

# Endocrine effects of tobacco smoke exposure during lactation in weaned and adult male offspring

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## Abstract

Children from pregnant smokers show more susceptibility to develop obesity in adult life. Previously, we failed to demonstrate a program for obesity in rat offspring only when the mothers were exposed to tobacco smoke during lactation. Here, we studied the short- and long-term effects of smoke exposure (SE) to both dams and their pups during lactation on endocrine and metabolic parameters. For this, we designed an experimental model where nursing rats and their pups were divided into two groups: SE group, exposed to smoke in a cigarette smoking machine (four times/day, from the third to the 21st day of lactation), and group, exposed to filtered air. Pups were killed at 21 and 180 days. At weaning, SE pups showed lower body weight (7%), length (5%), retroperitoneal fat mass (59%), visceral adipocyte area (60%), and higher subcutaneous adipocyte area (95%) with hypoinsulinemia (−29%), hyperthyroxinemia (59%), hypercorticosteronemia (60%), and higher adrenal catecholamine content (+58%). In adulthood, SE offspring showed higher food intake (+10%), body total fat mass (+50%), visceral fat mass (retroperitoneal: 55%; mesenteric: 67%; and epididymal: 55%), and lower subcutaneous adipocyte area (24%) with higher serum glucose (11%), leptin (85%), adiponectin (1.4-fold increase), total triiodothyronine (71%), free thyroxine (57%), TSH (36%), triglycerides (65%), VLDL cholesterol (+66%), and HDL cholesterol (91%) levels and lower corticosteronemia (41%) and adrenal catecholamine content (57%). Our present findings suggest that tobacco SE to both dams and their pups during lactation causes malnutrition in early life that programs for obesity and hormonal and metabolic disturbances in adulthood, only if the pups are submitted to the same smoke environment as the mother.

## Key Words

- ▶ lactation
- ▶ smoking
- ▶ hormones
- ▶ lipids
- ▶ adipose tissue
- ▶ programming

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## Introduction

Approximately 20% of women in the world are smokers (WHO 2010). This information awakens the interest about the effects of smoking on two important phases of woman's life: gestation and lactation. Some studies have

associated smoking during these periods with fetal growth restriction, preterm delivery, ectopic pregnancy, spontaneous abortion, and obesity development and its comorbidities, such as hypertension in childhood or

adult life (von Kries *et al.* 2002, Wideroe *et al.* 2003, Goldani *et al.* 2007, Einarson & Riordan 2009, Braun *et al.* 2010, Durmus *et al.* 2011).

Events or stimuli that occurred in the perinatal period (gestation or lactation) can permanently alter epigenetic markers such as histone acetylation/deacetylation or DNA methylation pattern, which can increase the risk of disease development in adult life. This association between early events (such as maternal smoking) and late consequences (such as obesity) exemplifies a phenomenon known as metabolic programming (de Moura *et al.* 2008, Lisboa *et al.* 2012). Recently, some studies have shown that tobacco exposure *in utero* is associated with changes in gene methylation profile (Suter *et al.* 2010, 2011), and the same event was observed in nicotine exposure (the main cigarette component)– changes the gene methylation pattern in fetal life are associated with long-term decreases in cortisol (Wang *et al.* 2011).

In rodents, it has been demonstrated that adult offspring from mothers who were exposed to nicotine during gestation and/or lactation become obese and have other metabolic disorders such as cardiovascular disorders, permanent  $\beta$ -cell loss, and insulin resistance (Bruin *et al.* 2007, Somm *et al.* 2009). Therefore, it seems that nicotine has the potential to act as an obesogenic factor in the offspring. In fact, our laboratory confirmed this hypothesis using an experimental model of maternal exposure exclusively to nicotine only during lactation. Nicotine is transferred by breast milk and promotes, in pups, higher body fat mass with metabolic alterations featured by hyperleptinemia, primary thyroid hypofunction, and higher adrenal catecholamine content and corticosterone levels (Oliveira *et al.* 2009, 2010). In adult life, it programs the progeny for overweight and higher adiposity associated with leptin and insulin resistance as well as secondary hypothyroidism with alterations in the leptin signaling pathway of hypothalamus–pituitary–thyroid axis (Oliveira *et al.* 2009, de Oliveira *et al.* 2010, Santos-Silva *et al.* 2010).

As there are other components besides nicotine in cigarettes, the association of nicotine with other tobacco smoke components may have different or combined effects in children/progeny compared with those effects previously observed in several studies when the mothers were exposed to nicotine alone. Thus, to test this hypothesis, we designed an experimental model where dams were exposed to tobacco smoke during lactation separated of their pups, i.e. we studied the effects of tobacco smoke components transferred only by milk. This model was justified because a considerable proportion of

smoking mothers relapse to drug use during lactation but avoid smoking in the same environment as the child. We detected higher cotinine levels in maternal milk, similar to those found in the model of maternal nicotine exposure. Also, there were important alterations in breast milk composition such as higher triglyceride and lactose levels (Santos-Silva *et al.* 2011). Again, these findings were similar to those observed in the model of maternal nicotine exposure (Oliveira *et al.* 2010). Pups from dams who were exposed to smoke presented, at weaning, hypertriglyceridemia, hyperinsulinemia, hypocorticosteronemia, lower adrenal catecholamine content, and no change in body weight or adiposity (Santos-Silva *et al.* 2011). Surprisingly, when adults, these offspring were programmed only for adrenal dysfunction, characterized by decreased total adrenal catecholamine content and tyrosine hydroxylase protein expression (Santos-Silva *et al.* 2012). These findings were different from those obtained from the maternal nicotine model (Oliveira *et al.* 2009, de Oliveira *et al.* 2010), suggesting that the combination of nicotine and other cigarette components programs for different outcomes than the maternal nicotine model.

Actually, children from smoking mothers are exposed to cigarette components not only through breast milk but also through the household environment. The WHO (2009) showed that 40% of children are submitted to cigarette components by environmental tobacco smoke, when at least one of their parents is a smoker. Children exposed to environmental tobacco present higher serum leptin, C-reactive protein, fibrinogen, and interleukin-6 levels (Nagel *et al.* 2009), which suggest more susceptibility to cardiovascular disease. As exclusive maternal smoke exposure (SE) fails to reproduce most of the programming effects of maternal nicotine exposure, and in order to try to reproduce what really can happen to children from smoker parents who are exposed to cigarette components both by breast milk and by direct inhalation, we design this study where both dams and pups were exposed to tobacco smoke during lactation to better understand the immediate and late repercussions of early environmental smoking on rat offspring's development and endocrine function.

## Materials and methods

Wistar rats were kept in a room with controlled temperature ( $25 \pm 1$  °C) and with artificial dark–light cycles (0700 h light:1900 h darkness cycle). Nulliparous 90-day-old female rats were caged with male rats at a proportion of 2:1. After mating, each pregnant rat was placed in an

individual cage with water and food *ad libitum* until delivery. The use of animals in our experimental design was approved by the Animal Care and Use of the Biology Institute of the State University of Rio de Janeiro (CEUA/016/2009). The handling of experimental animals was according to the principles adopted in Brazil according the Brazilian Law no. 11.794/2008 (Marques *et al.* 2009).

### Model of neonatal exposure to cigarette smoke during lactation

On the third day after delivery, lactating rats with their litters were randomly assigned to one of the following groups until weaning period (21 days of lactation):

- SE group ( $n=8$  dams) – dams and offspring were placed into a smoking machine (TE-10, Teague Enterprises, Davis, CA, USA) four times per day (1 h each exposure). This machine generated tobacco smoke from one research cigarette type 2R1F at a time (nicotine=1.74 mg/cigt; total particulate matter=28.6 mg/cigt; tar=23.4 mg/cigt; carbon monoxide=22.0 mg/cigt; Reference Cigarette Program, University of Kentucky, Lexington, KY, USA). A smoke mixture containing 89% sidestream smoke (smoke released from the burning end of a cigarette) and 11% mainstream smoke (smoke from the puff stream), as a surrogate for active smoking (Abreu-Villaça *et al.* 2010, 2012), was generated by the smoking machine in a staggered manner at the rate of a single 35 ml puff of 2-s duration each min. During exposure, the total suspended particulate was measured by weighing teflon-coated fiber filters (TX40H120-WW, Pallflex Products Co., Putnam, CT, USA) before and after a 5-min period, when air was collected from the chamber. There were 12 periods of collection, which generated levels of  $38.4 \pm 3.9$  mg/m<sup>3</sup> (mean  $\pm$  S.E.M.).
- Control (C group,  $n=8$  dams) – dams and offspring were exposed to filtered air in a similar chamber.

At birth, to maximize lactation performance, litters were adjusted for six male pups per SE or C dam. During lactation, pup's body weight was monitored daily and nose–rump length once a week. After weaning, pup's body weight and food intake were monitored every 4 days. We decapitated two pups per litter at weaning as well as at 180 days, with no prior anesthesia because anesthesia affects hormone and lipid metabolism. Blood samples, adipose tissue, and adrenal gland were collected and frozen at  $-20$  °C for further analysis.

### Detection of cotinine (nicotine metabolite)

At weaning, serum cotinine levels were determined using a cotinine assay kit from Orasure Technologies (Bethlehem, PA, USA) in accordance with the manufacture's recommendations. SE and C pups were killed (one pup/litter), the blood was collected, centrifuged at 2000 g for 20 min at 4 °C, and supernatant was stored at  $-20$  °C until assaying.

### Body composition evaluation

The central adiposity was determined at 21 and 180 days by weighing visceral fat mass (VFM) – mesenteric, epididymal, and retroperitoneal deposits. The carcass of C and SE offspring was weighed, autoclaved for 1 h, and homogenized in distilled water (1:1). The homogenate was stored at 4 °C for analysis to determine fat content gravimetrically (Oliveira *et al.* 2009). Three grams of sample were hydrolyzed on a shaking water bath at 70 °C for 2 h with 30% KOH and ethanol (3 ml each). Samples were acidified with 2.5 ml 6 M H<sub>2</sub>SO<sub>4</sub>. Total fatty acids and free cholesterol were removed by three successive washings with petroleum ether. After drying overnight in vacuum, all tubes were weighed and data were expressed as grams of fat by 100 g carcass. One gram of homogenate was used for determination of protein content by Lowry method (Lowry *et al.* 1951). Data were expressed as grams of protein by 100 g carcass.

### Morphological evaluation of the pup's adipose tissue

Samples of subcutaneous and retroperitoneal adipose tissue from six SE and six C offspring (21 and 180 days) were fixed in buffered formaldehyde. After 24 h of fixation, samples were dehydrated, cleared, and paraffin embedded. Sections of 5  $\mu$ m were obtained (microtome Microtec-CUT 4050, SC, USA), placed into slides, and stained with hematoxylin/eosin. A light microscope (model BX40 Olympus, Tokyo, Japan) coupled to a digital camera (Olympus DP7) was used to examine the morphology using a 20 $\times$  objective. Morphometric analysis was performed in captured images from ten slides per animal. For each slide, the area of ten adipocytes was randomly selected and analyzed using the IMAGE-J/NHI software (Wayne Rasband National Institute of Health, Bethesda, MA, USA).

### Serum biochemical parameters

Glucose was determined in blood samples from the tail vein of fasting rats using a glucometer (ACCU-CHEK

Advantage, Roche Diagnostics). Triglycerides, total cholesterol, and HDL cholesterol (HDL-C) fraction were analyzed using Biosystem commercial test kits with an automated A15 spectrophotometer (Biosystems S.A., Barcelona, Spain). LDL cholesterol (LDL-C) and VLDL cholesterol (VLDL-C) were calculated according to the Friedewald equation (Friedewald & Levy 1972):

$$\text{VLDL-C} = \text{triglycerides}/5.$$

$$\text{LDL-C} = (\text{total cholesterol} - \text{HDL-C} - \text{triglycerides})/5.$$

### Serum hormone quantification by RIA and enzyme immunoassay

Blood samples were centrifuged (1500 g for 20 min at 4 °C) to obtain sera, which were kept at –20 °C until the assay. All measurements were performed in one assay. The assay sensitivity, intra-assay variation, and kit provider are shown in Table 1.

### Adrenal catecholamine content measurement

For this, left adrenal gland was homogenized in 500 µl 10% acetic acid and centrifuged at 1120 g for 5 min. To assay, 50 µl of the supernatant/epinephrine standards were mixed with 250 µl buffer phosphate 0.5 M, pH 7.0, and 25 µl potassium ferricyanide 0.5%, followed by incubation (20 min; ice bath). Reaction was stopped with 500 µl ascorbic acid/NaOH 10 M (1:19 proportion). Parameters used in the fluorometer (Victor2, PerkinElmer, Waltham, MA, USA) were 420 nm excitation and 510 nm emission. Results were obtained by plotting the values into a linear regression of the standard epinephrine curve. Data were expressed in µM catecholamine/mg gland (Trevenzoli *et al.* 2007).

### Statistical analysis

GraphPad Prism 5 was used for statistical analyses and graphics (GraphPad Software, Inc., La Jolla, CA, USA). Results were reported as mean ± s.e.m. Changes in body weight and food intake were analyzed by two-way ANOVA and Newman–Keuls multiple comparison tests. The other experimental data were analyzed by unpaired Student's *t*-test with significance level set at  $P < 0.05$ . We studied two offspring from each mother per age. However, for the analyses, the litter was used as the experimental unit because we considered the average of values from animals of the same litter instead of using individual animal values.

## Results

### Changes in pups at weaning

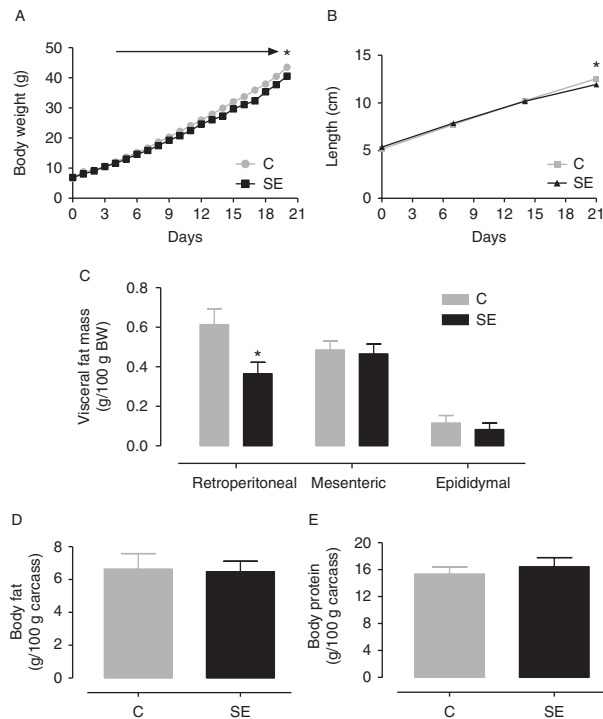
At weaning, SE pups presented high serum cotinine levels (145.3 ± 5.1 ng/ml) confirming tobacco exposure. Control pups had cotinine levels below the detection limit of the technique (<8 ng/ml). Cotinine levels found in SE animals are comparable with the levels detected in women heavy smokers (Eskenzazi & Bergmann 1995).

Figure 1 shows that SE pups presented significant lower body weight from the fourth day of life to weaning (–7% at 21 days;  $P < 0.05$ ), lower nose–rump length (–5% at 21 days;  $P < 0.05$ ), and lower retroperitoneal fat depot (–59%;  $P < 0.05$ ) compared with C pups at weaning without change in mesenteric and epididymal fat depots or in total body fat and total body protein. Figure 2 presents morphometric analysis of adipose tissue of SE and C pups at 21 days. SE pups showed lower retroperitoneal adipocyte area (–60%;  $P < 0.05$ ) and higher subcutaneous adipocyte area (+95%;  $P < 0.05$ ).

Table 2 shows the hormone profile of SE pups at weaning. These animals presented lower serum insulin

**Table 1** Assay sensitivity, intra-assay variation, and kit provider of RIA and enzyme immunoassay (EIA) used in the present study

	Assay sensitivity	Intra-assay variation (%)	Provider
Insulin	1 ng/ml	8.9	RIA kit (ImmuChem TM 125 I, coated tube, ICN Biomedicals, Inc., NY, USA)
Leptin	0.5 ng/ml	2.9	RIA kit (Linco Research, Inc., St Louis, MO, USA)
Corticosterone	50 ng/ml	7	RIA kit (ICN Biomedicals, Inc., Aurora, OH, USA)
Total triiodothyronine (TT <sub>3</sub> )	25 ng/dl	3.5	RIA kit (ICN Pharmaceuticals, Inc., Los Angeles, CA, USA)
Free thyroxine (FT <sub>4</sub> )	0.3 ng/dl	6.5	RIA kit (ICN Pharmaceuticals, Inc.)
Serum TSH	0.18 ng/ml	2.3	RIA kit expressed in terms of the reference preparation-3 (RP-3) supplied by NIH (USA)
ACTH	0.1 ng/ml	10	EIA kit (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA)
Adiponectin	0.155 ng/ml	2	EIA kit (Millipore, Billerica, MA, USA)



**Figure 1** Body weight (A), nose–rump length (B), white adipose tissue depots (C), total body fat (D), and total body protein (E) of weaned pups who were exposed to tobacco smoke (black) or not (gray) during lactation. Values represent mean and s.e.m. of eight pups per group. \* $P < 0.05$ .

(−29%;  $P < 0.05$ ), higher free thyroxine (FT<sub>4</sub>) (+59%;  $P < 0.05$ ), higher corticosterone (+60%;  $P < 0.05$ ), and higher adrenal catecholamine content (+58%;  $P < 0.05$ ) without changing the other analyzed parameters.

### Changes in pups in adult life

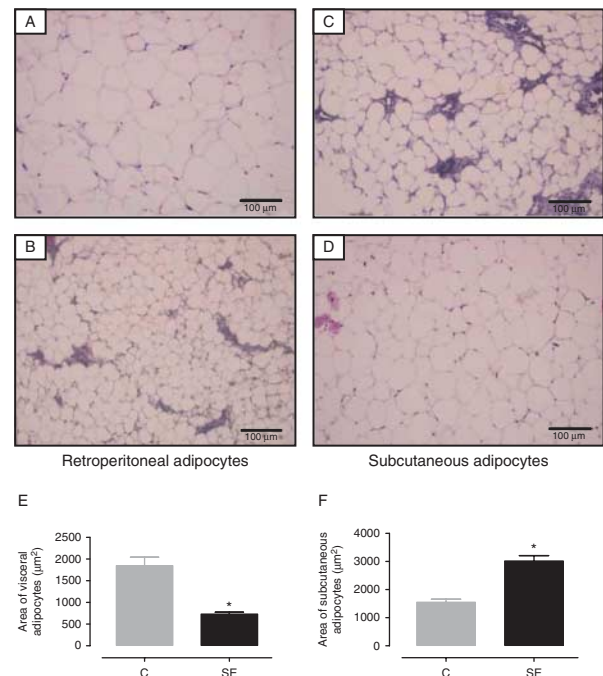
As depicted in Fig. 3, adult SE offspring presented no change in body weight when compared with C offspring, although they had higher cumulative food intake (+10%;  $P < 0.05$ ), which was confirmed by the significant difference of area under the curve (AUC) of temporal evolution of the food ingestion (+13%;  $P < 0.05$ ).

Regarding body composition, SE offspring presented higher fat mass in all visceral depots (retroperitoneal: +55%; mesenteric: +67%; and epididymal: +55%;  $P < 0.05$ ), higher total body fat (+50%;  $P < 0.05$ ) without change in total body protein. Figure 4 presents the morphometric analysis of adipose tissue at 180 days. The SE group only showed lower subcutaneous adipocyte area (−24%;  $P < 0.05$ ) than C group with no change in retroperitoneal adipocyte area.

The hormone profile at 180 days is depicted in Table 3. The SE group presented normoinsulinemia, hyperglycemia (+11%;  $P < 0.05$ ), hyperleptinemia (+85%;  $P < 0.05$ ), and hyperadiponectinemia (+1.40-fold increase;  $P < 0.05$ ), while both corticosteronemia and adrenal catecholamine contents were lower (41 and 57% respectively;  $P < 0.05$ ). Concerning the thyroid status, the SE group showed higher serum TSH (+36%;  $P < 0.05$ ), total triiodothyronine (TT<sub>3</sub>) (+71%;  $P < 0.05$ ), and FT<sub>4</sub> (+57%;  $P < 0.05$ ) levels compared with the C group. The lipid profile at 180 days is presented in Table 4. SE offspring showed higher triglycerides (+65%;  $P < 0.05$ ), HDL-C (+91%;  $P < 0.05$ ), and VLDL-C (+66%;  $P < 0.05$ ) than controls without changing the total cholesterol or LDL-C.

### Discussion

It is well known that normal conditions in early periods of life are essential for healthy development of humans, and several environmental factors, such as smoking exposure, at this stage can lead to complications in growth and future



**Figure 2** Photomicrography of adipose tissue at 21 days. (A) Retroperitoneal adipose tissue from control rats, (B) retroperitoneal adipose tissue from SE rats, (C) subcutaneous adipose tissue from control rats, (D) subcutaneous adipose tissue from SE rats, (E) morphometric analysis of retroperitoneal adipose tissue from pups who were exposed to tobacco smoke (black) or not (gray) during lactation, and (F) morphometric analysis of subcutaneous adipose tissue from pups who were exposed to tobacco smoke (black) or not (gray) during lactation. Values represent mean and s.e.m. of six pups per group. \* $P < 0.05$ .

**Table 2** Blood glucose and hormones of 21-day-old offspring who were exposed to tobacco smoke or not during lactation. Values represent mean  $\pm$  s.e.m. of eight rat pups per group

	Control group	Smoke group
Glycemia (mg/dl)	100.2 $\pm$ 3.1	98.7 $\pm$ 4.1
Serum insulin ( $\mu$ U/ml)	44.6 $\pm$ 3.1	32.0 $\pm$ 2.7 <sup>a</sup>
Serum leptin (ng/ml)	9.8 $\pm$ 1.5	8.3 $\pm$ 0.6
Adrenal catecholamine content ( $\mu$ M/mg)	0.62 $\pm$ 0.07	0.98 $\pm$ 0.09 <sup>a</sup>
Serum corticosterone (ng/ml)	267.2 $\pm$ 45.9	428.4 $\pm$ 28.0 <sup>a</sup>
Serum TT <sub>3</sub> (ng/dl)	121.6 $\pm$ 7.0	109.1 $\pm$ 11.7
Serum FT <sub>4</sub> (ng/dl)	0.76 $\pm$ 0.09	1.21 $\pm$ 0.13 <sup>a</sup>
Serum TSH (ng/ml)	0.22 $\pm$ 0.04	0.19 $\pm$ 0.01

TT<sub>3</sub>, total triiodothyronine; FT<sub>4</sub>, free thyroxine.

<sup>a</sup>Significant differences between groups.

metabolic disorders. For the first time, we evidenced that exposure to tobacco smoke in postnatal life through inhalation (direct exposure of second-hand) and lactational transfer of compounds in cigarette (due to maternal inhalation) is harmful for adiposity and endocrine function of the progeny. High serum cotinine concentrations in pups exposed to tobacco smoke during lactation confirms the effectiveness of our experimental model.

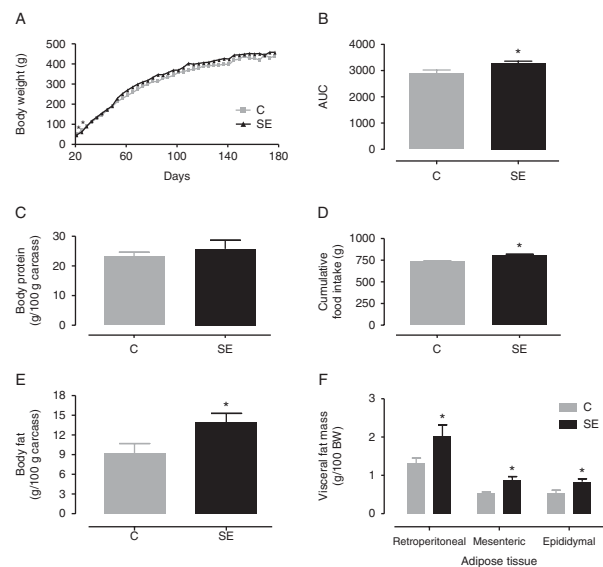
### Neonatal alterations

In our previous data, we failed to show changes in body weight of 21-day-old pups who were fed by mothers exposed to tobacco smoke (Santos-Silva *et al.* 2011). Here, pups who were directly exposed to smoke by inhalation and breast milk presented lower length, body weight, and retroperitoneal fat mass at weaning. A possible mechanism to explain these findings is the inhibitory effect of cigarette smoke in lipid accumulation (Shimada *et al.* 2009). It showed lower body weight and VFM associated with tobacco SE in adult rodents (Li & Kane 2003, Chen *et al.* 2007, 2008) and in humans (Klesges *et al.* 1998, Stice & Martinez 2005, Chiolero *et al.* 2008), probably due to the increase in metabolic rate and decrease in metabolic efficiency. However, we did not find any report of the tobacco exposure effect on the neonatal period.

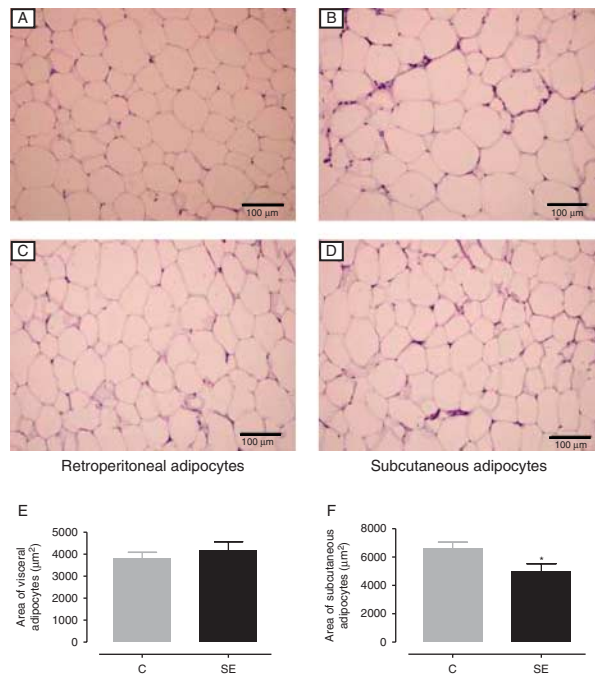
A relationship between prenatal nicotine exposure and higher epididymal adipocyte area in weaned rats was already shown (Somm *et al.* 2008). However, the effect of neonatal tobacco SE on the adipocyte morphology was still unknown.

The morphological analysis of the subcutaneous and retroperitoneal adipose compartments of SE pups at weaning showed distinct responses to tobacco SE, which can be explained by the concept that visceral and subcutaneous adipose tissues present distinct biological and

functional characteristics (Ibrahin 2010, Gil *et al.* 2011). The higher subcutaneous adipocyte area can be explained by its characteristic of triglyceride storage. As lactating dams who were exposed to smoke have higher milk triglyceride transfer (Santos-Silva *et al.* 2011), SE pups who were fed with a milk rich in triglycerides are likely to have a higher uptake of this lipid by subcutaneous adipocyte tissues. Distinctively, the lower retroperitoneal adipocyte area could be associated with the fact that this tissue is more sensitive to a lipolytic activity of catecholamines (Ibrahin 2010). In fact, we detected higher adrenal total catecholamine content in SE pups, and if it means a higher



**Figure 3** (A) Body weight, (B) AUC of food intake evaluation, (C) Total body protein, (D) Cumulative Food intake, (E) Total body fat and (F) White adiposetissue depots of adult rats who were exposed to tobacco smoke (black) or not (gray) during lactation. Values represent mean and s.e.m. of eight pups per group. \* $P < 0.05$ .



**Figure 4**

Photomicrography of adipose tissue at 180 days. (A) Retroperitoneal adipose tissue from control rats, (B) subcutaneous adipose tissue from control rats. (C) retroperitoneal adipose tissue from SE rats. (D) subcutaneous adipose tissue from SE rats, (E) morphometric analysis of retroperitoneal adipose tissue from pups who were exposed to tobacco smoke (black) or not (gray) during lactation, (F) morphometric analysis of subcutaneous adipose tissue from pups who were exposed to tobacco smoke (black) or not (gray) during lactation. Values represent mean and S.E.M. of six pups per group. \* $P < 0.05$ .

hormone release, this effect could help to explain the reduced area of retroperitoneal adipocytes.

Regarding glucose homeostasis, we recently evidenced in rats that dams exposed to tobacco smoke displayed lower insulin levels and unchanged glycemia (Santos-Silva *et al.* 2011). Here, at weaning, SE pups also presented hypoinsulinemia with no change in fasting serum glucose, reinforcing the association between direct tobacco SE and decrease in insulinemia that was observed in other models with adult mice (Chen *et al.* 2007, 2008). This alteration can suggest higher insulin sensitivity or a lower insulin secretion. This last possibility was previously described in pancreatic islets, which were acutely treated with nicotine (Yoshikawa *et al.* 2005).

The hypercorticosteronemia and higher catecholamine content in adrenal medulla of SE pups suggest higher stimulation of the adrenal gland function that can make the animals more sensitive to environmental stressor conditions. As these changes were quite similar to those previously observed in suckling pups whose

mothers were exclusively exposed to nicotine during lactation (Oliveira *et al.* 2010), we suggest that the changes induced by neonatal tobacco SE are due to the presence of nicotine, which can be inhaled and present in the milk. The higher adrenal catecholamine content in SE offspring can be due to a higher biosynthesis or a lower secretion. As those animals supposedly had higher visceral adipocyte lipolytic activity, it is more likely that the catecholamine secretion is increased due to a higher production. Also, those animals that had hypercorticosteronemia and glucocorticoids have a well-known stimulatory effect on phenylethanolamine *N*-methyltransferase in adrenal medullae, an enzyme that converts norepinephrine into epinephrine. Accordingly, the increase in catecholamine content may reflect a higher epinephrine production.

Despite the known anti-thyroid action described for some cigarette components such as thiocyanate and perchlorate (Dorea 2004, Steinmaus *et al.* 2007, Pearce & Braverman 2009), data from the literature addressing smoking and thyroid function are still controversial. Passive smoking induced lower  $T_3$ ,  $T_4$ , and TSH levels in adult women (Soldin *et al.* 2009) or higher  $T_3$  and  $T_4$  in adult men and women (Metsios *et al.* 2007); nicotine exposure did not change thyroid hormones and TSH levels in adult male rats (Colzani *et al.* 1998); and maternal smoking during pregnancy increased TSH levels in the blood cord (Shields *et al.* 2009). Here, the 21-day-old SE pups showed only higher serum  $T_4$ , suggesting that tobacco smoke components act on peripheral TH metabolism, such as extra-thyroidal deiodination or liver non-deiodinative metabolism (glucuronidation or desamination). This higher  $T_4$  without significant changes in TSH reflects an inappropriate pituitary

**Table 3** Blood glucose and hormones of 180-day-old offspring who were exposed to tobacco smoke or not during lactation. Values represent mean  $\pm$  S.E.M. of eight rat pups per group

	Control group	Smoke group
Glycemia (mg/dl)	69.9 $\pm$ 2.6	77.7 $\pm$ 1.8 <sup>a</sup>
Serum insulin ( $\mu$ IU/ml)	25.9 $\pm$ 2.4	24.3 $\pm$ 2.8
Serum adiponectin (ng/dl)	8.44 $\pm$ 2.0	20.24 $\pm$ 4.3 <sup>a</sup>
Serum leptin (ng/ml)	9.7 $\pm$ 1.8	17.9 $\pm$ 2.2 <sup>a</sup>
Adrenal catecholamine content ( $\mu$ M/mg)	1.4 $\pm$ 0.3	0.8 $\pm$ 0.1 <sup>a</sup>
Serum corticosterone (ng/ml)	347.9 $\pm$ 33.4	203.8 $\pm$ 47.9 <sup>a</sup>
Serum ACTH (ng/ml)	3.42 $\pm$ 0.31	4.49 $\pm$ 0.65
Serum $TT_3$ (ng/dl)	51.6 $\pm$ 5.0	88.4 $\pm$ 9.1 <sup>a</sup>
Serum $FT_4$ (ng/dl)	1.27 $\pm$ 0.21	2.00 $\pm$ 0.15 <sup>a</sup>
Serum TSH (ng/ml)	0.19 $\pm$ 0.01	0.26 $\pm$ 0.03 <sup>a</sup>

<sup>a</sup>Significant differences between groups.

**Table 4** Lipid profile of 180-day-old offspring who were exposed to tobacco smoke or not during lactation. Values represent mean  $\pm$  s.e.m. of eight rat pups per group

	Control group	Smoke group
Total cholesterol (mg/dl)	62.3 $\pm$ 3.9	86.5 $\pm$ 14.0
Triglycerides (mg/dl)	48.9 $\pm$ 3.5	80.7 $\pm$ 11.7 <sup>a</sup>
HDL-C (mg/dl)	21.7 $\pm$ 1.1	41.5 $\pm$ 2.6 <sup>a</sup>
LDL-C (mg/dl)	30.9 $\pm$ 3.7	43.3 $\pm$ 9.9
VLDL-C (mg/dl)	9.7 $\pm$ 0.7	16.2 $\pm$ 2.4 <sup>a</sup>

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very-low-density lipoprotein cholesterol.

<sup>a</sup>Significant differences between groups.

response to T<sub>4</sub>, which can occur in situations of chronic diseases or malnutrition, typically found in low T<sub>3</sub> syndrome. Despite the fact that serum T<sub>3</sub> is not significantly lower, it was 10% lower than the values observed in the control group. This hypothesis should be tested for further understanding of the relationship between tobacco smoke and thyroid dysfunction. We evidenced that neonate pups whose mothers were exposed to nicotine during lactation presented a primary hypothyroidism (Oliveira *et al.* 2009). Therefore, in this study, it is likely that the other cigarette compounds changed the effects of nicotine on thyroid function.

### Adulthood alterations

After weaning, the SE group presented a catch-up growth, because body weights of SE and control groups were similar in adult life. Furthermore, SE offspring showed a higher total and VFM that could not be explained only by the small increment of cumulative food intake. The development of late-onset obesity is frequently associated with maternal smoking during gestation in humans (von Kries *et al.* 2002, Wideroe *et al.* 2003, Goldani *et al.* 2007, Koshy *et al.* 2010, Durmus *et al.* 2011) and with maternal nicotine exposure during gestation/lactation in experimental studies (Holloway *et al.* 2005, Somm *et al.* 2008, 2009, Oliveira *et al.* 2009). Here, the same outcome was found in rats exposed to tobacco smoke exclusively in postnatal life both by transfer through the milk and direct inhalation (sidestream smoking).

Concerning adipocyte morphology, despite higher adiposity, adult SE offspring showed no change in visceral adipocyte area. However, these offspring were programmed for a lower subcutaneous adipocyte area. Some studies showed that subcutaneous abdominal adipocyte area is positively associated with unfavorable metabolic indexes (Imbeault *et al.* 1999). Larger adipocytes release

lower amounts of adiponectin (Skurk *et al.* 2007, Bambace *et al.* 2011). Thus, the lower subcutaneous adipocyte area may counterbalance the higher VFM, which could be responsible for hyperglycemia and hypertriglyceridemia, increasing adiponectin production. As previously proposed by Miyazaki *et al.* (2010), our findings suggest that reducing adipocyte size may be a potential strategy to decouple obesity from obesity-related diseases, as smoke-exposed adult animals were programmed for obesity but had higher adiponectin and HDL-C serum levels.

In adult life, higher body adiposity, hyperleptinemia and higher food intake are suggestive of central leptin resistance (de Oliveira *et al.* 2010), as physiologically hyperleptinemia causes hypophagia. Among the cigarette compounds, nicotine may have triggered leptin resistance because we previously showed that maternal nicotine exposure during lactation programmed for lower OB-R, lower JAK2, lower p-STAT3, and higher SOCS3, which is a well-known intracellular inhibitor of the leptin signaling pathway in the hypothalamus of the adult progeny, featuring a central leptin resistance (de Oliveira *et al.* 2010).

Different from that observed at weaning, adult SE offspring presented higher fasting glucose without changes in insulinemia, suggesting a glucose intolerance programming, also observed in adult rats exposed to nicotine on perinatal life (Somm *et al.* 2009, de Oliveira *et al.* 2010). It is possible that this inappropriate insulin secretion is the beginning of a pancreatic  $\beta$ -cell failure.

Again, in contrast to that observed at weaning, adult SE offspring had hypocorticosteronemia and lower adrenal catecholamine content. A similar profile was observed in an experimental model of nicotine withdrawal, in which CRH mRNA expression was unchanged, suggesting subsensitivity of the HPA axis immediately after smoking cessation (Semba *et al.* 2004). However, the adrenal profile of SE offspring is the opposite of the adult offspring programmed by nicotine during lactation, which presented hypercorticosteronemia and higher adrenal catecholamine content (Pinheiro *et al.* 2011). Therefore, regarding adrenal function, the isolated nicotine effects differ from those elicited by the combined tobacco smoke components.

Regarding thyroid function, adult SE offspring showed higher T<sub>3</sub>, T<sub>4</sub>, and TSH serum levels, showing that this higher thyroid hormone production is consequent to a thyrotrope hyperfunction, which is contrary to the secondary hypothyroidism found in adult rats whose mothers were exposed to nicotine during lactation (Oliveira *et al.* 2009). The hyperphagia found in adult SE



offspring suggests a functional hyperthyroidism, as  $T_3$  increases food intake through its action on energy expenditure and appetite circuits of the nervous system (Amin *et al.* 2011). The association between higher adiposity and higher thyroid hormones (that leads to an increase in energy expenditure) in the SE group may seem to be contradictory. However, some studies have described an increase in  $T_3$  and higher adiposity as an adaptive mechanism that occurs in obesity (Reinehr *et al.* 2006, 2008, Alevizaki *et al.* 2009, de Pergola *et al.* 2010). The hyperthyroid status of the SE group can be partially explained by hyperleptinemia as leptin has a well-known stimulatory action on the thyroid axis (Casanueva & Dieguez 1999, Nowak *et al.* 2002, Ortiga-Carvalho *et al.* 2002, de Oliveira *et al.* 2007). There is also an association between hyperthyroidism and hyperadiponectinemia (Yaturu *et al.* 2004, Seifi *et al.* 2012), which could help to explain the present findings in adult SE rats.

Even without ever having smoked, adults or adolescents exposed to environmental tobacco smoke have a higher risk of developing cardiometabolic diseases (Weitzman *et al.* 2005, Venn & Britton 2007, Xie *et al.* 2010). We evidenced that neonatal tobacco smoke programs for higher triglycerides, VLDL-C, and HDL-C in adult life. Initially, higher HDL-C seems to be contradictory as tobacco SE is associated with lower serum HDL-C in children (Nagel *et al.* 2009, Hirata *et al.* 2010). However, increased HDL-C was found in adult female rats who were exposed to tobacco smoke during gestation without change in total antioxidant capacity, suggesting that higher HDL-C is non-functional (Ng *et al.* 2009). Concerning higher triglyceride and VLDL-C levels in adult SE pups, an increase in these serum lipids has been described in both active and passive smokers (Whig *et al.* 1992). Thus, the changes in adiposity, hyperglycemia, and hypertriglyceridemia observed in SE adult progeny suggest that neonatal tobacco smoke acts as one imprinting/priming factor for future development of the metabolic syndrome. As adult offspring whose mothers were exposed to nicotine during lactation did not develop changes in lipid profile (Oliveira *et al.* 2010), the changes in lipid profile observed here were due to other cigarette components.

The comparison among distinct models of tobacco smoke and nicotine exposure leads to the following relevant inferences: i) the differences observed between the effects of tobacco SE exclusively through milk (indirect tobacco exposure) and tobacco SE both by breast milk and by direct inhalation are related to the serum cotinine levels of the offspring (indirect exposure model:  $17.9 \pm 4.2$  ng/ml vs milk transfer and inhalation exposure

model:  $145.3 \pm 5.1$  ng/ml), confirming the greater exposure to tobacco smoke in pups of the second experimental model; ii) in the tobacco SE model, we observed some similar effects compared with isolated nicotine (e.g. higher adrenal function at weaning and higher adiposity at adulthood); however, we also observed opposite effects between the two models (e.g. body weight at weaning and thyroid and adrenal functions at adulthood). Despite similar and/or different findings among the three experimental models (smoke component exposure only through milk, smoke chemical exposure via milk, and direct inhalation and isolated nicotine exposure), we cannot forget that the exposure to tobacco presented in this study is the model that best represents what happens to children exposed to environmental smoking. Also, the comparison between the different models is important to identify the factors and mechanisms that cause the changes associated with tobacco exposure. These factors could be better explored in future experiment performing, for instance, some dynamic tests such as GTT and CRH stimulation.

We conclude that neonatal tobacco exposure only during the lactation period (derived by either inhalation or milk transfer of constituents from cigarette smoke) was capable of promoting important metabolic changes since childhood. These metabolic disorders worsened during development, as adult animals were programmed for obesity, hyperleptinemia, hyperglycemia, dyslipidemia, hypocorticosteronemia, and thyroid dysfunction. Therefore, wide distribution of information about the importance of a tobacco-free environment is necessary to ensure adequate development in children.

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#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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