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Cholesterol synthesis in the lactating cow: Induced expression of candidate genes☆

Enrique Viturro*, Matthias Koenning, Angelika Kroemer, Gregor Schlamberger, Steffi Wiedemann, Martin Kaske, Heinrich H.D. Meyer

Physiology Weihenstephan, Technische Universitaet Muenchen, Weihenstephaner Berg 3, 85354 Freising, Germany

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ABSTRACT

Despite the extensive knowledge for other species, cholesterol metabolism in ruminants is nowadays still not clear. Huge differences in milk cholesterol concentration are observed between breeds, managing strategies, individuals and moment of the lactating cycle, but the genetic actors working in the process of cholesterol secretion into milk have not been identified. As ruminant diet contains no cholesterol, understanding the mechanisms and regulation of synthesis, transport and secretion into milk is crucial when trying to reduce the amount of this metabolite in dairy products. The present work aims to study the expression of candidate genes for these processes in the liver of *Bos taurus* during the lactating cycle.

Liver biopsies were obtained from 16 adult brown Swiss cows at different time points (2 weeks pre-partum and 0, 2, 4 and 8 weeks post-partum). After RNA extraction and reverse transcription, gene expression of candidate genes was studied using quantitative RT–PCR. Key enzymes of the cholesterol synthesis (3-hydroxy-methyglutaryl-coenzyme-A (HMG-CoA) synthase, HMG-CoA reductase and farnesyldiphosphat-farnesyltransferase (FDFT)) and gene expression feed-back regulators involved in lipid metabolism (sterol regulatory element binding proteins (SREBP1and 2) SREBP-cleavage activating protein (Scap) were selected as candidate genes.

HMG-CoA-reductase and FDFT showed a huge expression increase until week 2 post-partum ($p < 0.01$), most probably in response to the new requirements in the mammary gland. As well, and as a possible explanation for such modifications, an increase in the expression of the regulators SREBP1 and Scap was observed ($p < 0.01$ and $p < 0.05$ respectively). Most important, the whole synthesis machinery showed a coordinated regulation, as highly significant positive correlations were found between the expression levels of the above mentioned enzymes ($p < 0.01$). The increase of milk and blood cholesterol levels in *B. taurus* after parturition might be the result of a coordinated induction in the expression of key liver enzymes and their regulating factors.

1. Introduction

Milk is not only a source of nutrients in form of lactose, proteins and lipids, it also serves as an important delivery medium for other crucial molecules, such as vitamins, minerals, bioactive lipids and cholesterol. The milk lipid fraction constitutes the major source of energy and is known to be extremely variable among species, ranging from a reported 0% in rhinoceros milk to as much as 50% in whales. In addition to that, total lipid content of the milk also varies within species, depending on such factors as feed [1] or stage of lactation [2].

Particularly interesting among the milk lipid fraction is cholesterol, the major sterol in whole milk, with concentrations ranging 10–30 mg/dL (0.25–0.77 mM) [3]. This represents only 0.5% of the fat fraction, but because of the elevated consume of milk and dairy products in modern diet, these aliments range second on contribution to daily cholesterol intake, especially among infants [4], with the well-known consequences for cardiovascular health in older ages. For attempting a reduction of this ingredient in this first-need aliment, it is necessary to completely understand the physiological mechanisms of its homeostasis in the lactating cow.

Secretion of milk is a complicated process that depends on mobilisation of body reserves, endogenous synthesis and diet intake [5]. Taking into account that bovine diet will contain negligible amounts of cholesterol and that the cholesterol reserves in cellular membranes may not cover the huge requirements of this substance in the mammary gland (until 8 g/day), it is possible to hypothesize that the endogenous synthesis of cholesterol may play

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* Corresponding author. Fax: +49 8161 714421.
E-mail address: viturro@wzw.tum.de (E. Viturro).

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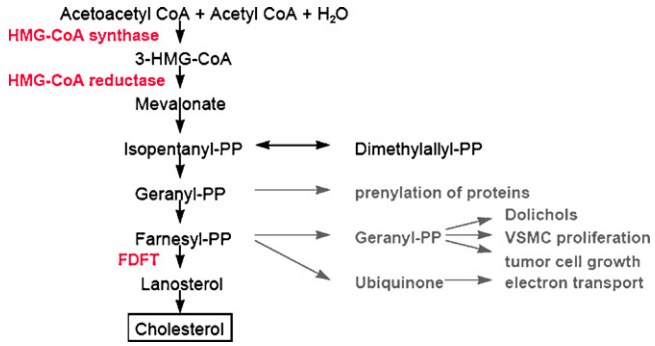

Fig. 1. Schematic representation of cholesterol biosynthesis (adapted from [11]). Only enzymes analyzed in this study are given.

a crucial role in this process in *Bos taurus*. Of special interest in this organism is to characterize the "transition period", defined as the interval of time between 3 weeks pre-partum and 3 weeks post-partum [6], during which the animal has to adapt its metabolism to the amazingly increasing new requirements.

Animal organisms achieve cholesterol homeostasis in part through control of genetic expression of the enzymes required for its biosynthesis, a process occurring mainly in liver. Most extensively studied are the genes for the two early sequential reactions, 3-hydroxy-methyglutaryl-coenzyme-A (HMG-CoA) synthase and HMG-CoA reductase. When sterols accumulate within cells, the amounts of mRNA produced by both genes are reduced, and conversely, when sterols are depleted, the amounts of mRNA increase and cholesterol synthesis is activated [7,8]. To achieve such a fine regulation cells employ a sophisticated sensor system, the sterol regulatory element binding proteins (SREBP) 1 and 2, transcription factors that can simultaneously activate a wide range of genes from the lipid metabolism [8,9]. These two membrane-embedded proteins are expressed in the endoplasmic reticulum together with their activator, the SREBP-cleavage activating protein (Scap). When cells are deprived from cholesterol, Scap escorts SREBP to the nucleus for achieving gene activation. On the other hand, excess cholesterol represses this transport by binding in the membrane to Scap and the biosynthesis machinery is not unnecessarily activated [10].

The aim of the present work is to analyze the gene expression of this range of enzymes and transcription factors that may play an important role in cholesterol level regulation during the lactating cycle in the liver of *B. taurus*. Among the above mentioned HMG-CoA reductase and synthase, the expression of a third enzyme of the biosynthetic pathway, the squalene synthase or farnesyl diphosphate farnesyl transferase (FDFT) will be studied. This enzyme acts on a later stage of the synthesis reactions and is the first step exclusively committed on cholesterol biosynthesis [11] (Fig. 1). Simultaneously, the gene expression of the regulating factors SREBP1, SREBP2 and Scap needs to be investigated, in order to test the hypothesis whether the genetic regulation of cholesterol synthesis during this period of time responds to a coordinated process.

2. Materials and methods

2.1. Animals and tissue collection

Liver biopsies were obtained from 16 adult brown Swiss cows on a normal milking regime and dry period of 56 days at the end of gravidity. Samples were collected using the Bard® MAGNUM™ Biopsy Instrument at different time points during the lactating cycle (2 weeks pre-partum and 0, 2, 4 and 8 weeks post-partum). Liver tissues were snap frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. RNA preparation and reverse transcription

Tissue samples of 50–80 mg weight were pulverized by grinding in frozen state, transferred into FastPrep tubes (MP Biomedicals) containing 400 mg of Matrix Green Beads (MP Biomedicals) and dissolved in 1 mL TriFast (Peqlab, Erlangen, Germany). This solution was snap frozen in liquid nitrogen and stored at −80 °C until the RNA extraction. After thawing on ice, the samples were homogenized in a BIO101 Thermo Savant Fast Prep FP120 Cell Disrupter (Qbiogene Inc.) at an intensity of 5.5 m/s for 30 s. Total RNA was isolated using the TriFast method (peqGold TriFast, Peqlab, Erlangen, Germany) following the manufacturer's instructions. RNA concentration was quantified at 260 nm (BioPhotometer, Eppendorf, Germany) and cDNA synthesis performed in a total reaction volume of 25 μL containing 1 μg of total RNA, 10 mM Tris–HCl (pH 9.0), 5 mM $MgCl_2$, 1 mM dNTP mix, 1 μg random primers and 50 U of M-MLV-RT RNase H (−) point mutant (Promega, Mannheim, Germany). Reverse transcription reaction was achieved by successive incubations at 25 °C for 10 min and 55 °C for 50 min, finishing with enzyme inactivation at 70 °C for 15 min (Gradient Cycler, Biometra, Göttingen, Germany). RT products were diluted 1:1 with PCR-grade water and stored at −20 °C.

2.3. Primer design and quantitative PCR

Primer sequences for amplification of bovine SREBP1 were obtained from the existing literature [12]. For the other genes, primer pairs were designed for each target gene using Primer3 Software [13] and the bovine genomic sequences available at the Bovine Genome Project (Baylor College of Medicine, http://www.hgsc.bcm.tmc.edu/projects/bovine/). Primers were designed to cover exon boundaries in order to avoid amplification of genomic DNA and validated for product specificity by routine PCR followed by electrophoresis in 1.5% agarose gel. Amplicons were isolated and commercially sequenced (AGOWA LGC Ltd., Teddington, UK). A complete list of primers used in this study

Table 1
List of primer pairs used for quantitative PCR measurements.

Gene	Forward primer	Reverse primer	Product
HMG-CoA synthase	5′-GATGGTCGCTATGCACTGGT-3′	5′-GCCCTCTCTCGAGGACCAGA-3′	132 bp
HMG-CoA reductase	5′-CTCTCTAAAATGATCAGCAT-3′	5′-TCAACTTTTCTTTCTCTGTTT-3′	246 bp
FDFT	5′-GAAATGCGCCATGCAGTA-3′	5′-GGAGATCGTTGGGAAGTCCT-3′	198 bp
SREBP1[a]	5′-CCAGCTGACAGCTCCATTGA-3′	5′-TGCGCGCCACAAGGA-3′	67 bp
SREBP2	5′-CAGGTCCTGGTACAGCCTCA-3′	5′–GCTCTTACCGGAACTTGCAG-3′	158 bp
SCAP	5′-GGTCACTTTCCGGGATGG-3′	5′-TGGGTAGCAGCAGGCTAAGA-3′	179 bp
Ubiquitin	5′-AGATCCAGCATAAGGAAGGCAT-3′	5′-GCTCCACCTCCAGGGTGAT-3′	198 bp
GAPDH	5′-GTCTTCACTACCATGGAGAAGG-3′	5′-TCATGGATGACCTTGGCCAG-3′	197 bp
β-Actin	5′-AACTCCATCATGAAGTGTGACG-3′	5′-GATCCACATCTGCTGGAAGG-3′	214 bp

[a] Primer sequence from ref. [12].

is present in Table 1. Quantitative PCR of mRNA in bovine liver biopsies was carried out using LightCycler DNA Master SYBR Green technology (Roche Diagnostics, Mannheim, Germany). After an initial denaturation step at 95 °C to ensure activation of the polymerase and complete denaturation of the cDNA, amplification reactions were performed in a final volume of 10 μL, including 1 μL of the LC FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 pmol of each primer, 3 mM $MgCl_2$, and 1 μL of cDNA. All PCR reactions were performed in duplicate with 45 amplification cycles and included a negative control consisting of pure PCR-grade water. Amplified products underwent melting curve analysis after the last cycle to ensure integrity of amplification.

2.4. Data analysis and statistics

Data were analyzed using the second derivate maximum method described in the LightCycler Relative Quantification Software, obtaining a crossing-point (CP) value for each gene on each animal at each timepoint. These values were translated to normalized expression quantities (ΔCP) using three reference genes in a form of normalization index (Ubiquitin, glyceraldehyde-3-phosphate dehydrogenase, and β-actin; see Table 1 for primer sequences). The normalization index was calculated as an arithmetic mean of the crossing-point values of the three reference genes. For graphical representation, a relative expression index (ΔΔCP) was generated for each timepoint by deducting the ΔCP value at baseline (week −2 pre-partum).

$$\text{reference index} = \text{mean}\,(CP_{\text{Ubiquitin}},\ CP_{\text{GAPDH}},\ CP_{\text{B-actin}})$$

$$\Delta CP = CP_{\text{target gene}} - \text{reference index}$$

$$\Delta\Delta CP = \Delta CP_{\text{timepoint}} - \Delta CP_{\text{week-2(baseline)}}$$

Between-group comparisons were conducted using one-way ANOVA followed by Bonferroni's multiple comparison tests. Differences were considered to be statistically significant at $p < 0.05$. Similarity in gene expression pattern was visualized by dot-plotting genes ΔCP pairwise and calculated as Pearson's correlation coefficient (r). All statistics and graphics were generated with SPSS 12.0 Software (SPSS Inc., Chicago, IL, USA).

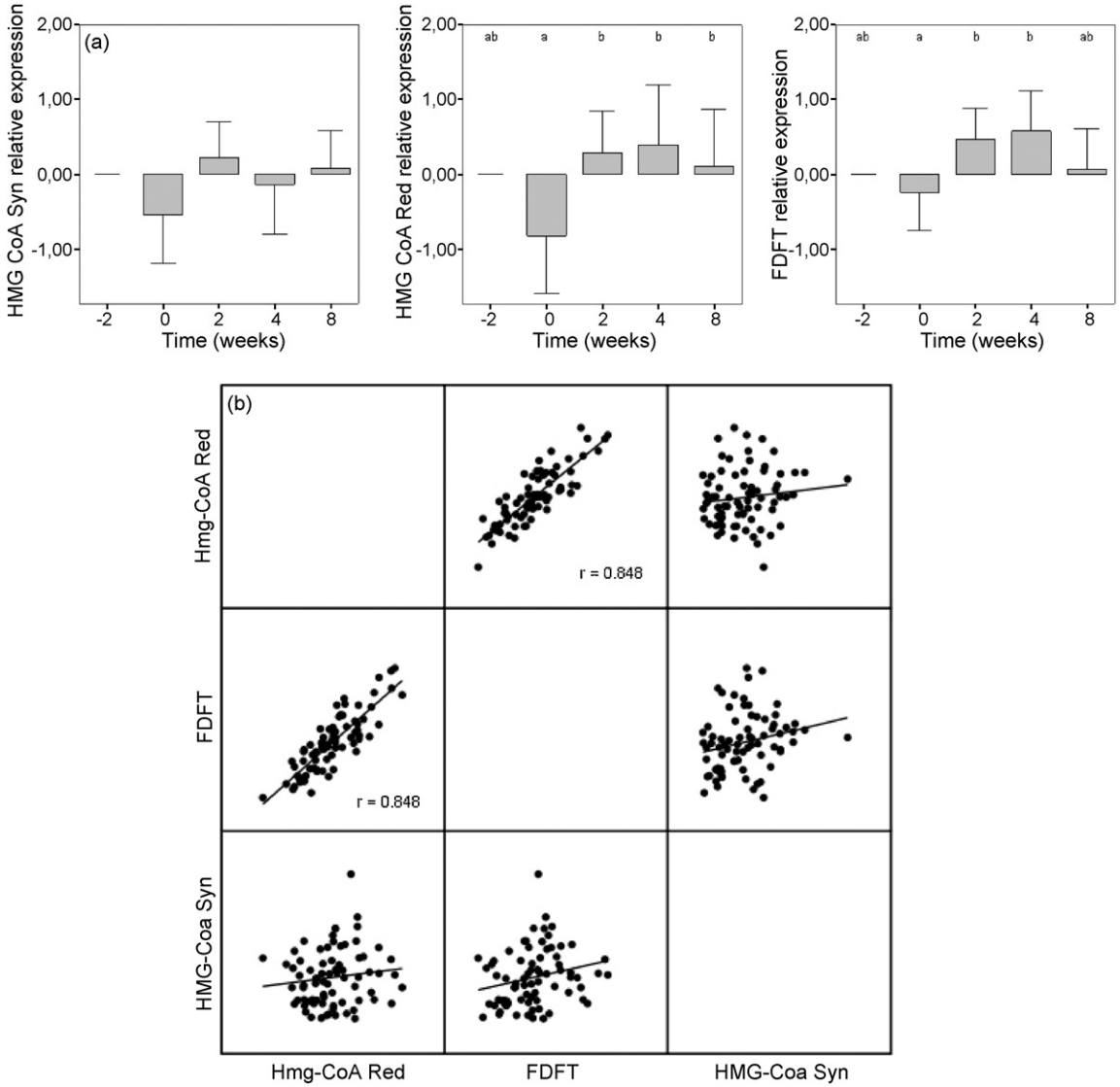

Fig. 2. (a) Relative expression (ΔΔCP) of cholesterol biosynthesis genes during the lactation cycle. Values represent means ± standard deviations. Time points carrying different superscripts are significantly different ($p < 0.05$). (b) Correlation of gene expression levels (ΔCP) of cholesterol biosynthesis genes. r values represent Pearson's correlation coefficient at $p < 0.01$.

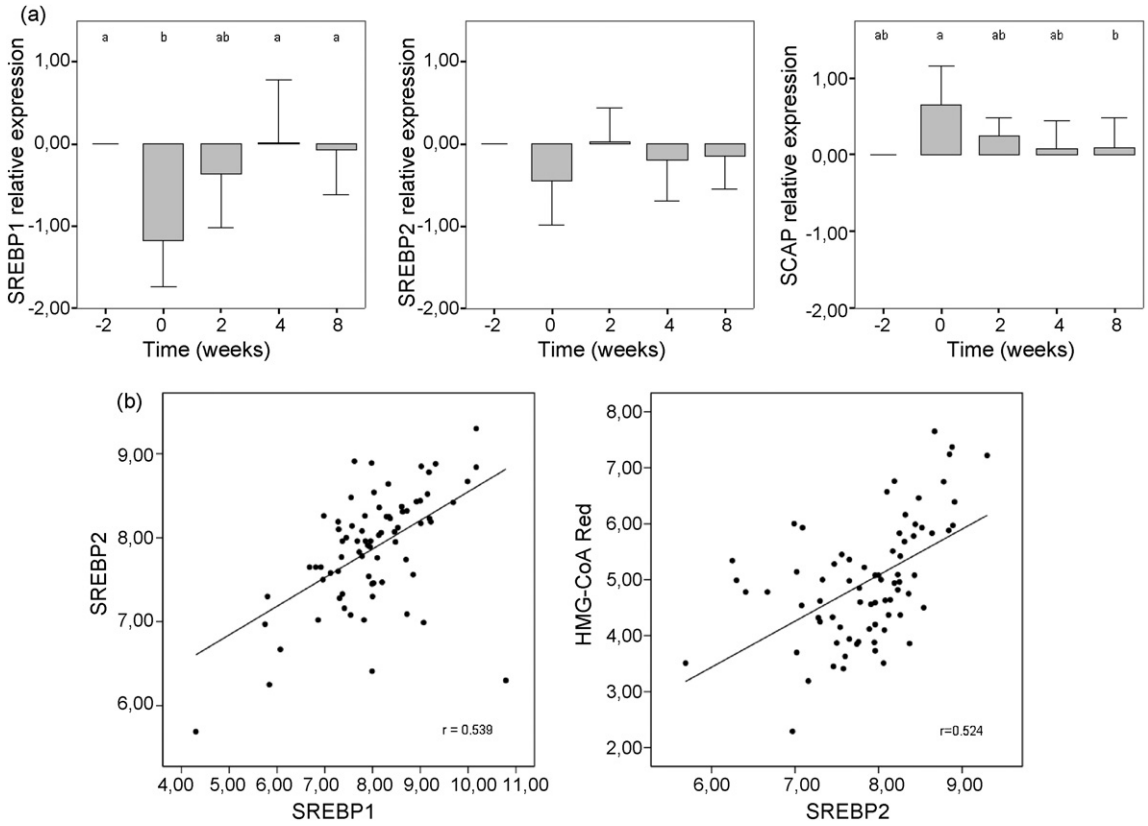

Fig. 3. (a) Relative expression ($\Delta\Delta$CP) of SREBP1, SREBP2 and Scap during the lactation cycle. Values represent means ± standard deviations. Time points carrying different superscripts are significantly different ($p < 0.05$). (b) Correlation of gene expression levels (ΔCP) of SREBP2 vs. SREBP1 and SREBP2 vs. HMG-CoA reductase. r values represent Pearson's correlation coefficient at $p < 0.01$.

3. Results

3.1. Primer testing and gel electrophoresis

The designed primer pairs demonstrated to be suitable for quantitative PCR analysis, showing single peaks and uniform melting curves, as well as a specific single band in high resolution agarose gel electrophoresis. The obtained product sequences matched completely the expected.

3.2. Cholesterol biosynthetic pathway

As shown in Fig. 2a, a highly significant regulation ($p < 0.01$) was observed for the expression of HMG-CoA reductase and FDFT mRNA: starting with down-regulation on the samples obtained immediately after parturition (week 0), a dramatic increase takes place on the following weeks, reaching the maximum expression level on week 4 post-partum. Similar tendencies were obtained for the HMG-CoA synthase, without reaching the statistical significance.

Expression levels of HMG-CoA reductase and FDFT demonstrated to be highly correlated ($r = 0.848$; $p < 0.01$), whereas HMG-CoA synthase did not show any significant correlation with neither of those genes (Fig. 2b).

3.3. Transcription activators

A similar gene expression pattern could be observed for the transcription factor SREBP1 (Fig. 3a), with a marked mRNA level decrease at parturition (week 0), followed by progressive increase during weeks 2 and 4 and expression stabilization at week 8 post-partum. SREBP2 showed analogous tendencies, without reaching statistical significance.

However, the expression pattern of Scap presented a very different pattern of level regulation as compared to the other studied genes: a strong increase in the expression occurs immediately at week 0, significantly different to the constant level of expression present at the rest of the studied period ($p < 0.05$). Significant correlations were found when comparing expression levels of these transcription activators (SREBP1 vs. SREBP2 ($r = 0.539$; $p < 0.01$), Scap vs. SREBP2 ($r = 0.282$; $p < 0.01$)) and transcription factors against synthesis enzymes (FDFT and HMG-CoA reductase vs. SREBP1 and SREBP2: $r = 0.282$–0.524; $p < 0.01$) (Fig. 3b).

4. Discussion

The objective of the present work was to deepen on the understanding on regulation of cholesterol production for milk synthesis by studying the changes in gene expression during the crucial transition period. Following the hypothesis that hepatic synthesis of cholesterol may play a crucial role in this mechanism and that hepatic adaptations to the onset of parturition might be characterized by long term molecular adaptation by gene expression changes [12], we studied the level of expression of three key enzymes of liver cholesterol synthesis (HMG-CoA reductase and synthase and FDFT). Although physiological mechanisms cannot be completely explained through RNA abundance, protein expression data have been extensively demonstrated to be largely concordant with gene transcription profiling [14]. Among the data on HMG-reductase and synthase, known key enzymes for this process, the

expression data of FDFT is of a great interest, since it catalyzes the first step committed exclusively to cholesterol synthesis. Agreeing with the extensive knowledge for other mammalian species, including human, in which cholesterol production in liver is largely associated with up- and down-regulation of these enzymes [15], HMG-CoA reductase and FDFT showed a significant up-regulation, especially at week 4 post-partum. Mammary gland has been shown to posses some ability on cholesterol production, but the amount of milk cholesterol synthesized in the mammary gland may only represent a 20% of the total [16] and the largest part of milk cholesterol might be produced in liver and transported in the blood to its goal [17]. As a probable result of this activation of liver cholesterol production in response to the new needs in the mammary gland, blood cholesterol levels increase dramatically on the weeks following parturition [18,19].

In our study, activation of the expression of the liver synthesis machinery did not occur until week 2 post-partum. A similar effect was observed in a recent work by Rudolph and co-workers [20] in mice: an increased expression of synthesis enzymes in the mammary gland takes place directly at secretary activation (in the present work, week 0), but mRNA levels fall one week later, when they are expressed at high levels in the liver. Interestingly, in the present work HMG-CoA synthase did not show significant changes in expression during the studied period, in agreement with previous data by Honda et al. [21], who stipulated that the responses of HMG-CoA reductase and synthase to different stimuli are different. This fact was remarked when plotting the expression of the three genes against each other: whereas HMG-CoA reductase and FDFT mRNA levels showed a notable positive correlation, HMG-CoA synthase expression did not appear to be related and can be hypothesized to either be constitutive or to respond to different signalling.

We were also interested in studying expression of the transcription regulatory proteins SREBP1 and SREBP2 and their activator Scap, a hot-topic on lipid research during the last years because of its central role in lipid metabolism [10,22]. In some works, it has been hypothesized that this family of transcription regulators may also play a significant role in lactation as critical regulators of secretory activation regarding lipid secretion [5] and their role in ruminant physiology is starting to be intensively studied [23,24]. Consistently with the increase in expression of the synthetic enzymes on week 2, results showed an earlier activation of expression of Scap at week 0, followed by progressive increases on expression of SREBP1 and SREBP2 at weeks 2 and 4 post-partum. Moreover, it could also be demonstrated that the expression level increases of these two key genes occurs co-ordinately and significantly correlate with those of the synthesis enzymes, remarking that these processes are all integrated on a complex regulatory mechanism that has to respond simultaneously and on the same direction to the different stimuli.

To our knowledge, this is the first work measuring mRNA levels of these genes in the lactating cow. Future experiments comprising the study of protein transport to the ER, Golgi and nucleus and regulation of the enzymatic activity might help to completely understand the mechanisms under which cholesterol levels in milk are regulated. As the post-transcriptional series of reactions leading to expression activation by SREBP1 and SREBP2 is extremely dependent on cholesterol levels and nutritional status, feeding and management trials might be of extreme importance when studying these homeostatic processes in cattle [24]. Also, and as existing for other similar pathways such as milk protein level regulation [25], the screening of these genes for common sequence variations might add important knowledge to the topic and help understanding the high interindividual variation observed [26].

In resume, the expression of key enzymes for liver de-novo cholesterol biosynthesis and the SREBP family of transcription factors is up-regulated after parturition in the dairy cow. This regulation occurs on a simultaneous way and might constitute the first line of action of the bovine organism upon lactation activation and the subsequent increased cholesterol requirements.

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