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RESEARCH PAPER

# Identification of early transcriptome-based biomarkers related to lipid metabolism in peripheral blood mononuclear cells of rats nutritionally programmed for improved metabolic health

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**Abstract** Moderate maternal calorie restriction during lactation protects rat offspring against obesity development in adulthood, due to an improved ability to handle and store excess dietary fuel. We used this model to identify early transcriptome-based biomarkers of metabolic health using peripheral blood mononuclear cells (PBMCs), an easily accessible surrogate tissue, by focusing on molecular markers of lipid handling. Male and female offspring of control and 20 % calorie-restricted lactating dams (CR) were studied. At weaning, a set of pups was killed, and PBMCs were isolated for whole-genome microarray analysis. The remaining pups were killed at 6 months of age. CR gave lower body weight, food intake and fat accumulation, and improved levels of insulin and leptin throughout life, particularly in females. Microarray analysis of weaned rat PBMCs identified 278 genes significantly differentially expressed between control and CR. Among lipid metabolism-related genes, expression of *Cpt1a*, *Lipe* and *Star* was increased and *Fasn*, *Lrp1* and *Rxrb* decreased in CR versus control, with changes fully confirmed by qPCR. Among them, *Cpt1a*, *Fasn* and *Star* emerged as particularly interesting. Transcript levels of *Cpt1a* in PBMCs correlated with their levels in WAT and liver at both ages examined; *Fasn* expression levels in PBMCs at an early age correlated with their expression levels in WAT; and early changes in *Star* expression levels in PBMCs correlated with their expression levels in liver and were sustained in adulthood. These findings reveal the possibility of using transcript levels of lipid metabolism-related genes in PBMCs as early biomarkers of metabolic health status.

**Keywords** Early biomarkers · PBMCs · Calorie restriction · Lactation · Metabolic programming

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## Introduction

The increasing prevalence of obesity has become a worldwide phenomenon, affecting both children and adults. Obesity and its related risks can be prevented by lifestyle changes and especially by changes in diet (Perk et al. 2012). Specific diets, foods and food components can contribute to the development of urgently needed therapeutic and preventive strategies. The development of such nutrition and food-based strategies is, however, severely hampered by the lack of predictive biomarkers, especially those that are accessible and quantify health (van Ommen et al. 2009). In order to identify such biomarkers, a model is needed that results in changes that reflect the later health status. Such a model is provided by moderate calorie restriction during lactation. Obesity and related pathologies can be programmed by maternal nutrition during the perinatal period (Pico et al. 2012; Sullivan and Grove 2010). While maternal calorie restriction during pregnancy has been associated with adverse health outcomes in adult offspring (Palou et al. 2010b,

2012), moderate maternal calorie restriction during lactation in rats has been shown to confer certain protection in the pups against development of obesity and related metabolic alterations associated with high-fat (HF) diet feeding, particularly dyslipidemia, insulin resistance and hyperleptinemia (Palou et al. 2010a). This provides an animal model that is suited to identify early biomarkers for metabolic health, in terms of a reduced tendency to develop overweight and its associated metabolic complications in adult life.

Suitability of biomarkers for efficacy substantiation requires that they can readily be assessed in humans. Most studies that mechanistically assess effects of diet and foods on health examine tissues such as adipose tissues, muscle or liver, which require invasive tissue biopsies (de Mello et al. 2012). Peripheral blood mononuclear cells (PBMCs) provide an attractive alternative that can be assessed in humans, because they can be easily and repeatedly collected in sufficient quantities (de Mello et al. 2012). Gene expression responses of PBMCs have been shown to reflect the liver environment (de Mello et al. 2012), as well as adipose tissue (Caimari et al. 2010a, b). Therefore, whole-genome transcriptome profiling of PBMCs of pups from calorie-restricted mothers during lactation may be used to identify early biomarkers, reflecting current and later health of metabolic tissues.

The aim of the current study was to identify early potentially predictive biomarkers of metabolic health by transcriptome profiling of PBMCs. As a model, we used the offspring of 20 % maternal calorie-restricted dams during lactation, which are less prone to obesity development compared to ad libitum fed controls. We also ascertained (1) to what extent the potential markers of optimized health identified at early ages continued to serve as potential markers in adulthood and (2) whether the changes occurring in blood cells reflected the metabolic environment in key tissues. We focused on selected genes of lipid metabolism and assessed these in relevant metabolic tissues, liver and white adipose tissue (WAT), at different ages.

## Materials and methods

### Animals and experimental design

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands (Resolution Number 1798. February 18th, 2009), and guidelines for the use and care of laboratory animals of the University were followed.

The study was conducted on male and female Wistar rats from 16 different litters following the protocol described below. All animals were housed under standard conditions, that is, controlled temperature (22 °C), the normal 12 h light and 12 h dark cycle, free access to tap water and a standard chow diet (3 kcal/g, with 8 % calories from fat; Panlab, Barcelona, Spain), unless specified otherwise. Briefly, 16 virgin female Wistar rats (body weight 225–260 g) were mated with male rats (Charles River Laboratories, Barcelona, Spain). After mating, each female was placed in an individual cage. On day 1 after delivery, excess pups in each litter were removed aiming for 10 pups per dam (five males and five females, when possible). Dams were assigned to either the control ($n = 11$ dams) or calorie-restricted ($n = 5$ dams) group. Control dams were fed ad libitum with standard chow diet (Panlab, Barcelona, Spain), while calorie-restricted dams were fed daily with a 20 % calorie-restricted diet throughout lactation, starting on day 1 after delivery until weaning (day 21) as previously described (Palou et al. 2012). During the lactating period, body weight of male and female offspring of control and calorie-restricted dams (control and CR, respectively) was followed.

At weaning, a set of animals made up of 24 pups from control (12 males and 12 females) and 24 from CR (12 males and 12 females) group were killed by decapitation under ad libitum feeding conditions. One half of pups ($n = 6$/group) were used to obtain different WAT depots (inguinal and retroperitoneal; iWAT and rWAT, respectively) and liver (which were rapidly removed, frozen in liquid nitrogen and stored at −70 °C until RNA analysis), as well as trunk blood samples (for peripheral blood mononuclear cells (PBMCs) isolation, as described in the next section). From the other half of pups ($n = 6$/group), blood samples were collected in heparinized containers and plasma was obtained by centrifugation at 700$g$ for 10 min.

Another set of animals, 28 control pups (12 males and 16 females) and 26 CR pups (14 males and 12 females), were kept alive. They were placed two per cage, paired with another animal of the same group and fed ad libitum with a normal-fat (NF) diet (3.8 kcal/g, 10 % calories from fat, Research Diets, Inc., NJ, USA) until the age of 6 months. Body weight and food intake of those animals were followed. Moreover, at the age of 6 months (prior to sacrifice), blood samples were collected at fed state and after 12 h fasting to obtain plasma. All the animals were decapitated under ad libitum feeding conditions at the age of 6 months, and samples of trunk blood for PBMCs isolation were collected. Body length (from the tip of the nose to the anus) and body composition (by EchoMRI-700TM, Echo Medical Systems, LLC, TX, USA) were measured in control and CR animals without anesthesia when animals were 21 days and 6 months old.

### Measurement of circulating parameters under fed/fasting conditions and calculation of the homeostatic model assessment for insulin resistance (HOMA-IR) at different ages

Blood samples collected at the ages of 21 days (under ad libitum feeding conditions) and 6 months (under ad libitum and 12 h fasting conditions) were used for analysis of circulating parameters. Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Peripheral hormones were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits: insulin concentration was determined using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and leptin with QuantikineTM Mouse Leptin Inmunoassay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Plasma non-esterified fatty acid (NEFA) and triglyceride (TG) levels were determined with a commercial enzymatic colorimetric kits (Wako Chemicals GmbH, Neuss, Germany and Triglyceride (INT) 20, Sigma Diagnostics, St Louis, MO, USA, respectively), following standard procedures. The homeostatic model assessment for insulin resistance (HOMA-IR) was used to assess insulin resistance. It is calculated from fasting insulin and glucose concentration using the formula of Matthews et al. (1985):

$$\text{HOMA-IR} = \text{fasting glucose}\ (\text{mmol/l}) \times \text{fasting insulin}\ (\text{mU/l})/22.5.$$

### PBMC isolation

Trunk blood samples of control and CR rats collected (at the age of 21 days and 6 months) under ad libitum feeding conditions were used to isolate PBMCs. Peripheral blood samples were collected using heparin in NaCl (0.9 %) as anticoagulant and then diluted with an equal volume of balanced salt solution. PBMCs were immediately isolated by Ficoll density-gradient separation according to the instructions of the manufacturer (GE Healthcare Bio Sciences, Barcelona, Spain).

### Total RNA isolation

Total RNA was extracted from iWAT, rWAT, liver and PBMCs of control and CR animals by EZNA® TOTAL RNA kit I (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Ins., Wilmington, DE), and its integrity confirmed using 1 % agarose gel electrophoresis (for iWAT, rWAT and liver).

### Microarray processing

For microarray analysis, RNA from PBMC samples obtained from male and female offspring of controls and CR animals at the age of 21 days was used ($n = 6$/group). RNA samples were analyzed on Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom). To assure the high quality of RNA, all samples used for microarrays had a RIN number $\geq 8$. Then, 0.04 μg of RNA from each sample was reverse transcribed to complementary DNA (cDNA) using the Agilent Low Input Quick Amp Labeling kit (Agilent Technologies, Inc., CA, USA), according to the manufacturer's protocol. Then, half of the cDNA sample (10 μl) was used for the linear amplification of RNA and labeling with cyanine-3 (Cy3) or Cy5. For these reactions, half of the amounts indicated by the manufacturer were used (van Schothorst et al. 2007). Transcription and labeling were carried out at 40 °C for 2 h. Then, the labeled and amplified cRNA samples were purified using Qiagen Rneasy MiniSpin columns (Qiagen, Venlo, the Netherlands). The incorporation of dyes and cRNA concentration was measured using the "microarray measurement mode" of the NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Ins., Wilmington, DE). Each sample containing 600 ng of cRNA labeled with Cy5 and 600 ng of Cy3 pool was hybridized on 4 × 44 K G4131F rat whole-genome Agilent microarrays (Agilent Technologies, Inc., Santa Clara, CA, USA) for 17 h at 65 °C in hybridization chambers in an oven rotating at 10 rpm (Agilent Technologies). After hybridization, the arrays were washed with "GE wash buffer 2" for 1 min at 37 °C, followed by acetonitrile for 1 min at room temperature, and finally with a solution for stabilization and drying for 30 s at room temperature, according to the manufacturer's protocol (Agilent Technologies).

### Microarray data analysis

The arrays were scanned with an Agilent Microarray Scanner (Agilent Technologies). Scanned images were examined for visible defects and proper grid alignment. The intensities of the signals from each spot were quantified, and the raw data were extracted using Feature Extraction Software version 10.5.1.1 (Agilent Technologies, Inc., Santa Clara, CA, USA). Quality control was performed for each of the arrays using LimmaGUI package in R from Bioconductor Software version 2.1. Only one of the arrays did not pass quality control based on MA plot and signal intensity distribution (Allison et al. 2006). Thus, in total, dataset from 23 arrays passed to the next step of analysis. Data were exported into GeneMaths XT 2.12 (Applied Mathematics, Sint-Martens-Latem, Belgium) for

background correction and normalization. Locally, weighted linear regression (lowess) analysis was chosen as a normalization method, which enables intensity-dependent effects in the log2 (ratio) values to be removed (Yang et al. 2002). Then, the values were converted to log2 values, and the target samples (Cy5) intensities were normalized against the intensities of reference samples (Cy3), as described previously (Pellis et al. 2003). Target signals with an average intensity lower than twofold above average background were discarded to increase accuracy of the data. Correction for multiple testing was not applied, as these corrections are often too strict to identify small effects, which are usually observed in nutritional studies (Keijer et al. 2010). Validity of data was assured by checking biological plausibility and by independent analysis by RT-qPCR (see below) in PBMCs and two metabolic tissues (liver and WAT).

To search for biomarkers of metabolic interest in both sexes, two-way analysis of variance (ANOVA) with factors of sex and experimental group was performed. The threshold of significance for this statistical test was set at $p \leq 0.01$. Moreover, fold change (FC) calculation between both groups of animals (CR vs control animals) was performed; FC equals the expression ratio between CR and controls in the case of increase, or equals −1/ratio in the case of decrease. The analyses were performed with SPSS for Windows (SPSS version 19.0, Chicago, IL). Subsequently, a statistically generated list of genes was manually analyzed in regard to their biological information, obtained with the use of available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways) based on key biological domains, such as molecular function and biological process. Some of these processes overlapped, and thus, they were collected, renamed, and all the unique genes were assigned into several biological processes according to their function.

## Real-time quantitative RT-polymerase chain reaction (RT-qPCR) analysis

To validate microarray data, mRNA expression levels of apolipoprotein B48 receptor (*Apob48r*), carnitine palmitoyltransferase 1 alpha (*Cpt1a*), fatty acid synthase (*Fasn*), hormone-sensitive lipase (*Lipe*), low density lipoprotein receptor-related protein 1 (*Lrp1*), phosphate cytidylyltransferase 2, ethanolamine (*Pcyt2*), retinoid X receptor beta (*Rxrb*), sortilin-related receptor, LDLR class A repeats-containing (*Sorl1*) and steroidogenic acute regulatory protein (*Star*) were measured by RT-qPCR in PBMC RNA samples of control and CR animals. Additionally, RT-qPCR was performed to analyze mRNA expression of aforementioned genes in PBMCs at the age of 6 months, and in iWAT, rWAT and liver, at the age of 21 days and 6 months. Regarding *Star*, its mRNA levels were only analyzed in liver, as its expression has not been described in WAT. We did not analyze *Apob48r* or *Sorl1* expression either in liver or in WAT, as both genes are expressed mainly in blood and neural cells.

For RT-qPCR analysis, 0.05 μg of PBMC total RNA was used for reverse transcription by using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, S.A., Madrid, Spain) according to the manufacturer's protocol. For iWAT, rWAT and liver, 0.25 μg of total RNA (in a final volume of 5 μl) was firstly denatured at 65 °C for 10 min and then reverse transcribed to cDNA with MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20 °C for 15 min, at 42 °C for 30 min, with a final step of 5 min at 95 °C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain).

Real-time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Each PCR was performed from 1/5 dilution of the cDNA product and forward and reverse primers (5 μM each). Primer sequences and products for the different genes are described in Table 1. All primers were purchased from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain). After an initial Taq activation at 95 °C for 10 min, PCR was performed using 40 two-temperature cycles with the following cycling conditions: 95 °C for 15 s and 60 °C for 1 min. To verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The values for the threshold (Ct) were calculated by the instrument's software (StepOne Software v2.2.2), and the relative expression of each mRNA was calculated as a percentage of male control rats, using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) with *β-actin*, GDP dissociation inhibitor 1 (*Gdi-1*) and TATA-Box Binding Protein (*Tbp*) as reference genes.

## Statistical analysis

All data were expressed as mean ± SEM. The statistical analysis of microarray data has been described in details in the section referred to microarray data analysis. Multiple comparisons were assessed by ANOVA repeated measures and two-way ANOVA to determine the effects of different factors (moderate maternal calorie restriction during lactation and sex). Single comparisons between groups were assessed by Student's *t* test and Paired *t* test. Person's correlation coefficient was used to determine the association between the expression pattern of lipid metabolism-related genes in PBMCs and other tissues. $P < 0.05$ was the threshold of significance, unless stated. The analyses were performed with SPSS for Windows (SPSS version 19.0, Chicago, IL).

**Table 1** Nucleotide sequences of primers and amplicon size used for RT-qPCR analysis of mRNA expression levels of selected genes in PBMCs, iWAT, rWAT and liver samples

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) | Amplicon (bp) |
|---|---|---|---|
| *β-actin* | *TACAGCTTCACCACCACAGC* | *TCTCCAGGGAGGAAGAGGAT* | 120 |
| *Apob48r* | *GGGCTACATCAGGCTTTGAG* | *TTCCTCCCCTACAACCTTCC* | 150 |
| *Cpt1a* | *GCTCGCACATTACAAGGACAT* | *TGGACACCACATAGAGGCAG* | 250 |
| *Fasn* | *CGGCGAGTCTATGCCACTAT* | *ACACAGGGACCGAGTAAT* | 222 |
| *Gdi-1* | *CCGCACAAGGCAAATACATC* | *GACTCTCTGAACCGTCATCAA* | 210 |
| *Lipe* | *TCACGCTACATAAAGGCTGCT* | *CCACCCGTAAAGAGGGAACT* | 169 |
| *Lrp1* | *GAGCAGGTTGTCAGTCAGCA* | *TAGGGTTTCCGATTTCCACA* | 187 |
| *Pcyt2* | *CCGACAGGGATGGGTCTG* | *TGGCTTCCTTCTTCTGATTCC* | 156 |
| *Rxrb* | *CCCTTCCCAGTCATCAGTTC* | *GGTGGCTTCACATCTTCAGG* | 152 |
| *Sorl1* | *CACCGTCTCATTGTCAGCAC* | *ATCTCGTAGCCCCTGGTTTC* | 123 |
| *Star* | *GGGTGGATGGGTCAGGTC* | *CTGCTGGCTTTCCTTCTTCC* | 168 |
| *Tbp* | *ACCCTTCACCAATGACTCCTATG* | *ATGATGACTGCAGCAAATCGC* | 190 |

*Apob48r* apolipoprotein B48 receptor, *Cpt1a* carnitine palmitoyltransferase 1alpha, *Fasn* fatty acid synthase, *Gdi-1* GDP dissociation inhibitor 1, *Lipe* hormone-sensitive lipase, *Lrp1* low density lipoprotein receptor-related protein 1, *Pcyt2* phosphate cytidylyltransferase 2, ethanolamine, *Rxrb* retinoid X receptor beta, *Sorl1* sortilin-related receptor, LDLR class A repeats-containing, *Star* steroidogenic acute regulatory protein and *Tbp* TATA-box binding protein

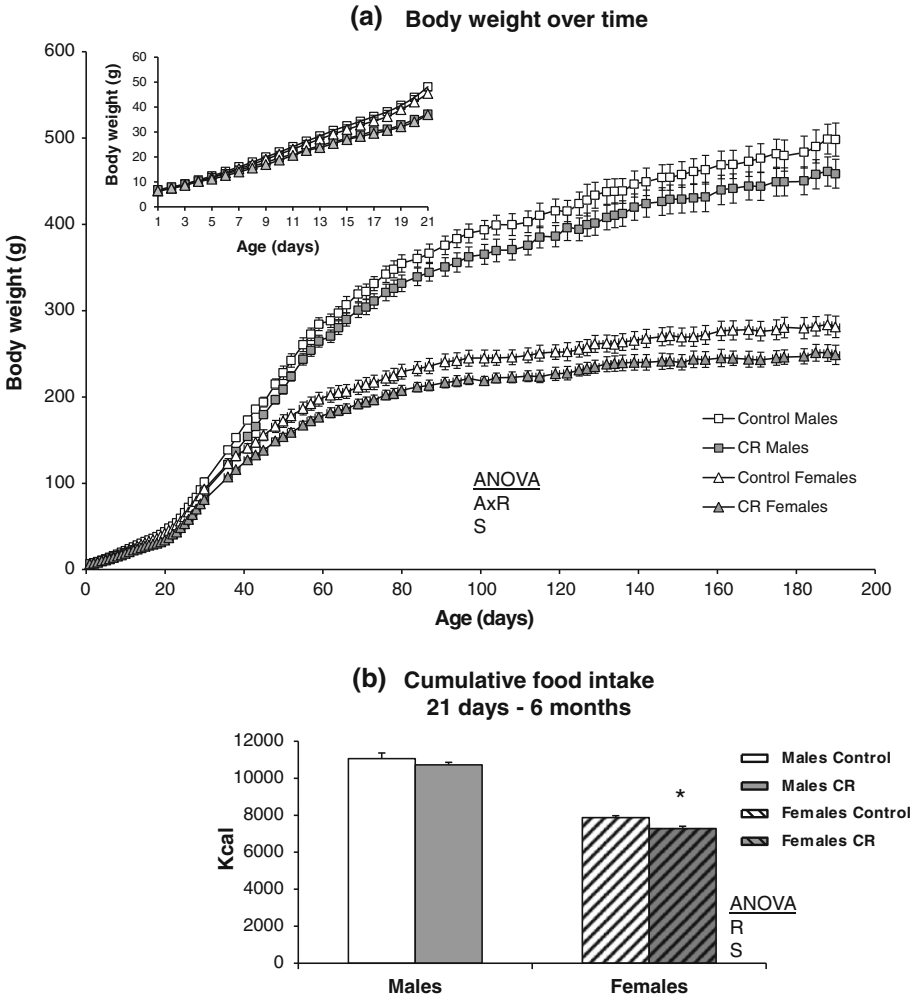



**Fig. 1** **a** Body weight with time of male and female offspring of control and calorie-restricted dams during lactation (CR) from day 1 until day 190 of age. The inset details body weight throughout the lactating period (from day 1 until day 21 of age). Data are mean ± SEM ($n = 12$–16 animals/group). Statistics: AxR, interaction between age and calorie restriction during lactation; S, effect of sex ($p < 0.05$; ANOVA repeated measures). **b** Cumulative food intake (Kcal) of male and female offspring of controls and calorie-restricted dams during lactation (CR) fed ad libitum with normal-fat diet from 21 days until the age of 6 months. Data are mean ± SEM ($n = 12$–16 animals/group until the age of 21 days; $n = 6$–8 animals/group until the age of 190 days). Statistics: R, effect of maternal calorie restriction during lactation; S, effect of sex ($p < 0.05$; two-way ANOVA). *, CR vs Controls ($p < 0.05$; Student's *t* test)

**Table 2** Morphological traits (a) and circulating parameters (b) in male and female offspring of control and calorie-restricted dams during lactation (CR) at weaning (21 days old) and at the age of 6 months

| | | Males | | Females | | ANOVA |
|---|---|---|---|---|---|---|
| | | Control | CR | Control | CR | |
| (a) Morphological traits | | | | | | |
| 21 Days | | | | | | |
| Body weight (g) | | 48.1 ± 2.1 | 37.1 ± 0.8* | 45.4 ± 1.1 | 36.9 ± 0.5* | R |
| Body length (cm) | | 11.5 ± 0.2 | 10.8 ± 0.2* | 11.0 ± 0.2 | 10.4 ± 0.1* | R, S |
| Body fat (%) | | 10.0 ± 0.4 | 8.68 ± 0.26* | 10.5 ± 0.3 | 8.87 ± 0.19* | R |
| iWAT (mg) | | 294 ± 35 | 187 ± 19* | 307 ± 33 | 186 ± 26* | R |
| rWAT (mg) | | 69.6 ± 9.9 | 40.1 ± 4.9* | 50.5 ± 3.7 | 24.9 ± 3.2* | R |
| Liver (g) | | 1.68 ± 0.05 | 1.25 ± 0.08* | 1.76 ± 0.06 | 1.29 ± 0.03* | R |
| 6 Months | | | | | | |
| Body weight (g) | | 498 ± 19 | 459 ± 17 | 281 ± 12 | 249 ± 11 | R, S |
| Body length (cm) | | 24.2 ± 0.5 | 24.0 ± 0.3 | 20.8 ± 0.3 | 20.3 ± 0.2 | S |
| Body fat (%) | | 20.7 ± 2.2 | 22.1 ± 1.6 | 22.1 ± 2.3 | 14.5 ± 1.2* | RxS |
| iWAT (g) | | 9.95 ± 1.20 | 10.7 ± 0.7 | 3.93 ± 0.37 | 2.14 ± 0.45* | RxS |
| rWAT (g) | | 12.8 ± 1.7 | 12.4 ± 1.0 | 4.53 ± 0.67 | 2.16 ± 0.23* | RxS |
| Liver (g) | | 16.0 ± 0.9 | 13.4 ± 0.6* | 8.13 ± 0.47 | 7.35 ± 0.28 | S, R |
| (b) Circulating parameters | | | | | | |
| 21 Days | | | | | | |
| Glucose (mg/dL) | Fed | 137 ± 7 | 120 ± 3* | 141 ± 5 | 116 ± 4* | R |
| Insulin (ng/L) | Fed | 0.111 ± 0.042 | 0.044 ± 0.010 | 0.105 ± 0.020 | 0.037 ± 0.004* | R |
| Leptin (ng/L) | Fed | 0.941 ± 0.154 | 0.408 ± 0.045* | 1.05 ± 0.11 | 0.585 ± 0.162* | R |
| NEFA (nM) | Fed | 1.46 ± 0.13 | 1.50 ± 0.14 | 1.37 ± 0.19 | 1.59 ± 0.13 | |
| TG (mg/mL) | Fed | 1.02 ± 0.14 | 0.893 ± 0.130 | 0.938 ± 0.094 | 0.851 ± 0.042 | |
| 6 Months | | | | | | |
| Glucose (mg/dL) | Fed | 108 ± 3 | 107 ± 6 | 105 ± 4 | 102 ± 4 | |
| | Fasting | 98 ± 8 | 95 ± 3# | 102 ± 3 | 90 ± 1*,# | R ($p = 0.059$) |
| Insulin (ng/L) | Fed | 2.77 ± 0.94 | 1.44 ± 0.26 | 0.835 ± 0.102 | 0.459 ± 0.050* | R ($p = 0.069$), S |
| | Fasting | 0.920 ± 0.379# | 1.06 ± 0.24# | 0.558 ± 0.121# | 0.287 ± 0.047# | |
| HOMA-IR | Fasting | 5.37 ± 2.38 | 5.77 ± 1.01 | 3.33 ± 0.75 | 1.67 ± 0.32 | S |
| Leptin (ng/L) | Fed | 15.8 ± 2.2 | 13.4 ± 1.1 | 6.07 ± 0.94 | 1.74 ± 0.19* | RxS |
| NEFA (nM) | Fed | 0.851 ± 0.152 | 0.910 ± 0.056 | 1.33 ± 0.24 | 0.866 ± 0.086 | |
| TG (mg/mL) | Fed | 1.61 ± 0.08 | 1.40 ± 0.19 | 1.02 ± 0.12 | 0.920 ± 0.073 | S |

Data are mean ± SEM ($n = 6$–8 animals/group) of male and female offspring of control and calorie-restricted dams during lactation (CR), under ad libitum feeding conditions (Fed) and after 12 h fasting (Fasting). Statistics: R, effect of maternal calorie restriction during lactation; F, effect of fasting conditions; S, effect of sex; FxR, interaction between fasting conditions and maternal calorie restriction during lactation; RxS, interaction between maternal calorie restriction during lactation and sex ($p < 0.05$; ANOVA repeated measures). *, CR vs Controls ($p < 0.05$; Student's *t* test); #, fasting vs fed conditions ($p < 0.05$; Paired *t* test). *iWAT* inguinal white adipose tissue, *rWAT* retroperitoneal white adipose tissue, *NEFA* non-esterified fatty acid, *TG* triglycerides

## Results

### Phenotypic characteristics and blood parameters throughout life

Maternal calorie restriction of 20 % during lactation resulted in lower body weight of both male and female offspring in comparison with control animals (Fig. 1a). This effect was significant from the age of 5 days and was persistent during the whole study period (6 months) ($p < 0.05$; two-way ANOVA). When animals were 6 months old, CR male and female rats weighed 7.8 and 11.4 % less than their controls, respectively (Table 2a).

Notably, cumulative calorie intake of animals from weaning (day 21) until the age of 6 months (Fig. 1b) was significantly lower in CR animals compared with controls (3.0 and 7.5 % less, in males and females, respectively; $p < 0.05$; two-way ANOVA). This may explain, at least in

part, the lower body weight occurring in CR animals, particularly females.

Other morphological traits of young and adult offspring are summarized in Table 2a. At weaning (21d), both male and female CR pups showed lower body length than the controls ($p < 0.05$; two-way ANOVA), but no significant differences were found in adult animals in either sex. In addition, at the age of 21 days, CR male and female animals showed lower body fat content (relative to their body weight) than control animals, as well as lower weight of inguinal and retroperitoneal WAT depots and of liver ($p < 0.05$; two-way ANOVA). In turn, at the age of 6 months, female CR animals, but not males, presented lower body fat content, as well as lower weight of iWAT and rWAT than controls (interactive effect between sex and calorie restriction, $p < 0.05$; two-way ANOVA), and both CR males and females displayed lower weight of liver ($p < 0.05$; two-way ANOVA).

Plasma circulating parameters of control and CR animals under ad libitum feeding conditions at weaning (21 days), as well as under ad libitum and 12 h fasting conditions at the age of 6 months, are shown in Table 2b. At weaning, CR pups displayed significantly lower plasma levels of glucose, insulin and leptin relative to their controls ($p < 0.05$; two-way ANOVA). No differences were found in NEFA and TG levels between control and CR pups.

At the age of 6 months, a tendency to lower glucose and insulin levels (under fasting and fed conditions, respectively) was found in CR animals versus controls ($p = 0.059$ and $p = 0.069$, respectively; two-way ANOVA). The decrease was particularly pronounced and statistically significant in females ($p < 0.05$; Student's $t$ test). CR females also showed lower leptin levels than their controls (the latter only under fed conditions) ($p < 0.05$; Student's $t$ test), but no significant differences were found between males. Fasting conditions resulted in a significant decrease in glucose levels, only in CR animals, and in insulin levels in the different groups of animals ($p < 0.05$; Paired $t$ test). No significant differences were found between control and CR animals concerning HOMA-IR index, although CR female animals showed a tendency to lower values than controls at 6 months ($p = 0.076$; Student's $t$ test). No significant differences were found concerning circulating NEFA and TG levels between control and CR adult animals.

## Lipid metabolism-related gene expression in PBMCs of pups at the age of 21 days based on whole-genome microarray analysis

In our microarray analysis, 45,018 probes were tested. Of them, those having an expression value of twice above the background (22,920) were further taken into account and normalized. In total, 310 probes were found to be significantly different between control and CR animals ($p \leq 0.01$; two-way ANOVA). Removal of duplicates resulted in 278 unique genes. Using available databases (Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways), these genes were classified into several biological processes according to their function. A total of 111 genes were unknown, thus were not included in any of the processes. From the remaining 167 known genes, 113 exhibited down-regulation and 54 up-regulation. As shown in Fig. 2, the processes with the highest number of genes differentially expressed were related to immune system, signaling, cell turnover, transcription machinery and transport (28, 24, 19, 16 and 12 genes, respectively). Other processes with a notable number of genes were related to metabolism of proteins, carbohydrates and lipids (11, 11 and 9, respectively). The remaining genes were related with the nervous system, redox metabolism, cytoskeleton, neural signaling, blood, cell communication, central metabolism, sensory perception and food intake control (9 or less genes involved in each of the processes).

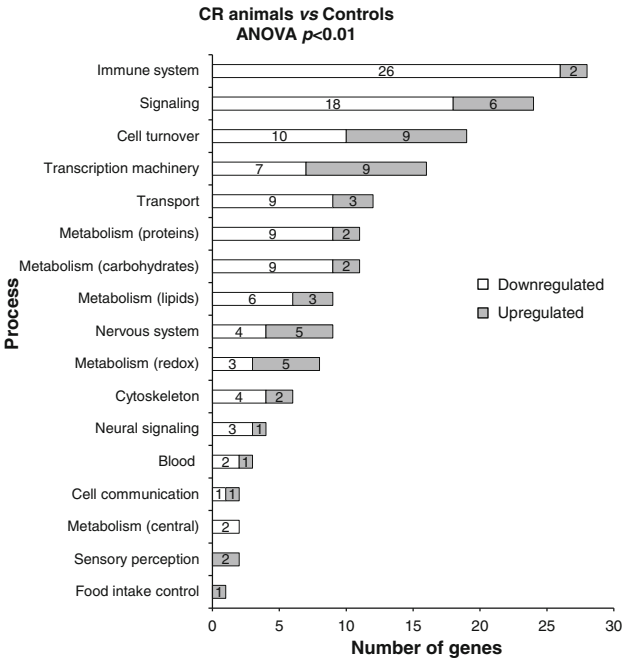



**Fig. 2** Classification into biological processes of the genes differentially expressed in PBMCs samples of male and female offspring of control and calorie-restricted dams during lactation (CR) at the age of 21 days. Statistical analysis was performed by considering males and females as a whole ($p \leq 0.01$; two-way ANOVA). The number of genes down- or up-regulated is indicated for each group of genes

Subsequently, genes involved in lipid metabolism were analyzed in detail (Table 3). Of the 9 genes involved in this process (*Apob48r*, *Cpt1a*, *Fasn*, *Lipe*, *Lrp1*, *Pcyt2*, *Rxrb*, *Sorl1* and *Star*), 6 showed down-regulation and 3 up-regulation. Down-regulated genes

were involved in lipogenesis/lipolysis, glycerophospholipid biosynthesis and low density lipoprotein uptake. Up-regulated genes were involved in β-oxidation, lipolysis and cholesterol transport.

## Confirmation of microarray results by RT-qPCR

To confirm gene array findings and to test whether the changes were consistent, RT-qPCR analysis of genes involved in the process of lipid metabolism was performed on the same RNA samples of 21-day-old male and female control and CR animals. Genes chosen for confirmation were as follows: *Fasn* (lipogenesis), *Rxrb* (lipogenesis/lipolysis), *Cpt1a* (β-oxidation) and *Lipe* (lipolysis); *Apob48r*, *Lrp1* and *Sorl1* (all involved in lipoprotein uptake); *Pcyt2* (glycerophospholipid biosynthesis); *Star* (cholesterol transport and uptake). RT-qPCR analysis confirmed most of the microarray data (Table 3), as differences in the expression levels of *Cpt1a*, *Lipe*, *Star* and *Fasn*, *Lrp1* and *Rxrb* between control and CR animals reached statistical significance ($p < 0.05$; two-way ANOVA) and followed the same pattern of up- and down-regulation as observed in the microarray analysis. Moreover, fold changes in those genes were similar using both techniques. Although RT-qPCR analysis of *Pcyt2* did not reveal significant differences between control and CR animals, single comparison between groups revealed that CR females exhibited a trend to lower *Pcyt2* mRNA levels relative to their controls ($p = 0.068$; Student's $t$ test). Differences for *Apob48* and *Sorl1* that were identified using the microarrays could not be confirmed by RT-qPCR analysis.

**Fig. 3** Comparison of mRNA expression levels of lipid metabolism-related genes in PBMCs, with the expression determined in inguinal and retroperitoneal WAT and liver of male and female offspring of control and calorie-restricted dams during lactation (CR) at the age of 21 days. mRNA levels were measured by RT-qPCR and expressed as a percentage of the mean value of males control. Data are mean ± SEM ($n = 6$–7 animals/group). Statistics: R, effect of maternal calorie restriction during lactation; S, effect of sex; RxS, interaction between the effect of maternal calorie restriction during lactation and the effect of sex ($p < 0.05$; two-way ANOVA). *, CR vs Controls ($p < 0.05$; Student's $t$ test); Corr, consistent positive correlations between mRNA expression levels of selected genes involved in lipid metabolism in PBMCs and mRNA expression levels of those genes in iWAT, rWAT and liver of male and female offspring of control and calorie-restricted dams during lactation (CR) at the age of 21 days; $r$, Pearson's correlation coefficient; $p$-value of the genetic correlation ($p < 0.05$); – indicates no correlations

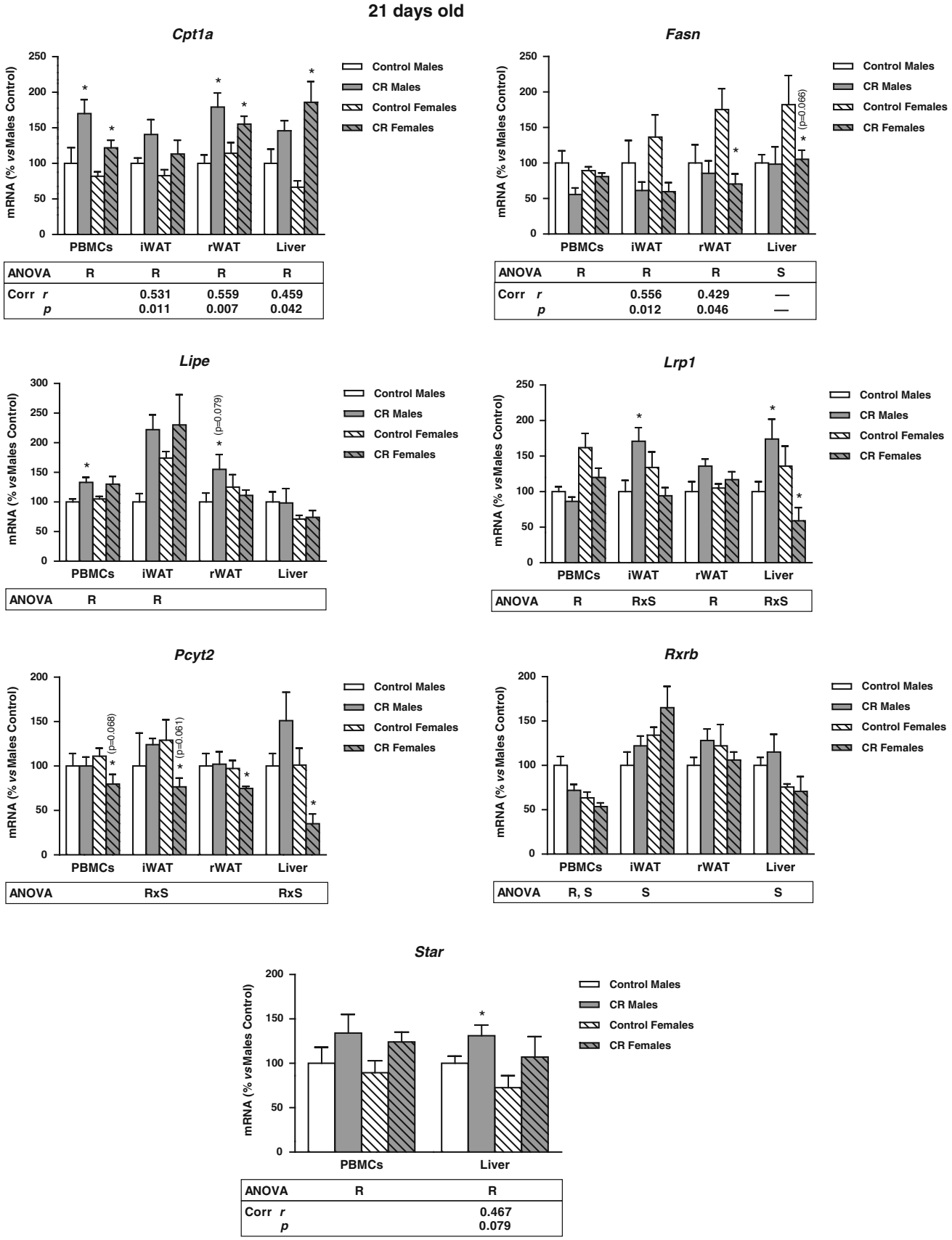
## Comparison of mRNA expression levels of genes involved in lipid metabolism in PBMCs with the expression measured in different tissues (iWAT, rWAT and liver) at the age of 21 days

To determine whether changes in mRNA levels of genes involved in lipid metabolism due to moderate maternal calorie restriction during lactation observed in PBMCs of

**Table 3** Microarray data of genes involved in lipid metabolism process and their validation by RT-qPCR in PBMCs samples of male and female offspring of control and calorie-restricted dams during lactation (CR) at the age of 21 days

| Related process | Gene name | Gene symbol | Sequence ID | Microarray | | | qPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $p$-value* | Fold change | | $p$-value** | Fold change | |
| | | | | | Males | Females | | Males | Females |
| Low density lipoproteins uptake | Apolipoprotein B48 receptor | *Apob48r* | NM_001109154 | 0.007 | −1.14 | −1.29 | 0.667 | +1.00 | +1.03 |
| β-oxidation | Carnitine palmitoyltransferase 1 alpha, liver | *Cpt1a* | NM_031559 | 0.004 | +1.22 | +1.15 | 0.002 | +1.17 | +1.13 |
| Lipogenesis | Fatty acid synthase | *Fasn* | NM_017332 | 0.002 | −1.11 | −1.25 | 0.025 | −1.19 | −1.03 |
| Lipolysis | Lipase, hormone sensitive | *Lipe* | NM_012859 | 0.010 | +1.14 | +1.1 | 0.004 | +1.09 | +1.07 |
| Low density lipoproteins uptake | Low density lipoprotein receptor-related protein 1 | *Lrp1* | NM_001130490 | 0.007 | −1.15 | −1.04 | 0.038 | −1.05 | −1.09 |
| Glycerophospholipid biosynthesis | Phosphate cytidylyltransferase 2, ethanolamine | *Pcyt2* | NM_053568 | 0.000 | −1.07 | −1.24 | 0.166 | −1.00 | −1.10 |
| Lipogenesis/lipolysis | Retinoid X receptor beta | *Rxrb* | NM_206849 | 0.002 | −1.33 | −1.17 | 0.026 | −1.10 | −1.05 |
| Low density lipoproteins uptake | Sortilin-related receptor, LDLR class A repeats-containing | *Sorl1* | NM_053519 | 0.002 | −1.08 | −1.34 | 0.238 | −1.07 | −1.03 |
| Cholesterol transport and uptake | Steroidogenic acute regulatory protein | *Star* | NM_031558 | 0.006 | +1.11 | +1.09 | 0.047 | +1.09 | +1.10 |

$p$-value * of microarray data and $p$-value ** of RT-qPCR data for statistical analysis (two-way ANOVA). Threshold of significance was set at $p \leq 0.01$ and $p < 0.05$ for microarray and RT-qPCR data. Ratios indicating fold changes in experimental group (CR vs Controls) are presented for microarray and RT-qPCR data. +, indicates up-regulation; –, down-regulation in CR animals of both sexes


21 days old
Cpt1a
mRNA (% vsMales Control)
Control Males
CR Males
Control Females
CR Females
PBMCs
iWAT
rWAT
Liver
ANOVA R R R R
Corr r 0.531 0.559 0.459
p 0.011 0.007 0.042
Fasn
(p=0.066)
ANOVA R R R S
Corr r 0.556 0.429 —
p 0.012 0.046 —
Lipe
(p=0.076)
ANOVA R R
Lrp1
ANOVA R RxS R RxS
Pcyt2
(p=0.068)
(p=0.061)
ANOVA RxS RxS
Rxrb
ANOVA R, S S S
Star
ANOVA R R
Corr r 0.467
p 0.079

21-day-old pups reflected the changes occurring in other tissues involved in lipid metabolism, we analyzed their mRNA expression levels in liver and in iWAT and rWAT, representative of subcutaneous and internal adipose tissue depots, respectively (Fig. 3). In accordance with findings in PBMCs, CR animals showed higher *Cpt1a* mRNA levels in both WAT depots and in liver compared with controls ($p < 0.05$; two-way ANOVA). *Fasn* expression levels in iWAT and rWAT were also significantly decreased in CR animals ($p < 0.05$; two-way ANOVA), in agreement with changes occurring in PBMCs. No significant changes between control and CR animals were found in liver, although a trend to lower *Fasn* mRNA levels was observed in CR females ($p = 0.066$; Student's *t* test). Differences found in PBMCs for *Lipe* mRNA expression levels were also found in iWAT and for *Star* in liver ($p < 0.05$; two-way ANOVA). Concerning *Pcyt2*, the trend to lower mRNA levels occurring in CR female animals with respect to their controls was also observed in both WAT depots and in liver by Student's *t* test.

However, changes found for *Rxrb* in PBMCs between control and CR animals were not related to changes in WAT or liver, where no changes in the expression levels of this gene could be shown as an effect of maternal calorie restriction during lactation. Finally, changes for *Lrp1* in PBMCs followed the same trend in iWAT and liver (however, only for females), but were in the opposite direction to those found in rWAT ($p < 0.05$; two-way ANOVA).

To evaluate how closely the changes in the lipid metabolism-related gene expression levels in PBMCs indicate those occurring in liver, iWAT and rWAT, correlation tests on the corresponding genes were performed (correlation values are indicated in Fig. 3). Notably, *Cpt1* mRNA levels in PBMCs were positively correlated with their expression levels in iWAT, rWAT and liver and also *Fasn* mRNA levels in PBMCs correlated positively with their expression levels in iWAT and rWAT. *Star* mRNA levels in PBMCs were slightly correlated with *Star* mRNA levels in liver. On the other hand, no associations were found in relative mRNA expression responses of the other genes studied in PBMCs (*Lipe*, *Lrp1*, *Pcyt2* and *Rxrb*) with those in WAT or liver (data not shown).

### mRNA expression levels of selected genes involved in lipid metabolism in PBMCs at the age of 6 months

We also examined how the mRNA pattern of PBMC lipid metabolism-related genes, which were significantly altered at the age of 21 days in CR rats, behaved in adult animals (Table 4). Our results show that overexpressed levels of *Star* in young CR males and females were maintained in adulthood, although without reaching statistical significance ($p < 0.077$; two-way ANOVA). Changes found for *Fasn* expression levels in young animals were only preserved in adulthood for females ($p < 0.05$; Student's *t* test; interactive effect between moderate maternal calorie restriction during lactation and sex, $p < 0.05$; two-way ANOVA). No changes in mRNA levels of *Apob48r, Cpt1a, Lipe*, *Lrp1*, *Pcyt2 and Sorl1* were observed between control and CR animals, and, contrary to what was found in young animals, *Cpt1a* mRNA levels were down-regulated in CR females relative to their controls ($p < 0.05$; Student's *t* test).

### Comparison of mRNA expression levels of selected genes involved in lipid metabolism in PBMCs with the expression measured in different tissues (iWAT, rWAT and liver) at the age of 6 months

We next determined whether the genes showing differential expression between control and CR adult animals in PBMCs, *Fasn*, *Cpt1a* and *Star*, also showed this in both fat depots (iWAT and rWAT) and in liver at an older age. We also included *Rxrb*, because its expression levels in control and CR animals showed different patterns dependent on the sex of animals (Fig. 4). Changes in *Cpt1a* mRNA levels observed in PBMCs of adult rats reflected those found in rWAT and liver (interactive effect between sex and calorie restriction, $p < 0.05$; two-way ANOVA), although in rWAT changes did not reach statistical significance. Concerning *Rxrb*, its mRNA levels in rWAT were differentially expressed in CR animals depending on sex, the same as observed in PBMCs (interactive effect between sex and calorie restriction, $p < 0.05$; two-way ANOVA). A similar trend was also found in iWAT for females ($p < 0.05$; Student's *t* test). *Star* expression profile observed in PBMCs was somewhat reflected in the liver, although without reaching statistical significance. Regarding *Fasn*, changes observed at mRNA levels of adult CR animals with respect to controls in PBMCs were not reflected either in fat depots or in liver.

As in young animals, we assessed the strength of association between the expression levels of these genes in PBMCs with those in iWAT, rWAT and liver of adult animals (Fig. 4) by correlation analysis. Similarly, to the findings in young animals, a positive significant correlation was observed between mRNA expression levels of *Cpt1a* in PBMCs and its expression levels in rWAT and liver at the age of 6 months. Moreover, a positive significant correlation was observed between expression levels of *Rxrb* in PBMCs and rWAT. No associations were found between mRNA levels of *Fasn* and *Star* in PBMCs and their expression levels in WAT or liver (data not shown).

## Discussion

Biomarkers derived from disease processes, such as those identified in obese subjects or in animal models of obesity,

**Table 4** mRNA expression levels of genes involved in lipid metabolism in PBMCs samples of male and female offspring of control and calorie-restricted dams during lactation (CR) at the age of 6 months

| | Males | | Females | | ANOVA |
|---|---|---|---|---|---|
| | Control | CR | Control | CR | |
| *Apob48r* | 100 ± 12 | 101 ± 12 | 100 ± 23 | 110 ± 20 | |
| *Cpt1a* | 100 ± 5 | 102 ± 8 | 114 ± 12 | 84.4 ± 3.6* | |
| *Fasn* | 100 ± 8 | 109 ± 8 | 126 ± 4 | 99.7 ± 4.8* | RxS |
| *Lipe* | 100 ± 13 | 107 ± 12 | 91.9 ± 5.2 | 92.4 ± 12.4 | |
| *Lrp1* | 100 ± 25 | 139 ± 39 | 73.8 ± 13.5 | 110 ± 13.2 | |
| *Pcyt2* | 100 ± 19 | 121 ± 28 | 84.1 ± 8.8 | 109 ± 22 | |
| *Rxrb* | 100 ± 6 | 115 ± 6 | 125 ± 10 | 103 ± 4 | RxS |
| *Sorl1* | 100 ± 21 | 96.5 ± 16.1 | 75.7 ± 2.5 | 103 ± 18 | |
| *Star* | 100 ± 15 | 137 ± 22 | 120 ± 10 | 167 ± 33 | R ($p = 0.077$) |

mRNA levels were measured under ad libitum feeding conditions at the age of 6 months by RT-qPCR and expressed as a percentage of the mean value of males control. Data are mean ± SEM ($n = 6$–8 animals/group). Statistics: R, effect of maternal calorie restriction during lactation; RxS, an interaction between the effect of maternal calorie restriction during lactation and the effect of sex ($p < 0.05$; two-way ANOVA). *, CR vs Controls ($p < 0.05$; Student's $t$ test)

might not serve to assess the potential benefits of bioactive compounds or changes in dietary habits aiming to decrease the risk of obesity and related metabolic alterations in healthy or at-risk individuals. Therefore, in this study, we took up this issue and aimed at identifying early transcript-related biomarkers of improved metabolic health, using an animal model programmed for reduced risk of obesity development and related metabolic alterations in adulthood. For reasons of accessibility and minimal invasiveness, a blood-derived RNA source, PBMCs, was used. Findings from this study reveal that transcript levels of lipid metabolism-related genes in PBMCs can be used as early biomarkers of metabolic health and potentially reflecting metabolic processes occurring in other tissues.

Early postnatal intervention based on moderate maternal calorie restriction during lactation brought about improvements in some phenotypic traits in the offspring, such as body weight and fat content, as previously described (Palou et al. 2011). The effect of lowering body weight was observed in both males and females from the fifth day of life and was persistent when animals were adult, although it was more pronounced in females than in males. At weaning, both males and females also displayed lower body fat content, as well as lower weight of fat depots, but differences were only maintained in later life in females. Despite changes in body weight, body length of adult animals was not affected. The decrease in body weight of offspring could be partly due to lower food intake. CR animals ate fewer calories than their controls, with the differences again more pronounced in females.

Circulating hormones, such as insulin and leptin, could also contribute to characterize the metabolic health of these animals in relation to a better ability to maintain energy homeostasis throughout life. Leptin and insulin are hormones related to central control of feeding behavior and energy expenditure (Schwartz et al. 2000). High leptin levels are associated with insulin resistance and metabolic syndrome (Esteghamati et al. 2009), whereas lower leptin levels have been associated with improvement in insulin sensitivity (Sanchez et al. 2008). At weaning, CR animals showed lower plasma levels of insulin and leptin. This healthier profile of circulating hormones was sustained in adulthood, particularly in females.

Regarding gene expression, taking both males and females into account, 278 genes were differentially expressed between control and CR animals in PBMCs at the age of 21 days. As PBMCs are a subset of white blood cells, it is not surprising that immune system was identified as the most affected process in terms of gene expression. This was followed by other processes such as signaling, cell turnover, transcription machinery. Interestingly, also the expression of genes involved in metabolism of proteins, carbohydrates as well as lipids, and redox and central metabolism was affected. In a previous study, using a similar animal model of 30 % calorie-restricted dams during lactation, the offspring showed an improved capacity to handle and store excess dietary fuel in adulthood (Palou et al. 2011). We therefore focused on genes related to lipid metabolism.

Fatty acid synthase (FASN) catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH. Some studies have evidenced that the expression of *Fasn* in PBMCs reflects metabolic adaptations of the organism to fasting/feeding conditions. *Fasn* expression levels were found to be decreased with fasting and increased with refeeding in normoweight rats; however, this response

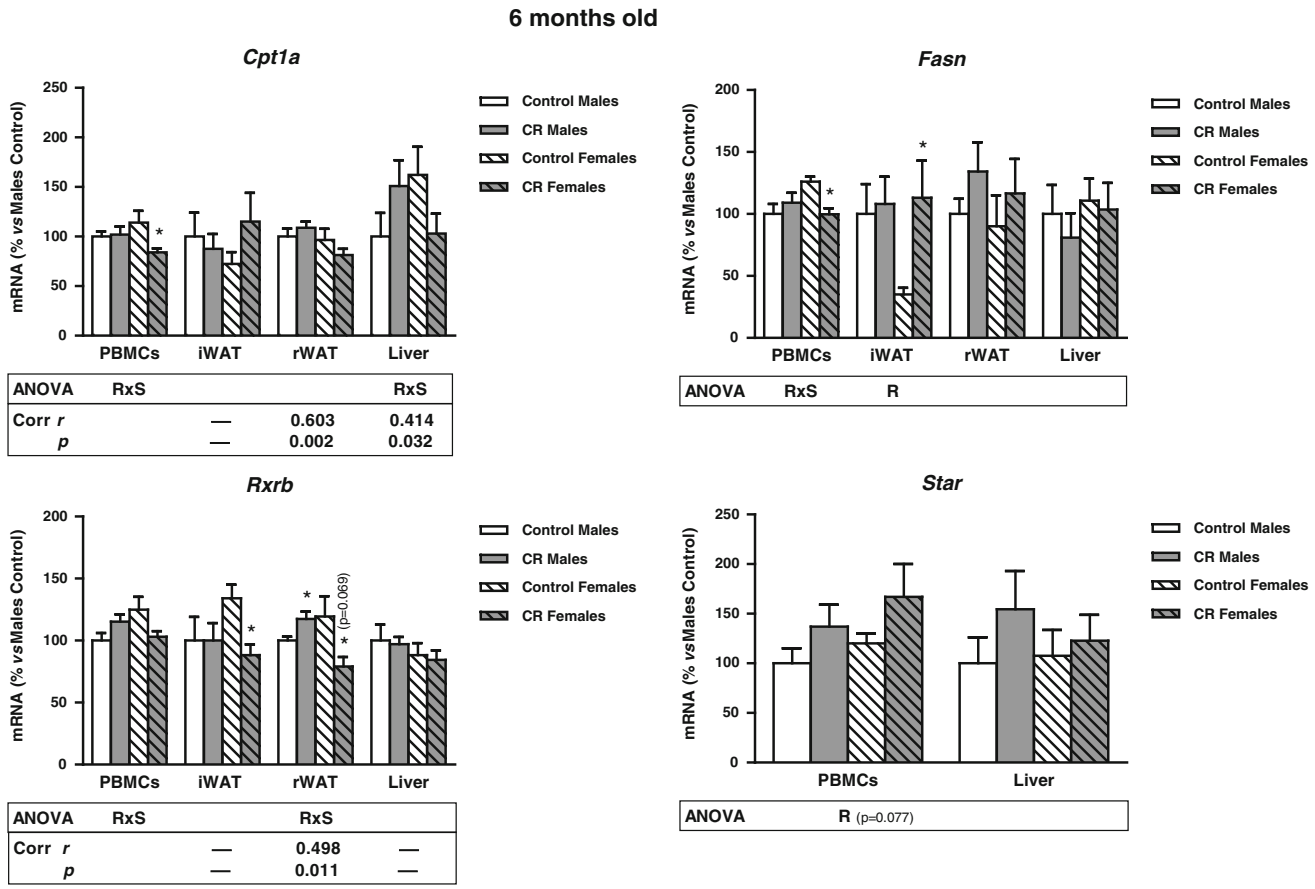



**Fig. 4** Comparison of mRNA expression levels of some lipid metabolism-related genes in PBMCs with the expression determined in inguinal and retroperitoneal WAT and liver of male and female offspring of control and at 6 months of age. Only those genes with significant differences between control and CR animals in PBMCs of adult animals are shown. mRNA levels were measured by RT-qPCR and expressed as a percentage of the mean value of males control. Data are mean ± SEM ($n = 6$–8 animals/group). Statistics: R, effect of maternal calorie restriction during lactation; RxS, an interaction between the effect of maternal calorie restriction during lactation and the effect of sex ($p < 0.05$; two-way ANOVA). *, CR vs Controls ($p < 0.05$; Student's $t$ test); Corr, consistent positive correlations between mRNA expression levels of selected genes involved in lipid metabolism in PBMCs and mRNA expression levels of those genes in iWAT, rWAT and liver of male and female offspring of control and calorie-restricted dams during lactation (CR) at the age of 6 months; $r$, Pearson's correlation coefficient; $p$-value of the genetic correlation ($p < 0.05$); – indicates no correlations

was impaired in obese rats (Caimari et al. 2010a; Oliver et al. 2013). Here, it is shown that weaned CR pups displayed lower *Fasn* mRNA levels than controls in PBMCs, and this decrease was also found in both WAT depots analyzed. This expression pattern was persistent in PBMCs at the age of 6 months but only in females, in accordance with their higher protection (compared with males) against fat accumulation. Therefore, lower mRNA levels of this gene might be proposed as a marker for a higher protection against fat accumulation in adulthood, in accordance with the metabolic role of FASN in lipid synthesis.

The hormone-sensitive lipase (LIPE, better known as HSL), encoded by the *Lipe* gene, is an enzyme that hydrolyzes acylglycerols, as well as several other lipids stored in adipose tissue (Yeaman 2004). Although transcription of *Lipe* was initially described as specific for adipocytes, subsequent studies have shown that it is expressed and has a role in lipid metabolism in multiple tissues, including liver and macrophages (Yeaman 2004). Its action has also been suggested to be linked with insulin secretion and insulin action (Kraemer and Shen 2002). Expression of this gene has also been described in PBMCs, where its level was found to be reduced in "at risk" obese subjects (Telle-Hansen et al. 2013). Interestingly, in the present study, we show that young CR animals exhibited higher *Lipe* mRNA levels in PBMCs and similarly in iWAT. Therefore, higher mRNA levels of this gene might be proposed as an early marker for a better capacity to regulate lipid metabolism in adulthood, possibly in association with improved insulin sensitivity. However, early changes were not persistent into adulthood, suggesting their potential usefulness as a biomarker only at early ages.

*Cpt1a* codes for carnitine palmitoyltransferase 1alpha (liver form). It mediates the transport of long-chain fatty

acids across the mitochondrial inner membrane and is rate limiting for their beta-oxidation. High expression levels of *Cpt1a* in PBMCs have been described in diet-induced obese rats (Caimari et al. 2010b). Similarly, *Cpt1a* mRNA levels in whole blood cells were higher in those overweight male children that seemed to be protected against the increase in plasma triglyceride levels associated with body fat accumulation (Sanchez et al. 2012). Conversely, lower expression of this gene in overweight male children was associated with higher HOMA index (Sanchez et al. 2012). In the present study, young CR animals showed higher mRNA levels of *Cpt1a* in PBMCs than controls. Moreover, this expression pattern was correlated with that occurring in liver and WAT (both inguinal and retroperitoneal depots). However, differences found at early ages were not sustained in adulthood, neither in PBMCs nor in the tissues that were investigated, although the expression profile of this gene in PBMCs of adult animals was also related to the expression in rWAT and liver. Altogether, these results suggest positive metabolic effects related with high expression levels of *Cpt1a* in PBMCs at early ages in relation to increased oxidative capacity.

*Rxrb* encodes a member of the retinoid X receptor (RXR) family of nuclear receptors, which is involved in mediating the effects of 9-cis-retinoic acid. This protein has been linked with lipid metabolism, with dual effects. On the one hand, RXRb heterodimerizes with peroxisome proliferator-activated receptor alpha (PPARα) and in this way cooperates in the induction of the acyl-CoA oxidase gene, which encodes the rate-limiting enzyme of peroxisomal β-oxidation of fatty acids (Keller et al. 1993). On the other hand, RXR may also induce FASN via formation of LXR/RXR heterodimers binding to their recognition sequences in the sterol regulatory element-binding protein 1c (SREBP-1c) promoter (Roder et al. 2007). The correlation between expression levels of this gene in PBMCs and rWAT in adulthood may be associated with the lower degree of adipogenesis that is observed.

The *Lrp1* gene encodes the low density lipoprotein receptor-related protein 1 (LRP1), an endocytic receptor which is ubiquitously expressed in a variety of organs, including WAT, liver and brain, and is involved in several cellular processes (Hussain et al. 1999). Notably, *Lrp1* has been described to be up-regulated in human and mouse obese adipose tissue, and silencing of *Lrp1* expression in 3T3F442A murine preadipocytes brought about reduction in cellular lipid level that was associated with an inhibition of adipogenesis (Olivier et al. 2009). These observations suggest that changes in *Lrp1* expression may have important consequences for fat accumulation and obesity, which agrees with the protection against fat accumulation in CR animals.

Other genes, whose expression was changed in CR versus control animals, include *Pcyt2* and *Star*, which were down-regulated and up-regulated, respectively. *Pcyt2* encodes CPT: phosphoethanolamine cytidylyltransferase, the main regulatory enzyme in the de novo ethanolamine phospholipids synthesis (Pavlovic and Bakovic 2013). The meaning of underexpression of this gene in PBMCs at an early stage of life, as occurring in CR animals, remains to be determined. Nonetheless, it must be mentioned that microarrays results of this gene were confirmed by RT-qPCR only in females; a similar decrease was also found in WAT and liver of CR females, but not males, and these changes were not maintained in adult rats. Hence, its function as a potential biomarker appears more limited. The *Star* gene encodes for the steroidogenic acute regulatory protein (STAR), which is a transport protein that modulates cholesterol transfer within mitochondria for the production of adrenal and gonadal steroids in steroidogenic tissues, representing the limiting step (Lin et al. 1995). This gene has also been described to be expressed in monocytes, macrophages and human aortic tissue (Taylor et al. 2010). In macrophages, *Star* overexpression impacts positively on the lipid-related phenotype of these cells, since it represses a number of genes involved in cholesterol biosynthesis and LDL uptake and markedly increases the expression of ABCA1, strongly suggesting that *Star* increases sterol efflux to apoAI (Taylor et al. 2010). This protein has also been shown to be a protective molecule for endothelial dysfunctions in aortic endothelium (Tian et al. 2012). Interestingly, presence of STAR protein has also been described in human liver cells, where it appears to be involved in cellular cholesterol homeostasis, representing a potential therapeutic target in the management of hyperlipidemia (Hall et al. 2005). Therefore, results from this study suggest that increased transcript levels of *Star* in blood cells of young CR animals, also reflecting changes occurring in liver, may be indicative of improved cholesterol metabolism and hence improved cardiovascular health. Notably, a trend to higher transcript levels of *Star* in PBMCs was also shown in CR adult animals. Hence, it would be interesting to assess the relationship between PBMC transcript levels of *Star* and the ability to maintain normal blood cholesterol levels under appropriate dietary insults, or in an animal model prone to develop hypercholesterolemia, since in the conditions of this study, no differences were found between groups concerning blood cholesterol (data not shown).

All in all, despite the fact that nutritional-induced changes in PBMC gene expression are generally expected to be smaller than the effects induced by diseases (Bouwens et al. 2007), early programming effects occurring in CR animals due to calorie restriction during lactation were reflected in a substantial number of changes. Among genes related with lipid metabolism, it is noteworthy that expression levels of *Cpt1a*, *Fasn* and *Star* in PBMCs at

early ages were significantly correlated with expression profiles in WAT and/or liver.

To summarize, the findings from this study, using an animal model that confers certain protection in the pups against development of obesity and related metabolic alterations by dietary intervention in lactating dams, reveal the possibility of using transcript levels of lipid metabolism-related genes in PBMCs, particularly *Fasn*, *Cpt1a* and *Star*, as early biomarkers of metabolic health, potentially providing a valid biological readout for the study of metabolic processes in humans.

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**Conflict of interest** None.

**Ethical standard** All institutional and national guidelines for the care and use of laboratory animals were followed.

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