depot weight | | | | |
| Mean SC depot | | 4.86 ± 0.83 | 2.98 ± 0.51 | 0.123 |
| VC depots | | | | |
| Omental | | 9.11 ± 1.12 | 7.40 ± 1.06 | 0.347 |
| Mesenteric | | 5.87 ± 0.68 | 4.48 ± 0.50 | 0.184 |
| Retroperitoneal | | 5.78 ± 0.91 | 4.27 ± 0.87 | 0.311 |
| CON vs. CLA | | *P*-value[2] | | |
| EBW | | 0.836 | 0.418 | |
| Depot weight | | | | |
| Mean SC depot | | 0.169 | 0.449 | |
| VC depots | | | | |
| Omental | | 0.297 | 0.546 | |
| Mesenteric | | 0.360 | 0.980 | |
| Retroperitoneal | | 0.363 | 0.403 | |

[ab]Within a row, means with different superscript letters differ at $P < 0.05$.

[1]General linear model followed by Bonferroni post-hoc tests; significant *P*-value ($P < 0.05$) is highlighted in boldface type.

[2]Student's *t*-test.

**Table 3: Adipocyte sizes (µm²) of different fat depots in control (CON) and conjugated linoleic acid (CLA) supplemented dairy cows during the first 105 DIM**

| Item | DIM 1 | DIM 42 | DIM 105 | *P*-value[1] |
|---|---|---|---|---|
| CON (n = 15; means ± SEM) | | | | |
| Subcutaneous (SC) depots | | | | |
| Tailhead | 6,300 ± 448[ab] | 5,442 ± 408 | 6,103 ± 325 | 0.389 |
| Withers | 6,258 ± 451[ab] | 5,110 ± 407 | 4,521 ± 390 | 0.060 |
| Sternum | 5,782 ± 243[b] | 5,180 ± 314 | 4,831 ± 454 | 0.257 |
| Visceral (VC) depots | | | | |
| Omental | 6,381 ± 372[ab] | 6,077 ± 289 | 5,851 ± 286 | 0.587 |
| Mesenteric | 6,172 ± 294[ab] | 6,222 ± 267 | 5,784 ± 436 | 0.682 |
| Retroperitoneal | 7,945 ± 208[a,y] | 6,634 ± 234[yz] | 6,500 ± 414[z] | **0.019** |
| CLA (n = 10; means ± SEM) | | | | *P*-value[2] |
| SC depots | | | | |
| Tailhead | | 4,573 ± 291[ab,y] | 3,233 ± 127[z] | **0.005** |
| Withers | | 3,976 ± 332[b] | 3,021 ± 583 | 0.239 |
| Sternum | | 3,959 ± 87[b] | 3,452 ± 329 | 0.220 |
| VC depots | | | | |
| Omental | | 4,750 ± 277[ab] | 3,997 ± 410 | 0.211 |
| Mesenteric | | 4,235 ± 296[ab] | 3,902 ± 207 | 0.433 |
| Retroperitoneal | | 5,552 ± 407[a] | 4,316 ± 335 | 0.070 |
| CON vs. CLA | | *P*-value[2] | | |
| SC depots | | | | |
| Tailhead | | 0.160 | **0.001** | |
| Withers | | 0.090 | 0.092 | |
| Sternum | | **0.010** | 0.059 | |
| VC depots | | | | |
| Omental | | 0.018 | **0.011** | |
| Mesenteric | | **0.002** | **0.008** | |
| Retroperitoneal | | 0.074 | **0.006** | |

[a,b]Within a column, means with different superscript letters differ at $P < 0.05$.

[yz]Within a row, means with different superscript letters differ at $P < 0.05$ for GLM and $P < 0.017$ for Student's *t*-test.

[1]GLM, followed by Bonferroni post-hoc tests; significant *P*-value ($P < 0.05$) is highlighted in boldface type.

[2]Student's *t*-test, after the Bonferroni α-correction for multiple comparisons; significant differences ($P < 0.017$) are highlighted in boldface type.

**Table 4: Coefficients of significant correlations between adipocyte sizes of different fat depots and plasma concentrations of NEFA and BHBA in control (CON) and conjugated linoleic acid (CLA) supplemented dairy cows during the first 105 DIM**

| | NEFA | | | | BHBA | | | |
|---|---|---|---|---|---|---|---|---|
| CON | Mean CON[1] | 1 DIM | 42 DIM | 105 DIM | Mean CON[1] | 1 DIM | 42 DIM | 105 DIM |
| | | n = 5 each | | | | n = 5 each | | |
| Subcutaneous (SC) depots | | | | | | | | |
| tailhead | - | - | - | - | - | - | - | - |
| withers | - | - | – 0.990*** | - | - | - | - | - |
| sternum | - | - | - | - | - | - | - | - |
| Mean SC depot | - | - | - | - | - | - | - | - |
| Visceral (VC) depots | | | | | | | | |
| Omental | - | - | - | - | - | - | - | 0.879* |
| Mesenteric | - | - | - | - | - | - | - | - |
| Retroperitoneal | 0.698** | - | - | - | - | - | - | - |
| Mean VC depot | 0.602* | - | - | - | - | - | - | - |
| CLA | Mean CLA[2] | | | | Mean CLA[2] | | | |
| SC depots | | | | | | | | |
| tailhead | - | | - | - | - | | - | 0.989** |
| withers | - | | - | - | - | | - | - |
| sternum | - | | - | - | 0.651* | | - | 0.880* |
| Mean SC depot | - | | - | - | 0.657* | | - | 0.901* |
| VC depots | | | | | | | | |
| Omental | - | | - | - | - | | - | - |
| Mesenteric | - | | - | - | - | | - | - |
| Retroperitoneal | - | | - | - | - | | - | - |
| Mean VC depot | - | | - | - | - | | - | - |

[1]Mean CON comprising the control fat supplemented cows from 1, 42, and 105 DIM (n = 15).

[2]Mean CLA comprising the CLA supplemented cows from 42 and 105 DIM (n = 10).

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

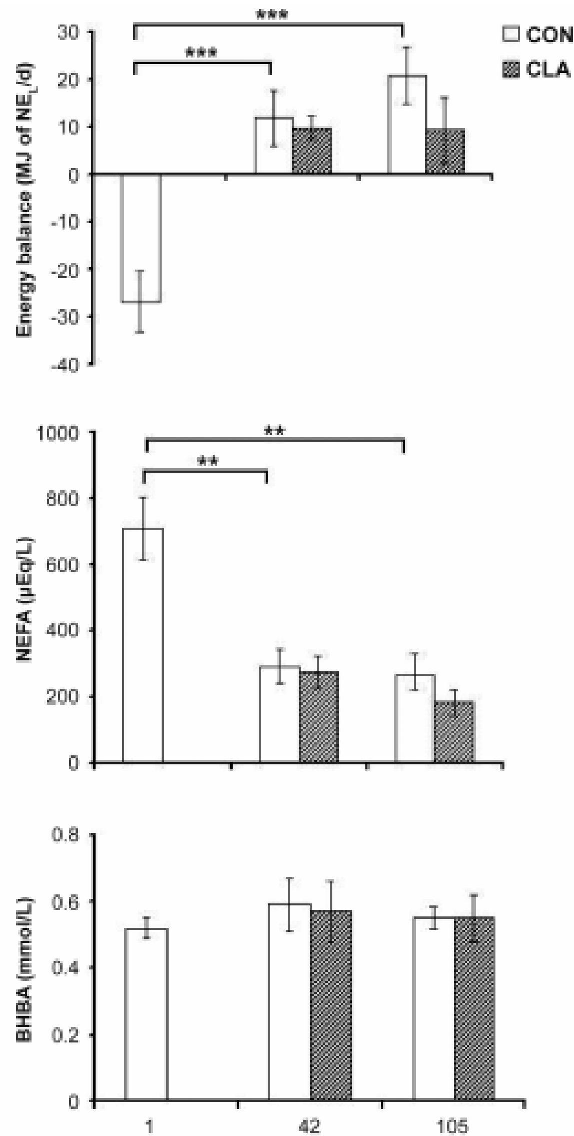


**Fig. 1:** **Energy balance and plasma concentrations of NEFA and BHBA of control (CON) and conjugated linoleic acid (CLA) supplemented dairy cows during the first 105 DIM.** Each group represents the mean ± standard error of the means of 5 animals. ** $P < 0.01$; *** $P < 0.001$. Net energy balance (MJ $NE_L$/d) = energy intake (MJ $NE_L$/d) – [$NE_M$ (MJ $NE_L$/d) + $NE_L$ (MJ $NE_L$/d)].

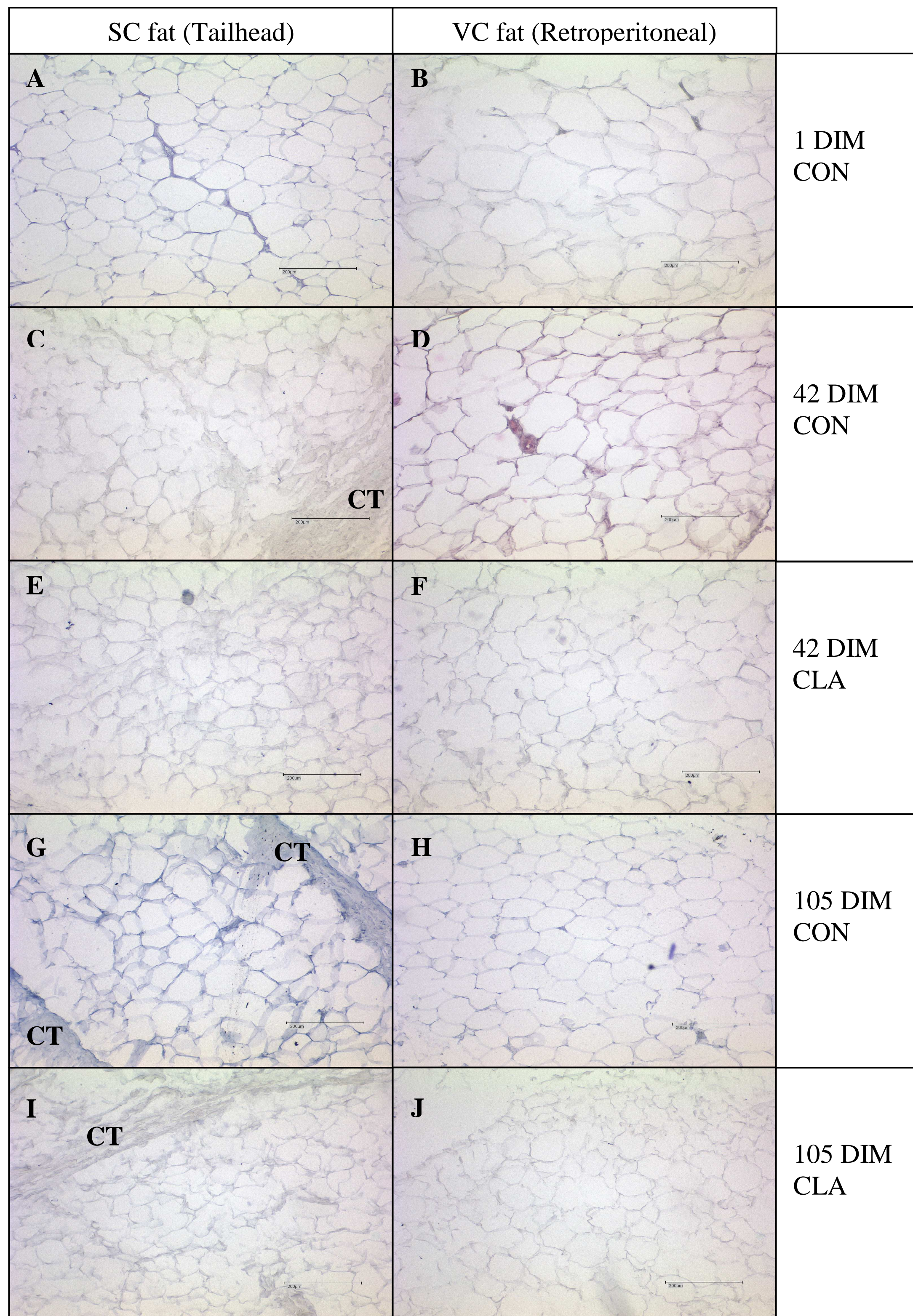


**Fig. 2:** **Histological sections of the subcutaneous (SC) fat depot from the tailhead (left column) and of the retroperitoneal visceral (VC) fat depot (right column) from control (CON) and conjugated linoleic acid (CLA) supplemented dairy cows at 1, 42, and 105 DIM.** Higher portions of connective tissue fibers were found in SC depots than in VC depots. Hematoxylin-eosin staining. Original magnification 100×; Scale bar = 200 µm; CT = connective tissue fibers.

# 3 Manuscript 2 (submitted to: Journal of Dairy Science 2011)

## Immunohistochemical characterization of phagocytic immune cell infiltration into different adipose tissue depots of dairy cows during early lactation

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## Abstract

This present study aimed to investigate whether phagocytic immune cells infiltrate into bovine adipose tissue (AT) and to study the effects of lactation and conjugated linoleic acid (CLA) supplementation on the invasion of phagocytic immune cells into different subcutaneous (SC) and visceral (VC) fat depots of primiparous dairy cows during the first 105 days in milk (DIM). German Holstein-Friesian cows (HF; n = 25) with a mean body condition score (BCS) of 3.0 were divided into a control (CON) and a CLA group. From 1 DIM until sample collection, CLA cows were fed 100 g of CLA supplement/d (about 6% of *c*9,*t*11 and *t*10,*c*12 isomers each), whereas the CON cows received 100 g/d of a fatty acid mixture instead of CLA. The CON cows (n = 5 each) were slaughtered at 1, 42, and 105 DIM, and the CLA cows (n = 5 each) were slaughtered at 42 and 105 DIM. Adipose tissues (AT; n = 150) from three SC (tailhead, withers, and sternum) and three VC (omental, mesenteric, and retroperitoneal) depots were sampled. In addition, SC tailhead biopsies were collected by repeated surgical biopsies (three samplings within 7 wk; n = 36) from 12 non-pregnant, non-lactating Simmental heifers (SM; mean BCS = 5.0) fed diets of varying energy density to compare the changes in phagocytic immune cell infiltration with early-lactating cows. Immunohistochemical analyses of different fat depots revealed a low incidence of phagocytic immune cell infiltration in early-lactating cows. The portion of infiltrating macrophages (CD68+) in a few positive AT samples of HF cows was slightly lower in SC versus VC fat and was positively correlated with both empty body weight and adipocyte size. However, no differences with regard to DIM and CLA supplementation were observed in HF cows. Increased accumulation of phagocytic immune cells, albeit at low cell numbers in non-pregnant, over-conditioned SM heifers might be related to larger adipocytes secreting higher amounts of chemoattractant adipokines compared with the early-lactating cows. In conclusion, the extent of fatness in HF cows may not be high enough to stimulate significant infiltration of phagocytic cells in AT and therefore, these immune cells might have no major role in the immunologic and metabolic adaptations during early lactation.

Key words: adipose tissue, dairy cow, early lactation, immune cell infiltration

## Introduction

During early lactation, dairy cows undergo coordinated metabolic adaptations, mainly due to the excessive energy demands imposed by the lactating udder (Bauman and Currie, 1980). High-yielding cows then undergo comprehensive metabolic adaptations with fat mobilization, decreased insulin sensitivity, and immunosuppression being major issues (Butler and Smith, 1989; Mallard et al., 1998). The energy requirements at this period can not entirely be met through dietary intake, and energy has to be mobilized from body reserves, mainly from adipose tissue (AT), thus resulting in a state of negative energy balance (NEB). Besides its function as an energy storage depot, AT secretes endocrine factors that exert a dichotomic function both in metabolism and immunoregulation; AT thus forms a gateway connecting metabolism with innate immunity (Schäffler and Schölmerich, 2010). The major phagocytic cells of the innate immune system include neutrophils, monocytes, and macrophages, which originate from the myeloid progenitors in the bone marrow (Noorman et al., 1997; Weisberg et al., 2003). Neutrophils circulate in the blood and are readily attracted to sites of inflammation (Schymeinsky et al., 2007). Monocytes are also circulating cells, which eventually evade into tissues and differentiate into macrophages (Hume et al., 2002). These immune cells have the ability to phagocytize directly; among them, macrophages are highly effective phagocytes acting in innate immunity and tissue remodeling via cytokine secretion and apoptotic body clearance (Duffield, 2003; Saillan-Barreau et al., 2003). Tissue macrophages can be identified via flow cytometry or immunohistochemical staining by their specific expression of a number of proteins including CD68, CD14, CD11b, and CD11c (Khazen et al., 2005). CD68 is the main macrophage-specific marker; however, Noorman et al. (1997) reported that commonly used macrophage markers are also expressed on monocytes (CD14; CD11b; CD11c) or on granulocytes (CD11b). In the context of obesity and high-fat diet, humans and mice exhibit increased infiltration of AT macrophages (ATM), particularly in visceral (VC) depots to form a proinflammatory micro-milieu causing local and systemic insulin resistance (Weisberg et al., 2003; Xu et al., 2003; Nishimura et al., 2009). This phenomenon is associated with changing adipokine profiles and positively correlates with adipocyte size and body fat mass (Weisberg et al., 2003). Moreover, AT

neutrophils transiently infiltrate prior to the infiltration of ATM in diet-induced obese mice (Elgazar-Carmon et al., 2008).

Conjugated linoleic acid (CLA) designates a group of naturally occurring dienoic derivatives of linoleic acid. *Cis*-9, *trans*-11 (*c*9,*t*11) is the predominant natural CLA isomer occurring exclusively in ruminant meat and milk, whereas commercially available CLA preparations contain this isomer together with the *trans*-10, *cis*-12 (*t*10,*c*12) in equimolar concentrations (Poirier et al., 2006). Isomers of CLA have a wide range of beneficial effects, such as anticarcinogenic, antiatherogenic, antidiabetic, and antiobesity (Bauman et al., 2001). *In vitro* and *in vivo* studies also demonstrate that CLA modulates immune function (O'Shea et al., 2004). Supplementation of dairy cows with CLA has recently been reported to decrease milk fat but left body fat mostly unaffected in terms of depot mass (von Soosten et al., 2011). However, when considering adipocyte sizes, CLA supplemented cows had smaller adipocytes both in subcutaneous (SC) and VC fat depots compared with control (CON) cows (Akter et al., 2011). Nevertheless, the *t*10,*c*12 isomer promotes ATM infiltration (Poirier et al., 2006), whereas the *c*9,*t*11 isomer decreases this phenomenon in mice (Moloney et al., 2007).

So far, most research on ATM infiltration has focused on their contribution to the development of obesity-associated pathologies through systemic effects in nonruminant species (Bouloumié et al., 2005; Schäffler and Schölmerich, 2010). To our knowledge, no report exists about phagocytic immune cell infiltration into bovine AT. Therefore, we aimed to investigate: 1) whether phagocytic immune cells infiltrate into SC and VC fat depots of primiparous dairy cows during the first 105 DIM and 2) whether or not dietary CLA supplements would have any effect on this infiltration phenomenon. Immunohistochemistry was performed on AT cryosections to characterize the portion of CD68+, CD14+, CD11b+, and CD11c+ cells in AT of early-lactating dairy cows. In addition, the changes in the portion of phagocytic immune cells in AT of early-lactating cows were compared with non-pregnant, over-conditioned heifers and fat steers.

## Materials and methods

### *Animals, experimental designs, and diets*

***Trial 1: Early-lactating cows.*** The animal experiment was conducted according to the European Union guidelines and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany (File number 33.11.42502-04-071/07). The details of this study have been described previously (von Soosten et al., 2011). In brief, German Holstein-Friesian cows (HF; n = 25) with a mean BCS of 3.0 (scale = 1 to 5) were housed in a free-stall barn at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Braunschweig, Germany. The animals were fed according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). Water was provided ad libitum. Before parturition, cows received a diet consisting of a partial mixed ration (PMR, 60% corn silage and 40% grass silage on a DM basis; 6.7 MJ $NE_L$/kg DM) for ad libitum intake and 2 kg concentrate/d (6.7 MJ $NE_L$/kg DM). After parturition, they were fed a PMR (25% grass silage, 38% corn silage and 37% PMR-concentrate on a DM basis) for ad libitum intake (7.5 MJ $NE_L$/kg DM). Additionally, 4 kg of concentrate (DM basis; 8.9 MJ $NE_L$/kg DM for CON cows and 8.7 MJ $NE_L$/kg DM for CLA cows), which contained the control fat supplement or the CLA supplement, was provided by the computerized concentrate feeding stations (type RIC; Insentec, B.V., Marknesse, the Netherlands) in pelleted form and water was available ad libitum from parturition until slaughter.

The cows were randomly allocated to either the CON (n = 15) or the CLA (n = 10) group. From 1 DIM until sample collection, the CLA cows were fed 100 g/d of a rumen-protected (lipid encapsulation technique) CLA supplement (Lutrell Pure, BASF SE, Ludwigshafen, Germany) and the CON cows received 100 g/d of a fatty acid mixture (Silafat, BASF SE). The pelleted CLA-concentrate contained about 6% each of the *t*10,*c*12 and *c*9,*t*11 isomers (calculated proportion in the CLA-concentrate). The CON animals (n = 5 each) were slaughtered at 1, 42, and 105 DIM, whereas the CLA cows (n = 5 each) were slaughtered at 42 and 105 DIM.

***Trial 2: Non-pregnant, over-conditioned heifers.*** The animal experiment was approved by the North Rhine-Westphalia State Agency for Nature, Environment, and Consumer Protection (LANUV-NRW), Recklinghausen, Germany (File number 8.87.51.05.20.10.103). Adult, non-pregnant, non-lactating Simmental heifers (SM; n = 12) with a mean BCS of 5.0 were housed in a tie-stall barn bedded with straw at the Frankenforst experimental research farm of the Faculty of Agriculture, University of Bonn, Germany. The animals were fed with grass silage for ad libitum intake. To investigate the effects of a moderate weight loss, the heifers were allocated to 2 groups (n = 6 each) of similar mean BW (692 ± 43 kg). One half of the heifers was assigned to a ration in which grass silage was blended with a hay/straw mixture (ratio 37:63 on a DM basis; n = 6), whereas the other animals remained on the grass silage diet. The grass silage contained 10.6% CP, 54.8% NDF (ash-free), and 34.6% ADF (ash-free), and the hay/straw-silage mixture contained 8.5% CP, 63.2% NDF (ash-free), and 37.0% ADF (ash-free) on a DM basis. The estimated ME concentrations of the grass silage and hay/straw mixture were 9.5 and 8.1 MJ/kg of DM, respectively. After 4 wk of differential feeding, all animals were fed again with grass silage for further 3 wk. To avoid potential effects of divergent stages of estrus cycle, all animals received a progesterone-releasing intravaginal device (PRID-alpha, Ceva Sante Animale, Libourne, France) that was renewed every third week.

In addition, to investigate the phagocytic immune cell infiltration in male animal with high BW, tailhead fat biopsies (n = 3) were sampled from 13-yr-old, rumen-fistulated, fat German Red Pied steers (RP; n = 3), with a mean BW of 1300 kg. The animals were housed in a free-stall barn at the Frankenforst experimental research farm of the Faculty of Agriculture, University of Bonn, Germany. They had ad libitum access to pasture during summer and to medium quality grass silage during winter.

***Tissue sample collection and analysis***

In trial 1, AT samples from three SC depots (tailhead, withers, and sternum) and from three VC depots (omental, mesenteric, and retroperitoneal fat) were collected immediately after slaughter. The mesenteric fat was collected from the fat around the jejunum and the retroperitoneal fat was sampled from the pararenal fat, located superficial to the renal fascia. The AT samples were then dissected and weighed. The

empty BW (EBW) was calculated as sum of the weights of all body parts, which were recorded during the slaughter process without claws cut off, ingesta and contents of urinary and gall bladder. For trial 2, SC tailhead fat samples (n = 36) were collected by repeated surgical biopsies (1 samplings at the end of each the consistent grass silage feeding, the differing feeding and the refeeding period) from the tailhead area according to the methods of Smith and McNamara (1989). In brief, after cleaning and disinfecting the sampling area and local lumbar anesthesia with 2% procaine hydrochloride (Procasel 2%, Selectavet; Dr. Otto Fischer, Weyarn-Holzolling, Germany), s.c. fat biopsy was excised through a 5-cm incision from the base of the tail. Subcutaneous tailhead fat biopsies were taken collected from RP steers in the same method. All the tissue samples were then cut into small pieces (approximately 1 $cm^3$), rinsed in 0.9% sodium chloride and immediately snap frozen in liquid nitrogen. They were transported on dry ice to the laboratory and then stored at -80°C until analyses.

### *Immunohistochemical analyses*

Adipose tissue cryosections (14 to 18 µm) were cut on a cryostat (Leica, Wetzlar, Germany), mounted on SuperFrost Plus slides (Menzel, Braunschweig, Germany) and stored at -20°C until further staining. Frozen tissue sections were fixed in chloroform-methanol mixture (Applichem, Darmstadt, Germany; 1:1, vol/vol) at room temperature (RT) for 4 h, the mixture was stored at -20°C before use. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide (Roth¸ Karlsruhe, Germany) in methanol at RT for 15 min, the sections were incubated with normal goat serum at a dilution of 1:10 at RT for 20 min to block non-specific binding. The sections were then incubated with the primary monoclonal antibodies (mAb) at 4°C overnight, as listed in Table 1. The mAb against CD68 was detected by a secondary peroxidase-coupled goat-anti-mouse antibody (Dako), whereas the mAb against CD14, CD11b, and CD11c were detected with a biotinylated secondary goat anti-mouse antibody (Southern Biotech, Birmingham, AL) in PBS at a dilution of 1:200 at RT for 30 min. After incubating the slides with the biotinylated secondary antibody, the sections were additionally incubated with horseradish peroxidase-conjugated streptavidin (Southern Biotech) at a dilution of 1:1000 (1:1600 for CD14) at RT for 30 min. Antigen-antibody complexes were visualized by 3-amino-9-ethylcarbazole (Biozol, Eching, Germany). The sections were

counterstained with Mayer's hematoxylin and mounted with Kaiser's glycerol gelatine (both from Merck, Darmstadt, Germany). Bovine lymphnode samples were used as positive and negative controls to the entire procedure to test specificity of immunostaining. The sections used as negative controls were incubated with PBS instead of the primary mAb.

### *Histoplanimetry*

Bright-field pictures were taken from 10 representative fields per sample at 100× magnification using a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) and JVC digital color camera KY-F75U (Hachioji Plant of Victor Company, Tokyo, Japan). Adipocyte areas ($\mu m^2$) were measured as described previously (Akter et al., 2011). The numbers of immunopositive cells and adipocytes per $mm^2$ were counted (10 fields/sample) using a grid (900 × 700 µm) with an image analysis software (DISKUS version 4.0; Hilgers, Königswinter, Germany). The portion of CD68+ cells for each sample was calculated as the sum of the number of CD68+ cells divided by the total number of cells per section and then multiplied by 100. The same procedure was used to calculate the portion of CD14+, CD11b+, and CD11c+ immune cells in different AT depots. For the CD68 immunostaining, in total 150 samples (from 25 HF cows) were tested. However, for the detection of additional CD14, CD11b, and CD11c markers in HF cows, six AT samples were taken from two cows per group at 1, 42, and 105 DIM (n = 60), including 9 CD68+ samples. In addition, these markers were also used for the remaining one CD68+ sample belonging to a cow at 1 DIM (in total n = 61).

### *Statistical analyses*

Data for all variables were tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variances with the Levene's test. The Student's t-test was used to analyze the differences between the adipocyte sizes of HF cows and SM heifers; differences were considered significant at $P < 0.05$. Pearson correlation was used to examine the relationship between the percentage of CD68+ cells with both EBW and

adipocyte size. All analyses were done using SPSS software, version 19.0 (SPSS, Inc., Chicago, Illinois). Values are given as means ± SEM.

## Results

The hypothesis of the present study was that the portion of phagocytic cells might be small in AT of early-lactating dairy cows. To identify and quantify these immune cells into AT, sections were immunohistochemically stained by using mAbs against CD68, CD14, CD11b, and CD11c. Immunopositive cells were found in only few samples of HF cows during the first 105 DIM. Macrophages (CD68+) were immunolocalized as uniformly small, isolated cells around the cytoplasm of mature adipocytes. Representative pictures of the SC (tailhead) and the VC (mesenteric) fat depots are shown in Fig. 1. The same staining patterns were observed for CD14, CD11b, and CD11c; however, in contrast to CD68+ staining, most of the positive cells were observed in the stromal-vascular fraction (SVF) rather than around adipocytes.

### *Immune cell infiltration into adipose tissue of early-lactating cows*

***CD68.*** CD68 is the main cell surface marker of macrophages. With the exception of 10 samples from eight cows, no ATM positive staining was observed in the remaining 140 samples (Table 1). The average portion of CD68+ ATM in the few positive samples was slightly but insignificantly lower in SC depot (3.7 ± 0.7%, n = 3) than in VC depot (24.7 ± 5.2%, n = 7).

***CD14.*** CD14 is used as a marker of macrophages and monocytes. The immunostaining was positive only in one (out of 61) samples. The portion of CD14+ immune cells was 2.0% in the SC tailhead depot (Table 1).

***CD11b.*** CD11b is used as a marker of macrophages, monocytes, and granulocytes. The immunostaining was positive in eight (out of 61) samples from six cows, among which five animals were coincidently immunopositive for CD68 (Table 1). The average portions of CD11b+ immune cells in the positive samples were 1.9 ± 0.6% (n = 3) and 1.6 ± 0.7% (n = 5) in SC and VC depots, respectively.

***CD11c.*** CD11c is commonly used as a marker of macrophages and monocytes. With the exception of two samples from one cow, no CD11c positive staining was observed in the remaining 59 samples. The average portion of CD11c+ immune cells in the positive samples was 3.7 ± 1.7% (n = 2). The SC depot from tailhead and sternum, but not the VC depot showed positive signal for this marker. These two samples were also immunopositive for CD68.

***Effects of lactation time and CLA supplementation on the invasion of immune cells into adipose tissue***

A total of nine animals showed positive immunostainings in a few positive AT depots, irrespective of DIM and CLA supplementation (Table 1). No positive staining was found in any of the SC depots of CON cows at 1 DIM. For the visceral depots, positive staining both for the CD68 and the CD11b markers were detected coincidently in omental and mesenteric fat in two CON cows of 1 DIM. At 42 DIM, two CD68+ samples were found both in the SC withers depot and in the omental fat from the same CON cow. At 105 DIM, one positive CD68 staining in the retroperitoneal fat and three positive CD11b immunostainings in the SC fat from withers and sternum as well as in the retroperitoneal fat were observed from the same CON cow. Considering CLA supplementation, four positive immunostainings for the CD68, CD14, CD11b, and CD11c markers in the SC tailhead depot and two positive immunostainings for the CD68 and CD11c markers in the SC sternum depot were detected coincidently in the same CLA fed cow at 42 DIM. However, no differences with regard to either DIM or CLA supplementation were observed in the few positive samples for all the immunostainings.

***Comparison of the portion of phagocytic immune cells in adipose tissues from early-lactating cows, non-pregnant, over-conditioned heifers, and fat steers***

We herein found that the average portions of ATM (CD68+) in the SC tailhead depot were only marginal both in non-pregnant SM heifers and the early-lactating HF cows (Table 3), although the adipocyte size of this depot was larger in SM heifers than in HF cows ($P < 0.001$), with mean areas of 8,230 ± 240 µm² and 5,146 ± 491 µm²,

respectively. However, no significant effect of differential feeding was observed in the SM heifers. The portion of CD68 positive samples (SC tailhead) in the SM heifers (64%, i.e., 23 out of 36 samples) was 16 times higher than in the corresponding depots from the HF cows (4%; i.e., 1 out of 25 samples). Moreover, only 10% of the samples showed positive signals for each of the CD14, CD11b, and CD11c markers in HF cows, whereas they were 47, 33, and 58%, respectively in SM heifers (Table 3). In fat RP steers, no CD68 and CD11c positive staining were observed in the SC fat, whereas the portions of CD14+ and CD11b+ samples were 100 and 33%, respectively.

### *Correlations between the portion of ATM (CD68+) with EBW and adipocyte size*

To investigate the relationship between the portion of ATM (CD68+) in positive samples with EBW and adipocyte size, we calculated the correlations. Strong positive correlations were found between the portion of ATM with both EBW (r = 0.788, $P$ = 0.007, n = 10) and mean adipocyte area (r = 0.684, $P$ = 0.029, n = 10). Data collected from the 10 positive samples out of 150 analyzed, are shown in Fig. 2. Some samples comprising large adipocytes still did not show positive signal for CD68 marker. However, no significant relationships were detectable between the portion of ATM and adipocyte size in SC tailhead depots from the SM heifers.

## Discussion

In the present study, different fat depots of dairy cows were characterized in terms of the number of phagocytic immune cells present. In this context, metabolic adaptations to early lactation and dietary CLA related changes were of particular interest. For identifying phagocytic immune cells in AT, we used mAbs directed against CD markers i.e., CD68, CD14, CD11b, and CD11c that are surface markers for monocytes, macrophages, and granulocytes (neutrophils). We herein present immunohistochemical evidence against an appreciable infiltration of phagocytic immune cells in bovine SC and VC fat depots. Several previous reports have been presented with regard to the localization and distribution of such cells in AT of obese mice and humans by using various mAbs (Weisberg et al., 2003; Xu et al., 2003; Curat et al., 2004; Cinti et al., 2005; Elgazar-Carmon et al., 2008). In the context of obesity and high-fat diet,

macrophages are one of the most prominent infiltrating cell types into AT. Most research on ATM infiltration in monogastric species is limited to obese individuals; however, early-lactating cows are not 'obese' and may thus lack significant infiltration of macrophages into different AT depots.

We herein characterized specific immunohistological features of infiltrating macrophages in different bovine AT depots. In early-lactating cows, the sparsely observed CD68+ macrophages were mostly localized around mature adipocytes. They probably correspond to the number of resident ATM that may eventually respond to local inflammation. This finding is similar to the study of infiltrating macrophages into the SC fat in lean humans (Cancello et al., 2005). The ATM of early-lactating cows were uniformly small, isolated, and dispersed among the adipocytes. This observation agrees with the study of infiltrating macrophages into the SC fat of lean mice (Weisberg et al., 2003). In contrast, ATM localize to crown-like structures around individual adipocytes in VC fat both in lean and obese mice, which increase in frequency with obesity (Cinti et al., 2005). These structures were also found within the SC and VC fat in human obese patients, but were less frequent than in mice (Cancello et al., 2005; Zeyda et al., 2007).

***Evidence against an appreciable infiltration of phagocytic immune cells into bovine adipose tissue during early lactation***

It is well known that metabolic adaptations in bovine AT form a critical part in establishing and maintaining lactation (Smith and McNamara, 1990; McNamara, 2010). During early lactation, the state of NEB contributes to the immunocompromised situation in dairy cows, and thus leads to increased susceptibility to infectious diseases. In the present study, a low incidence of infiltrating phagocytic immune cells was observed in different fat depots of primiparous dairy cows during the first 105 DIM. We herein found that the portion of the CD68 positive samples was 6.6% in HF cows. Although CD68 is the main cell surface marker for macrophages, some other additional known macrophage markers have been reported to be expressed on monocytes (CD14; CD11b; CD11c) or on granulocytes (CD11b). Therefore, CD14, CD11b, and CD11c markers were combined in the present study and the portions of positive samples were 1.6, 13, and 3.3%, respectively. Weisberg et al. (2003) estimated that the portion of

ATM ranges from under 10% in lean mice to over 50% in extremely obese, leptin-deficient mice, where AT depots from all mice contained F4/80 expressing cells (macrophages). However, in the present study, only 32% of the early-lactating HF cows showed marginal infiltration of CD68+ ATM in different fat depots, irrespective of DIM and CLA supplementation.

In the present study, the average portion of CD68+ ATM was numerically higher in VC than in SC depot, which is in agreement with other studies in obese mice (Weisberg et al., 2003) and humans (Tchoukalova et al., 2004; Harman-Boehm et al., 2007). This may be due to the fact that VC fat is more metabolically active and is thought to differ from SC fat in terms of adipokine production (Altomonte et al., 2003). Several other authors have suggested that the differences in the local production of chemokines may account for such a discrepancy (Takahashi et al., 2003; Bruun et al., 2004; Skurk et al., 2005). This is in line with the view that adipocytes found in different depots have different properties (Wajchenberg et al., 2002; Cartwright et al., 2007). However, herein we did not observe any effect of DIM on the invasion of phagocytic immune cells into bovine AT (Table 2).

Immunohistochemical analyses of different SC and VC fat depots revealed that the portion of CD68+ ATM (from 10 positive samples out of 150 analyzed) was positively correlated with both EBW and adipocyte size. The positive linear correlation found between adipocyte size and CD68+ ATM suggests that large adipocyte size probably triggers macrophage infiltration. However, macrophage motility is characterized by its ability to respond to chemokine gradients emanating from AT (Gruen et al., 2007; described in the next section). In mice, ATM content in SC, perigonadal, perirenal, and mesenteric depots correlates positively with criteria for adiposity such as body mass index, percent body fat and adipocyte size (Weisberg et al., 2003). A similar positive correlation has been observed between ATM content in human SC fat depot and adiposity (Curat et al., 2004).

### *Mechanisms of infiltration of phagocytic immune cells into adipose tissue*

The mechanism behind the migration of phagocytic immune cells into AT is not well clarified. It is thought that with the expansion of AT during weight gain, induction of a range of signaling pathways activates the adipose SVF, allowing monocytes to extravasate through the endothelial cell layer into the AT where they differentiate into macrophages. These macrophages localize predominantly around dead adipocytes (Neels and Olefsky, 2006). Several reports suggest that the diverse biological activity of macrophage is mediated by functionally distinct subpopulations that are phenotypically polarized by their microenvironment and by exposure to inflammatory mediators. These divergent ATM subpopulations are broadly classified into two major groups: classically activated M1-type and alternatively activated M2-type macrophages. M1-types are induced by inflammatory agents, whereas M2-types are induced by IL-4 and IL-13 (Lumeng et al., 2007; Zeyda et al., 2007; Bourlier et al., 2008). M1-type macrophages display a cytotoxic, proinflammatory phenotype, whereas M2-type macrophages suppress immune and inflammatory responses and participate in wound repair and angiogenesis (Laskin, 2009). The ATM phenotypically resemble the antiinflammatory M2-type of macrophages in humans and mice (Zeyda and Stulnig, 2007). The changes in the expression of pro- and anti-inflammatory factors after weight loss in obese individuals have been associated with a shift in the activation type of the tissue resident macrophages from the M1-type to M2-type macrophages (Clément et al., 2004). However, metabolic changes of AT in obesity may stimulate the chemokines production, contributing to macrophage infiltration into AT (Wellen and Hotamisligil, 2005). On a quantitative basis, the vast majority of these chemokines come from the SVF of AT (Fain, 2006). For example, ATM produce the majority of TNF-α in AT, when compared to adipocytes or other cells in the SVF (Weisberg et al., 2003) and TNF-α expression increases with obesity (Hostamisligil et al., 1993). In SC fat of dairy cows, the mRNA abundance of TNF-α is higher at 1 DIM and at week 5 postpartum, compared with week 8 antepartum (Sadri et al., 2010).

Surface marker characteristics of ATM are important for their quantification. Most experimental data on AT inflammation originate from mouse experiments and in general F4/80-expressing cells in AT are referred to as ATM. Murine ATM have been shown to be CD14 negative and CD11c has been found particularly as a marker for diet-

induced obesity-associated inflammatory macrophages (M1-type) in murine AT (Lumeng et al., 2006), whereas CD206, CD209, and CD163 are well known M2 markers (Mantovani et al., 2002; Gordon and Taylor, 2005). Human ATM differs from murine ATM by expressing CD14; however, CD11c is only poorly expressed on human ATM (Curat et al., 2004; Zeyda et al., 2007). In the present study, 58% samples showing positive signals for CD11c in SC fat of non-pregnant, over-conditioned SM heifers, might represent the M1-type of macrophages.

In our previous report (Akter et al., 2011), we demonstrated that the experimental HF cows experiencing NEB at first sampling on 1 DIM, but were returned to positive energy balance at the day of second slaughter series i.e., 42 DIM irrespective of CLA supplementation. During early lactation, the state of NEB results in increased plasma concentrations of both NEFA and BHBA which in turn exert inhibitory effects on leukocytes and thus contribute to the immunocompromised situation in ewes and dairy heifers (Lacetera et al., 2002, 2004). The portions of CD68+, CD14+, CD11b+, and CD11c+ samples in SM heifers were consistently higher than in samples from HF cows (Table 3). The present data suggest that the increased accumulation of CD68+, CD14+, CD11b+, and CD11c+ cells, albeit at low cell numbers, in SM heifers could be related to large adipocytes secreting higher amounts of chemoattractant adipokines. In this context, several reports showed that AT production of monocyte chemoattractant protein-1, a chemoattractant specific for monocytes and macrophages is increased in obese, leptin-deficient mice compared with lean mice (Lu et al., 1998; Sartipy and Loskutoff, 2003). Moreover, leptin is secreted exclusively by adipocytes and is capable of linking metabolism and immune homeostasis (Matarese and La Cava, 2004). It has been reported to be also a chemoattractant for neutrophils (Ottonello et al., 2004) as well as for monocytes and macrophages (Gruen et al., 2007). In our present study, the larger adipocyte size of SC tailhead fat that we observed in SM heifers was associated with higher concentration of plasma leptin (data not shown) compared with HF cows. However, plasma leptin concentrations may reflect increased overall secretion, but they do not provide detailed information regarding local concentration gradients formed in AT. For example, leptin is a chemoattractant at concentrations as low as 1 pg/ml, with maximal effects at 1 ng/ml. However chemotaxis tended to decline at concentration of 10 and 100 ng/ml (Gruen et al., 2007). Our data also indicate that in fat RP steers, the portions of CD14 and CD11b positive samples were higher than the HF cows, although

no CD68 and CD11c positive staining were observed in the SC fat in fat RP steers (Table 3). Possible reasons for this difference might be due to sex hormone differences immanent in the comparison of intact females versus male long-term castrates. Comparing intact male and female mice, Weisberg et al. (2003) reported that the portion of mature F4/80-expressing macrophages was slightly lower for the SC fat of males regardless of body condition.

***Effects of CLA on the invasion of phagocytic immune cells into bovine adipose tissue***

In the present study, 4 positive immunostainings for the CD68, CD14, CD11b, and CD11c were detected coincidently in the same tissue (SC tailhead) and in the same CLA fed HF cow at 42 DIM. The same animal showed positive staining for the CD68 and CD11c in the SC depot from sternum at 42 DIM (Table 2). Although we hypothesized that in bovine AT, phagocytic immune cell infiltration might be affected by dietary CLA supplementation, no obvious differences with regard to the CLA supplementation were observed in HF cows.

Several authors reported that the *t*10,*c*12 isomer promotes ATM infiltration and decreases leptin and adiponectin secretion, which in turn contributes to insulin resistance and hyperinsulinemia in CLA-fed humans and animals (Pérez-Matute et al., 2007; Clément et al., 2002; Poirier et al., 2006). The *t*10,*c*12 isomer rapidly induced inflammatory factors, such as TNF-α and IL-6 in AT (Ahn et al., 2006, Poirier et al., 2006). *In vitro* studies with 3T3-L1 and human adipocytes have also shown that the *t*10,*c*12 isomer directly stimulates IL-6 secretion via a nuclear factor-kappa B-dependent mechanism (Poirier et al., 2006). In contrast, supplementation with *c*9,*t*11 isomer results in low ATM infiltration with concomitant decrease in inflammatory cytokines, which may in turn improve insulin sensitivity in mice (Moloney et al., 2007). However, the present study did not focus on the individual CLA isomer, but tested mixture at a ratio of 50:50 for the main *c*9,*t*11 and *t*10,*c*12 isomers.

## Conclusions

The portion of phagocytic immune cells both in SC and VC fat depots was marginal in early-lactating cows. The average portion of ATM (CD68+) in few positive samples was slightly lower in SC versus VC fat depots. No significant alterations in this infiltration phenomenon, with regards to DIM and CLA supplementation were observed during early lactation. Increased accumulation of phagocytic immune cells, albeit at low cell numbers, in non-pregnant, over-conditioned heifers could be related to larger adipocytes compared with the early-lactating cows. Based on the small portion of positive samples, we concluded that the extent of fatness in early-lactating dairy cows may not be high enough to stimulate significant infiltration of phagocytic cells and therefore, these immune cells might have no major role in the immunologic and metabolic adaptations during early lactation.

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## Table 1: Primary mouse monoclonal antibodies used in this study

| Targets | Clones | Sources | Dilutions | Specificity | References |
|---|---|---|---|---|---|
| CD68 (human) | EBM11 | DakoCytomation, Glostrup, Denmark | 1:100 | Macrophages | Bielefeldt-Ohmann et al., 1988 |
| CD14 (bovine) | CAM36A | VMRD, Pullman, WA., USA) | 1:100 | Macrophages, monocytes | Miyazawa et al., 2006 |
| CD11b (canine) | CA16.3E 10 | Biozol, Eching, Germany | 1:25 | Macrophages, monocytes, granulocytes | Danilenko et al., 1992. |
| CD11c (bovine) | BAQ153 A | VMRD | 1:25 | Macrophages, monocytes, | Miyazawa et al., 2006 |

**Table 2:** **Portion (%) of positive cells expressing CD68, CD14, CD11b, and CD11c markers in different fat depots of Holstein cows during the first 105 DIM** (to allow for tracing back individual positive sample, respective individual animal identification numbers are given in brackets)

| Item | Markers | 1 DIM | 42 DIM | 105 DIM |
|---|---|---|---|---|
| Control group (n = 15) | | | | |
| Subcutaneous (SC) depots | | | | |
| Tailhead | CD68: | - | - | - |
| | CD14: | - | - | - |
| | CD11b: | - | - | - |
| | CD11c: | - | - | - |
| Withers | CD68: | - | 4.5 (657) | - |
| | CD14: | - | - | - |
| | CD11b: | - | - | 1.8 (647) |
| | CD11c: | - | - | - |
| Sternum | CD68: | - | - | - |
| | CD14: | - | - | - |
| | CD11b: | - | - | 0.7 (647) |
| | CD11c: | - | - | - |
| Visceral (VC) depots | | | | |
| Omental | CD68: | 14.3 (635) | 12.8 (657) | - |
| | CD14: | - | - | - |
| | CD11b: | 0.7 (635), 4.7 (664) | - | - |
| | CD11c: | - | - | - |
| Mesenteric | CD68: | 41.6 (619), 27.2 (664) | - | - |
| | CD14: | - | - | - |
| | CD11b: | 0.4 (664) | - | - |
| | CD11c: | - | - | - |
| Retroperitoneal | CD68: | - | - | 46.8 (647) |
| | CD14: | - | - | - |
| | CD11b: | - | - | 0.8 (640), 1.5 (647) |
| | CD11c: | - | - | - |
| Conjugated linoleic acid group (n = 10) | | | | |
| SC depots | | | | |
| Tailhead | CD68: | | 2.0 (668) | - |
| | CD14: | | 2.0 (668) | - |
| | CD11b: | | 3.3 (668) | - |
| | CD11c: | | 1.2 (668) | - |
| Withers | CD68: | | - | - |
| | CD14: | | - | - |
| | CD11b: | | - | - |
| | CD11c: | | - | - |
| Sternum | CD68: | | 4.7 (668) | - |
| | CD14: | | - | - |
| | CD11b: | | - | - |
| | CD11c: | | 6.3 (668) | - |
| VC depots | | | | |
| Omental | CD68: | | - | 7.2 (655) |
| | CD14: | | - | - |
| | CD11b: | | - | - |
| | CD11c: | | - | - |
| Mesenteric | CD68: | | - | - |
| | CD14: | | - | - |
| | CD11b: | | - | - |
| | CD11c: | | - | - |
| Retroperitoneal | CD68: | | 23.2 (666) | - |
| | CD14: | | - | - |
| | CD11b: | | - | - |
| | CD11c: | | - | - |

**Table 3: Portion (%) of immune cells in the subcutaneous tailhead fat in early-lactating primiparous (HF) cows, non-pregnant, over-conditioned (SM) heifers and in fat steers (RP) (means ± SEM)**

| Item | CD68 | CD14 | CD11b | CD11c |
|---|---|---|---|---|
| HF cows (n = 25) | 2.0 (n = 1)* | 2.0 (n = 1)*† | 3.3 (n = 1)*† | 1.2 (n = 1)*† |
| SM heifers (n = 36) | 1.9 ± 0.4 (n = 23) | 1.7 ± 0.4 (n = 17) | 2.2 ± 0.6 (n = 12) | 2.1± 0.7 (n = 21) |
| RP steers (n = 3) | - | 2.5 ± 0.8 (n = 3) | 2.1 (n = 1) | - |

*Indicates the tissue sample coincidently from same animal (ear tag 668)

†Indicates n = 10 from early-lactating HF cows were tested.

n = number of samples.

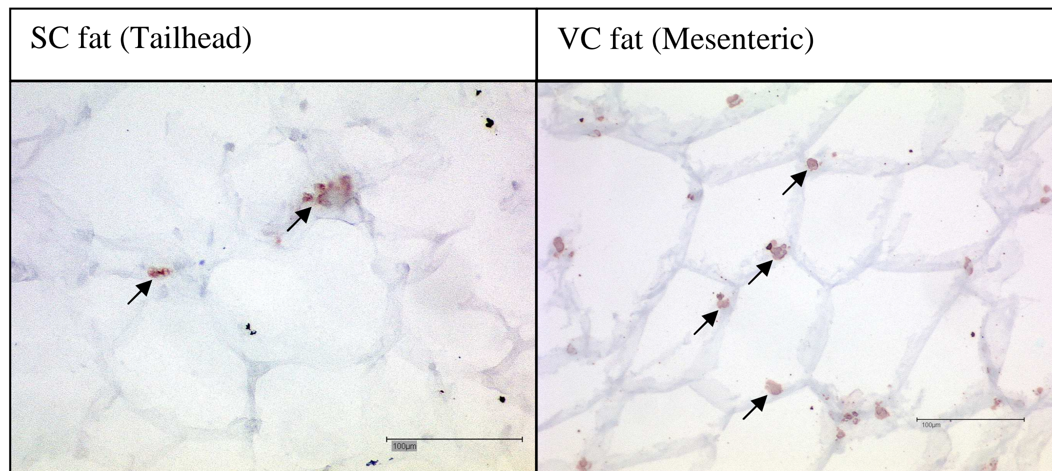


**Fig. 1:** **Immunohistochemical localization of adipose tissue macrophages in subcutaneous (SC) fat from the tailhead and visceral (VC) fat from the mesenteric depot in early-lactating cows, using antibody against CD68.** Representative positive cells (arrows designate examples) appear as red staining around mature adipocytes. Macrophages were more frequent in mesenteric fat than in the SC tailhead depot. Original magnification 200×; Scale bar = 100 µm.

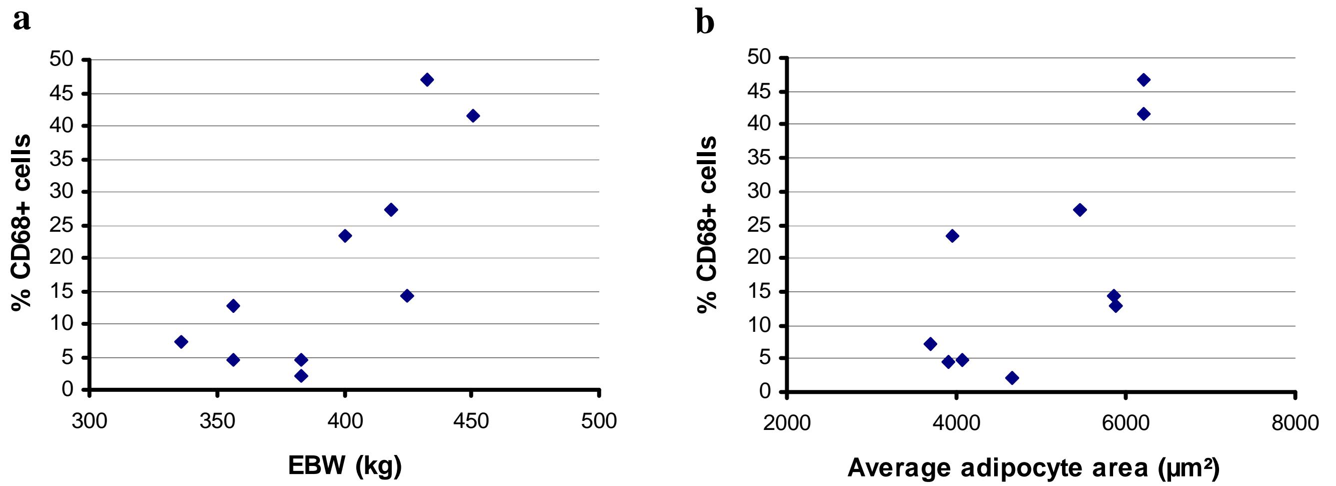


**Fig. 2:** **Correlations between the portion of CD68+ cells with (a) empty body weight (EBW) and (b) adipocyte size.** Strong positive correlations were found between the portion of CD68+ cells with both EBW (r = 0.788, *P* = 0.007, n = 10) and mean adipocyte area (r = 0.684, *P* = 0.029, n = 10). Data included herein were collected from all positive immunostained samples of early-lactating Holstein-Friesian cows.

# 4 General discussion and conclusions

The present research was carried out to compare different SC and VC fat depots of dairy cows in terms of adipocyte size and number of phagocytic immune cells present. Furthermore, the effects of CLA on adipocyte size and on the invasion of phagocytic immune cells in different fat depots were investigated in dairy cows up to the first 105 DIM. This is the first report on the effects of CLA on bovine AT cellularity. Herein, we consider potential changes within AT and put it into relation with concomitant changes in the entire organism during early lactation.

Early lactation is attended by massive fat mobilization. In the present study, the concomitant changes of adipocyte size were non-uniform in different SC and VC fat depots during early lactation. For CON animals, adipocyte sizes were decreased at 42 and 105 DIM compared to 1 DIM in almost all the SC and VC fat depots examined, but the decrease in the retroperitoneal fat only tended to be significant at 105 DIM. Previous studies on the cellularity of bovine AT have reported a decrease in the mean adipocyte size of the SC fat during early lactation (Pike and Roberts, 1980, 1981). The present study confirms the cellularity changes found in these previous reports and further characterizes cellularity variable in different anatomical locations of SC and VC fat up to 105 DIM. Moreover, in a companion study (von Soosten et al., 2011), the authors found that the retroperitoneal fat depot weight for CON animals was significantly decreased at 42 DIM and numerically decreased at 105 DIM compared to 1 DIM. Combining the results from this study with that reported in the aforementioned companion study, we herein confirm that the retroperitoneal fat might have undergone the greatest changes during early lactation. In the same study, von Soosten et al. also reported that supplementation of dairy cows with CLA decreases milk fat but leaves body fat mostly unaffected in terms of depot mass. However, when considering adipocyte sizes of different SC and VC fat depots of dairy cows, we herein observed decreasing effects of CLA up to 105 DIM, albeit these alterations were not mirrored by the plasma NEFA concentrations. However, the positive correlation between adipocyte size and plasma BHBA, indicate that the processes of lipolysis might be increased in AT in a depot-specific manner both in CON and CLA supplemented cows. We hereby speculate that the CLA-induced decreases in adipocyte size may indicate lipolytic and/or antilipogenic effects of CLA on AT in primiparous dairy cows.

Human and mouse studies suggest that increased body fat mass is associated with increased infiltration of ATM, and induces AT inflammation and insulin resistance (Weisberg et al., 2003; Xu et al., 2003). However, during early lactation, the state of NEB provides more plasma NEFA and BHBA that exert inhibitory effects on leukocytes and thus contribute to the immunocompromised situation in ewes and dairy heifers (Lacetera et al., 2002, 2004). We herein present immunohistochemical evidence against an appreciable infiltration of phagocytic immune cells into different AT depots of dairy cows during early lactation. The average portion of ATM (CD68+) in a few positive samples was numerically lower in SC versus VC fat depot. This may be due to the fact that VC fat is more metabolically active and is thought to differ from SC fat in the production of adipokines (Altomonte et al., 2003). In addition, increased accumulation of phagocytic immune cells (albeit at low cell numbers) in SC fat of non-pregnant, over-conditioned heifers might be related to larger adipocytes secreting higher amounts of chemoattractant adipokines compared with the early-lactating cows.

In conclusion, dietary CLA supplements have site-specific effects on adipocyte size of different fat depots in dairy cows. The results suggest that dairy cows respond to CLA in terms of a decrease in adipocyte size in the same way as other monogastric species such as rodents and pigs. The present data also suggest that CLA had no effect on the invasion of phagocytic immune cells in bovine AT. Early-lactating dairy cows are not 'obese' and may thus lack significant infiltration of phagocytic cells in bovine AT. Therefore these immune cells might have no major role in the immunologic and metabolic adaptations during early lactation. However, the present study did not focus on individual CLA isomers, since a mixture at a ratio of 50:50 *c*9,*t*11 and *t*10,*c*12, the two major isomers, was used as feed supplement. Further research is required on the action of individual *c*9,*t*11 and *t*10,*c*12 isomers in order to better understand and evaluate their physiological effects on body fat depots in dairy cows. Moreover, recent study put in evidence a clear effect of CLA on the differentiation of bovine preadipocytes (Lengi and Corl, 2010). Therefore, another line of investigation should focus on characterizing bovine AT in terms of the number and size of preadipocytes. Furthermore, typization of ATM subpopulations would be potentially interesting for further research.

# 5 Summary

High-yielding dairy cows experience a period of NEB during early lactation and are then susceptible to metabolic disorders, compromised immune response and fertility. Dietary intake at this period lags behind the increase of nutrient needs; therefore energy has to be mobilized from body reserves mainly from the WAT. Nevertheless, WAT is not a functionally homogeneous tissue; rather, it consists of different depots with considerable functional and morphologic differences. Besides its main function as an energy storage depot, WAT is currently considered as an endocrine organ capable of producing and secreting adipokines. The adipose-derived hormones affect a wide range of different processes including appetite regulation, glucose metabolism, insulin sensitivity, lipid metabolism, inflammation, and immune functions. In the context of obesity and high-fat diet, humans and mice exhibit increased infiltration of ATM, particularly in VC depots to form a proinflammatory micromilieu causing local and systemic insulin resistance. However, the metabolic stress situation in early-lactating dairy cows is similar to some symptoms observed in obese individuals suffering from metabolic syndrome, for example, decreased insulin sensitivity in peripheral tissues. Furthermore, the state of NEB elicits increased release of plasma NEFA and BHBA that exert inhibitory effects on leukocytes and thus contribute to the immunocompromised situation in early lactation, leading to increased susceptibility to infectious diseases. To our knowledge, no reports exist about body fat-decreasing effects of CLA in dairy cows. Moreover, evidences for phagocytic immune cell infiltration into bovine AT, in particular addressing the heterogeneity of SC versus VC fat depots are lacking.

Conjugated linoleic acid, a naturally occurring fatty acid, derived from beef and dairy products has been found to decrease milk fat excretion in lactating animals. Dietary CLA supplements also decrease body fat mass in several monogastric species, such as rodents and pigs. Herein, we hypothesized that dietary CLA supplements have site-specific effects on adipocyte size of different fat depots in dairy cows, with regards to the concomitant changes during early lactation. It was also hypothesized that low incidence of AT phagocytic immune cell infiltration might occur in early-lactating dairy cows, since they are certainly not ‘obese’. Therefore, the experiments conducted herein were aimed: 1) to characterize different SC and VC fat depots of dairy cows in terms of adipocyte size, 2) to investigate phagocytic immune cell infiltration in bovine AT, 3) to

evaluate the effects of CLA on adipocyte size of different fat depots in dairy cows during the first 105 DIM, and 4) to investigate the potential effects of CLA on the invasion of phagocytic immune cells into bovine AT.

German Holstein cows (n = 25; mean BCS = 3.0) were randomly allocated to either the CON (n = 15) or the CLA (n = 10) group. From the first DIM until sample collection, the CLA cows were fed 100 g of CLA/d (containing about 6% each of the *c*9,*t*11 and *t*10,*c*12 isomers), whereas CON cows received 100 g/d of a fatty acid mixture without CLA. The CON cows (n = 5 each) were slaughtered at 1, 42, and 105 DIM, and the CLA cows (n = 5 each) were slaughtered at 42 and 105 DIM. Adipose tissues (n = 150) from three SC depots (tailhead, withers, and sternum) and from three VC depots (omental, mesenteric, and retroperitoneal) were sampled. In addition, SC tailhead AT samples were collected by repeated surgical biopsies (3 samplings within 7 weeks, n = 36) from non-pregnant, non-lactating Simmental heifers (n = 12; mean BCS = 5.0) fed diets of varying energy density. Hematoxylin-eosin staining was done on 10-µm-thick paraffin-embedded tissue sections (n = 150). Adipocyte area ($\mu m^2$) of 100 adipocytes per section was measured at magnification 100×. Immunohistochemistry was used to quantify CD68, CD14, CD11b, and CD11c positive cells on AT cryosections (14 to 18 µm). The portion of immunopositive cells per sample was calculated as the sum of the number of immunopositive cells divided by the total number of cells per section and then multiplied by 100 (magnification 100×, 10 fields/sample).

We herein demonstrated that significant changes related to duration of lactation were limited to retroperitoneal fat: adipocyte sizes were significantly smaller at 105 DIM compared to 1 DIM in CON cows. Adipocyte sizes were decreased in SC depots from the tailhead at 105 DIM and from the sternum at 42 DIM in CLA versus CON cows, whereas for VC depots adipocyte sizes were decreased in mesenteric fat at 42 and 105 DIM, and in omental and retroperitoneal fat at 105 DIM in CLA versus CON cows. Within the CLA group, adipocyte sizes were smaller in the SC depot from the tailhead at 105 DIM compared to 42 DIM. Adipocyte sizes and depot weights were significantly correlated in SC depot ($r = 0.795$, $P < 0.01$) in the CLA group and in retroperitoneal fat both in the CON ($r = 0.698$, $P < 0.01$) and the CLA ($r = 0.723$, $P < 0.05$) group. The present long-term study suggested for the first time that dietary CLA supplements decrease adipocyte sizes of both SC and VC fat depots to different extent in dairy cows. Moreover, the positive correlation between adipocyte size and plasma BHBA,

suggesting that the processes of lipolysis might be increased in AT in a depot-specific manner both in CON and in CLA supplemented cows. The results indicated that CLA might have lipolytic and/or anti-lipogenic effects on bovine AT, similar to what is known from CLA supplemented monogastrics. However, immunohistochemical analyses of different fat depots revealed a low incidence of phagocytic immune cell infiltration in early-lactating cows. The average portion of ATM (CD68+) in a few positive samples was slightly but nonsignificantly higher in VC versus SC fat. In addition, increased accumulation of phagocytic immune cells (albeit at low cell numbers) in SC fat of non-pregnant, over-conditioned heifers might be related to larger adipocyte size secreting higher amounts of chemoattractant adipokines compared with the early-lactating cows. The results indicated that the extent of fatness in early-lactating cows may not be high enough to stimulate significant infiltration of phagocytic cells. Therefore, these immune cells might have no importance for the immunologic and metabolic adaptations during early lactation in dairy cows. The present data also suggested that CLA had no effect on the invasion of these cells into bovine AT.

The results are of general importance to Animal Science and provide a general basis for the regulatory role of AT in ruminants. The present dissertation also contributes to improve the knowledge about the long-term effects of dietary CLA supplements on body fat depots in dairy cows. Further research on the effects of individual *c*9,*t*11 and *t*10,*c*12 isomers on body fat depots of dairy cows should be carried out. Since dietary CLA supplementation has effects on bovine preadipocytes differentiation, another line of investigation should focus on characterizing bovine AT in terms of the number and size of preadipocytes. Furthermore, typization of ATM subpopulations would be potentially interesting for further research.

# 6 Zusammenfassung

Während der Frühlaktation kommt es bei hochleistenden Milchkühen zu einer Phase der NEB, in der sie für Stoffwechselerkrankungen anfällig und sowohl die Immunantwort als auch die Fruchtbarkeit eingeschränkt sind. Der erhöhte Energiebedarf kann nicht durch die Futteraufnahme ausgleichen werden, sondern wird durch die Mobilisierung von Körperfettreserven, vor allem dem weißen Fettgewebe (WAT), kompensiert. Das WAT besteht aus verschiedenen Depots mit erheblichen funktionellen und morphologischen Unterschieden. Neben seiner Hauptfunktion als Energiespeicher, ist das WAT als endokrines Organ angesehen, das Adipokine produziert und sekretiert. Die aus dem Fettgewebe stammenden Hormone beeinflussen verschiedene Prozesse wie z.B. Appetit, Glukosestoffwechsel, Insulinsensitivität, Fettstoffwechsel, Entzündungsprozesse und Immunfunktionen. Bei Übergewicht sowie einer fettreichen Diät, weisen Menschen und Nager eine erhöhte Infiltration von Makrophagen (ATM) vor allem ins VC Fettgewebe auf, was zur Ausbildung eines proinflammatorischen Mikromilieus und einer lokalen und systemischen Insulinresistenz führt. Einige Symptome, die während der Stoffwechsel-belastenden Situation bei Kühen während der Frühlaktation auftreten, ähneln denen des metabolischen Syndroms, beispielsweise die verringerte Insulinsensitivität in peripheren Geweben. Des Weiteren kommt es während der NEB zu einer erhöhten Freisetzung von Plasma-NEFA und -BHBA, wodurch die Leukozytenbildung gehemmt wird, was schließlich zu einem verschlechterten Immunstatus in der Frühlaktation beiträgt. Die Anfälligkeit für Infektionskrankheiten wird dadurch erhöht. Bislang sind keine Studien über die Körperfett reduzierenden Effekte von CLA bei Milchkühen bekannt. Des Weiteren fehlen Nachweise über die Infiltration phagozytierender Immunzellen in das bovine AT, insbesondere bezüglich der Unterschiede zwischen SC zu VC Fettdepots.

Konjugierte Linolsäuren, die natürlicherweise in Fleisch- und Milchprodukten vorkommen, reduzieren die Milchfettexkretion bei laktierenden Tieren. Daneben reduzieren CLA-Supplemente die Körperfettmasse bei verschiedenen Monogastriern, z.B. bei Nagetieren und Schweinen. Dies führte zu der Annahme, dass CLA-Supplemente während der Frühlaktation gewebsspezifische Effekte auf die Adipozytengröße in verschiedenen Fettdepots von Milchkühen vermitteln. Außerdem nahmen wir an, dass eine geringe Infiltration phagozytierende Immunzellen in das AT bei frühlaktierenden Milchkühen auftritt, da diese nicht „übergewichtig“ sind. Daher

hatte die vorliegende Arbeit folgende Ziele: 1) die Adipozytengröße in den verschiedenen SC und VC Fettdepots bei Milchkühen zu charakterisieren, 2) den Einfluss von CLA auf die Adipozytengröße verschiedener Fettdepots von Milchkühen während der ersten 105 Tage der Laktation zu evaluieren, 3) die Infiltration phagozytierender Immunzellen in das bovine AT zu untersuchen, 4) die möglichen Effekte von CLA auf die Infiltration phagozytierender Immunzellen in das bovine AT zu ermitteln.

Deutsche Holstein Kühe (n = 25; mittlerer BCS = 3,0) wurden zufällig einer CON (n = 15) und einer CLA (n = 10) Gruppe zugeteilt. Ab dem ersten Laktationstag bis zur Probenahme wurden die CLA-Kühe mit 100 g CLA/Tag (mit je 6% *c*9,*t*11- und *t*10,*c*12-Isomer) gefüttert, während die CON-Kühe 100 g/Tag eines Fettsäuregemisches ohne CLA erhielten. An Tag 1, 42 und 105 der Laktation wurden je fünf CON-Tiere geschlachtet. Die CLA-Tiere (n = 5) wurden an Tag 42 und 105 der Laktation geschlachtet. Es wurden Gewebeproben (n = 150) aus 3 SC (Schwanzansatz, Widerrist, Brust) und 3 VC Fettdepots (omental, mesenteric, retroperitoneal) entnommen. Zusätzlich wurden wiederholt chirurgische Biopsien (3 Probenahmen innerhalb von sieben Wochen, n = 36) von SC Fettgewebsproben aus der Region des Schwanzansatzes von nicht-trächtigen, nicht-laktierenden Fleckvieh-Färsen (n = 12; mittlerer BCS = 5,0), deren Futter unterschiedliche Energiedichten aufwiesen, entnommen. Paraffinschnitte (10 µm, n = 150) wurden mit Hämatoxylin-Eosin gefärbt. Die Adipozytengröße (µm²) von 100 Adipozyten je Schnitt wurde bei 100-facher Vergrößerung ausgezählt. Mittels Immunhistochemie wurde die Anzahl positiver CD68, CD14, CD11b, CD11c Zellen auf AT Gefrierschnitten (14 bis 18 µm) bestimmt. Der prozentuale Anteil positiver Zellen wurde aus der Summe der immunpositiven Zellen auf die Gesamtzellzahl pro Schnitt bezogen und mit dem Faktor 100 multipliziert (100-fache Vergrößerung, 10 Ausschnitte pro Probe).

Signifikante Unterschiede bezüglich des Laktationsverlaufes waren auf das retroperitoneale Fett begrenzt waren: die Adipozytengröße war bei den CON-Tieren an Tag 105 der Laktation im Vergleich zu Tag 1 der Laktation signifikant kleiner. Die Adipozytengröße in SC Schwanzfettproben an Tag 105 der Laktation und dem Brustfett an Tag 42 der Laktation waren bei CLA-Tieren im Vergleich zu den CON-Tieren verringert. Im VC Depot war die Adipozytengröße im mesenterialen Fett an Tag 42 und 105 der Laktation, sowie im omentalen und retroperitonealen Fett an Tag 105 der Laktation in den CLA-Tieren im Vergleich zu den CON-Tieren verringert. Innerhalb

der CLA-Gruppe war die Adipozytengröße an Tag 105 der Laktation im SC Schwanzfett kleiner als an Tag 42. Die Adipozytengröße und das Depotgewicht korrelierten im SC Depot ($r = 0{,}795$, $P < 0{,}01$) in der CLA-Gruppe und im retroperitonealen Fett sowohl in der CON ($r = 0{,}698$, $P < 0{,}01$) als auch in der CLA ($r = 0{,}723$, $P < 0{,}05$) Gruppe signifikant. Diese Langzeitstudie zeigt zum ersten Mal, dass CLA-Supplemente bei Milchkühen die Adipozytengröße von SC und VC Fettdepots in unterschiedlichem Ausmaß verringern können. Weiterhin weist die positive Korrelation zwischen Adipozytengröße und Plasma-BHBA darauf hin, dass die Lipolyse im AT sowohl in den CON als auch in den CLA-supplementierten Kühen depotspezifisch erhöht sein könnte. Die Ergebnisse lassen vermuten, dass CLA ähnlich wie bei Monogastern, lipolytische und/ oder antilipogene Effekte auf das bovine AT haben könnten. Es konnte jedoch lediglich eine geringe Infiltration phagozytierender Immunzellen in verschiedene Fettgewebe der frühlaktierenden Kühe nachgewiesen werden. In den wenigen positiven Proben war die Anzahl von ATM (CD68+) leicht, aber nicht signifikant höher in den VC als in den SC Fettdepots. Außerdem könnte die erhöhte Ansammlung phagozytierender Immmunzellen (unabhängig ihrer geringen Anzahl) im SC Fett nicht-tragender, überkonditionierter Färsen, mit der erhöhten Adipozytengröße und der damit einhergehenden erhöhten Sekretion von Chemokinen zusammenhängen. Der Körperfettgehalt bei Kühen in der Frühlaktation ist eventuell nicht hoch genug, um eine signifikante Infiltration phagozytierender Zellen zu bewirken. Daher sind diese Immunzellen in der immunologischen und metabolischen Anpassung bei Milchkühen während der Frühlaktation offenbar nicht von Bedeutung. CLA scheinen zudem keinen Effekt auf die Infiltration dieser Zellen in das bovine AT zu haben.

Die Ergebnisse sind von allgemeiner Bedeutung für die Nutztierwissenschaften und liefern grundlegende Erkenntnisse für das Verständnis der Regulation des AT bei Wiederkäuern. Die hier vorliegende Dissertation trägt dazu bei, das Wissen über Langzeiteffekte von CLA-Supplementen auf die Körperfettdepots von Milchkühen zu verbessern. Um individuelle Effekte der *c*9,*t*11 und *t*10,*c*12 Isomere auf die Körperfettdepots von Milchkühen zu untersuchen, sind weitere Studien notwendig. Da CLA-Gaben die Differenzierung von bovinen Präadipozyten beeinflussen können, sollten weitere Studien zur Charakterisierung des bovinen AT im Zusammenhang mit Anzahl und Größe von Präadipozyten durchgeführt werden. Weiterhin wäre die Typisierung von ATM-Subpopulationen für die weitere Forschung wichtig.

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