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# Feeding level regulates the expression of some genes involved with programed cell death and remodeling in goat and sheep mammary tissue

Eleni Tsiplakou[1], Christina Mitsiopoulou[1], Dimitrios Skliros[2], Alexandros Mavrommatis[1] and Emmanouil Flemetakis[2]

[1]Department of Animal Science, School of Animal Biosciences, Agricultural University of Athens, Iera Odos 75, GR-11855, Athens, Greece and [2]Laboratory of Molecular Biology, Department of Biotechnology, School of Applied Biology and Biotechnology, Agricultural University of Athens, Iera Odos 75, GR-11855, Athens, Greece

## Research Article

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**Author for correspondence:**
Eleni Tsiplakou, Email: eltsiplakou@aua.gr

## Abstract

Mammary tissue (MT) turnover is characterized by programed cell death and remodeling which might be affected by both feeding level and animal species. Thus, twenty-four dairy goats and the same number of sheep were assigned to three homogenous sub-groups per animal species and fed the same diet in quantities which met 70% (FL70), 100% (FL100) and 130% (FL130) of their daily energy and crude protein requirements. Individual MT samples were taken by biopsy from the animals on the 30$^{th}$ and 60$^{th}$ experimental day. The results showed, in the first sampling time, a significant reduction in the mRNA abundance for selected genes involved in programed cell death in both FL 70 fed goats (*STAT3* and *BECN1*) and sheep (*CASPASE8* and *BECN1*) compared with the respective FL100 groups. The FL130, in comparison with the FL100, caused a significant increase in transcripts accumulation of *STAT3* gene in both sampling times and *CASPASE8* gene in the second sampling time in goat MT, while the opposite happened for the mRNA expression of *CASPASE8* and *BECN1* genes in sheep MT, but only in the first sampling time. Moreover, a significant up regulation in the mRNA levels of *MMP2* gene in MT of FL130 fed sheep was observed. The FL130, in comparison with the FL70, caused an enhancement in the mRNA expression levels of *BECN1, CASPASE8, BAX and STAT3* genes in goat MT only. It was also shown that apoptosis and autophagy can be affected simultaneously by the feeding level. Overfeeding affects MT programed cell death and remodeling by a completely different way in goats than sheep. In conclusion, feeding level and animal species have strong effects on both MT programed cell death (apoptosis and autophagy) and remodeling but the molecular mechanisms need further investigation.

In addition to genetic and nutritional factors, milk production potential is shaped by the number of mammary epithelial cells and by the mammary tissue (MT) organization (Knight, 2000; Capuco *et al*., 2003; Yart *et al*., 2013). The mammary secretory tissue organization is modulated by the ratio between cell proliferation and apoptosis (programed cell death, PCD) as well as by the metalloproteinase activity, in a procedure known as mammary cell turnover (Stefanon *et al*., 2002). Although the majority of mammary cell turnover is taking place during late pregnancy and prior to lactation, cell proliferation and apoptosis have also been observed, in cows (Capuco *et al*., 2001), goats (Knight and Peaker, 1984) and rodents (Tucker, 1969), during established lactation (Wall and McFadden, 2012).

Numerous genes are involved in the regulation of mammary cell turnover. More specifically, the signal transducer and activator of transcription 3 (encoded by the *STAT3* gene) (Chapman *et al*., 2000; Colitti and Farinacci, 2009; Piantoni *et al*., 2010), the caspase 8 (encoded by the *CASPASE8* gene) (Gajewska *et al*., 2005) as well as the pro-apoptotic bcl-2-like protein 4 (encoded by the BAX gene) (Schorr *et al*., 1999; Walton *et al*., 2001) are proteins critical for the initiation of apoptosis. However, lysosomal cell death has also received attention in the last years (Boya and Kroemer, 2008; Aits *et al*., 2015) and is independent of the executioner caspases, but does require the activity of *STAT3* in order to upregulate the expression of lysosomal proteases such as cathepsin B (encoded by the *CTSB* gene) (Kreuzaler *et al*., 2011). Moreover, autophagy is another type of cell death, with beclin-1 protein (encoded by the *BECN1* gene) to be considered as one of its most reliable markers (Gajewska *et al*., 2005). Finally, the matrix metalloproteinase 2 (encoded by the *MMP2* gene) and 9 (encoded by the *MMP9* gene) respectively, break down the extra-cellular matrix, resulting in a second wave of apoptosis and MT remodeling (Green and Lund, 2005).

Many research studies in dairy ruminants, using immunohistochemical techniques, have shown that nutrient availability, and more specifically energy restriction, reduce the number of mammary epithelial cells (Colitti *et al*., 2005; Dessauge *et al*., 2011), increase (Dessauge



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*et al*., 2011) or do not modify apoptosis (Nørgaard *et al*., 2005) and inhibit mammary cell proliferation at 8 wk, but not at 16 wk postpartum (Nørgaard *et al*., 2005). However, to the best of our knowledge, the impact of feeding level on the molecular regulation of mammary cell turnover remains poorly documented. Up to now, only the effect of negative energy balance on the mRNA abundance of genes encoding proteins, indicative of mammary cell turnover, has been studied in cows (Dessauge *et al*., 2011) and goats (Ollier *et al*., 2007). Moreover, both Boutinaud *et al*. (2008) and Sigl *et al*. (2008) studied the impact of feed restriction in the expression of some genes involved in the apoptosis of bovine mammary epithelial cells purified from milk. Thus, the objective of this study was to investigate the effect of feeding level on the mRNA expression of some genes involved in programed cell death (*STAT3*, *CASPASE8*, *BAX*, *CTSB*, *BECN1*) and remodeling (*MMP2* and *MMP9*) in goat and sheep mammary tissue.

## Materials and methods

### Experimental design

Twenty-four 3 to 4-years-old Friesian crossbred dairy sheep and twenty-four 3 to 4-years-old Alpine cross bred dairy goats were maintained at the animal house of the Agricultural University of Athens. Housing and care of the animals conformed to Ethical Committee guidelines of Faculty of Animal Science and to EU standards for the protection of animals used for scientific purposes and/or feed legislation.

Three months post-partum (90 ± 8 d in milk) both animal species were assigned into three homogenous sub-groups ($n = 8$) balanced by their body weight (BW) and fat corrected milk yield. The average initial BW and milk yield was 59.1 ± 4.1 and 1.01 ± 0.20 kg/d respectively for sheep, and 53.1 ± 2.1 and 0.70 ± 0.08 kg/d for goats. Each animal of both groups was fed individually throughout the experimental period which lasted 60 d. The three groups (treatments) of both animal species were fed with the same diet which covered 70% (FL70), 100% (FL100) and 130% (FL130) of their daily individual energy and crude protein requirements, respectively (National Research Council, 1981; Zervas, 2007). The quantities of food offered to the animals were adjusted on a group basis at 0, 12, 24, 31, 39 and 52 experimental day in order to meet the 70%, 100% and 130% of animal's requirements of each group, respectively. The diet given to both animal species consisted of alfalfa hay and concentrates with a forage/concentrate ratio of 50/50. The alfalfa hay and concentrates used were from the same batch throughout the experimental period. The concentrate diet (g/kg) consisted of: maize grain, 360; barley grain, 360; soybean meal, 160; wheat middlings, 110; calcium phosphate, 15; common salt, 3; mineral and vitamins premix, 2. The mineral and vitamin premix contained (per kg as mixed): 150 g Ca, 100 g P, 100 g Na, 100 mg Co, 300 mg I, 5000 mg Fe, 10 000 mg Mn, 20 000 mg Zn, 100 000 mg Se, 5 000 000 IU retinol, 500 000 IU cholecalciferol and 15 000 mg α-tocopherol. The full experimental designs have been described in detail for sheep and goats in the studies of Tsiplakou *et al*. (2012*a*) and Tsiplakou *et al*. (2012*b*) respectively.

### Mammary tissue

Mammary tissue (MT) samples were taken by biopsy on the 30$^{th}$ and 60$^{th}$ experimental day, which correspond to 120 and 150 d in milk respectively, of each dietary treatment after the morning milking. Before the biopsy, the udder of the animals was shaved and cleaned, and local anesthesia was achieved by subcutaneous injection of 2 ml lidocaine hydrochloride (xylocaine 2%, AstraZeneca, Athens, Greece). A 2-mm incision was made to facilitate the insertion of the biopsy needle. Both biopsy samples were taken from the right mammary gland using a Bard Magnum® Biopsy instrument (BARD, Athens, Greece) in which the biopsy needle (14G) was adapted. The length of the sample notch was about 1.9 cm and approximately 15 mg tissue was collected from a depth of 3–5 cm. After the tissue samples were taken, a stapler (Leukoclip SD, Smith and Nephew, England) was used to close the wound and the site of sampling received a prophylactic treatment with a disinfecting powder (Terramycin, w/Polymyxin, Pfizer, Athens, Hellas, containing 33.812 mg oxytetracycline hydrochloride and 1.457 mg Polymyxin B sulfate as active ingredients) and then covered with spray (Oxyvet spray, Provet, Athens, Greece, containing 2.2 g oxytetracycline HCL). Immediately after the biopsy sampling, all animals received antibiotic prophylaxis with 5 ml of Terramycin Long Acting (Pfizer, Athens, Hellas, containing 217.40 mg oxytetracycline dihydrate).

### Determination of transcript abundance using real-time RT-qPCR assay

Total RNA was isolated from 15 mg of MT using the Trizol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol. DNase treatment used DNase I (Promega, Madison, WI) at 37°C for 60 min to remove all traces of genomic DNA. The RNA integrity was evaluated with agarose gel (3%) after isolation and after DNase treatment of the samples. Discrete bands were monitored in all samples representing 28s and 18s ribosomal RNAs respectively, showing little or no RNA hydrolysis. Quantity of RNA was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was reverse transcribed from 2 µg of DNase-treated total RNA, using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. The resultant cDNA was diluted to a final volume of 100 µl, and SYBR green-labeled PCR fragments were amplified using gene-specific primers (online Supplementary Table S1) designed from the transcribed region of each gene using Primer Express 1.5 software (Applied Biosystems, Darmstadt, DE). Consensus primers were designed in order to amplify target-gene regions for both animals. RT-PCR reactions were performed on a Stratagene MX3005P real-time PCR using iTaq Fast SYBR Green Supermix with ROX (BioRad, Hercules, CA) at a final volume of 15 µl, gene-specific primers at a final concentration of 0.2 µM each and 1 µl of cDNA. PCR cycling started at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis. The geometrical mean of the expression levels of RPS9 and UXT genes was used as internal standard (Bionaz and Loor, 2007). Relative transcript levels of the gene of interest ($X$) were calculated as a ratio to the geometrical average of RPS9 and UXT ($C$), as $(1 + E)^{-\Delta C_t}$, where $\Delta C_t$ was calculated as $(C_t^X - C_t^C)$. PCR efficiency ($E$) for each amplicon was calculated employing the linear regression method on the Log(Fluorescence) per cycle number data, using the LinRegPCR software (Ramakers *et al*., 2003). While some studies have identified references genes for cow (Bionaz and Loor, 2007) and goat (Bonnet *et al*., 2013) MT during lactation there is scarce information, to the best of our knowledge,

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**Table 1.** The mean relative transcript accumulation of genes in goat mammary tissue of the three dietary treatments (FL70, FL100, FL130), at the two sampling times (30th and 60th experimental day). The units in the Table are arbitrary

| | | Dietary treatment | | | | Effects | | |
|---|---|---|---|---|---|---|---|---|
| | | FL70 | FL100 | FL130 | SEM | Diet | Time | Diet × Time |
| *STAT3* | 30th day | 0.12$^{a}$ | 0.19$^{b}$ | 0.21$^{b}$ | 0.017 | * | | |
| | 60th day | 0.16$^{b}$ | 0.15$^{b}$ | 0.21$^{a}$ | 0.012 | ** | | |
| | Overall mean | 0.14$^{a}$ | 0.17$^{b}$ | 0.22$^{c}$ | 0.014 | ** | NS | * |
| *CASPASE 8* | 30th day | 0.03 | 0.03 | 0.04 | 0.003 | NS | | |
| | 60th day | 0.02$^{ab}$ | 0.02$^{a}$ | 0.04$^{b}$ | 0.004 | * | | |
| | Overall mean | 0.02$^{a}$ | 0.02$^{a}$ | 0.04$^{b}$ | 0.002 | * | NS | NS |
| *BAX* | 30th day | 0.02 | 0.02 | 0.02 | 0.003 | NS | | |
| | 60th day | 0.02$^{a}$ | 0.02$^{a}$ | 0.03$^{b}$ | 0.003 | * | | |
| | Overall mean | 0.02$^{a}$ | 0.02$^{ab}$ | 0.03$^{b}$ | 0.003 | * | NS | NS |
| *CTSB* | 30th day | 0.61 | 0.69 | 0.95 | 0.102 | NS | | |
| | 60th day | 0.80 | 0.87 | 0.69 | 0.074 | NS | | |
| | Overall mean | 0.71 | 0.80 | 0.82 | 0.084 | NS | NS | NS |
| *BECN1* | 30th day | 0.13$^{a}$ | 0.19$^{b}$ | 0.20$^{b}$ | 0.018 | ** | | |
| | 60th day | 0.21 | 0.20 | 0.23 | 0.012 | NS | | |
| | Overall mean | 0.16$^{a}$ | 0.19$^{b}$ | 0.21$^{b}$ | 0.011 | * | ** | * |
| *MMP2* | 30th day | 0.35 | 0.29 | 0.23 | 0.056 | NS | | |
| | 60th day | 0.35 | 0.37 | 0.20 | 0.092 | NS | | |
| | Overall mean | 0.37 | 0.34 | 0.22 | 0.071 | NS | NS | NS |
| *MMP9* | 30th day | 0.02 | 0.02 | 0.03 | 0.008 | NS | | |
| | 60th day | 0.02 | 0.03 | 0.02 | 0.006 | NS | | |
| | Overall mean | 0.02 | 0.03 | 0.02 | 0.007 | NS | NS | NS |

Means with different superscript (a, b) in each row (between dietary treatments) for each gene differ significantly ($P \leq 0.05$).
*$P < 0.05$, ** $P < 0.01$, and *** $P < 0.00$, NS, Non significant.

for sheep. So, based on the fact that the UXT has been proposed both in cows and goats as a stable reference gene for the MT during lactation (Bionaz and Loor, 2007; Bonnet *et al.*, 2013) it was also used in the present study. Additionally, the UXT has been used as a reference gene in a study with sheep MT (Carcangiu *et al.*, 2013). As the choice of RPS9 is concerned, it was done taking into account that it has been characterized also by high stability in the MT of cows (Bionaz and Loor, 2007) while in goats, Finot *et al.* (2011) concluded that in the choice of reference genes should be included at least one ribosomal protein gene.

### *Statistical analysis*

The experimental data were analyzed using the SPSS statistical package (version 16.0) using a general linear model (GLM) for repeated measures analysis of variance (ANOVA) with dietary treatments ($T$) (FL70; FL100; FL130) and sampling time ($S$) as fixed effects and their interactions ($T \times S$) according to the model:

$$Y_{ijk} = \mu + T_i + S_j + (T \times S)_{ij} + A_k + e_{ijk}$$

where $Y_{ijk}$ is the dependent variable, $\mu$ the overall mean, $T_i$ the effect of dietary treatment, $S_j$ the effect of sampling time, $(T \times S)_{ij}$ the interaction between dietary treatments and sampling time, $A_k$ the animal's effect and $e_{ijk}$ the residual error. Multiple comparisons were obtained using Tukey's test. Significance was set at $P < 0.05$.

## Results and discussion

Underfeeding (FL70 compared with FL100) caused a significant reduction in the mRNA expression of *STAT3* and *BECN1* genes in MT at both sampling times and in the first sampling time, respectively, in goats (Table 1) and in the mRNA expression of *CASPASE 8* and *BECN1* genes in the first sampling time in sheep (Table 2). For completeness, the overall mean values for each dietary treatment (disregarding time) and for each time point (disregarding diet) are given in the online Supplementary Table S2. *STAT3* was postulated as a death factor in differentiated mouse mammary epithelium (Chapman *et al.*, 2000), thus, an upregulation of the mRNA expression of *STAT3* gene has usually been observed during mammary involution in cows (Singh *et al.*, 2008; Piantoni *et al.*, 2010) and sheep (Colitti and Farinacci, 2009). In agreement with our results in sheep, Ollier *et al.* (2007) reported a significant decline in the mRNA expression of *CASPASE8* and *BECN1* genes in the MT of 48 h food-deprived goats at first stage of lactation. Moreover, Moyes *et al.* (2011) indicated that several genes associated with cell death, other than

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**Table 2.** The mean relative transcript accumulation of genes in sheep mammary tissue of the three dietary treatments (FL70, FL100, FL130), at the two sampling times (30th and 60th experimental day)

| | | Dietary treatment | | | | Effects | | |
|---|---|---|---|---|---|---|---|---|
| | | FL70 | FL100 | FL130 | SEM | Diet | Time | Diet × Time |
| *STAT3* | 30th day | 0.23 | 0.24 | 0.20 | 0.024 | NS | | |
| | 60th day | 0.20 | 0.17 | 0.21 | 0.015 | NS | | |
| | Overall mean | 0.22 | 0.22 | 0.20 | 0.018 | NS | * | NS |
| *CASPASE 8* | 30th day | 0.07[a] | 0.12[b] | 0.09[a] | 0.009 | * | | |
| | 60th day | 0.07 | 0.07 | 0.08 | 0.004 | NS | | |
| | Overall mean | 0.07[a] | 0.10[b] | 0.09[ab] | 0.006 | * | ** | * |
| *BAX* | 30th day | 0.05[a] | 0.07[b] | 0.04[a] | 0.004 | * | | |
| | 60th day | 0.05 | 0.05 | 0.06 | 0.007 | NS | | |
| | Overall mean | 0.05 | 0.06 | 0.05 | 0.006 | NS | NS | ** |
| *CTSB* | 30th day | 0.85 | 0.77 | 1.06 | 0.117 | NS | | |
| | 60th day | 0.76 | 0.80 | 0.99 | 0.098 | NS | | |
| | Overall mean | 0.82 | 0.80 | 1.02 | 0.095 | NS | NS | NS |
| *BECN1* | 30th day | 0.27[a] | 0.48[b] | 0.32[a] | 0.030 | ** | | |
| | 60th day | 0.27 | 0.29 | 0.33 | 0.022 | NS | | |
| | Overall mean | 0.27[a] | 0.40[b] | 0.32[a] | 0.024 | * | * | ** |
| *MMP2* | 30th day | 0.19 | 0.12 | 0.18 | 0.033 | NS | | |
| | 60th day | 0.16 | 0.13 | 0.26 | 0.035 | NS | | |
| | Overall mean | 0.17[ab] | 0.13[a] | 0.22[b] | 0.033 | * | * | * |
| *MMP9* | 30th day | 0.003 | 0.001 | 0.002 | 0.001 | NS | | |
| | 60th day | 0.002 | 0.001 | 0.001 | 0.001 | NS | | |
| | Overall mean | 0.003 | 0.001 | 0.001 | 0.001 | NS | NS | NS |

The units in the Table are arbitrary.
Means with different superscript (a, b) in each row (between dietary treatments) for each gene differ significantly ($P \leq 0.05$).
*$P < 0.05$, ** $P < 0.01$, and *** $P < 0.00$, NS, Non significant.

those included in this study, were downregulated in the MT of cows with negative energy balance during mid-lactation. Thus, it can be assumed that the downregulation in the expression of genes involved with the programed cell death (PCD), which was observed in the MT of FL70 animals, could be part of a molecular mechanism intended to maintain their milk production as much as possible. Indeed, the fact that the down regulation in the above genes was more intense (statistically significant) at the first sampling time (after 30 experimental days) might show that the molecular mechanisms cannot preserve the milk production when the animals consume a diet which covers 70% of their nutritional requirements for more than two months. A positive correlation between milk yield and mRNA expression of *BECN1, CASPASE 8 and BAX* genes in goats was found (Fig. 1). Moreover, the fact that the mRNA accumulation of both *CASPASE8* and *BECN1* genes was affected simultaneously in the MT of FL70 fed sheep, may indicate that apoptosis and autophagy can be exhibited simultaneously, pointing out a complex regulation of these pathways.

On the contrary, significantly higher mRNA expression of *CASPASE3* and *CTSB* genes in the MT of feed restricted cows, which were at 11 weeks of lactation, has been reported by Dessauge *et al.* (2011). Moreover, Nørgaard *et al.* (2008) observed that the transcript level of *CASPASE3* was not affected by a low feeding level in the MT of cows which were at approximately 9 months of lactation. No difference in the epithelial cell apoptosis between cows fed with either low or high energy density diets, when the animals were at 8 weeks postpartum, has been observed by Boutinaud *et al.* (2008). The different response of negative energy balance on the expression of genes related with the PCD between cows and small ruminants may be attributed to animal species differences and to the level and duration of feed restriction. In addition, in contrast to small ruminants, in cows there is a characteristic overlapping between periods of lactation and pregnancy which may affect mammary PCD in a completely different way. Besides, the high levels of pregnancy hormones stimulate the development of new secretory tissue which may oppose the stimuli for mammary involution initiated by milk stasis (Gajewska *et al.*, 2013). Moreover, the elevated levels of sex steroids during pregnancy may also affect the MT autophagy in cows. Indeed, the high autophagy in bovine mammary epithelial cells, when they are cultured in fetal bovine serum-deficient media in the presence of E2 or P4 *in vitro*, suggests that these hormones additionally stimulate the induction of this process (Sobolewska *et al.*, 2009).

The regulation of autophagy in bovine mammary epithelial cells is affected also by a combination of factors including auto/paracrine apoptogenic peptides as well as lactogenic hormones

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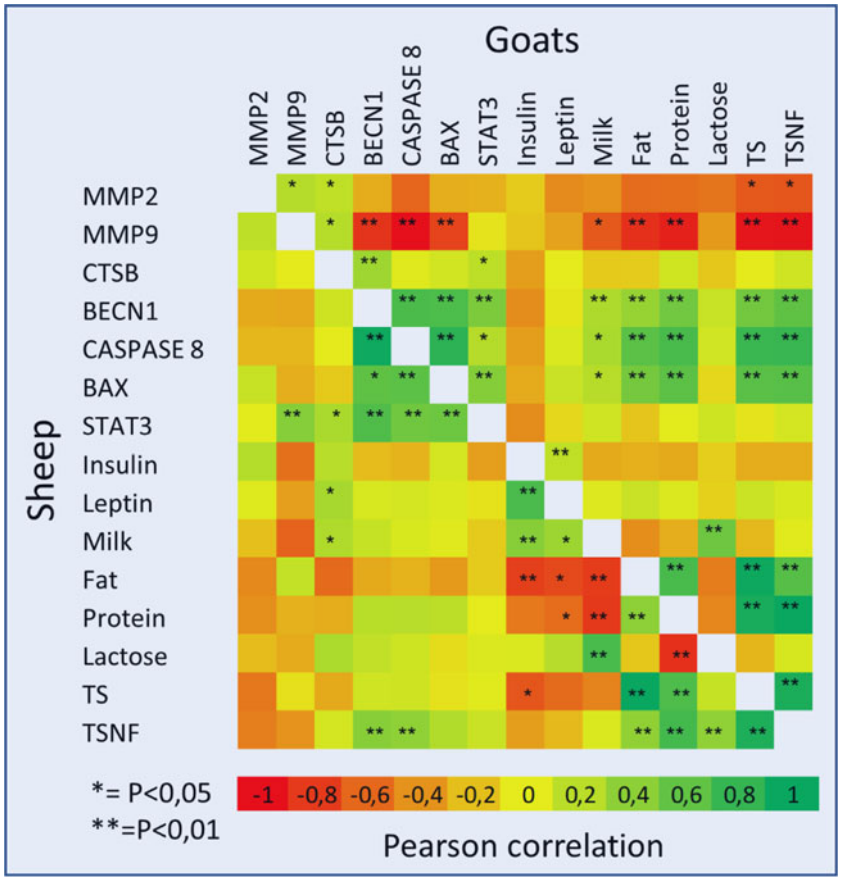


**Fig. 1.** Pearson correlation between apoptotic genes in mammary tissue, milk yield and composition and blood hormones in sheep and goats.

(Motyl *et al.*, 2007; Boutinaud *et al.*, 2012; Lollivier *et al.*, 2015). It has been shown in sheep that the lactation stage itself has an effect on the expression of genes related with mammary proliferation and PCD (Colitti *et al.*, 2009). Moreover, feeding a low energy-density diet to cows inhibits mammary cell proliferation at 8, but not at 16, week post partum (Nørgaard *et al.*, 2005). Thus, the impact of low feeding level on mammary cell proliferation and apoptosis, further to the animal species differences, is probably dependent on the lactation stage as well.

The mRNA transcripts of *STAT3* and *CASPASE8* genes were significantly higher in the MT of overfed goats (FL130 *vs.* FL100) at the second sampling time (Table 1). These results show that prolonged consumption (more than 2 months) of a diet which covers 130% of goats' nutritional requirements causes a significant up regulation of the expression of genes involved in PCD. It has been reported that obesity (Ozcan *et al.*, 2004) enhances the endoplasmic reticulum stress in mice MT and, as a consequence, activation of apoptotic mechanisms (Lin *et al.*, 2007). In accordance with our findings, Hennigar *et al.* (2015) found significantly higher *CASPASE*3 accumulation, determined by immunoblotting, in the MT of obese lactating mice. A significant increase in caspase activation and apoptosis in adipose tissue from both mice with diet-induced obesity and obese humans has also been observed (Alkhouri *et al.*, 2010; Pintus *et al.*, 2012). Thus, excess feed intake may impair respiratory capacity and prime cells for apoptosis, increasing cellular susceptibility to additional stress.

Overfeeding sheep (FL130 *vs.* FL100) had a completely different effect when compared with goats. Specifically, at the first sampling time, a significant decrease in the mRNA transcript accumulation of *CASPASE8* and *BECN1* genes in the MT of FL130 fed sheep, in comparison with the FL100, was observed (Table 2). This reduction in the expression of genes involved with PCD may be due to the significant rise in the mRNA expression of *MMP2* gene which was also found (Table 2). It has been reported that *MMP2* promotes endbud invasion into the stroma of MT by suppressing epithelial apoptosis (Wiseman *et al.*, 2003). Moreover, the fact that the sheep of our study had higher body fat accumulation compared with the goats (documented in Tsiplakou *et al.*, 2012*a*, 2012*b*), might mean that their MT contained more adipose tissue which would result in increased leptin production. It has been shown that the plasma leptin response to feeding level is strongly dependent on body fatness (Daniel *et al.*, 2002). Indeed, the leptin concentration in blood plasma was higher in FL130 fed sheep (2.22 ng/ml) compared with goats (1.57 ng/ml) (Tsiplakou *et al.*, 2012*a*, 2012*b*). Obesity significantly increased mRNA expression of leptin in mice MT (Kamikawa *et al.*, 2009). Thus, it was hypothesized that leptin could also participate in the control of mammary epithelial cell growth and survival. It has been shown that leptin up-regulates the expression factors which are associated with the extra-cellular matrix, induces the expression of anti-apoptotic genes and reduces the expression of apoptotic genes in human mammary epithelial cells (MCF-7) (Perera *et al.*, 2008).

In the MT of FL130 fed goats significantly higher mRNA expression levels of *STAT3*, *BAX*, *CASPASE8* and *BECN1* genes, compared with the FL70 ones, were found (Table 1). As mentioned earlier, obesity creates endoplasmic reticulum stress (Ozcan *et al.*, 2004) which may tip the phenotype toward cell death. In contrast, no significant changes in the expression of

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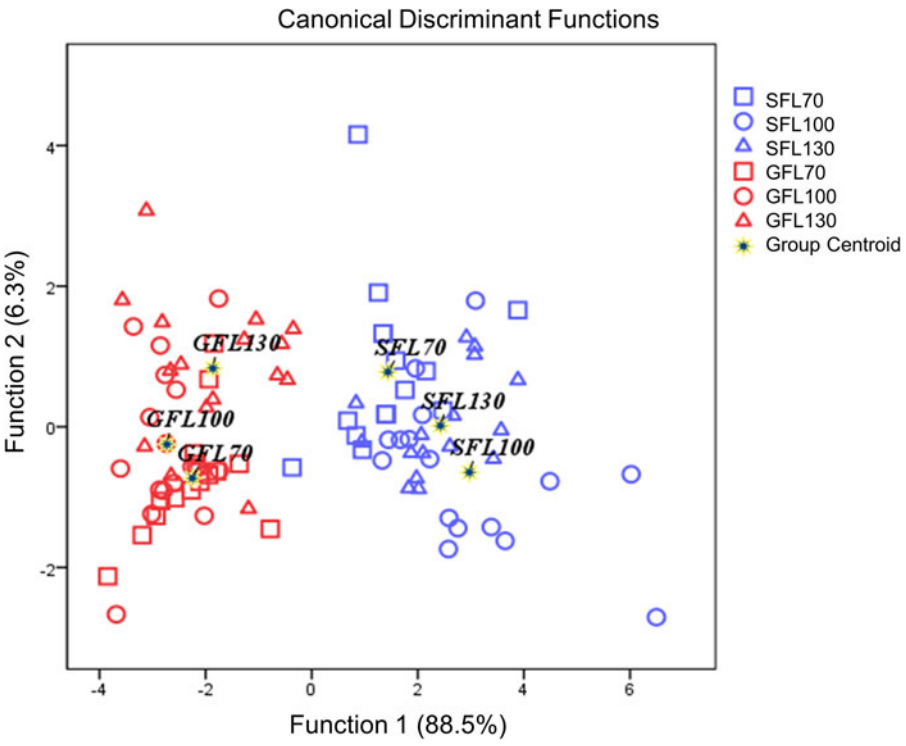


**Fig. 2.** Discriminant plot separating the samples by the dietary treatments (FL70, FL100, FL130) and animal species (goat in red, sheep in blue).

genes involved either in programed cell death or with the extracellular matrix components were observed in the MT of sheep fed either with FL130 or FL70 (Table 2). These findings indicate that in ruminant MT the regulation of genes involved with apoptosis, autophagy and remodeling is controlled by complex mechanisms which remain to be studied in depth. Moreover, once again these results may indicate differences in the molecular mechanisms governing the PCD between sheep and goats even under the same dietary treatments.

A discriminant analysis was applied to pooled data of relative gene expressions in order to investigate if the samples can be distinguished not only based on the dietary treatments but also on animal species (Fig. 2). Seven variables were entered to develop a model to discriminate the ninety-six samples of each case. The percentages of the samples that were classified into the correct group, according to the dietary treatment and animal species were 66.7%. Wilks' lambda was observed at 0.076 for Function 1 ($P < 0.001$) and 0.516 for Function 2 ($P < 0.001$) and the relative expressions of *CASPASE8* and *STAT3* were the variables that contributed the most.

In conclusion, the mRNA accumulation of selected genes (*STAT3*, *BECN1* and *CASPASE8*), involved in either apoptosis or autophagy in sheep and goat MT, was significantly downregulated in the FL130 fed animals compared with the FL100 ones. The FL130, compared with the FL100, induced apoptosis (by increasing mRNA expression of *STAT3* and *CASPASE8*) in goats MT, while the opposite happened in the case of sheep (by reducing mRNA expression of *BECN1* and *CASPASE8*). Inhibition of programed cell death (apoptosis and autophagy) in the MT of FL130 fed sheep compared with the FL100 one, was accompanied by an enhancement in the remodeling process, indicated by the increase of *MMP2* transcripts. The FL130, compared with the FL70 had different impact in the mRNA accumulation of some genes involved with either programed cell death (apoptosis and autophagy) or remodeling in sheep and goat MT which underlines animal species differences, details of which remain to be elucidated. Finally, apoptosis and autophagy can be affected simultaneously by the feeding level indicating complex molecular mechanisms linking the respective processes.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S002202992000103X.

**Author contributions.** Dr E. Tsiplakou designed the study, wrote the paper and performed the analyses. Both Dr G. Karalias and D. Skliros were involved in the laboratory analyses. Dr E. Flemetakis participated in writing and in the interpretation of the results.

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