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Proliferation and apoptosis in subcutaneous adipose tissue of lactating cows with different genetic merit for milk yield

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ABSTRACT

The aim of this study was to investigate the adipocyte size and fate in subcutaneous fat (scAT) of cows diverging for genetic merit at mid lactation stage, when anabolic activity increases and animals are in a state of positive energy balance. Twenty mid lactation cows (180 ± 20 days in milk) grouped according to the Estimated Breeding Values (EBV) for milk yield in plus (EBVp) and minus (EBVm) variants were selected. Average of adipocytes area, proliferation and apoptotic labelling index as well as DLK-1 expression, a marker of pre-adipocytes, were immunohistochemically evaluated in scAT biopsies. In EBVp cows, the BCS was lower ($P < 0.01$) whereas milk yield, protein, fat yield ($P < 0.001$) and plasma free fatty acid concentration ($P < 0.05$) were higher. The scAT of EBVp cows showed a significantly ($P < 0.001$) higher frequency between 500 and 3000 μm^2 classes in comparison to EBVm cows, that showed a significantly ($P < 0.01$) higher apoptotic labeling index. The immunohistochemical reaction showed DLK-1 positivity in scAT of EBVp cows. Taking together, the data indicate a link between milk yield genetic merit of cows, scAT morphology and function, suggesting greater dynamics and metabolic flexibility in EBVp cows.

1. Introduction

Adipose tissue (AT) is a tissue that changes its mass during the adult life. The cellular development and dynamics of AT is a result of proliferation of cell number (hyperplasia) and of an increase of cell size (hypertrophy) (Hausman et al., 2001). Although mature adipocytes should not have the capacity to divide, there are studies that reported the replication of mature adipocytes *in vitro* (Loffler et al., 1994). Also partially differentiated cells, such as pre-adipocytes, are able to duplicate prior to differentiation (Prins and O'Rahilly, 1997). Proliferation is under hormonal, nervous and paracrine control (Hausman et al., 2001) and it has been also demonstrated that region-specific differences in fat cell precursors could contribute to differential adipose tissue growth (Dijan et al., 1983). Delta-like 1 homolog (DLK-1) is a well-established marker of pre-adipocytes (Sul, 2009). DLK-1 is a transmembrane glycoprotein with epidermal growth factor (EGF)-like repeats in the extracellular domain (Sul, 2009). Membrane-bound DLK-1 acts as an inhibitor of *in vitro* pre-adipocyte differentiation and inhibits pre-adipocyte proliferation by regulating their entry into the G1/S-phase of the cell cycle (Mortensen et al., 2012). The membrane tethered DLK1 can be cleaved by tumor necrosis α converting enzyme (TACE), generating a biologically active soluble form (Hudak and Sul, 2013) that maintains proliferating cells in an undifferentiated state during development (Wang et al., 2010). Instead, apoptosis, in human AT, has been considered as a result of macrophage recruitment in visceral AT of obese patients (Aron-Wisnewsky et al., 2009), although about 20% apoptosis was observed also in *in vitro* differentiating human visceral pre-adipocytes (Pomari et al., 2015; Colitti and Stefanon, 2016). In subcutaneous and visceral fat of cows at early lactation, only marginal infiltration of these inflammatory cells was reported (Akter et al., 2012).

Considering the peculiar fat metabolism in dairy cows, it is likely that other regulatory mechanisms are involved in cell death of adipocytes in cattle.

A large body of research has been devoted to the study of fat metabolism and its implication to physiological regulation and healthy or pathologically conditions of dairy cows (Summer et al., 2005; Graugnard et al., 2012; Rocco and McNamara, 2013). The energy deficit during early lactation in high yielding dairy cows is accompanied by fat mobilization from AT. In a condition of negative energy balance (NEB), reduced lipogenesis and increased lipolysis is described in discrete AT depots (McNamara and Hillers, 1986). When the energy balance reaches positive values, the energy is stored in AT depots, which are increasingly refilled by means of lipogenesis and by adipogenesis, thus preparing the organism for

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the upcoming energy deficit in the subsequent lactation (Hausman et al., 2001; McNamara, 1995).

Adipose tissue responds to biochemical and nervous signals and it plays an active role during lactation through the secretion of signaling molecules (Colitti, 2015; Hovey and Aimo, 2010). Diet composition and plan of nutrition, parity and milk yield strongly influence the extent of AT accumulation and losses and it has been observed that also genetic merit of the cow can influence the mobilization of fat depots (Sgorlon et al., 2015a). Adipose tissue mass is determined by balancing lipolysis, lipogenesis, and adipocyte proliferation (Della-Fera et al., 2001) and it is likely that genetic make-up of the animal regulates the nutrient fluxes among tissues and modulates the expression of genes which orchestrate the metabolism and the fate of adipose cells (Khan et al., 2013; Rocco and McNamara, 2013).

In this study, size, cell proliferation and apoptosis were measured in subcutaneous adipose tissue (scAT) of dairy cows in the mid stage of lactation to evaluate if the genetic merit for milk yield can be related to variations of adipocyte functions.

2. Material and methods

2.1. Animals and sampling

Twenty mid lactation and pregnant Simmental cows (180 ± 20 days in milk, DIM) from a commercial farm were selected from a herd of 280 lactating cows. Cows were housed in the farm in free-stall barn, had free access to fresh water and fed *ad libitum* a total mixed ration (TMR) regularly twice a day, at around 07.00 a.m. and 17.00 p.m. Diets were formulated to cover the nutrient requirements for lactation (NRC, 2001) and further details can be found in Sgorlon et al. (2015b). All animals included in the study were clinically healthy, at parity 3, had somatic cell count in the milk lower than 200,000/ml and a body condition score (BCS; Edmonson et al., 1989) between 2.75–3.25. Moreover, cows were selected according to their Estimated Breeding Values for milk yield (EBV), provided by Italian Simmental (ANAPRI; www.anapri.eu) breed association. The EBV is an approximation of the genetic merit of a cow for a defined phenotype and is estimated by animal model using data records and kinships. The genetic merit is simply the additive effect of an individual's genotype on the trait expressed relative to the population mean phenotype. (Wilson et al., 2011). Ten animals with the extreme positive and 10 animals with the extreme negative EBV values for milk, fat and protein yields were included in the study (Table 1). The selected animals were in the plus or minus 10% of the population.

On the day of official milk recording, during morning milking (from 5.00 a.m. to 6.00 a.m.), 50 ml of milk from each cow was sampled into a tube with preservative (Bronopol, 0.02% w/v). Milk protein, fat, lactose contents as well as somatic cell count were predicted using the mid infrared spectroscopy method (Fourier Transform Instrument, FT6000, Foss Electric, Hillerød, Denmark). After milking right before the morning meal, blood was sampled from the coccygeal vein in 10 ml vacuum tubes with lithium heparin or K3-EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium). The blood was centrifuged within 1 h at 3000 RPM for 10 min at 20 °C and the plasma samples were stored at −20 °C until further analysis. The analyses of glucose (mmol/L), urea (mmol/L) and cholesterol (mmol/L) were performed using a Roche Cobas® 6000 analyzer and proprietary kits (Hoffmann-La Roche AG, Basel Switzerland). Free fatty acids (FFA, mmol/L) and beta-hydroxy-butyrate acid (BHBA, mmol/L) were measured with Randox kits (Randox Laboratories Limited, Crumlin UK).

Biopsies of subcutaneous adipose tissue (scAT) were collected from the dorsal pelvic region as described by McNamara and Hillers (1986), using a 20 gauge biopsy needle. Tissue samples were fixed in 10% (w/v) neutral formalin for 24 h at room temperature, processed for paraffin embedding and cut at a thickness of 5–7 μm. Sections meant for a morphometric analysis were stained with haematoxylin-eosin, observed and photographed at 20× under Leica DM750 microscope equipped with Leica ICC50 HD (Leica Microsystems, Milan, Italy).

The study complies with the national regulation on the use of animals in research and was approved by the bioethical committee of University of Udine.

Table 1
Mean (± standard error, SE) of body condition score (BCS), days in milk (DIM), milk yield, milk composition and hematological variables in the two groups of cows diverging for estimated breeding value (EBV) for milk yield. Ten cows with the highest EBV (EBVp) and 10 cows with lowest EBV (EBVm) were selected within the herd.

Item	EBVp mean ± SE	EBVm mean ± SE	*P* value
BCS	2.81 ± 0.11	3.25 ± 0.05	**
DIM	175.6 ± 6.96	162.8 ± 7.06	n.s.
EBV milk yield	1077.4 ± 26.33	−492.7 ± 23.99	***
EBV fat yield	34.5 ± 1.39	−17.7 ± 1.01	***
EBV protein yield	33.7 ± 0.66	−16.0 ± 0.95	***
Milk			
Yield (Kg)	31.02 ± 1.39	19.84 ± 1.88	**
Fat (%)	3.79 ± 0.15	3.89 ± 0.21	n.s.
Protein (%)	3.71 ± 0.09	3.72 ± 0.15	n.s.
Fat yield (kg/d)	1.16 ± 0.05	0.75 ± 0.06	***
Protein yield (kg/d)	1.15 ± 0.05	0.74 ± 0.07	***
Plasma			
Glucose (mmol/L)	3.92 ± 0.06	3.98 ± 0.11	n.s.
Cholesterol (mmol/L)	6.04 ± 0.52	5.37 ± 0.75	n.s.
Urea (mmol/L)	6.00 ± 0.46	6.55 ± 0.31	n.s.
FFA (mmol/L)	0.14 ± 0.03	0.08 ± 0.01	*
BHBA (mmol/L)	0.71 ± 0.09	0.63 ± 0.08	n.s.

n.s. = not significant; * = P < 0.05; ** = P < 0.01; ***P < 0.001.
FFA: Free Fatty Acids.
BHBA: Beta Hydroxy Butyrate Acid.

2.2. Histomorphology

Adipocytes Tool, a macro of ImageJ1.50b software (http://rsb.info.nih.gov/ij/), was used to measure adipocyte area (in μm^2) (Baecker, 2012). After running the macro, the scale was settled for magnification and the global checkbox on the set scale dialog was chosen. The options in the 'p' and 's' buttons of macro were settled and measurements were checked by the roi manager. The areas of the adipocytes were measured on ten different field for each sample slide. Cell area of 490.2 ± 62.7 (mean ± se) cells was measured in EBVp group and 413.0 ± 86.2 (mean ± se) cells was measured in EBVm group.

To label macrophages sections were stained with periodic acid-Schiff (PAS) according to standard protocol (Sigma, Milan, Italy).

2.3. Immunohistochemistry analyses

Immunohistochemical investigations were performed as described more fully by Colitti (2015). The avidin-biotin-peroxidase complex (ABC) method was performed using the Vectastain ABC kit (PK-4000, Vector Laboratories, Burlingame, CA, USA). Primary antisera were horse anti-Ki-67 (Novocastra, U.K.) and rabbit anti-DLK (Abcam, Cambridge UK). Ki-67 and DLK were visualized using the 3,30-diaminobenzidine tetrachloride (DAB solution, Vector Laboratories, Burlingame, CA) as a chromogen.

In the control sections, blocking solution was substituted for the primary antibody. Lymph node and adrenal gland were used as positive controls for Ki-67 and DLK reactions respectively.