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# Antenatal corticosteroid therapy modulates hepatic AMPK phosphorylation and maternal lipid metabolism in early lactating rats

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ABSTRACT

Antenatal corticosteroid therapy is used to reduce neonatal mortality in preterm infants but it is currently unknown whether this intervention affects lipid metabolism at the peripartum. This study aimed to evaluate if antenatal corticosteroid therapy in pregnant rats and women affects lipid metabolism during early lactation. We evaluated women at risk of preterm delivery that received corticosteroid therapy (CASE) and women that were not exposed to corticosteroid and were not at risk of preterm delivery (CONTROL). Samples were collected to measure serum and milk triacylglycerol (TAG) three days after delivery. Rats were treated with dexamethasone (DEX) between the 15th and the 20th days of pregnancy. Samples were collected at different days after delivery (L3, L8 and L14). TAG was measured in serum, liver and mammary gland (MG). TAG appearance rates were measured after tyloxapol injection and gavage with olive oil. We also evaluated the expression of key genes related to lipid metabolism in the liver and in the MG and hepatic phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). CASE volunteers delivered earlier than CONTROL but presented unaltered milk and serum TAG concentrations. Early lactating DEX rats exhibited increased TAG in serum, MG and milk. No changes in CD36 and LPL were detected in the MG and liver. Early lactating DEX rats displayed increased TAG appearance rate and reduced hepatic AMPK/ACC phosphorylation. Our data revealed that antenatal corticosteroid therapy reduces hepatic AMPK/ACC phosphorylation during early lactation that reflects in increased TAG concentration in serum, MG and milk.

## 1. Introduction

Antenatal corticosteroid therapy with betamethasone (BET) or dexamethasone (DEX) is widely used as a strategy for reducing neonatal morbidity and mortality of preterm infants with less than 34 weeks. The benefits of this intervention are associated with the reduction in the incidences of respiratory distress syndrome (RDS) and intraventricular hemorrhage (IVH) [1]. Despite the well-known positive outcomes of antenatal corticosteroid therapy for the newborn, there is evidence pointing to adverse metabolic effects in women exposed to glucocorticoids at the end of pregnancy. Antenatal BET has been shown to elicit glucose intolerance in non-diabetic mothers at risk of preterm delivery [2,3]. Corticosteroid therapy in pregnant women with gestational diabetes also led to an increased demand for insulin in order to control glucose levels [4].

At the metabolic point of view, the pregnancy-to-lactation transition is a singular period for maternal lipid homeostasis. Lipoprotein lipase (LPL) is respectively increased in the mammary gland (MG) and reduced in adipose tissue allowing the shunt of the circulating pool of triacylglycerol (TAG) to the milk synthesis [5]. An appropriate amount of

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non-esterified fatty acid (NEFA) for milk synthesis by MG is also assured by the upregulation of key metabolic steps of *de novo lipogenesis* (DNGL) and fatty acid re-esterification into TAG [6]. The liver plays a relevant role in this scenario by secreting VLDL that will be further used as a substrate for LPL in the MG [7].

Although it is well recognized that exogenous glucocorticoids modulate adipose tissue LPL activity and enhance hepatic secretion of TAG containing newly synthesized NEFA [8–10], it is particularly challenging to discern if antenatal corticosteroid therapy *per se* affects maternal lipid metabolism during the peripartum. While ethical issues preclude the use of BET or DEX in mothers that are not at risk of preterm delivery, observational studies also face the limitation that term and preterm mothers at a given time point after delivery are not at the same postmenstrual age.

In the present study, we collected data from human and animal models with the attempt to clarify if the exposure to corticotherapy during late pregnancy affects maternal lipid metabolism during early lactation. Part of the study was based on an observational approach that enrolled pregnant volunteers at risk of preterm delivery. Additionally, pregnant rats treated with DEX were subjected to the analysis of multiple biochemical pathways that mediate lipid metabolism in the MG and in the liver.

## 2. Materials and methods

### 2.1. Animals and treatment

The experiments were performed after approval by the Committee for Ethics in Animal Experimentation at the State University of Campinas, Brazil (protocol No. 4963–1/2018). Female and male Wistar rats were provided by the Animal Breeding Center at the University of Campinas (Campinas, Sao Paulo, Brazil). All rats were housed in animal care facility with controlled temperature (22 ± 2 °C) and light-dark cycle (lights on at 7:00 AM and off at 7:00 PM). The rats were kept with standard chow (Nutrilab, Colombo, PR, Brazil) and water *ad libitum*.

The females and the male were housed together for 3 days (two females with one male per cage) upon reaching the age of 12 weeks. In some experiments, age-matched female rats were kept unmated to be used as a virgin control group (CTL). The females were isolated on the first day of pregnancy, which was assumed when spermatozoa and estrous cells were simultaneously observed in the vaginal lavage. Treatments started on the 15th day of pregnancy. The pregnant rats were assigned to two groups. One group received untreated tap water, and a second group received 0.1 mg/kg/day of water-soluble DEX diluted in the drinking water for 6 days. Details of this treatment were described elsewhere [11].

### 2.2. Tissues sampling and biochemical analysis

Unless otherwise stated, samples were obtained from fed dams without previous separation from the litter. Blood samples were collected from tail puncture and processed for serum extraction. Milk samples were obtained with the use of capillary glass tubes 20 min after a subcutaneous injection containing 5 IU of oxytocin (UCBVet, Jaboticabal, SP, Brazil) [12].

Serum TAG and cholesterol were measured with enzymatic colorimetric assays (LABORLAB, Sao Paulo, SP, Brazil). Glucose levels were assessed in whole blood using a glucometer (Accu-Chek Active, Roche, Basel, Switzerland). Inguinal mammary glands (MG), liver and milk samples were stored at −80 °C for later processing.

Total milk was also used for determination of total protein concentration. To this end, samples were slightly homogenized and diluted in 0.9% NaCl (1:100). Subsequently, samples were assayed with a colorimetric dye (Cat. # 500–0006; Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. A standard curve with bovine albumin was set to serve as reference. Absorbance was red at 595 nm.

### 2.3. TAG and cholesterol determination in liver, mammary gland and milk

Lipids were extracted from liver, MG and milk samples as previously described [13]. Briefly, samples were weighed and homogenized in a solution containing chloroform and methanol and subjected to overnight shaking at 4 °C. After the addition of a 0.6% NaCl solution, the samples were centrifuged (2000 ×*g* for 20 min) and the organic layer was collected and dried at 45 °C for 24 h. The lipids were solubilized in isopropanol and quantified with the kits used for serum samples.

### 2.4. In vivo measurement of TAG appearance rate

Tests to measure *in vivo* TAG appearance rate were performed as previously described [14]. Briefly, overnight fasted lactating rats on the third day after delivery (L3) received an intraperitoneal injection with the LPL inhibitor tyloxapol (Triton WR-1339; Sigma Aldrich, St Louis, USA) dissolved in 0.9% NaCl (500 mg/kg). Blood samples were collected from tail puncture before and 1, 2, 3 and 6 h after tyloxapol injection. Serum was extracted from blood samples and subjected to TAG quantification. The litters were kept with their respective dams during the overnight fasting and the test. Linear regressions of the curves (TAG concentration *vs.* time) were presented as TAG appearance rate ($h^{-1}$).

### 2.5. Olive oil gavage test

Lactating rats received an oral gavage with extra virgin olive oil (5 ml/kg) on the third day after delivery (L3). Blood samples were collected from tail puncture before and 90, 180, and 360 min after the gavages. Serum samples from blood were used for TAG determination. The litters were kept with their respective dams during the test.

### 2.6. RNA extraction and qPCR

Total RNA was extracted and processed for reverse transcription and quantitative PCR as previously described [15]. The sequences of the primers were as follow: *Dgat1* (NM_053437) sense: 5′-AGGATGTTCCGCCTTTGGG-3′ and antisense: 5′-CGTGGACA TACATGAGCACAGC-3′; *Dgat2* (NM_001012345) sense: 5′-AAGCCCAT CACCACCGTTG-3′ and antisense: 5′-TTCCTTCCAGGAGCTGGCAC-3′; *Fasn* (NM_017332) sense: 5′-TGGTGAAGCCCAGAGGGATC-3′ and antisense: 5′-CACTTCCACACCCATGAGCG-3′; *Me1* (NM_012600) sense: 5′-ACTGATGGAGAGCGAATCCTCG-3′ and antisense 5′-TTTCTGTG CCCACGTCCAAAG-3′; *Sec22b* (NM_001025686) sense: 5′-CGTGCTCGG AGAAATCTCGG-3′ and antisense: 5′-AACACGGCTACTGCTGCAAGC-3′; *Mttp* (NM_001107727) sense: 5′-TATGACCGTTTCTCCAAGAGTGG-3′ and antisense: 5′-TCAAGGTTCTCCTCTCCCTCATC-3′; *ApoB* (NM_019287) sense: 5′-CTGCGGTGGCAGAAATAACG-3′ and antisense: 5′-CCTTGAGCAAACCTTAGGTAGGG-3′; *Rpl37a* (X14069) sense: 5′-CA AGAAGGTCGGGATCGTCG-3′ and antisense: 5′-ACCAGGCAAGTCT CAGGAGGTG-3′.

### 2.7. Western blotting

Inguinal mammary gland and liver fragments were processed for Western blotting as previously described [16]. Fifty micrograms of protein were subjected to SDS-PAGE using 6.5% or 10% gels (%T) and transferred to nitrocellulose membranes. The primary antibodies used were anti-phospho-acetyl-CoA carboxylase (pACC) (cat. # 07–303) from Millipore (Temecula, CA, USA), anti-CD36 (CD36) (cat. # sc-9154) and anti-lipoprotein lipase (LPL) (cat. # sc-373759) from St. Cruz Biotechnology (Dallas, TX, USA), anti-acetyl-CoA carboxylase (ACC) (cat. # 3662) and anti-phospo-AMP-activated protein kinase alpha (pAMPKα) (cat. # 2531) from Cell Signaling Technology (Danvers, MA, USA),

anti-sterol regulatory element binding proteins 1/2 (SREBP-1/2) (cat. # bs-1402R) from Bioss Inc. (Boston, MA, USA) and anti-peroxisome proliferator- activated receptor gamma (PPARγ) (cat. # ab191407) from Abcam (Cambridge, UK). Secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) were used for chemiluminescent detection of the bands through visualization on a ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA). Target protein intensities were quantified by optical densitometry analysis using ImageJ software (https://imagej.nih.gov/ij), and the results were normalized by the amount of protein transferred to the membranes as indicated by Ponceau S staining.

### 2.8. Human data

Pregnant women that attended to the Centro de Atenção Integral à Saúde da Mulher – Hospital da Mulher J.A. Pinotti (CAISM) and agreed to volunteer were included in the study (Approved by the local Ethics Committee on Research: No. 59666316.0.0000.5404). Exclusion criteria were: chronic use of glucocorticoids, diagnosis of systemic lupus erythematosus or rheumatoid arthritis, Type 1 or Type 2 Diabetes Mellitus, Gestational Diabetes Mellitus, chronic hypertension, gestational hypertension and pre-eclampsia.

Volunteers at risk of preterm delivery that received antenatal corticosteroid therapy with BET were assigned to the CASE group. Volunteers that delivered at term and did not receive antenatal corticosteroid therapy with BET were assigned to the CONTROL group. We evaluated 30 volunteers in each group.

Upon agreement, blood and milk samples were collected from each volunteer in the morning (from 6:00 AM to 8:00 AM) before breakfast. The samples were collected between 48 h and 72 h after delivery. We also assessed the medical records and the gestational age was calculated by the Capurro method.

### 2.9. Statistical analysis

Animal data are presented as the means ± standard error (S.E.). The normality of the data was confirmed using the Shapiro-Wilk test. Parametric data were compared using two-way ANOVA or one-way ANOVA, followed by a Tukey's multiple comparison test. When making comparisons between two groups, the unpaired Student's *t*-test was used for parametric data, and Mann-Whitney test was used for non-parametric data.

Human data were presented as means ± standard deviation (S.D.) or median with the interquartile range (non-parametric data). Continuous variables with parametric distribution were compared with Student's *t*-test, while continuous variables with non-parametric distribution were compared with Mann-Whitney. Categorical data were expressed as absolute numbers and percentage, and were compared with Fisher test.

Statistical analyses were conducted with GraphPad Prism software version 8.4 (GraphPad Software, Inc.) and SigmaPlot 12.0 (Systat Software, Inc.). Results with $P$ values that were $< 0.05$ were considered significant.

## 3. Results

### 3.1. Concentrations of TAG in serum and milk are not altered when comparing term to preterm mothers at the third day after delivery

We evaluated some ponderal and biochemical parameters with the attempt to elucidate if antenatal corticotherapy could modulate maternal lipid metabolism soon after delivery. We enrolled volunteers that were at risk of preterm delivery and received antenatal corticosteroid therapy with betamethasone (CASE). Their data were compared to those from women that delivered at term and did not receive antenatal corticosteroid therapy (CONTROL). Data of both groups were matched according to the postnatal day as they were exclusively collected at the third after delivery.

Volunteers of both groups had similar age, body mass and BMI. The majority of the volunteers assigned to the CASE group reached the preterm condition (delivery before the end of the 37th week). As a consequence, the median gestational age at the delivery was lower in the CASE (35 weeks *vs.* 39 in the CONTROL group; $P < 0.001$) and their newborn had lower birth weight (2.45 kg *vs.* 3.15 kg of those born to CONTROL volunteers; $P < 0.0001$).

The frequencies of cesarean sections were similar between the two groups but the percentage of primigravida was lower in the CASE group (30% *vs.* 56% in the CONTROL group; $P = 0.04$). Volunteers assigned to both CASE and CONTROL groups had similar serum concentrations of total cholesterol and TAG and milk TAG (Table 1).

### 3.2. Circulating TAG levels are increased in lactating rats treated with DEX during pregnancy

Our observational approach revealed similar levels of TAG in the milk and in the serum collected at the third day after delivery from volunteers that received and did not receive antenatal corticotherapy. However, as the median gestational ages were different between the groups, our human data did not rule out a possible modulation of maternal lipids by antenatal glucocorticoids.

In order to address this issue without the bias of differences in gestational length, we used a model of rats subjected to DEX treatment during pregnancy. Untreated undisturbed pregnant rats delivered at term 22.3 ± 0.42 (n = 6) days after copulation. The duration of pregnancy in DEX treated rats (22.2 ± 0.36) was similar to the untreated (n = 7; $P = 0.94$).

Pregnant rats at the 15th day of pregnancy (P15) that were either untreated or treated with DEX were heavier than age-matched CTL (respectively 19% and 15% heavier; $P < 0.05$). As gestational age advanced, untreated pregnant rats continued to gain weight but no similar increase was noted in pregnant rats treated with DEX. Consequently, pregnant rats treated with DEX at P19 were 13% heavier than age-matched CTL ($P < 0.05$) but 23% lighter than untreated pregnant rats at the same gestational age ($P < 0.01$).

Both groups of pregnant rats experienced a rapid reduction in body weights after delivery so that they reached similar values at L3. At this moment of lactation, the body weights of rats that received and did not

**Table 1**
General characteristics and biochemical parameters of the volunteers.

| | Control (n = 30) | CASE (n = 30) | *P* |
|---|---|---|---|
| **General characteristics** | | | |
| Maternal age (years) | 25 ± 4 | 26 ± 6 | 0.84 |
| Maternal body mass (kg) | 58.94 ± 1.42 | 59.31 ± 1.53 | 0.86 |
| Maternal BMI ($kg/m^2$)$m^2$ | 21.97 ± 0.45 | 22.77 ± 0.60 | 0.29 |
| Gestational age at the delivery (weeks+days) | 39 + 2 (39 +1–40 +0) | 35 + 5 (34 +1–38 +0) | < 0.001 |
| Body mass of the newborn (kg) | 3.149 ± 0.08 | 2.456 ± 0.11 | < 0.0001 |
| Primigravida, n (%) | 17 (56.7) | 8 (30.0) | 0.04 |
| Cesarean section, n (%) | 8 (26.7) | 15 (50.0) | 0.11 |
| Twin births, n (%) | 0 (0) | 5 (16.7) | 0.05 |
| **Biochemical parameters** | | | |
| Maternal serum total cholesterol (mg/dl) | 189.0 ± 6.5 | 194.1 ± 6.1 | 0.57 |
| Maternal serum TAG (mg/dl) | 222.7 (168.8–270.2) | 187.2 (124.1–290.8) | 0.24 |
| Milk TAG (mg/dl) | 988.2 (635.3–1929.4) | 917.6 (564.7–1718) | 0.42 |

BMI, body mass index; TAG, triacylglycerol. Continuous variables with parametric distribution were expressed as mean ± S.D. Continuous variables with non-parametric distribution were expressed as median (interquartile range). Categorical variables were expressed as absolute number (%). Student's *t*-test or Mann-Whitney were used for continuous variables, and Fisher test was used to compare categorical data.

receive DEX during pregnancy were similar to age-matched CTL. As the lactation proceeded, both groups of lactating dams exhibited a similar weight gain. Consequently, the body weights of rats that received and did not receive DEX during pregnancy were similarly increased at L14 (approximately 15% higher than age-matched CTL; $P < 0.05$) **(**Fig. 1**A)**.

The daily food intake assessed between the P15 and P20 were similar in rats being treated and not being treated with DEX. For both cases, the daily food intakes were elevated when compared to age-matched CTL (approximately 30% higher; $P < 0.001$) (Fig. 1B). Daily liquid intake between P15 and P20 in both groups of pregnant rats were not different than that of CTL (Fig. 1C).

Although both groups of lactating dams exhibited a progressive decline of glycemia during lactation reaching minimal values at the 14th day of lactation (L14) (approximately 10% lower than L3; $P < 0.001$), only the rats treated with DEX during pregnancy had glucose levels lower than age-matched CTL at L14 (9% lower; $P < 0.05$) (Fig. 1D).

An increase in circulating cholesterol levels during lactation was noted exclusively in rats treated with DEX during pregnancy (values at L14 were 34% higher than that at L3; $P < 0.05$). At L14, the levels of circulating cholesterol were also increased when compared to age-matched CTL (36% higher; $P < 0.05$) (Fig. 1E).

Lactating rats that were not treated with DEX during pregnancy

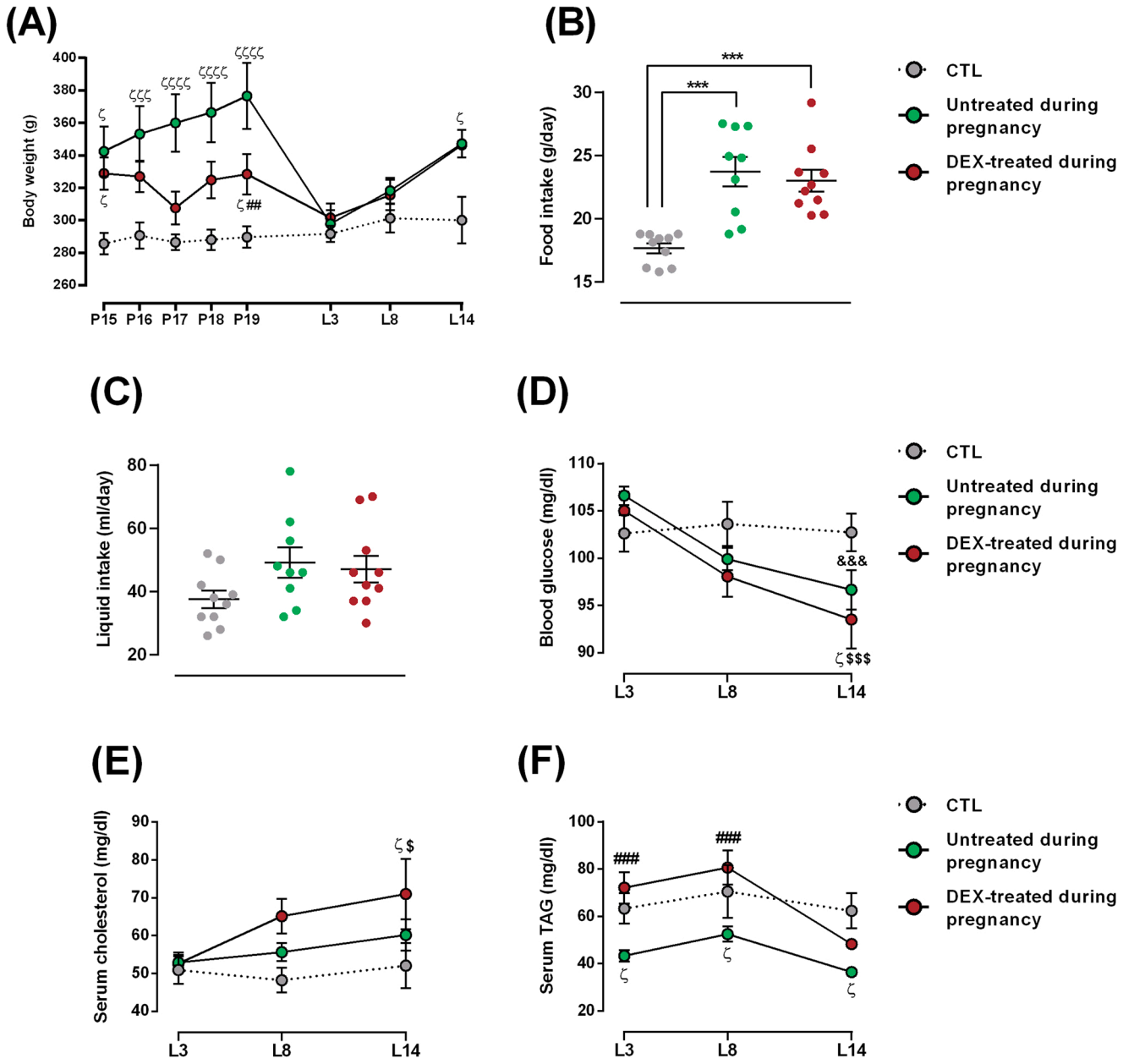


**Fig. 1.** Body weight, food and liquid intake and biochemical characteristics in rats treated with DEX during pregnancy. Female rats were assigned to three groups. Two groups were mated. Half of the mated rats were treated with DEX between the 15th and the 20th days of pregnancy. A third group was kept unmated (CTL). Body weight was assessed on the 15th, 16th, 17th, 18th and 19th days of pregnancy (P15, P16, P17, P18 and P19), and on the 3rd, 8th and 14th days of lactation (L3, L8 and L14, respectively) (A). Food (B) and liquid (C) intakes were measured between P15 and P20. Blood glucose (D) and serum cholesterol (E) and TAG (F) were assessed at L3, L8 and L41 in non-fasted dams. Data are shown as mean ± S.E. $^{\zeta}P < 0.05$, $^{\zeta\zeta}P < 0.01$, $^{\zeta\zeta\zeta}P < 0.001$ and $^{\zeta\zeta\zeta\zeta}P < 0.0001$ *vs.* CTL at the same time point; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* untreated during pregnancy at the same time point. $^{\$}P < 0.05$ and $^{\$\$\$}P < 0.001$ *vs.* DEX-treated during pregnancy at L3; $^{\&\&\&}P < 0.001$ for L14 *vs.* L3 within those untreated during pregnancy; $^{***}P < 0.001$ (Body weight: n = 5; Food and liquid intake; n = 10 for CTL and DEX-treated pregnant, and n = 9 for untreated pregnant; Blood glucose and serum TAG and cholesterol: n = 15 for CTL, n = 31 for untreated pregnant and n = 18 for DEX-treated pregnant. Data were analyzed with two-way ANOVA).

exhibited reduced levels of circulating TAG at L3, L8 and L14 (respectively 32%, 25% and 42% lower than age-matched CTL; $P < 0.05$). Such reduction was not observed in lactating rats treated with DEX during pregnancy. Moreover, TAG levels at L3 and L8 were significantly increased in rats that were exposed to DEX during pregnancy when compared to those that were not (respectively 67% and 55% higher; $P < 0.001$) (Fig. 1F).

### 3.3. Antenatal DEX-induced increase in circulating TAG during early lactation is associated with increased TAG appearance rate after whole-body inhibition of LPL

In order to clarify if the increased TAG levels seen in lactating rats exposed to DEX during pregnancy were associated to modulations in hepatic lipids stores, we assessed the hepatic contents of TAG and cholesterol in the different groups. Interestingly, both rats that were treated and not treated with DEX during pregnancy had similar hepatic TAG content at L3. At this specific moment of lactation, the hepatic TAG contents of both groups of lactating mothers were also similar to that of CTL. Moreover, hepatic TAG content exhibited a progressive and similar decline in both groups of lactating rats. At L14, the hepatic TAG content in both groups of lactating rats was approximately 80% lower than in CTL rats ($P < 0.0001$) (Fig. 2A). Hepatic cholesterol levels in both groups at L3 were also similar to that of the CTL rats. At L14, both the rats that were untreated and treated with DEX had reduced hepatic cholesterol levels (approximately 40% lower than CTL; $P < 0.01$) (Fig. 2B).

Because the data on hepatic TAG content was not coincident with the increased circulating TAG seen in early lactating rats treated with DEX during pregnancy, we decided to evaluate the kinetics of serum TAG levels after a challenge with olive oil. The lactating rats treated with DEX during pregnancy was the only group that experienced an increase in the circulating levels of TAG 90 min after the oral challenge with olive oil (167% higher than their respective basal levels; $P < 0.0001$). At this specific time-point, the circulating TAG levels in lactating rats treated with DEX during pregnancy were also higher than those not treated with DEX and the CTL group (212% and 111% higher, respectively: $P < 0.0001$ and $P < 0.001$) (Fig. 2C). Accordingly, we have found that AUC values (of the curves of circulating TAG concentration *vs.* time) were increased in the lactating rats treated with DEX (149% higher than untreated lactating rats and 77% higher than CTL; $P < 0.01$ and $P < 0.05$) (Fig. 2D).

In order to discern if the higher TAG values detected in lactating rats treated with DEX during pregnancy were due to increased hepatic TAG secretion, we performed an *in vivo* assay with whole-body inhibition of LPL. This assay consisted of evaluating TAG kinetics after an i.p. injection with tyloxapol [17].

Our experiments revealed that TAG concentrations in L3 rats were 63% lower than that of CTL rats 6 h after tyloxapol injection ($P < 0.0001$). On the other hand, TAG concentrations in L3 rats exposed to DEX during pregnancy were similar to that of CTL rats 2, 3 and 6 h after tyloxapol injection (Fig. 2E). Accordingly, the values of the linear regressions obtained from the kinetics of circulating TAG (TAG appearance rate) were reduced in L3 rats not treated with DEX during pregnancy (55% lower than those from L3 rats treated with DEX during pregnancy; $P < 0.05$) (Fig. 2F).

### 3.4. TAG content is increased in the mammary glands of early lactating rats treated with DEX during pregnancy

Previous publication by Tedstone and colleagues suggested that lower tyloxapol-induced increase in circulating TAG in the lactating rats could be due to an ineffective inhibition of LPL expressed in the MG [18].

Aiming at clarifying if the increase TAG appearance rat seen in L3 rats treated with DEX during pregnancy was a consequence of increased hepatic VLDL production or impaired TAG uptake by the MG, we next evaluated key aspects of the milk composition.

The milk of the early lactating rats that were treated with DEX during pregnancy had a higher content of cholesterol and TAG (respectively 56% and 159% higher than that of untreated rats at L3; $P < 0.001$) (Fig. 3A and B) but unaltered protein concentration (Fig. 3C). On the other hand, MG expression of the enzymes that catalyze diacylglycerol esterification into TAG, acyl CoA:diacylglycerol acyltransferase 1 (*Dgat1*) and *Dgat2* as well as the protein contents of the fatty acid transported CD36 and LPL were not altered by antenatal exposure to DEX (Fig. 3D-G). Moreover, as example of the milk samples, TAG content in MG was increased in early lactating rats that were treated with DEX during pregnancy (91% higher than that of untreated rats at L3; $P < 0.01$) (Fig. 3H).

### 3.5. Increased TAG in the mammary glands, milk and serum of early lactating rats treated with DEX during pregnancy are associated to reduced hepatic AMPK phosphorylation

As our data on milk composition and MG transcription profile were not consistent with a possible impairment of TAG uptake, we then hypothesized that the exacerbated TAG appearance rate seen in early lactating rats treated with DEX during pregnancy would result from increased hepatic VLDL production.

To reinforce such interpretation, we measured the expression of key genes involved in VLDL synthesis and secretion. Our findings demonstrated that the expression of microsomal triglyceride transfer protein (*Mttp*), apolipoprotein B (*Apob*) and SNAP receptor SEC22 (*Sec22*) were similar between the two groups of lactating rats at L3, irrespective of DEX treatment during pregnancy (Fig. 4A–C). The hepatic expression of fatty acid synthase (*Fasn*) and malic enzyme 1 (*Me1*), two limiting steps for *de novo* lipogenesis (DNGL) were similar when comparing L3 rats that were either treated or not treated with DEX during pregnancy (Fig. 4D–E). Accordingly, the protein content of two transcription factors the govern the expression of DNGL enzymes, the sterol regulatory element-binding proteins 1/2 (SREBP-1/2) and the peroxisome proliferator-activated receptor gamma (PPARγ), and the protein content of CD36 were also not altered in the liver of early lactating rats treated with DEX during pregnancy (Fig. 5A–C).

As we found no obvious modulation in the expression of proteins classically implicated in VLDL production and hepatic DNLG, we decided to focus on possible changes in the AMPK pathway. Our experiments revealed that the levels of AMPKα and ACC phosphorylation were reduced in the liver early lactating rats treated with DEX during pregnancy (respectively 31% and 44% lower than CTL; $P < 0.05$) (Fig. 5D and E).

## 4. Discussion

The present investigation was undertaken to test if antenatal corticotherapy would affect maternal lipid metabolism soon after delivery. Our initial strategy consisted of an observational approach that selected women at a risk of preterm delivery prescribed with BET. As a control group, we selected women without risk of preterm delivery and therefore not prescribed with antenatal BET. As our design excluded cases of gestational diabetes mellitus (GDM), chronic hypertension, gestational hypertension and pre-eclampsia from both groups, we initially expected a reduced number of preterm deliveries among the volunteers assigned to the BET group.

Our data revealed that volunteers assigned to CTL and BET groups had similar serum cholesterol and serum and milk TAG levels 3 days after delivery. However, as the deliveries occurred, we found a higher number of preterm deliveries in the BET (resulting in a median gestational age of 35 weeks at delivery). We thus concluded that our inclusion criteria strategy could not dissociate the antenatal corticotherapy from preterm delivery and, therefore, was not appropriate to rule out the

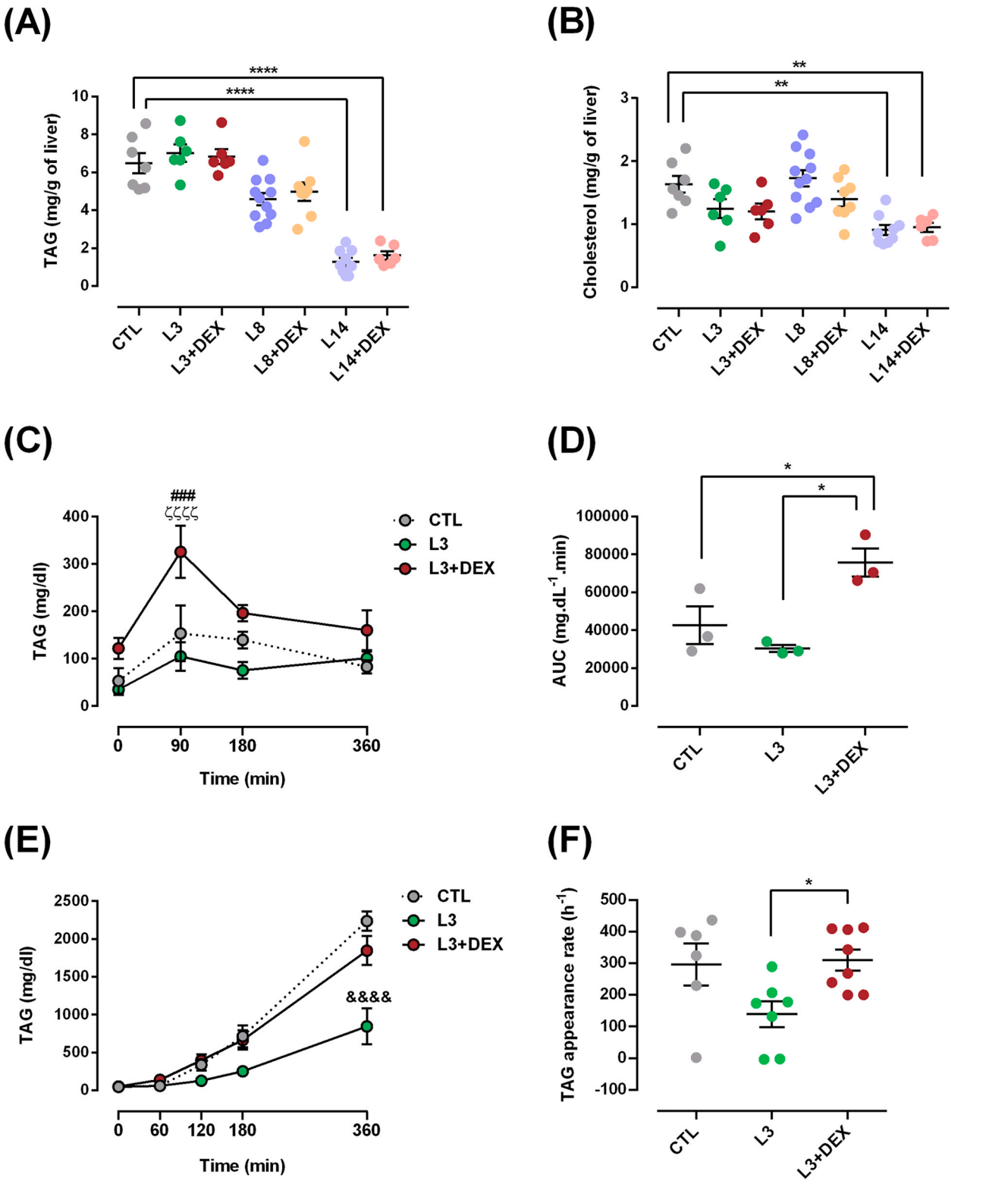


**Fig. 2.** TAG appearance after tyloxapol injection or *gavage* with olive oil in lactating rats treated with DEX during pregnancy. Female rats were assigned to three groups. Two groups were mated. Half of the mated rats were treated with DEX between the 15th and the 20th days of pregnancy. A third group was kept unmated (CTL). Hepatic contents of TAG (A) and cholesterol (B) were assessed on the 3rd, 8th and 14th days of lactation (L3, L8 and L14 and L3 +DEX, L8 +DEX and L14 +DEX). Lactating rats at the third day after delivery (L3 and L3 +DEX) and the CTL rats were subjected to a gavage with olive oil and circulating TAG were assessed (C), and the AUC was calculated (D). TAG concentrations were also measured before and after tyloxapol injection in CTL and lactating rats on the 3rd day of lactation (L3 and L3 +DEX) (E). The linear regression was calculated as TAG appearance rate (F). Data are shown as mean ± S.E. $^{\zeta\zeta\zeta\zeta}P < 0.0001$ *vs.* basal levels; $^{\#\#\#}P < 0.001$ *vs.* CTL and untreated pregnant rats during pregnancy at the same time point; $^{\&\&\&\&}P < 0.0001$ *vs.* CTL and L3 +DEX at the same time point; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$ (TAG concentration after tyloxapol and TAG appearance rate: n = 6 for CTL, n = 7 for L3 and n = 8 for L3 +DEX; Hepatic TAG and cholesterol: n = 6–11; Serum TAG concentration after olive oil gavage and AUC: n = 3. Data on TAG concentration in different time points after tyloxapol and after olive oil analyzed with two-way ANOVA. The remaining data were analyzed with one-way ANOVA).

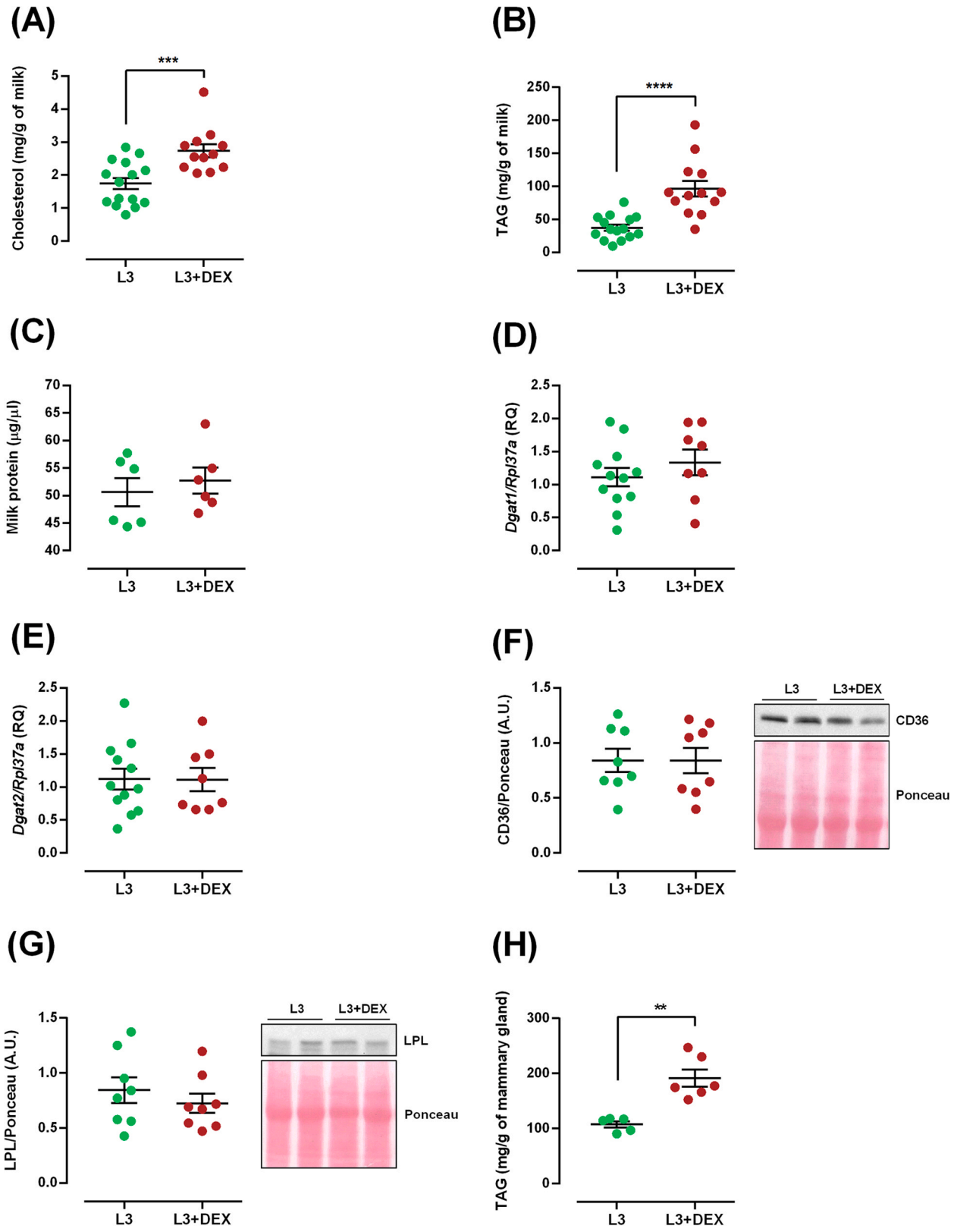


**Fig. 3.** TAG concentration in the milk and gene expression in the MG of lactating rats treated with DEX during pregnancy. Pregnant female rats were assigned to two groups. Half of mated rats were treated with DEX between the 15th and the 20th days of pregnancy. Cholesterol (A), TAG (B) and protein (C) concentrations were measured in the milk at the 3rd day of lactation (L3). The MG of L3 and L3 +DEX rats were processed for RNA extraction and mRNA quantification of *Dgat1* (D) and *Dgat2* (E). Protein was extracted for western blot detection of CD36 (F) and LPL (G). MG was also used to assess TAG content (H). Data are shown as mean ± S.E. $^{**}P < 0.01$; $^{***}P < 0.001$; * ** * $P < 0.0001$ (Cholesterol and TAG concentration in the milk: n = 15 for L3 and n = 12–13 for L3 +DEX; Protein concentration in the milk: n = 6 for L3 and L3 +DEX; qPCR: n = 12 for L3 and n = 8 for L3 +DEX; Western blot: n = 8 for L3 and L3 +DEX; TAG concentration in the MG: n = 5 for L3 and n = 6 for L3 +DEX). Data were analyzed with Student's *t*-test.

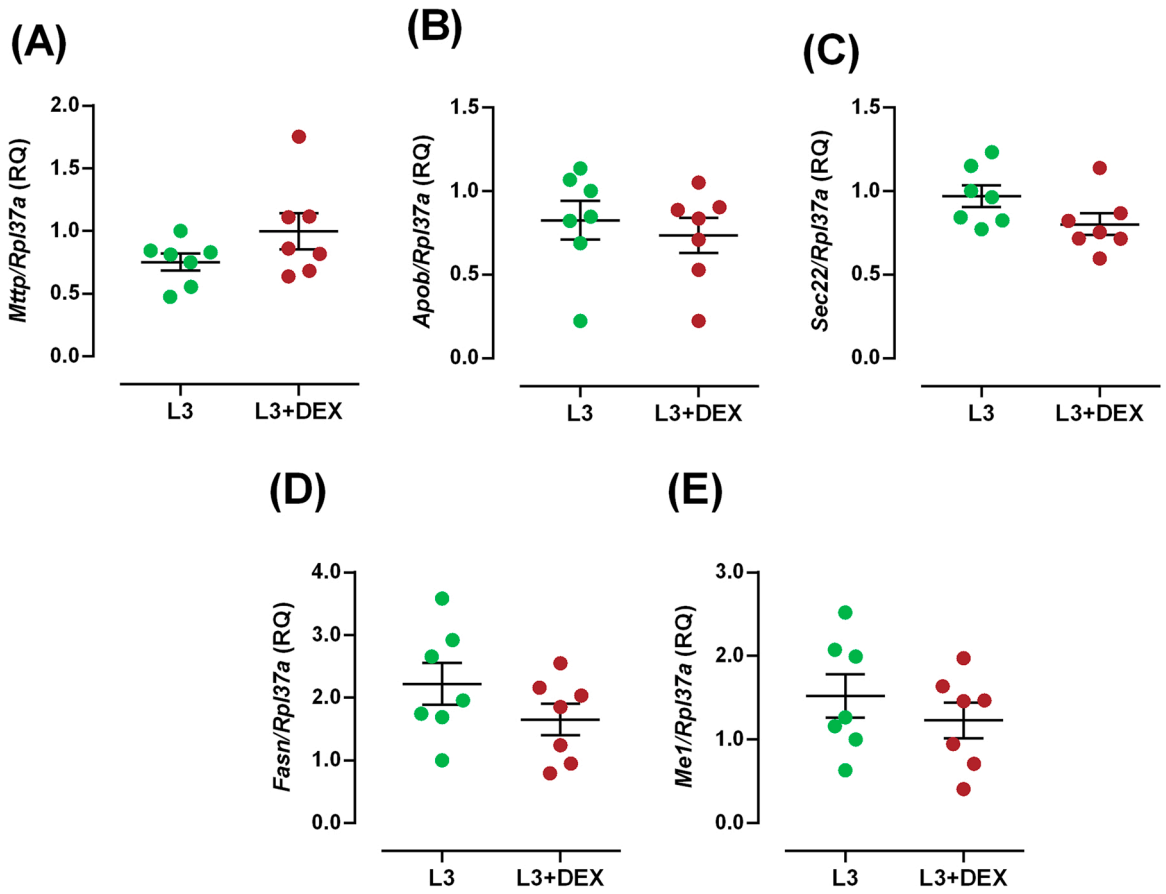


**Fig. 4.** Expression of genes related to VLDL production and *de novo* lipogenesis in the liver of lactating rats treated with DEX during pregnancy. Pregnant female rats were assigned to two groups. Half of mated rats were treated with DEX between the 15th and the 20th days of pregnancy. Liver samples were obtained at L3 and processed for RNA extraction and qPCR detection of *Mttp* (A), *Apob* (B), *Sec22* (C), *Fasn* (D) and *Me1* (E). Data are shown as mean ± S.E. (n = 7 for L3 and L3 +DEX). Data were analyzed with Student's *t*-test.

hypothesis that antenatal corticotherapy modulates maternal lipid metabolism during the peripartum.

Preterm delivery is a relevant variable that has to be taken into account when evaluating maternal lipid metabolism at the peripartum. Fumeaux and colleagues demonstrated that very preterm mothers (28–32 weeks) have higher milk total fat during the first week after delivery when compared to term volunteers [19]. Accordingly, a meta-analysis including 23 studies with very preterm mothers showed that milk fat content is higher than in term mothers at the 3rd day after delivery [20]. Albeit we have found that milk TAG was not increased in preterm mothers subjected to antenatal corticotherapy, the following characteristics differ our volunteers from those of the above-mentioned studies: (i) we have excluded cases of GDM and (ii) our volunteers were moderate to late preterm. Moreover, these observational investigations referenced above do not describe the extent of the use of BET in preterm mothers.

With the attempt to investigate maternal lipid metabolism during early lactation in a model that we could dissociate antenatal corticotherapy from preterm delivery, we performed a series of experiments using pregnant rats treated with DEX. DEX and BET have identical clinical efficacy and are both recommended by the world health organization in cases of risk of preterm delivery [21]. We choose to treat pregnant Wistar rats with DEX because it was previously reported that this synthetic glucocorticoid does not affect the duration of pregnancy in rodents [22]. Importantly, this characteristic of the model was reproduced in our experiments.

The experiments with pregnant rats treated with DEX allowed us to conclude that antenatal corticotherapy increases circulating TAG during early lactation (L3 and L8). Accordingly, previous publication from our group has also described that this model of DEX treatment increased circulating TAG during pregnancy [23]. Interestingly, lactating rats are well recognized to experience a reduction in adipose tissue and skeletal muscle LPL activity [24,25] simultaneously to an increase in the MG tissue [5]. Such synchronized changes allow an appropriate supply of circulating TAG to milk synthesis and result in a transitory state of reduced circulating TAG levels [26].

The body of data presented herein allows the conclusion that increased circulating TAG during early lactation (L3 and L8) in rats treated with DEX during pregnancy is unlike to result from an impaired uptake by MG. This conclusion is supported by the findings showing increased TAG content in the milk and MG of early lactating rats of the DEX group. In contrast, we have not found any change in CD36, LPL, *Dgat1* and *Dgat2* in the MG of early lactating rats of the DEX group. Noteworthy, changes in LPL protein content induced by DEX in rat adipocytes *in vitro* were already reported to correlate with changes LPL activity [8]. Collectively, these data also allow the conclusion that increased TAG in the milk of rats exposed to antenatal corticotherapy is probably a consequence of increased circulating TAG, and not a result of the action of DEX directly in the MG.

Taking into the account the proposition above, we interpreted our data showing increased TAG appearance rate and higher circulating TAG after gavage with olive oil early in lactating rats exposed to DEX during pregnancy as a consequence of increased VLDL secretion.

It is noteworthy that previous studies have shown that fatty acid uptake and DNLG are increased in the liver from lactating rats. Instead of allocating it for re-esterification, the liver of the lactating rat uses the

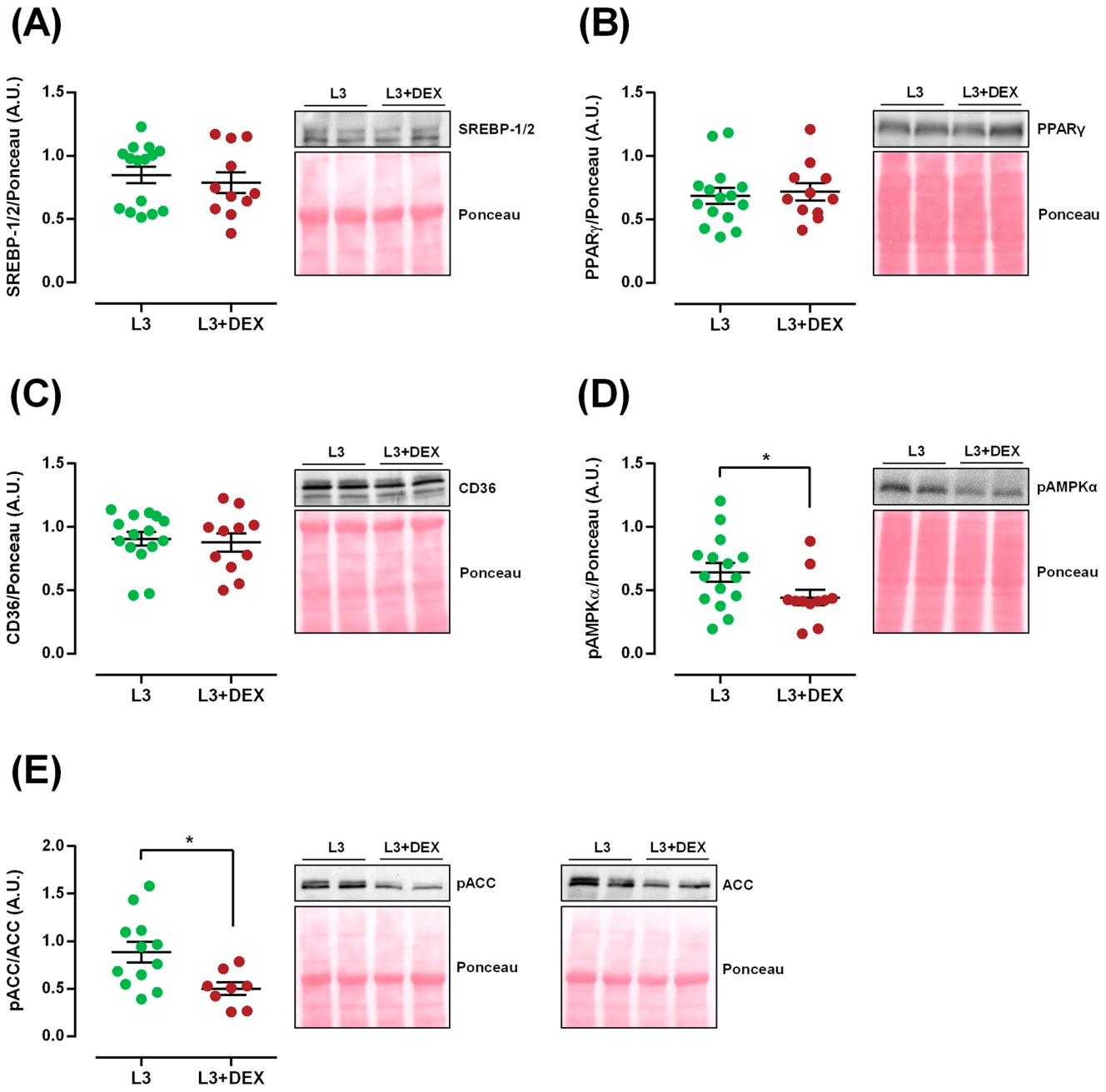


**Fig. 5.** Content of proteins related to lipid metabolism and AMPK pathways in the liver of lactating rats treated with DEX during pregnancy. Pregnant female rats were assigned to two groups. Half of mated rats were treated with DEX between the 15th and the 20th days of pregnancy. Liver samples were obtained at L3 and processed for western blot detection of SREBP-1/2 (A), PPARγ (B), CD36 (C), phosphorylated AMPKα (D) and ACC and phosphorylated ACC (E). Data are shown as mean ± S.E. *$P < 0.05$. (n = 12–15 for L3, and n = 8–11 for L3 +DEX). Data on pAMPKα were analyzed with Mann-Whitney test. The remaining data were analyzed using Student's *t*-test.

NEFA preferentially for the synthesis of ketone bodies [6,27]. We have not found changes in hepatic transcripts that conciliate the proposition that antenatal DEX could increase hepatic FFA uptake or DNLG at L3. In addition, as the expression of *Mttp*, *Apob* and *Sec22* were not altered in the liver of the lactating rats subjected to DEX treatment, we also conclude that the increased hepatic TAG secretion seen at L3 is not due to a higher production of VLDL, but instead to an enrichment of secreted lipoproteins with TAG.

We then hypothesized that downregulation of pathways mediating the synthesis of ketone bodies could be taking place to allow the shunt of NEFA to re-esterification in the liver of the lactating rat subjected to antenatal DEX.

Hepatic AMP-activated protein kinase (AMPK) acts to phosphorylate and inhibit acetyl-CoA carboxylase (ACC). ACC inhibition in the liver reduces malonyl-CoA, what leads to an increase in fatty acid oxidation and ketogenesis [28]. Accordingly, AMPK activation correlates with increased ketone bodies concentration and reduced circulating TAG [29–32]. Conversely, AMPK inhibition correlates with reduced hepatic ketogenesis and lipolysis and higher VLDL secretion rate [33,34].

We thus conclude that lower hepatic AMPK/ACC phosphorylation may contribute to the increased circulating TAG seen in early lactating rats treated with antenatal DEX. In consonance with our interpretation, it was previously demonstrated glucocorticoids can reduce hepatic AMPK activity and that DEX increases circulating TAG through a

mechanism dependent on reduced hepatic AMPK/ACC phosphorylation [35–37].

Altogether, the present experiments collect experimental evidence to support the hypothesis that antenatal exposure to synthetic glucocorticoids affects the lipid metabolism of the lactating dam by increasing hepatic TAG secretion and consequently TAG levels in the milk. The present data also suggest that reduced hepatic AMPK phosphorylation may mediate the metabolic effect of antenatal corticotherapy in early lactating rats.

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## CRediT authorship contribution statement

**Fernanda Ballerini Hecht:** Validation, Investigation, Data curation, Formal analysis. **Caio Jordão Teixeira:** Validation, Investigation, Writing – original draft, Writing – review & editing. **Dailson Nogueira de Souza:** Investigation. **Filiphe de Paula Nunes Mesquita:** Investigation. **Ryana Elyzabeth do Val Roso:** Investigation. **Frhancielly Shirley Sodré:** Investigation. **Vanessa Barbosa Veronesi:** Investigation. **Deborah Fabiana da Rocha:** Investigation. **Yan Guida Dantas de Menezes:** Investigation. **Mariana Rodrigues Pioli:** Investigation. **Silvana Bordin:** Funding acquisition, Writing – original draft, Writing – review & editing. **José Guilherme Cecatti:** Conceptualization, Supervision. **Adriana Gomes Luz:** Conceptualization, Supervision. **Gabriel Forato Anhê:** Conceptualization, Formal analysis, Data curation, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

## Conflict of interest statement

The authors have no conflict of interest to declare

## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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