

Paola Signorelli, Laura Avagliano, Marta R. Reforgiato, Nadia Toppi, Josefina Casas, Gemma Fabriàs, Anna Maria Marconi, Riccardo Ghidoni and Anna Caretti*

De novo ceramide synthesis is involved in acute inflammation during labor

DOI 10.1515/hsz-2015-0213

Received July 23, 2015; accepted October 20, 2015; previously published online October 23, 2015

Abstract: Gestation is regulated by an inflammatory process that allows implantation and parturition. The comprehension of such inflammatory switches is important for the identification of therapeutic targets in pregnancy defects. Sphingolipids are a class of structural membrane components with important signaling functions. Among sphingolipids, ceramide is a well-known mediator of stress signals and pro-inflammatory responses. In this paper, we evaluated the association between ceramide increase and the inflammatory process of labor, comparing placentas from vaginal deliveries, including both spontaneous and induced labor, versus elective cesarean. We demonstrated that: (i) the inflammatory marker IL-6 is upregulated in labored placentas; (ii) IL-6 content inversely correlates with labor duration; (iii) ceramide content and expression of serine palmitoyl transferase (SPT, rate limiting enzyme for *de novo* ceramide synthesis) are increased in labored placentas; (iv) the expression of SPT directly correlates with inflammation and inversely with labor duration. These observations suggest that ceramide metabolism and signaling may be implicated in controlling important inflammatory mechanisms driving gestation: we hypothesize that ceramide can be a therapeutic target in inflammatory complications of parturition.

Keywords: IL-6; serine palmitoyl transferase; sphingolipids.

Introduction

Gestation is a unique condition involving the maternal immune system, requiring the suppression of the own immune response for the acceptance of a semi-allogenic fetus, as well as the functionality of immune system for maternal and fetal protection from infection during pregnancy. A highly complex balance of signals drives the innate immune response through-out pregnancy: a pro-inflammatory phase allowing blastocyst implanting into the uterus, a successive anti-inflammatory phase allowing fetal development and growth and a final acute inflammatory phase allowing cervical ripening, uterine contraction and parturition (Mor et al., 2011). Excessive, persistent or deregulated maternal inflammatory responses result in adverse pregnancy outcomes and preterm birth is associated with increased production of pro-inflammatory cytokines, chemokines and prostaglandins (Guleria and Pollard, 2000; Kramer et al., 2009; Kunzmann et al., 2013). Likewise, Fetal Growth Restriction (Cotechini and Graham, 2015) and preeclampsia are associated with an exaggerated maternal inflammatory response. Moreover, a reduced production of anti-inflammatory mediators was detected in association with infertility, spontaneous sporadic or recurrent miscarriages and fetal undergrowth (Marzi et al., 1996; Piccinni et al., 1998; Kwak-Kim et al., 2003; Hossein et al., 2004; Fukui et al., 2008; Hanzlikova et al., 2009; Jin et al., 2011; Rolle et al., 2013). Thus, the comprehension of the regulation of inflammatory switches represents an important acquisition for the identification of therapeutic targets in pregnancy defects as well as early markers of adverse outcomes. Pro-inflammatory lipids are functional components of the uterine stromal compartment remodeling, occurring in the early gestation (Christiaens et al., 2008). Sphingolipids are a broad class of structural membrane components endowed with important signaling activities. Among sphingolipids, ceramide is a well-known mediator of stress signals and pro-inflammatory responses. Ceramide can accumulate mainly in response to an external stress, inducing the catabolism of membrane sphingolipid, or to an endogenous stress leading to its increased *de novo* synthesis.

*Corresponding author: Anna Caretti, Department of Health Sciences, University of Milan, San Paolo Hospital, via A. di Rudinì 8, I-20142 Milan, Italy, e-mail: anna.caretti@unimi.it

Paola Signorelli, Laura Avagliano, Marta R. Reforgiato, Nadia Toppi, Anna Maria Marconi and Riccardo Ghidoni: Department of Health Sciences, University of Milan, San Paolo Hospital, via A. di Rudinì 8, I-20142 Milan, Italy

Josefina Casas and Gemma Fabriàs: Research Unit on BioActive Molecules, Catalan Institute of Advanced Chemistry (IQAC/CSIC), Department of Biomedical Chemistry, Jordi Girona 18, E-08034 Barcelona, Spain

Sphingolipid altered metabolism occurs in cancer, degenerative and inflammatory diseases and few sphingolipid metabolism inhibitors are used in clinical trials (i.e. fingolimod, amitryptiline) (Nährlich et al., 2013; Kappos et al., 2015). Sphingolipid metabolic rate is enhanced in early pregnancy in the first inflammatory implantation phase (Kaneko-Tarui et al., 2007). Similarly, at the end of pregnancy, the acute inflammation of labor and delivery was shown to involve sphingolipids signaling. Ceramide is a known activator of NF- κ B, a transcription factor that controls the expression of many labor-associated genes including cyclooxygenase 2, prostaglandin-endoperoxide synthase 2 and the oxytocin receptor, together with other key inflammatory genes (Sykes et al., 2014). It was also shown that ceramide accumulation mediates the inflammatory response in placenta by stimulating prostaglandins 2 (PGE2) formation (Nakamura et al., 2001; Kawano et al., 2004; Sykes et al., 2014). Our group recently demonstrated that *de novo* synthesis of ceramide is not only enhanced in acute lung inflammation but also supports it (Caretti et al., 2014). We proved that inflammatory stimuli induce an increased transcription of the rate limiting enzyme of ceramide synthesis (serine palmitoyl transferase, SPT) and that the pharmacological inhibition of this activity down regulates inflammatory response. SPT is a heterodimer composed of three subunits (SPTLC1, 2, 3). Among the three subunits of SPT, forming the catalytically active site, SPTLC3 was shown to be highly expressed in placenta (Hornemann et al., 2006). We here hypothesize that up-regulation of ceramide synthesis actively takes part to the acute inflammatory phase driving labor. In this manuscript we showed that ceramide content and SPT expression are increased in placentas from vaginal delivery (both spontaneous and induced parturition) in respect with placentas from cesarean section. Moreover, the transcriptional control of SPT directly correlates with inflammation and inversely with labor duration.

Results

Inflammation is increased in placentas from vaginal delivery compared to elective cesarean section

Human labor is fundamentally an inflammatory process, characterized by the sudden expression of pro-inflammatory cytokines and chemokines, eliciting multiple downstream biological consequences that culminate in parturition. While elective cesarean section allows a delivery without

placenta inflammation, spontaneous and induced vaginal delivery implies the labor related inflammation process. To investigate the inflammatory profile of human placenta from vaginal deliveries, we analyzed the mRNA and protein expression of IL-6 and TNF- α cytokines. As control, we chose placenta samples from elective cesarean section (with no labor occurrence). As expected, IL-6 mRNA (Figure 1A, left graph) was over-expressed in all vaginal deliveries, with the induced group showing higher expression than the spontaneous one, picking at threefold increase (*; vs. cesarean section, $p < 0.05$, Dunnett post-test). In agreement with the transcriptional activity, the protein level of IL-6, measured by immunoassay technique in homogenated tissue, was increased in the induced versus cesarean section group (Figure 1A, right graph, **; $p < 0.01$, Dunnett post-test).

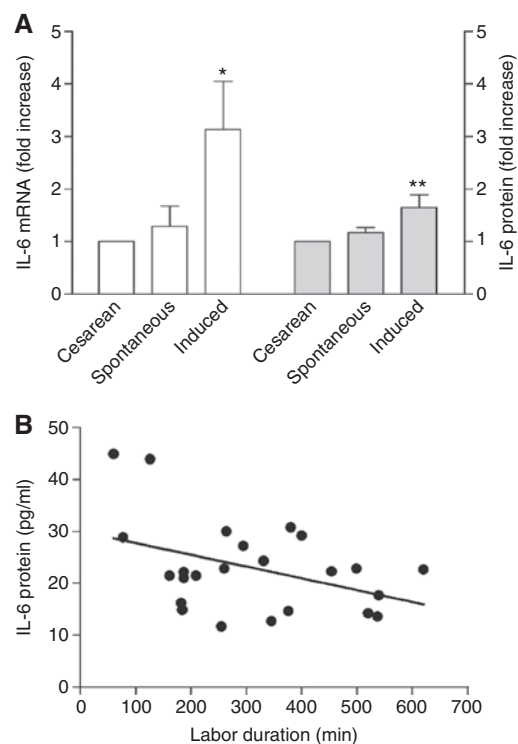


Figure 1: Labor-related inflammation increases in placentas from spontaneous and induced as compared to cesarean parturition. IL-6 mRNA expression (panel A, left) and cytokine content (panel B, right) in placentas collected from women with spontaneous or induced onset of labor or from women who underwent cesarean parturition. RT-PCR and Elisa determinations of each sample have been performed in triplicate. Data on the graphs are expressed as fold increase vs. ct (cesarean parturition, stated as =1). Significance was evaluated by one-way ANOVA: $p = 0.016$ for mRNA expression; $p = 0.014$ for cytokine content. * $p < 0.05$ and ** $p < 0.01$ vs. ct (Dunnett post-test). Panel (B) represents IL-6 protein expression (pg/ml) inversely correlated with the corresponding labor duration (min). Significance was evaluated by Linear correlation: $p = 0.045$, $r^2 = 0.17$, $n = 24$.

TNF- α mRNA expression was unaffected by the different parturition settings and the related protein was almost undetectable and considered ‘out of range below’ (data not shown). Moreover, IL-6 cytokine expression level inversely correlates with labor duration ($p=0.045$) suggesting that inflammation is probably an acute event characterizing the labor process (Figure 1B).

Total ceramide content increases in placentas from vaginal delivery compared to elective cesarean section

In order to investigate a possible role of the pro-inflammatory sphingolipid ceramide in parturition,

we evaluated the ceramide species content by LC-MS in placentas. As shown in Figure 2A, total ceramide content almost doubled in vaginal deliveries vs. elective cesarean section, moving from 4.5 ± 0.5 to 7.5 ± 1.2 and 7.8 ± 0.8 , in spontaneous and induced group, respectively ($p<0.05$, Dunnett post-test). Single ceramide species expression is differently modulated. The less representative ceramides, namely C18:0 and C20:0 acyl-chain bearing ceramides (Figure 2B), are down-regulated in the spontaneous and induced group. Among the major ceramide species (Figure 2C), C16:0, C24:0 and C24:1 acyl-chain bearing ceramides are all significantly up-regulated ($p<0.05$; $**p<0.01$ vs. cesarean, Dunnett post-test) thus accounting for the overall ceramide accumulation. No significant changes in sphingomyelin, hexosyl-ceramides, lactosyl

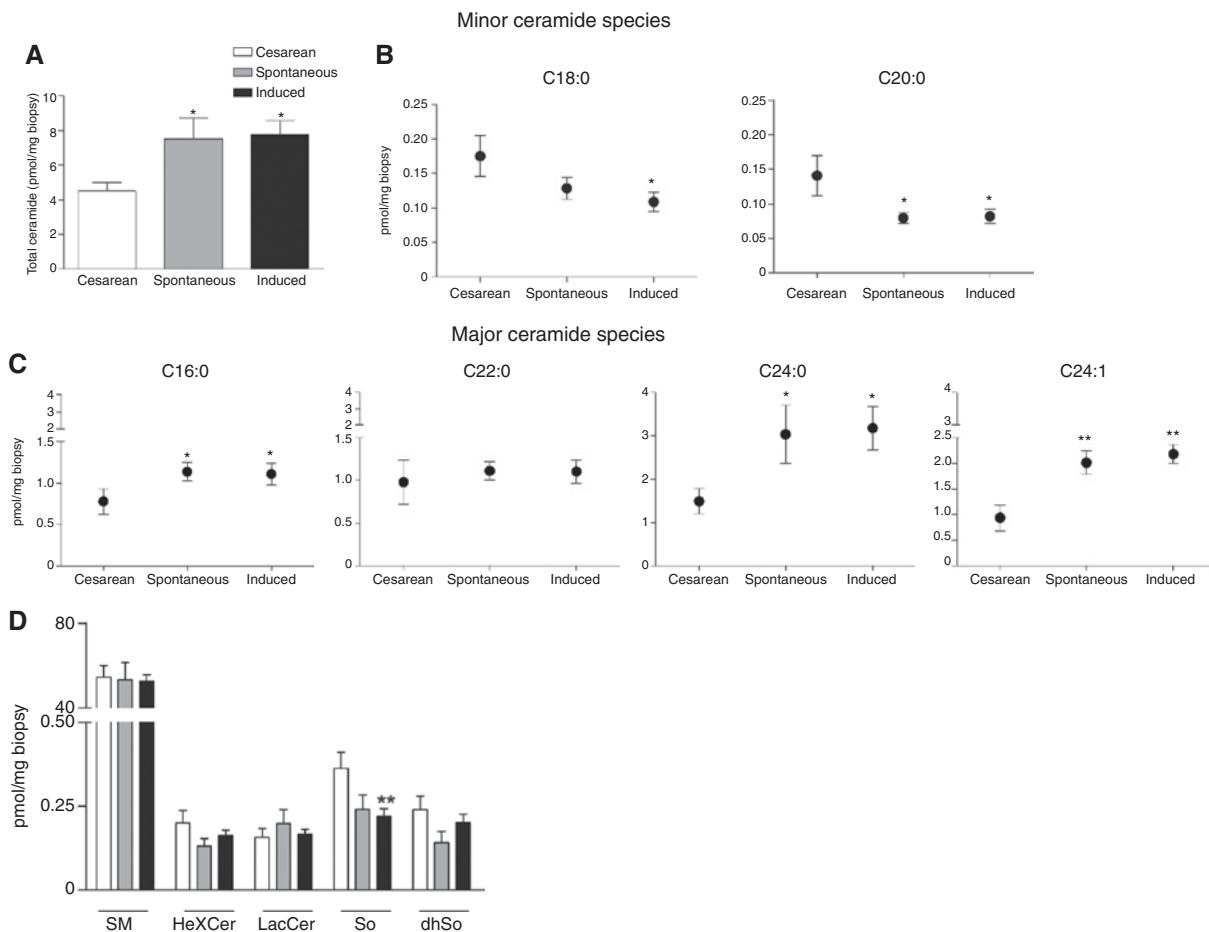


Figure 2: Total ceramide content is upregulated in placentas from spontaneous and induced as compared to cesarean parturition. Total ceramide content (panel A), single ceramide species expression (panels B and C) and sphingomyelin (SM), hexosyl-ceramides (HexCer), lactosyl ceramide (LacCer), sphingosine (So), dihydrosphingosine (dhSo), panel (D) in placentas collected from women with spontaneous or induced onset of labor or from women who underwent cesarean parturition, considered as the control. Note the different scale graph values between the minor (panel B) and the major (panel C) ceramide species represented. Ceramides were analyzed by LC-MS and expressed as pmol/mg biopsy. Significance was evaluated by one-way ANOVA: $p=0.035$, for total ceramides; $p=0.041$, for C16:0; $p=0.048$, for C18:0; $p=0.041$, for C20:0; $p=NS$, for C22:0; $p=0.044$, for C24:0; $p=0.001$, for C24:1. $*p<0.05$ and $**p<0.01$ vs. ct (Dunnett post-test).

ceramide and dihydrosphingosine were identified among groups. A slight decrease in sphingosine was observed in the inflamed placentas.

Placenta ceramide accumulation is associated to enhanced transcription of serine palmitoyl transferase (SPT), the enzyme controlling its *de novo* synthesis

SPT activity is related to a dimer formed mainly by SPTLC1 and either SPTLC2 or 3. First, we evaluated the basic expression of SPT subunits in placentas from control elective cesarean section. Given that SPTLC1 is ubiquitously expressed, whereas the other two subunits are differentially expressed in the various tissues, we evaluated the mRNA expression of the SPTLC2 and 3 in respect with SPTLC1. We observed that mRNA ratio SPTLC3:SPTLC1 was 23-fold higher than the ratio SPTLC2:SPTLC1, suggesting that placentas are enriched in the SPTLC3 subunit, as previously reported (Hornemann et al., 2006) (Figure 3A). As expected, when evaluating the protein levels, SPTLC1 was higher than the other subunits (Figure 3B), as determined by densitometric analysis of Western blotting bands. Second, at the aim of evaluating the contribution of *de novo* synthesis of sphingolipids to the measured ceramide accumulation, we compared the transcription of the three subunits in placentas from vaginal deliveries (both spontaneous and induced group) versus elective cesarean section (Figure 3C). We observed that in the induced group, SPTLC1, 2 and 3 mRNA levels were up-regulated by more than twofold ($*p < 0.05$; $**p < 0.01$ vs. cesarean, Dunnett post-test) while the spontaneous group increased by three-fold in SPTLC3 mRNA level only. The transcript upregulation correlates with a significant raise in protein expression (Western blotting analysis) for both SPTLC1 and 3 subunits of the enzyme (Figure 3D; $*p < 0.05$; $**p < 0.01$ vs. cesarean, Dunnett post-test).

SPT mRNA and protein expression inversely correlate with labor duration

We have observed that labor-related inflammation inversely correlates with the labor duration (Figure 1B) and that SPT is upregulated in the inflammatory process (Figure 3). Therefore, we investigated whether there was a correlation between SPT subunits expression and the duration of labor as well as the inflammatory status. SPTLC1 mRNA level (Figure 4A) significantly decreased along with

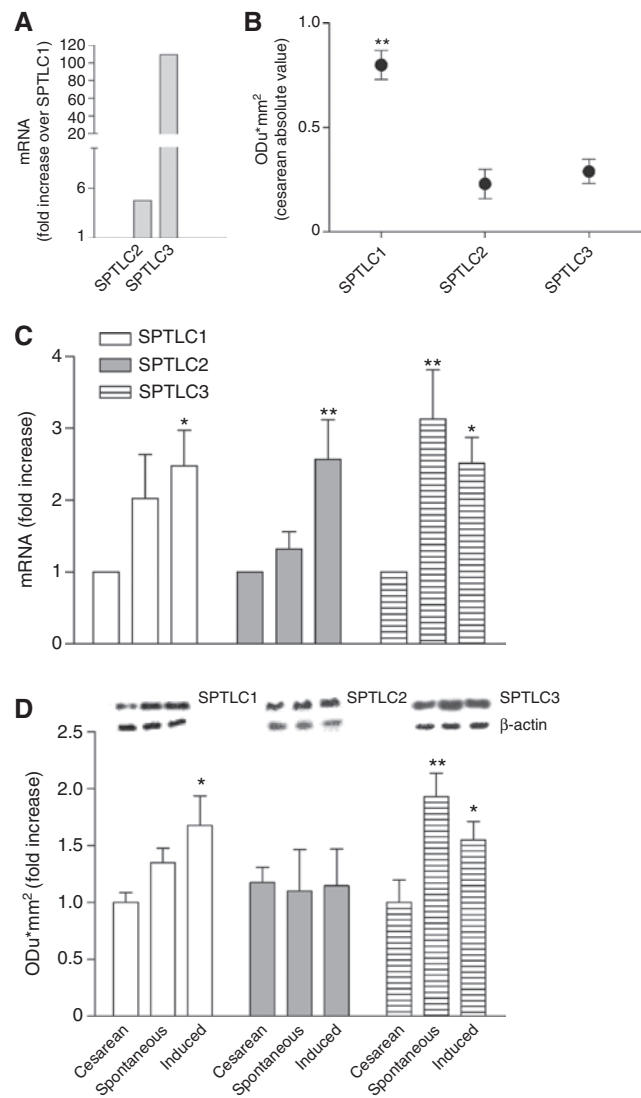


Figure 3: Serine palmitoyl transferase (SPT) subunits are over-expressed in placentas from spontaneous and induced as compared to cesarean parturition.

Panel (A) reports the normalization of mRNA value of SPTLC2 and 3 subunits to the expression level of SPTLC1 mRNA in the control cesarean group. In panel (B), the basal protein expression of the three subunits in the elective cesarean group is represented. SPTLC1, 2, 3 mRNA expression (panel C) and protein content (panel D, expressed as $ODU \cdot mm^2$ from optical densitometry of Western Blotting protein bands) in placenta collected from women with spontaneous or induced onset of labor or from women who underwent cesarean parturition. Data on the graphs (C, D) are expressed as fold increase vs. ct (cesarean parturition, stated as =1). Western blotting images referred to the most representative ones out of two/three independent analysis. RT-PCR determination of each sample was performed in triplicate. Significance was evaluated by one-way ANOVA: panel (B), $p = 0.0001$; panel (C), $p = 0.044$ for SPTLC1 and $p = 0.006$ and for both SPTLC2 and SPTLC3 mRNA; panel (D), $p = 0.048$ and $p = 0.002$ for SPTLC1 and SPTLC3 protein, respectively; $*p < 0.05$ and $**p < 0.01$ vs. ct (Dunnett post-test).

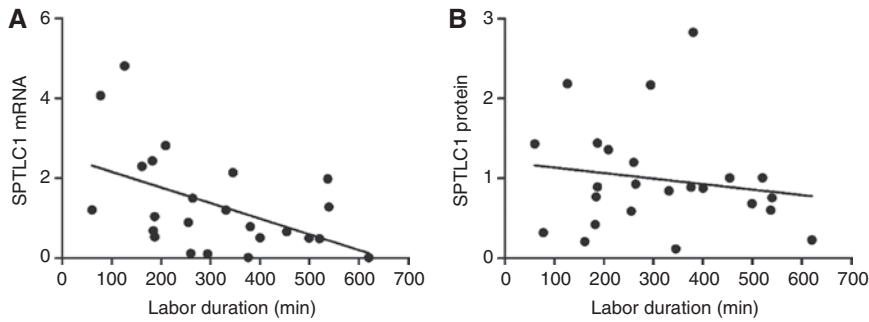


Figure 4: Serine palmitoyl transferase (SPT) mRNA expression correlates with labor duration. SPTLC1 mRNA (panel A) and protein (panel B) expression from placentas collected from women with spontaneous or induced onset of labor inversely correlates with the corresponding labor duration, expressed as minutes. Significance was evaluated by Linear correlation: $p=0.013$, $r^2=0.24$, $n=24$ for SPTLC1 mRNA; $p=NS$, for SPTLC1 protein.

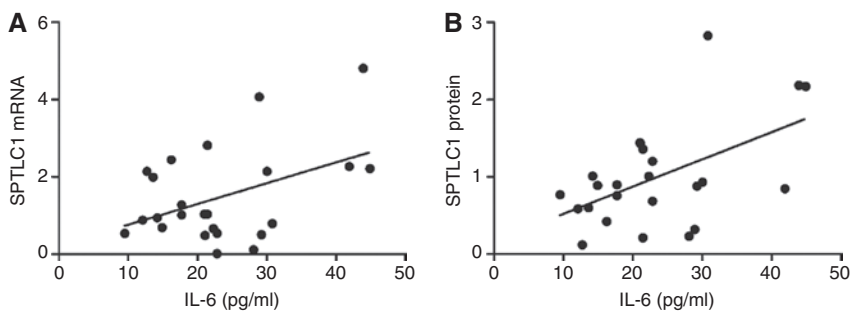


Figure 5: Serine palmitoyl transferase (SPT) mRNA and protein expression directly correlates with IL-6 cytokine content. SPTLC1 mRNA (panel A) and protein (panel B) expression from placentas collected from women with spontaneous or induced onset of labor directly correlates with the corresponding IL-6 content, expressed as pg/ml. Significance was evaluated by Linear correlation: $p=0.032$, $r^2=0.19$, $n=24$ for SPTLC1 mRNA expression; $p=0.008$, $r^2=0.27$, $n=24$ for SPTLC1 protein expression.

labor duration ($p=0.013$) and the protein expression level (Figure 4B) showed the same trend, though statistically not significant. Furthermore, both mRNA and protein levels increased accordingly to the protein content of IL-6, one of the prevalent marker of inflammation in placenta (Figure 5A; $p=0.032$, Figure 5B; $p=0.008$). We did not find any correlation with either the duration of labor nor the level of IL-6 protein for SPTLC2 and 3 subunits at both transcriptional and transductional level (data not shown).

Discussion

To the best of our knowledge, we reported for the first time that ceramide content and expression of SPT (rate limiting enzyme for *de novo* ceramide synthesis) are increased in labored with respect to the cesarean placentas and that SPT expression directly correlates with inflammation and inversely with labor duration.

Parturition is characterized by a complex of transcriptionally regulated events that include NF- κ B activation

and inflammatory mediators as well as multiple contractile genes transcription (Haddad et al., 2006; Christiaens et al., 2008; Eddama et al., 2009). Dysregulation of this mechanism and abnormal, increased or early inflammation characterize parturition complications, such as pre term birth and preeclampsia (Subramaniam et al., 2013; Zhang et al., 2013). During normal pregnancy, the maternal immune response is highly controlled by a complex of cytokines that protects the fetus and promotes the development of the placenta. The resolution of inflammation, that characterizes the first and the last stage of gestation, plays an important role in the success of pregnancy. As reviewed by Chatterjee et al. (2014), this anti-inflammatory activity is largely mediated by IL-4 and IL-10.

Under the control of progesterone, IL-4 is produced by T-helper 2 (Th-2) immune cells of the placenta, by maternal decidua and by maternal and fetal endothelial cells. IL-10 is constitutively expressed in placental villous trophoblast and additionally it is released by uterine NK cells, monocytes and T-regs in the decidua. It increases in the

first and second trimesters and it decreases prior to labor and delivery of the fetus.

Data presented in this manuscript assess the concept of a transcriptional program, involving the enhancement of the sphingolipid synthesis and its related ceramide accumulation, regulating the inflammatory reaction of labor and delivery. We demonstrated that the inflammatory marker IL-6 is upregulated in labored placentas, collected from women with vaginal delivery, compared to placentas from women who underwent elective cesarean section. Phillips et al., demonstrated a negative correlation between inflammatory prostaglandins synthetic enzymes expression and the duration of labor in term and preterm gestations, suggesting that transcriptionally regulation of inflammatory related genes affects parturition in terms of prolonged stressful labor (Phillips et al., 2014). In agreement with the hypothesis of an acute and self resolving inflammation characterizing the physiology of labor, we showed that IL-6 cytokine expression inversely correlates with labor duration, ranging from 1 h to 10 h. This observation suggests that a robust inflammatory mediators release facilitates the expulsion of a viable offspring. It has been reported that sphingolipid metabolism is finely regulated at the maternal-embryonic interface and that sphingolipid mediators take part to the first inflammatory phase of blastocyst implantation and to the acute late inflammation of labor and delivery (Kaneko-Tarui et al., 2007). Accordingly, in placenta from spontaneous and induced onset of labor, we observed a significant increase in ceramide content and we found that labor associated increase of IL-6 expression correlates with total ceramide content up-regulation. In addition we observed a slight decrease in sphingosine in inflamed placentas and no significant changes in the other sphingolipids, such as sphingomyelins and hexosyl-ceramides, among the different patient groups, confirming the hypothesis that ceramide accumulation occurs mainly because of an increased synthesis instead of catabolism of more complex sphingolipids. The accumulation of ceramide likely derives from a transcriptionally regulated enhancement of *de novo* sphingolipids synthesis, since we observed a significant increase of mRNA and protein expression of SPT (the enzyme responsible for the rate-limiting step in the *de novo* synthesis of ceramide) in placenta from spontaneous and induced parturition. SPT is composed of the SPTLC1 subunit forming an active heterodimer with SPTLC 2 or 3. It has been suggested that, while SPTLC1 acts as an anchor for the other subunits, SPTLC2 and SPTLC3 combine alternatively in the complex, play specific functional role and offer a limiting factor for the enzymatic activity depending on their relative amount in

the tissue (Hornemann et al., 2006). Here we reported that both SPTLC1 and SPTLC3 subunit expression increased in labored placentas, suggesting that the formation of this heterodimer occurs and SPTLC3 might represent the limiting factor of SPT activity. Although placentas are enriched in SPTLC3, we observed that SPTLC1 is the most representative, at the protein level, among the three subunits in placentas from cesarean section. To note, SPTLC1 is also the unique subunit whose transcript and protein content are highly dependent on the labor acute inflammation (IL-6 cytokine content). According to this hypothesis, we have recently demonstrated (Caretti et al., 2014) that *de novo* sphingolipid synthesis is involved in acute inflammation and that pharmacological inhibition of SPT activity down regulates the inflammatory response. The present results showed that both SPT and IL-6 enhanced transcription are inversely related to labor length. This is particularly interesting when considering that fast labor is desirable for both mother and fetus and that preterm labor is initiated by inflammatory cascades (Romero et al., 2014). The data presented are underpinning a complex molecular mechanism driving not only labor development but also the whole inflammatory processes along gestation. Placenta expression of PGE2 at labor onset is under control of pro-inflammatory transcriptional factors (such as NF- κ B) that can be activated by ceramide, among other mediators. In this view, transcriptional activation of ceramide synthesis can be hypothesized as part of the early events orchestrating the inflammatory response of parturition. Our data raise the question of possible association between altered sphingolipid metabolism and gestation/parturition defects and most important provide new perspective for investigation of a potential molecular target for the therapy of preterm birth.

Materials and methods

Case selection

Thirty-one normal-shaped, singleton placentas were obtained at the time of uncomplicated term delivery from nonsmoking women with uneventful pregnancies. Seven placentas were from women who underwent an elective cesarean section ('cesarean' group), considered as the control group for all further analysis. Six placentas were from women with spontaneous onset of labor ('spontaneous' group). Eighteen placentas were collected from women with induction of labor ('induced' group) performed according to the Bishop score. Exclusion criteria were gestational age <37 weeks and any maternal or fetal diseases or labor complication. Cesarean section were made for fetal breech presentation or for uterine scar due to previous surgery. Cases selection was made by matching patients for maternal

Table 1: Clinical characteristics.

| | Cesarean | Spontaneous | Induced |
|--|--------------|--------------|-------------|
| Sampling | n=7 | n=6 | n=18 |
| Maternal age (years) | 32.4±2.8 | 29.7±2.4 | 32.1±1.2 |
| Gestational age (weeks) | 38.6±0.3 | 39.0±0.3 | 40.0±0.3 |
| Pre-pregnancy BMI (kg/m ²) | 21.6±1.7 | 22.6±1.5 | 22.3±0.7 |
| Duration of active labor (min) | – | 230.7±87.9 | 333.7±34.4 |
| Neonatal birth-weight (g) | 3221.4±170.6 | 3271.4±203.8 | 3339.4±84.7 |
| Neonatal blood pH at birth | 7.33±0.01 | 7.25±0.04 | 7.26±0.01 |

Clinical characteristics of the three experimental groups: cesarean group (women who underwent an elective cesarean section), spontaneous group (women with spontaneous onset of labor) and induced group (women with induction of labor). Data are expressed as mean±SEM. Data among the three experimental groups do not differ significantly ($p=NS$, one-way ANOVA test). BMI, Body mass index.

and fetal characteristics. Maternal age, pre-pregnancy body mass index, and neonatal birth weight were similar between the three groups (Table 1). No woman received medications during pregnancy and/or epidural analgesia and/or antibiotics during labor. For each woman who had vaginal delivery (both spontaneous and induced group) we evaluated the duration (expressed in minutes) of the active phase of labor.

Placental sampling

Samples from grossly unremarkable placental parenchyma were randomly collected immediately after delivery in midway between the chorionic and basal plates; samples were washed in phosphate-buffered saline solution to remove maternal blood, immediately frozen in liquid nitrogen, and stored at -80°C for further processing.

Reagents and antibodies

The synthetic oligonucleotides used in this study were purchased from M-Medical (Milan, Italy). The following primary antibodies and dilutions were used: anti-SPTLC1, 1:1000 (ab176706, Abcam, Cambridge, UK); anti-SPTLC2, 1.5 µg/ml (ab23696, Abcam, Cambridge, UK); anti-SPTLC3, 1:250 (HPA044247, Sigma, St. Louis, MO, USA); anti-β-actin, 1:5000 (A5316, Sigma, St. Louis, MO, USA). The secondary antibodies (1:10 000) were from Jackson Laboratories (Bar Harbor, ME, USA). All reagents were of the maximal available purity degree.

IL-6 and TNF-α cytokine determination

Human placenta samples were homogenized in phosphate saline buffer (PBS) containing protease inhibitors (Roche Italia, Milan, Italy) by using a tissue homogenizer. Part of the homogenates was centrifuged at 1200 g for 30 min at 4°C and the supernatants were used for cytokines analysis. IL-6 and TNF-α were determined by LaboSpace (Milan, Italy) by means of biomarker multiplex immunoassays that measure the levels of multiple proteins in a single sample volume using the bead-based multiplex solution by the Luminex® Platform. Determinations have been performed in triplicate.

LC-MS analysis

Sphingolipids from human placenta samples (ceramide, sphingomyelins and cerebroside) were extracted by the Bligh-Dyer extraction method (Bligh and Dyer, 1959). Sphingolipid extracts, fortified with internal standards (N-dodecanoylsphingosine, N-dodecanoylglucosylsphingosine, and N-dodecanoylsphingosylphosphorylcholine, 0.2 nmol each), were prepared as previously described (Merrill et al., 2005) and analyzed. The liquid chromatography-mass spectrometer consisted of a Waters Acquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters, Millford, MA, USA), operated in positive electrospray ionization mode. Full scan spectra from 50 to 1500 Da were acquired and individual spectra were summed to produce data points each 0.2 s. Mass accuracy and reproducibility were maintained by using an independent reference spray by the LockSpray interference. The analytical column was a 100 mm 2.1 mm i.d., 1.7 µm C8 Acquity UPLC BEH (Waters). The two mobile phases were phase A: methanol; phase B: water, both contained 0.2% formic acid (v/v) and 2 mM ammonium formate. A linear gradient was programmed: 0.0 min: 20% B; 3 min: 10% B; 6 min: 10% B; 15 min: 1% B; 18 min: 1% B; 20 min: 20% B; 22 min: 20% B. The flow rate was 0.3 ml/min. The column was held at 30°C. Quantification was carried out using the extracted ion chromatogram of each compound, using 50 mDa windows. The linear dynamic range was determined by injecting standard mixtures. Positive identification of compounds was based on the accurate mass measurement with an error <5 ppm and its LC retention time, compared to that of a standard (2%).

Western blotting

Human placenta samples were homogenized in phosphate buffered saline (PBS) containing proteases inhibitors (Roche Italia, Milan, Italy) by using a tissue homogenizer and spun at 1200 g for 30 min at 4°C. An aliquot of the homogenate was used for protein quantification by means of the Coomassie Blue kit (Bio-Rad Laboratories Italia, Milan, Italy) according to manufacturer's instructions. The remaining homogenate was resuspended in Laemmli buffer, boiled for 8 min and stored at -20°C. Equal amount of proteins (50 µg for SPTLC 1, 2, 3) were separated on 8% acrylamide gels by SDS-electrophoresis and transferred onto nitrocellulose membranes. After blocking unspecific binding sites with 5% dry skimmed milk in TBS-Tween 0.1% (TBST),

the membranes were incubated (4°C/overnight) with primary antibodies diluted in TBST, followed by incubation (room temperature/1 h) with the appropriate HRP-secondary antibody diluted in TBST-5% dry skimmed milk. The same membranes were immunoblotted against β -actin for data normalization. Proteins were detected by chemiluminescence and bands intensity was quantified by Gel Doc 2000, using Quantity One Software (BioRad Life Science, Hercules, CA, USA). Western blotting analysis has been repeated twice/three-fold for all the samples belonging to the three experimental groups.

RNA extraction and quantitative RT-PCR

Total RNA was extracted and reverse transcribed according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, placenta's biopsies were homogenized in 175 μ l of lysis buffer by using a tissue homogenizer, placed in a heated Thermoblock at 70°C for 3 min and then centrifuged at 1200 *g* for 10 min at room temperature. The lysates were then cleared by 95% ethanol, transferred to spin basket assembly, centrifuged and the eluate discarded. After incubation at room temperature for 15 min with DNase mix, the lysates were washed, centrifuged and the eluate discarded. Nuclease free water was then applied to the membrane to elute the RNA, stored at -80°C until used. The amplification of target genes (IL-6, TNF- α , SPTLC 1, SPTLC 2, SPTLC 3: primer sequences as previously reported (Hornemann et al., 2006; Dehecchi et al., 2011) was performed using the Syber Green system (Qiagen, Milan, Italy). Relative mRNA expression of target genes was normalized to the endogenous GAPDH control gene and results calculated by $2^{-\Delta\Delta Ct}$ method (Arocho et al., 2006), as labored vs. control cesarean placentas. RT-PCR determinations were performed in triplicate.

Statistical analysis

The reported results are the mean value obtained from all the placenta samples belonging to each experimental patient group. In case of Western blotting, we showed the most representative images. Data significance was evaluated by one-way ANOVA followed by the Dunnett post-test (all vs. cesarean group) when significant ($p < 0.05$). Linear correlation analysis was performed by the parametric Pearson method (significant when $p < 0.05$). Data are expressed as mean \pm SEM. Data analysis was performed by GraphPad Instat (La Jolla, CA, USA) software.

Acknowledgments: Financial support from the institutional grants of the University of Milan and the PhD program in 'Molecular and Translational Medicine' of the University of Milan, Italy, is acknowledged.

References

Arocho, A., Chen, B., Ladanyi, M., and Pan, Q. (2006). Validation of the $2^{-\Delta\Delta Ct}$ calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. *Diagn. Mol. Pathol.* 15, 56–61.

- Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917.
- Caretti, A., Bragonzi, A., Facchini, M., De Fino, I., Riva, C., Gasco, P., Musicanti, C., Casas, J., Fabrias, G., Ghidoni, R., et al. (2014). Anti-inflammatory action of lipid nanocarrier-delivered myricocin: therapeutic potential in cystic fibrosis. *Biochim. Biophys. Acta* 1840, 586–594.
- Chatterjee, P., Chiasson, V.L., Bounds, K.R., and Mitchell, B.M. (2014). Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. *Front Immunol.* 5, 253.
- Christiaens, I., Zaragoza, D.B., Guilbert, L., Robertson, S.A., Mitchell, B.F., and Olson, D.M. (2008). Inflammatory processes in preterm and term parturition. *J. Reprod. Immunol.* 79, 50–57.
- Cotechini, T. and Graham, C.H. (2015). Aberrant maternal inflammation as a cause of pregnancy complications: a potential therapeutic target? *Placenta.* 36, 960–966
- Dehecchi, M.C., Nicolis, E., Mazzi, P., Cioffi, F., Bezzerri, V., Lampronti, I., Huang, S., Wiszniewski, L., Gambari, R., Scupoli, M.T., et al. (2011). Modulators of sphingolipid metabolism reduce lung inflammation. *Am. J. Respir. Cell. Mol. Biol.* 45, 825–833.
- Eddama, O., Petrou, S., Schroeder, L., Bollapragada, S.S., Mackenzie, F., Norrie, J., Reid, M., and Norman, J.E. (2009). The cost-effectiveness of outpatient (at home) cervical ripening with isosorbide mononitrate prior to induction of labour. *BJOG* 116, 1196–1203.
- Fukui, A., Kwak-Kim, J., Ntrivalas, E., Gilman-Sachs, A., Lee, S.K., and Beaman, K. (2008). Intracellular cytokine expression of peripheral blood natural killer cell subsets in women with recurrent spontaneous abortions and implantation failures. *Fertil. Steril.* 89, 157–165.
- Guleria, I. and Pollard, J.W. (2000). The trophoblast is a component of the innate immune system during pregnancy. *Nat. Med.* 6, 589–593.
- Haddad, R., Tromp, G., Kuivaniemi, H., Chaiworapongsa, T., Kim, Y.M., Mazor, M., and Romero, R. (2006). Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. *Am. J. Obstet. Gynecol.* 195, 394 e391–324.
- Hanzlikova, J., Ulcova-Galova, Z., Malkusova, I., Sefrna, F., and Panzner, P. (2009). TH1-TH2 response and the atopy risk in patients with reproduction failure. *Am. J. Reprod. Immunol.* 61, 213–220.
- Hornemann, T., Richard, S., Rutti, M.F., Wei, Y., and von Eckardstein, A. (2006). Cloning and initial characterization of a new subunit for mammalian serine-palmitoyltransferase. *J. Biol. Chem.* 281, 37275–37281.
- Hossein, H., Mahroo, M., Abbas, A., Firouzeh, A., and Nadia, H. (2004). Cytokine production by peripheral blood mononuclear cells in recurrent miscarriage. *Cytokine* 28, 83–86.
- Jin, L.P., Fan, D.X., Zhang, T., Guo, P.F., and Li, D.J. (2011). The costimulatory signal upregulation is associated with Th1 bias at the maternal-fetal interface in human miscarriage. *Am. J. Reprod. Immunol.* 66, 270–278.
- Kaneko-Tarui, T., Zhang, L., Austin, K.J., Henkes, L.E., Johnson, J., Hansen, T.R., and Pru, J.K. (2007). Maternal and embryonic control of uterine sphingolipid-metabolizing enzymes during murine embryo implantation. *Biol. Reprod.* 77, 658–665.

- Kappos, L., O'Connor, P., Radue, E.W., Polman, C., Hohlfeld, R., Selmaj, K., Ritter, S., Schlosshauer, R., von Rosenstiel, P., Zhang-Auberson, L., et al. (2015). Long-term effects of fingolimod in multiple sclerosis: the randomized FREEDOMS extension trial. *Neurology* 84, 1582–1591.
- Kawano, Y., Fukuda, J., Nasu, K., Matsumoto, H., Narahara, H., and Miyakawa, I. (2004). Synergistic effect of interleukin (IL)-1 α and ceramide analogue on the production of IL-6, IL-8, and macrophage colony-stimulating factor by endometrial stromal cells. *Fertil. Steril.* 82 (Suppl 3), 1043–1047.
- Kramer, B.W., Kallapur, S., Newnham, J., and Jobe, A.H. (2009). Prenatal inflammation and lung development. *Semin. Fetal Neonatal Med.* 14, 2–7.
- Kunzmann, S., Collins, J.J., Kuypers, E., and Kramer, B.W. (2013). Thrown off balance: the effect of antenatal inflammation on the developing lung and immune system. *Am. J. Obstet. Gynecol.* 208, 429–437.
- Kwak-Kim, J.Y., Chung-Bang, H.S., Ng, S.C., Ntrivalas, E.I., Mangubat, C.P., Beaman, K.D., Beer, A.E., and Gilman-Sachs, A. (2003). Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF. *Hum. Reprod.* 18, 767–773.
- Marzi, M., Vigano, A., Trabattoni, D., Villa, M.L., Salvaggio, A., Clerici, E., and Clerici, M. (1996). Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clin. Exp. Immunol.* 106, 127–133.
- Merrill, A. H., Jr., Sullards, M.C., Allegood, J.C., Kelly, S., and Wang, E. (2005). Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods* 36, 207–224.
- Mor, G., Cardenas, I., Abrahams, V., and Guller, S. (2011). Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann NY Acad Sci.* 1221, 80–87.
- Nahrlich, L., Mainz, J.G., Adams, C., Engel, C., Herrmann, G., Icheva, V., Lauer, J., Deppisch, C., Wirth, A., Unger, K., et al. (2013). Therapy of CF-patients with amitriptyline and placebo – a randomised, double-blind, placebo-controlled phase IIb multicenter, cohort-study. *Cell Physiol. Biochem.* 31, 505–512.
- Nakamura, S., Kawano, Y., Kamihigashi, S., Sugano, T., Narahara, H., and Miyakawa, I. (2001). Effect of ceramide analogs on interleukin-1 α -induced production of prostaglandin E2 by amnion-derived (WISH) cells. *Acta Obstet Gynecol Scand* 80, 12–17.
- Phillips, R.J., Fortier, M.A., Lopez Bernal, A. (2014). Prostaglandin pathway gene expression in human placenta, amnion and choriodecidua is differentially affected by preterm and term labour and by uterine inflammation. *BMC Preg. Childb.* 14, 241.
- Piccinni, M.P., Beloni, L., Livi, C., Maggi, E., Scarselli, G., and Romagnani, S. (1998). Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat. Med.* 4, 1020–1024.
- Rolle, L., Memarzadeh Tehran, M., Morell-Garcia, A., Raeva, Y., Schumacher, A., Hartig, R., Costa, S.D., Jensen, F., and Zenclussen, A.C. (2013). Cutting edge: IL-10-producing regulatory B cells in early human pregnancy. *Am. J. Reprod. Immunol.* 70, 448–453.
- Romero, R., Dey, S.K., and Fisher, S.J. (2014). Preterm labor: one syndrome, many causes. *Science* 345, 760–765.
- Subramaniam, A., Cliver, S.P., Andrews, W.W., Faye-Petersen, O.M., Goldenberg, R.L., and Biggio, J.R. (2013). Effect of corticosteroid interval on markers of inflammation in spontaneous preterm birth. *Am. J. Obstet. Gynecol.* 209, 379 e371–376.
- Sykes, L., MacIntyre, D.A., Teoh, T.G., and Bennett, P.R. (2014). Anti-inflammatory prostaglandins for the prevention of preterm labour. *Reproduction* 148, R29–40.
- Zhang, Z., Gao, Y., Zhang, L., Jia, L., Wang, P., Zhang, L., and Li, H. (2013). Alterations of IL-6, IL-6R and gp130 in early and late onset severe preeclampsia. *Hypertens. Pregnancy* 32, 270–280.