



UCP1 and UCP2 expression in different subcutaneous and visceral adipose tissue deposits in 30 days old goat kids and effect of fatty acid enriched diets



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ABSTRACT

Beside its importance in the first hours of life, brown adipose tissue has also significant roles in the following stages of growth and in adults by regulating energy metabolism, but its identification in adult ruminants is still controversial. Quantitative PCR, followed by histological confirmation, was used to investigate UCP expression and brown and white adipocytes' distribution in 30-day-old goat kids. The influence of maternal diet enriched with either fish oil or stearic acid was investigated as well. Results showed the differential expression of both UCP1 and UCP2 genes between subcutaneous and visceral adipose tissues, suggesting a different thermogenic activity between the two macro areas. The maternal diet influenced neither UCP1 nor UCP2 gene expression. The presence of multilocular adipocytes in 1-month goat kids is remarkable, as suggests thermogenic activity in non-newborn animals. Further insights into characteristics and functions of adipose tissue in young and adult goats are worth exploring.

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1. Introduction

Adipose tissue (AT) is a loose connective tissue, mainly composed by adipocytes. In the past decades, only two types of adipocytes were recognized, distinguishable according to their function and structure. White adipocytes are responsible for energy storage as triglycerides. White adipose tissue (WAT) is distributed throughout the body and can be found at subcutaneous level, such as sternum and base of the tail or around internal organs (visceral AT), such as heart, kidney, stomach, liver and intestines (Sauerwein et al., 2014). Visceral and subcutaneous deposits are demonstrated to have different metabolic characteristics and ability to release inflammatory cytokines (O'Rourke et al., 2009). Brown adipocytes' main function, instead, is to dissipate energy through the production of body heat and muscle metabolism (Cannon and Nedergaard, 2004; Harwood, 2012). Uncoupling Protein 1 (UCP1) is an important regulator of adaptive thermogenesis in mammals and exclusively expressed in brown adipose tissue (BAT) (Damlé and Marín-García, 2010; Mailloux and Harper, 2011). In precocious animals such as cow, sheep and goat, BAT starts developing in the fetal life, from myogenic

precursors Myf5+, during late gestation up to term (Symonds et al., 2012a), and has the maximum activity in the first hours/days after birth, fulfilling the main thermoregulatory heat production activity, which is of paramount importance for the metabolic adaptation to the extra-uterine environment (Symonds and Lomax, 1992). Indeed, maternal diet can influence newborn's performances, especially in term of adipose tissue's development (Bispham et al., 2003) and fatty acid composition (Berthelot et al., 2012). Besides its importance in newborns, BAT has also a significant role in adults, by regulating energy metabolism (Lee et al., 2013). The presence of BAT in adult mammals is a recent discovery and has been demonstrated in cow (Asano et al., 2013) and sheep (Henry et al., 2010), but only one old study is available in goat, showing the absence of UCP1 mRNA in newborns at ≥ 2.5 days (Trayhurn et al., 1993). Recently, a new typology of adipocyte has been identified, namely brown-in-white, brite or beige adipocytes, that can be induced to form within white fat by cold and β_3 -adrenergic receptor stimulation (Petrovic et al., 2010). These brown inducible adipocytes show an expression panel partially typical of white adipocytes, but have a role in thermogenesis via expression of UCP1 (Wu et al., 2012). Although these cells express UCP1, the characteristic brown fat marker, they are Myf5-, suggesting that they derive from a distinct population of cell precursors compared to brown adipocytes (Mueller, 2014). Function, origin and localization of beige adipocytes are still not fully described and the literature is limited to human and mice. It is now clear that adipogenesis and adipocyte differentiation are complex

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processes involving a temporally regulated set of gene-expression events and there are fundamental differences between small and large mammals. A central role in the transcriptional cascade that regulates adipogenesis is that of PPAR γ , whose expression is necessary not only for promoting adipogenesis, but also for maintaining adipocytes' differentiated state. PPAR γ expression is regulated by several pro- and anti-adipogenic factors (Rosen and MacDougald, 2006), as polyunsaturated fatty acid (PUFA) (Hardwick et al., 2009), which can induce the expression of UCP1 in humans and mice (Petrovic et al., 2010; Tiraby et al., 2003). Due to their nutraceutical activity, PUFA and saturated fatty acid (SFA) are often included in periparturient goat diets. PUFA can positively influence goat immune defenses as well (Agazzi et al., 2004; Lecchi et al., 2011, 2013; Pisani et al., 2009; Thanasak et al., 2004). Both PUFA and SFA included in ewe's diets can modulate BAT development in newborn lambs up to 24 h of age (Chen et al., 2007), as confirmed by Ojha and coworkers (2013), but no information is available in older animals. Particularly in goats, studies on adipose tissue development, composition and diet influence are still scarce.

The main aim of the present study was to describe adipose tissue characteristics in 30-day-old goat kids, especially focusing on the presence and localization of BAT in different subcutaneous and visceral adipose tissue deposits, by means of quantitative PCR measurement of UCP1 gene expression and confirmation by histological and immunohistochemistry analyses. Together with UCP1, the expression of UCP2 was evaluated. UCP2 is an uncoupling protein mainly expressed by WAT and skeletal muscle. Its mRNA expression has a peak at 30 days of postnatal age in sheep, can be modulated by maternal diet restriction as well and a role in adipose tissue development has been suggested (Gnanalingham et al., 2005). Given the background of the maternal diet's influence on newborn BAT development, we also investigated whether diets integrated with PUFA and SFA may influence the distribution of brown and white adipocytes.

2. Materials and methods

The experimental protocol used in this study was approved by the ethics committee of the University of Milan (Protocol No. 5/11, 18 January 2011).

2.1. Animals, diets and tissue sampling

Adipose tissue samples were obtained from ten 29.8 \pm 2.8-day-old healthy suckling kids, which were part of a larger experiment aimed to evaluate the influence of the maternal diet on peripartum and goat kids' performances. A group of 26 multiparous Alpine goats, homogeneous for parity and milk production during the previous lactation, were fed with different diets enriched with fatty acids, either saturated (ST, 69:26 percentages ratio of stearic acid (C18:0) and palmitic acid (C16:0)) or unsaturated (FO, fish oil containing 10.22% of EPA = 20:5 and 7.65% of DHA = 22:6), starting from a week before kidding until slaughtering of the kids. A third group of animals fed a control diet without any specific diet integration was also used as control (CTRL). FO and ST goats' diets were adapted for the dry period (supplemented with 30 g of fatty acids) and lactation period (supplemented with 50 g of fatty acids). Additional information regarding maternal diets can be found in Table 1. Goats were housed in single boxes and kids were individually fed by their own mothers. From this larger group, ten male kids, distributed among controls (n = 4) and treated (n = 3 + 3), were randomly selected in order to be included in the present experiment. Samples were collected only from these animals. Subcutaneous fat was taken from sternum, armpit cavity, base of the tail and withers, while visceral fat was taken from perirenal, omental and pericardial depots. Samples for molecular biology analysis were snap frozen in liquid nitrogen and

Table 1

Ingredients and chemical composition of the experimental diets.

Ingredient % of DM	Experimental diets					
	Pre-kidding			Post-kidding		
	CTRL	FO	ST	CTRL	FO	ST
Alfalfa hay	0.0	0.0	0.0	31.2	29.8	30.7
Mixed hay	62.3	59.6	61.4	15.3	14.6	15.1
Concentrate mixture	31.9	30.5	31.4	46.8	44.8	46.2
Corn meal	5.3	5.0	5.2	6.2	5.9	6.2
Fish oil	0.0	4.4	0.0	0.0	4.3	0.0
Stearate	0.0	0.0	2.0	0.0	0.0	1.9
CaCO ₃	0.5	0.5	0.0	0.5	0.5	0.0
DM, %	88.4	88.7	88.6	89.3	89.5	89.4
CP	12.3	11.9	12.2	17.8	17.2	17.5
EE	2.9	4.9	4.5	3.2	5.2	4.8
NDF	43.9	43.8	43.3	33.7	34.0	33.2
Ashes	6.3	6.5	6.0	7.2	7.3	6.8
Ca	0.8	0.8	0.9	1.1	1.1	1.2
P	0.4	0.4	0.4	0.8	0.8	0.8
Nel (Mcal/d)	1.61	1.66	1.67	1.67	1.72	1.72

stored at -80°C while samples for histological and immunohistochemical analyses were placed in 10% buffered formalin immediately after removal, fixed for 7 days and further processed.

2.2. UCP1 and UCP2 mRNA expression

Total RNA was extracted using a commercial kit specific for all kind of tissues (RNeasy Plus Universal Mini Kit – Qiagen, Milano, Italy), and treated with DNase (Rnase-Free Dnase Set – Qiagen, Milano, Italy). Retrotranscription was performed on 1.5 μg RNA, using the iScript cDNA Synthesis kit (Biorad, Segrate (MI), Italy) and the resulting cDNA was used as template for qualitative and quantitative PCRs. A pool was generated using 2 μl of cDNA of each sample. Qualitative PCRs were performed in 10 μl final volume with 1 μl buffer (Vivantis Technologies, Analytical Service SRL, Cassina De' Pecchi (MI), Italy), 1.5 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate (dNTP – Vivantis Technologies, Analytical Service SRL, Cassina De' Pecchi (MI), Italy), 1 μM each primer (Primm, Milano, Italy) and 0.025 U Taq polymerase (Vivantis Technologies, Analytical Service SRL, Cassina De' Pecchi (MI), Italy). no-RT reactions were performed for each target, as negative control. Primers and length of the amplified fragments are listed in Table 2. PCRs were performed on all samples at the same conditions: 35 cycles at 96 $^{\circ}\text{C}$ for 30 s, 60 $^{\circ}\text{C}$ for 30 s, 72 $^{\circ}\text{C}$ for 45 s and results were visualized on a 1.6% agarose gel stained with ethidium bromide.

Quantitative real time PCRs were carried out in 15 μl Eva Green mix (Biorad, Segrate (MI), Italy), 400 nM GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 450 nM LRP10 (low-density lipoprotein receptor-related protein 10) and 500 nM HPCAL1 (hippocampin-like 1), UCP1 and UCP2 primers, using the iQ5 Real Time RT-PCR system (BioRad, Segrate (MI), Italy). Primers and length of the amplified fragments are listed in Table 2. One microliter of cDNA was used for each sample. Samples were tested in duplicate and non-reverse transcribed controls and no template controls were included in the assays. The thermal profile for each gene was 95 $^{\circ}\text{C}$ for 90 s, 40 cycles at 95 $^{\circ}\text{C}$ for 5 s and 60 $^{\circ}\text{C}$ for 10 s; the melting curve was created running the samples at 55 $^{\circ}\text{C}$ for 60 s and 80 cycles starting at 55 $^{\circ}\text{C}$ up to 95 $^{\circ}\text{C}$, increasing 0.5 $^{\circ}\text{C}$ every 10 s. Relative quantification of both UCP1 and UCP2, toward the pool sample, was calculated using the comparative delta-delta-Ct method (Giulietti et al., 2001). The PCR efficiency was evaluated by creating a standard curve with 3-fold serial dilutions of the pooled cDNA and HPCAL1, GAPDH and LRP10 were used for normalization of the mRNA abundance, as the most stable reference genes for adipose tissue

Table 2

List of UCP genes under analysis and reference genes used for qPCR, including sequence accession numbers, primer sequence and length. The Efficiency values are included between 90.9 and 109.6 and the R^2 values are included between 0.991 and 0.999.

Sequence name	Symbol	Accession number	Primer forward (5'–3')	Primer reverse (5'–3')	Length (bp)	Reference
Uncoupling protein 1	UCP1	X14064	TCCTGTCITTTGATCGCCTCT	GAACAGTCCATGTGCCAGTG	112	Yonezawa et al. (2009)
Uncoupling protein 2	UCP2	AF127029	GCATAGGCATCCAGGAATCA	TAGGACGCTTCTGTCTCC	112	Yonezawa et al. (2008)
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_001034034	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	119	Lecchi et al. (2012)
Hippocalcin-like 1	HPCAL1	NM_001098964	CCATCGACTTCAGGGAGTTC	CGTCGAGGTCATACATGCTG	99	Hosseini et al. (2010)
Low density lipoprotein receptor-related protein 10	LRP10	BC149232	CCAGAGGATGAGGACGATGT	ATAGGGTTGCTGTCCTGTG	139	Hosseini et al. (2010)

(Hosseini et al., 2010). The MIQE guidelines (Bustin et al., 2009) were followed.

2.3. Histological analysis

In order to evaluate the histological differences of subcutaneous and visceral AT in 30-day-old goat kids, a histological analysis, with hematoxylin and eosin staining, was performed. The distribution of areas of white adipocytes in different AT deposits has been evaluated, as well. The analysis was performed on formalin fixed samples. Tissues were routinely processed and paraffin embedded. Two 4 μ m thickness, serial sections from each block were mounted on glass slides and stained with hematoxylin and eosin. Pictures of 3 consecutive 20 \times power fields were captured. Image analysis software (Image J) was used to select areas of white adipocytes, non-white adipocytes (e.g. brown or beige adipocytes) and non-adipocytes' areas (e.g. connective tissue, air bubbles, blood vessels, nerves). Ratios between white and non-white adipocytes' areas and perimeters were calculated. Western blot and immunohistochemical analysis were performed as well (Supplementary Material).

2.4. Statistical analysis

All data were evaluated for normal distribution using the Kolmogorov–Smirnov test. All data from quantitative PCR and histological evaluation were log-transformed and elaborated with an analysis of variance using the statistical software SAS (SAS Inst. Inc., Cary, NC). Post-hoc tests were carried out on parametric data

using the Tukey–Kramer method. In addition, Pearson correlations analyses have been performed in order to evaluate the goat-kid age influence on UCP1 and UCP2 expression and to correlate the histological analysis with gene expression.

3. Results

3.1. mRNA expression of UCP1 and UCP2 is higher in visceral deposits

Qualitative PCRs revealed the presence of both UCP1 and UCP2 genes in all samples under analysis (data not shown). Quantitative real time PCR demonstrated that UCP1, as marker of BAT, is differentially expressed between visceral and subcutaneous AT deposits ($p < 0.05$), with higher expression in the visceral ones (Fig. 1A). A greater expression ($p < 0.05$) of UCP2 gene was detected in visceral deposits as well, as compared to subcutaneous ones (Fig. 1B). Highest expression of both UCP1 and UCP2 was demonstrated in perirenal areas.

3.2. White and brown adipocytes distribution in subcutaneous and visceral adipose tissue deposits

The presence of brown adipocytes, as determined by hematoxylin–eosin staining, was clearly identified in all adipose tissue depots within the non-white adipocytes' areas (Fig. 2A). Brown adipocytes were identified as containing small lipid droplets, rich cytoplasm and central nucleus, whereas white adipocytes were identified as containing one big lipid droplet, poor cytoplasm and peripheral nucleus. The different

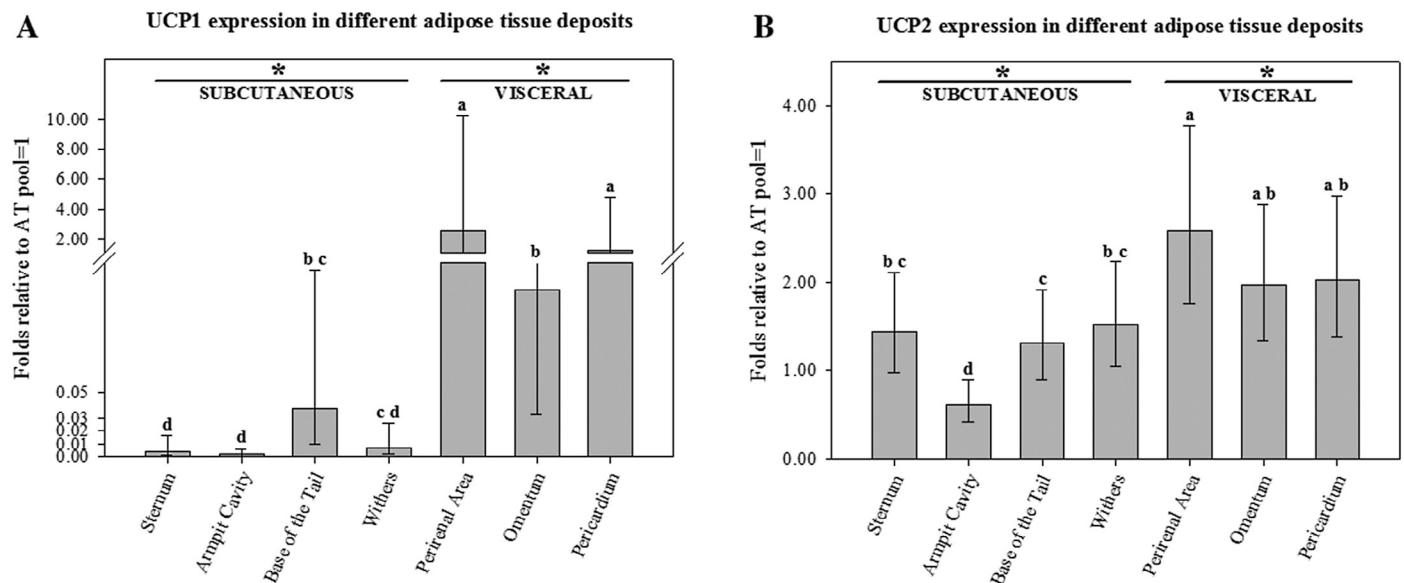
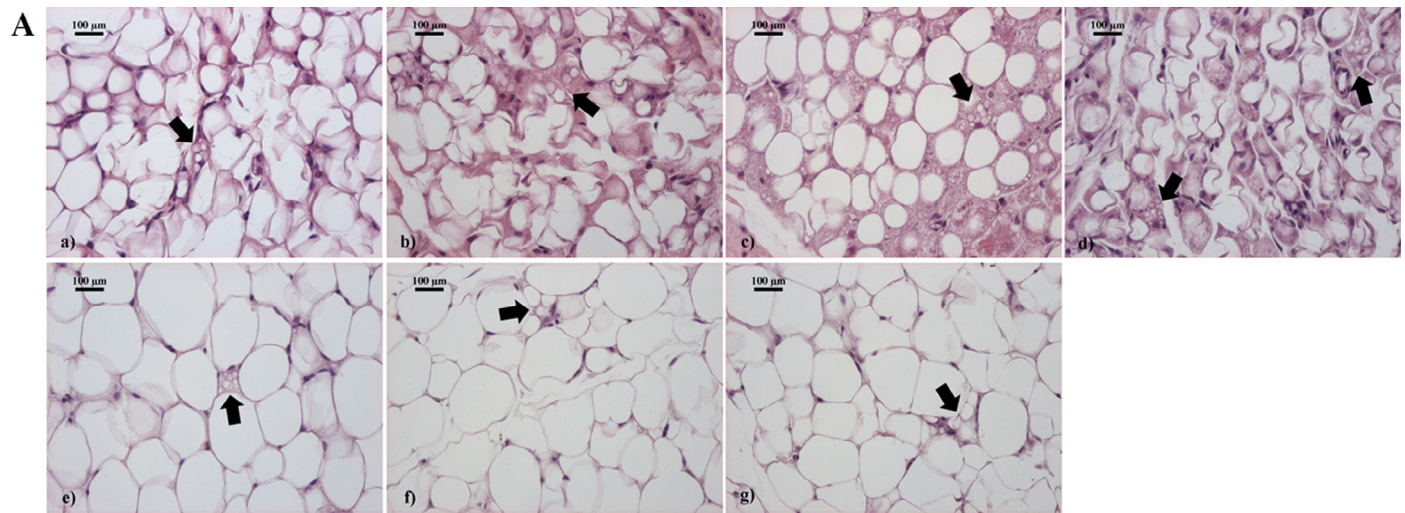


Fig. 1. UCP expression in different subcutaneous and visceral deposits. (A) UCP1 mRNA was found in all adipose tissue samples under analysis. Together with UCP2 (B), it shows a higher expression in visceral deposits than in subcutaneous' ones ($*p < 0.05$). Back-transformed least-squares means and 95% confidence limits are shown.



B White adipose tissue areas (WAT) in subcutaneous and visceral deposits

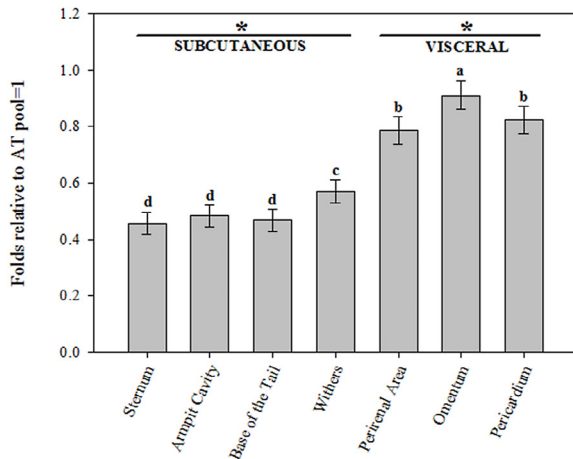


Fig. 2. Histological analysis results. (A) Multilocular adipocytes were identified in all tissues under analysis and are pinpointed by the black arrows. White adipocytes show different characteristics in subcutaneous or visceral deposits: (a) sternum, (b) armpit cavity, (c) base of the tail, (d) withers, (e) perirenal area, (f) omentum, (g) pericardium. (B) White areas were analyzed using Image J Pro Plus and show statistically significant differences between subcutaneous and visceral deposits ($p < 0.05$).

distribution patterns of the white adipocytes' areas between subcutaneous and visceral deposits were confirmed by statistical analysis ($p < 0.05$) (Fig. 2B).

3.3. Effects of the goat kids age on the gene expression and correlation with the histological analysis

Statistical analysis revealed a correlation between UCP expression and goat kids age ($p < 0.05$). UCP1 expression proved to be inversely proportional to kids' age, while on the contrary UCP2 expression got higher with the increase of kids' age. In addition, UCP2 expression was directly correlated with white adipocyte areas identified after statistical analysis. Indeed, UCP2 expression was higher when white adipocyte areas were bigger.

3.4. Effect of the maternal diet on UCP1 and UCP2 expression and adipose tissue' composition

In the last part of the study, the possible influence of maternal diet on BAT goat kids was investigated. Both quantitative real time PCR and histological analysis were used for this purpose. As shown in Fig. 3A, no statistically significant differences in the expression

of either UCP1 or UCP2 genes were detected among control and fatty acid enriched diets or between the two diets. Histological analysis confirmed these results (Fig. 3B).

4. Discussion

Brown adipose tissue is of paramount importance for newborn organisms and their capability to adjust the body temperature to the new environment, but also for adults and muscle metabolism regulation. Involved in AT functions is UCP1, exclusively expressed by BAT. Although essential for the survival, information about BAT distribution and activity in ruminant species are still scarce, and somewhat controversial. The aim of this study was to partially cover this gap in goats. The age of the goat kids' involved in the experiment represents a key point in this study as it is demonstrated that BAT, and therefore UCP1 expression, decreases with the animal growth. But while Pope and coworkers (2014) proved its absence in 30 day old lambs, Yuan and collaborators described it expressed in lambs from 2 up to 12 months age (2012).

In our study we demonstrated the expression of UCP1 in 30 day old goat kids in all subcutaneous and visceral samples included in the experiment (although extremely low in armpit cavity and

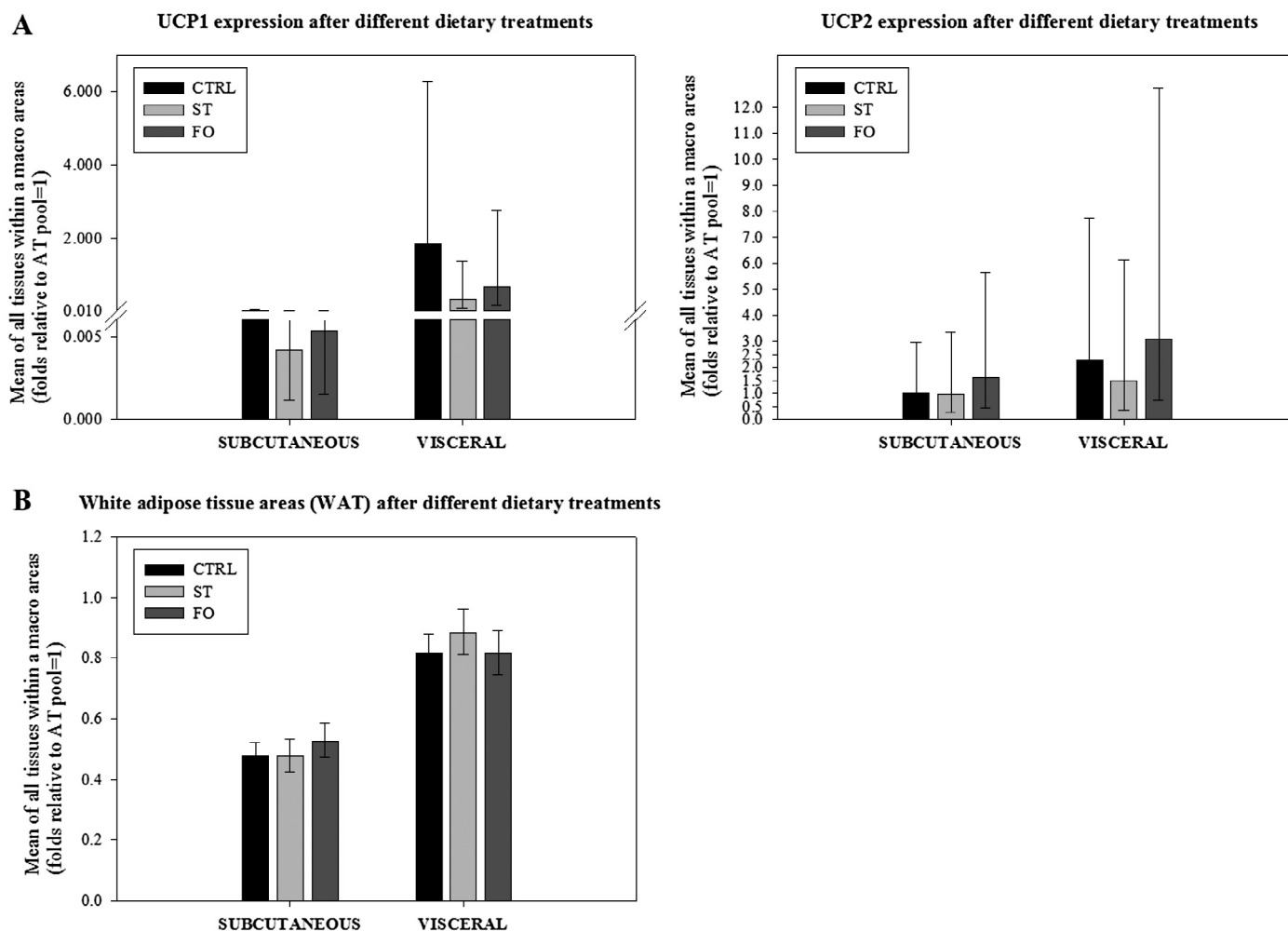


Fig. 3. UCP expression (A) and white adipose tissue distribution (B) after different maternal dietary treatments. The comparison among maternal control diet (CTRL), stearic acid (ST) and fish oil (FO) enriched diets showed no statistically significant differences in the expression of both UCP1 and UCP2 genes and in white adipocytes distribution ($p > 0.05$). Back-transformed least-squares means and 95% confidence limits are shown.

withers), confirming what previously described in lambs by Yuan and coworkers (2012) and by a previous study (Smith et al., 2004).

Consistent with earlier findings in sheep (Henry et al., 2010), we also demonstrated that UCP1 and UCP2 genes are differentially expressed between subcutaneous and visceral adipose tissues, both genes being, in our study, highly expressed in visceral deposits (especially perirenal), compared to subcutaneous ones. This is not surprising considering that visceral adipocytes are more metabolically active as compared to subcutaneous ones (Ibrahim, 2010), that perirenal and omental areas are the major adipose tissue deposits in newborn lambs and goats (Clarke et al., 1997a; Skarda, 2000) and the major source of BAT in the fetus is around the central organs (Clarke et al., 1997b).

Since literature referring to goat adipose tissue is scarce, in order to comment and explain our results, it is necessary to look at the discrete number of studies that have investigated AT behavior in other species such as human, mice or lambs.

As described by Clarke and coworkers (1997b), BAT around internal organs (i.e. perirenal and pericardial) is replaced by white fat after birth. Indeed, UCP1 expression in perirenal AT has a peak at 4 days up to 7 days from the birth, afterwards UCP1 expression decreases rapidly until 30 days when it reaches basal values. A comparable depot in older lambs, 3 months old, has been identified by Symonds and coworkers (2012b) in sternum and clavicular areas, in which UCP1 gene expression is greater than all other adipose

tissue locations. Compared to lambs, our results, instead, demonstrated a delay in UCP1 expression changes after birth in goat, as its mRNA is still higher in visceral depots in 30 day old animals, compared to subcutaneous ones. These observations are supported by the statistical analysis demonstrating an inverse relation between goat kids' age (expressed in days) and UCP1 expression, suggesting that the thermoregulatory potential of adipose tissue is decreasing. Another aspect to take into consideration, when explaining our results, is the possibility that UCP1 mRNA may be expressed not only by brown, but also by beige adipocytes. These cells have a very low basal level of UCP1 expression, but they have the ability to activate UCP1 expression after cold exposure or different types of stimulation and it has been observed that brown fat in adult humans has the molecular characteristics of murine beige rather than brown adipose cells (Wu et al., 2012).

To confirm the UCP1 expression data we decided to investigate the anatomical differences between subcutaneous and visceral adipose tissue deposits, in terms of BAT and WAT localization. In adults, while the localization of BAT in mouse or human is clearly distinguishable (e.g. interscapular region), in farm animals a gross distinction between BAT and WAT is not possible (Symonds et al., 2012b). Therefore, in our study the identification of multilocular adipocytes in sternum, base of the tail, perirenal, omental and pericardial deposits confirmed UCP1 gene expression analysis, and it is consistent with previous data in goats and other ruminant species

(Smith et al., 2004; Trayhurn et al., 1993), while brown adipocytes' presence in armpit cavity and withers has never been investigated before. Quantitative gene expression of UCP2 basically confirmed anatomical findings. Moreover, the different white adipocytes distribution between subcutaneous and visceral deposits supports the hypothesis that these two major adipose tissue depot groups display several anatomical and functional differences as previously suggested (Ibrahim, 2010). Even though the Western blotting analysis identified the UCP1 protein in both perirenal and omental adipose tissues, as well as sternum and base of tail, although in very little amount (data not shown), the detection of UCP1 presence by immunohistochemistry gave no informative results (Supplementary Material).

The histological analysis per se did not clarify the origin of our multilocular adipocytes, as it cannot distinguish between single brown and beige cell. However at least for the subcutaneous depots, we can speculate a beige lineage, as multilocular cells are dispersed within unilocular white cells and the UCP1 mRNA expression is very low (Keipert and Jastroch, 2014). On the contrary, it is possible that the high expression of UCP1 in visceral tissues is due to the presence of brown adipocytes that are disappearing as the animal grows, together with beige cells dispersed within white adipose tissue.

Given the background of PUFA relationship with PPAR γ , and how PPAR γ can influence BAT development, the second part of this study was focused on demonstrating the hypothesis that dietary supplementation with saturated or polyunsaturated fatty acids during late gestation would increase UCP1 gene expression, thus increasing the thermogenic capability of newborn kids. However, fish oil did not enhance PPAR α , and related hepatic gene mRNA levels in transition dairy goats (Agazzi et al., 2010). The present results extended previous experiments (Ebrahimi et al., 2013), which showed a different gene expression of PPAR α and PPAR γ as a consequence of different diet administration, but investigate neither UCP1 expression nor the maternal diet influence.

Quantitative PCR data on UCP1 and UCP2 expression in adipose tissue showed no statistically significant differences between CTRL and fatty acids supplemented kids or between FO and ST animals, thus demonstrating that feeding goats with PUFA or saturated fatty acid starting from a week before kidding up to 1 month after kid's birth does not influence BAT activity in the offspring. This information is therefore in contrast with other studies in rat (Priego et al., 2013), and somewhat different with an experiment carried out in sheep (Chen et al., 2007), which presented the evidence that supplementing prepartal diets of ewes with PUFA for about 1 month decreased cold tolerance in newborn lambs (6 and 22 h of age), by decreasing UCP1 gene expression. If this happened to our animals, the effects did not last up to 30 days of age of the animals. It must also be said that the adipose tissue metabolism of sheep and goats have been shown to be sensibly different (Tsiplakou et al., 2011).

5. Conclusions

In conclusion, studies on adipose tissues in farm animals, especially goats, are still scarce and comparison with other species is sometimes difficult. An efficient development of this tissue during the fetal period and the first days of life is of particular relevance for both animal health and economical gains, as demonstrated by several studies in calves (Carstens et al., 1997), lambs (Slee, 1981) and goat kids (Mellado et al., 2000). However, understanding these mechanisms is generally difficult, due to the cellular heterogeneity of the tissue, and further investigation are highly needed. Our study demonstrated for the first time the presence of brown adipose tissue in goat 1 month after birth as determined by the expression of UCP1 gene and this differs between subcutaneous and visceral depots. In addition, we confirmed that UCP1 expression is

inversely related to the age of the animal. We moreover identified multilocular adipocytes dispersed within white cells and both are differently distributed among subcutaneous and visceral deposits and this fact can reflect the different functions of the two macro areas, but further investigation is needed. Finally, we proved that fatty acid enriched diets administered to goats starting 1 week before kidding have no influence on UCP1 expression or brown and white adipose tissue distribution in suckling kids. The presence of UCP1 and therefore of thermogenic activity in 1 month goat kids is remarkable and suggests that further insights into the function of BAT in young and adult goats are worth exploring.

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Appendix: Supplementary material

Supplementary data to this article can be found online at [doi:10.1016/j.rvsc.2015.03.014](https://doi.org/10.1016/j.rvsc.2015.03.014).

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