Postnatal overnutrition programs the thyroid hormone metabolism and function in adulthood

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Abstract

Early overnutrition (EO) during lactation leads to obesity, leptin resistance and lower thyroid hormone (TH) levels during adulthood. To better understand the biological significance of this thyroid hypofunction, we studied the long-term effects of postnatal EO on both the function of hypothalamic–pituitary–thyroid (HPT) axis and the metabolism and action of TH. To induce EO, the litter size was reduced to three pups per litter (small litter (SL) group) on the third day of lactation. In the controls (normal litter group), litter size was adjusted to 10 pups per litter. Rats were killed at PN180. TRH content and in vitro TSH were evaluated. Iodothyronine deiodinase (D1 and D2) activities were measured in different tissues. Mitochondrial α-glycerol-3-phosphate dehydrogenase (mGPD), uncoupling protein 1 (UCP1) and TH receptor (TRβ1) were evaluated to assess TH action. The SL group presented lower TRH, intra-pituitary and released TSH levels, despite unchanged plasma TSH. They presented lower D1 activity in thyroid, muscle and white adipose tissue (WAT) and higher D2 activity in the hypothalamus, pituitary, brown adipose tissue (BAT) and WAT, which confirmed the hypothyroidism. UCP1 in BAT and TRβ1 in WAT were decreased, which can contribute to a lower catabolic status. Despite the lower TH, the D2 activity in the thyroid, heart and testes was unchanged. Hepatic D1, mGPD and TRβ1 were also unchanged in SL rats, suggesting that the TH conversion and action were preserved in the liver, even with lower TH. Thus, this model indicates that postnatal EO changes thyroid function in adult life in a tissue-specific way, which can help in the understanding of obesogenesis.

Key Words

- overnutrition
- lactation
- hypothyroidism
- deiodinase
- programming

Introduction

The high prevalence of childhood obesity is considered a worldwide health problem. In addition to genetic factors, epigenetic factors can contribute to this epidemic. Epidemiological, clinical and experimental studies have shown that the nutritional, hormonal and environmental factors observed in the early stages of development have long-term effects on hormonal and metabolic homeostasis. This association can be characterized as a metabolic programming phenomenon, which is defined as an association between physical and chemical stimuli in early life and future functional status (de Moura et al. 2008).

Overnutrition during lactation represents a risk factor for obesity, diabetes and cardiovascular diseases. Animals raised in small litters have hyperphagia, obesity, hypertriglyceridemia and hyperinsulinemia at adulthood.
Thus, these animals make good models for metabolic syndrome programmed by early-life overnutrition (Plagemann et al. 1992). Our group proved that postnatal early overnutrition (EO) programs for thyroid dysfunction, which is characterized by low T₄ and T₃ serum levels, which may, in turn, be related to the changes in the leptin-signaling pathway in hypothalamic–pituitary–thyroid (HPT) axis (Rodrigues et al. 2009). It is well known that a disturbance in thyroid function is associated with marked changes in both energy expenditure and body weight. However, to date, there has been no evidence regarding whether this hypothyroidism has biological significance in the EO model.

The enzyme 5′-iodothyronine deiodinase is responsible for the conversion of T₄ to T₃. In other words, this enzyme regulates tissue and serum TH availability. Based on some functional criteria, tissue-specific distribution and protein sequences, 5′-deiodinases are classified as two enzymes: type 1 (D1) and 2 (D2). In rodents, D1 is mainly detected in the liver, thyroid and kidney, and it generates most of the circulating T₃, whereas D2 is mainly found in target tissues, such as the brain, pituitary and brown adipose tissue (BAT), and catalyzes the local T₄ to T₃ conversion. However, according to our current understanding, D1 and D2 make similar contributions to plasma T₃ production. In hypothyroidism, a decrease in D1 and an increase in D2 occur; conversely, the opposite effect was found to occur in hyperthyroidism (Bates et al. 1999, Bianco & Kim 2006, St Germain et al. 2009). To our knowledge, there are no data regarding the profiles of deiodinases in EO animals.

Therefore, we aimed to investigate the long-term effects of postnatal EO on thyroid hormone (TH) metabolism by evaluating the D1 and D2 activities in different tissues. By investigating these long-term effects, we can enhance our understanding regarding the degree of hypothyroidism previously reported in EO animal models. Because circulating thyrotropin (TSH) was unaltered in adult small litter (SL) rats, we evaluated the thyrotropin release hormone (TRH) in the hypothalamus, the TSH in the pituitary gland and the in vitro TSH secretion both under basal conditions and after TRH stimulation. Additionally, we studied the uncoupling protein 1 (UCP1) in BAT and the mitochondrial α-glycerol-3-phosphate dehydrogenase (mGPD) activity in the liver, which are both TH-dependent proteins, as well as the expression of β1 isoform of the TH receptors (TRβ1) to better address the TH action in liver and adipose tissue in this programming model. We hypothesized that changes in TH metabolism, action and function can lead to a lower metabolic rate in this experimental model, which can help explain the development of obesity.

Materials and methods

Our experimental protocol was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, according to the Brazilian Law no. 11.794/2008. Wistar rats were housed in a temperature-controlled room with 12 h light:12 h darkness cycle. Four-month-old virgin female rats were caged with a single male rat (3:1). During pregnancy and lactation, mothers were housed in individual cages and were administered water and a standard commercial diet ad libitum. To induce postnatal EO, 3 days after delivery, the litter size was adjusted to three male rats per each litter (SL) (Plagemann et al. 1992, Rodrigues et al. 2009). Litters containing ten pups per mother served as controls (normal litter, NL). After weaning (postnatal day-PN 21), all pups received standard chow. Body weight and food intake were measured until the day of sacrifice. One animal was randomly chosen from each litter and was used in the subsequent experiments, thus giving a total of 12 pups per group from 24 different litters (SL = 12 and NL = 12). Both groups were killed at PN180 by rapid decapitation with no prior anesthesia to collect blood, white adipose tissue (WAT), BAT, soleus (skeletal muscle), liver, hypothalamus, pituitary, thyroid, heart and testes.

Visceral fat content

Mesenteric, epididymal and retroperitoneal WAT were weighed for evaluation of central adiposity (Rodrigues et al. 2009).

Plasma hormones measurement

Blood was centrifuged (1000 g, 30 min, 4 °C) and plasma was stored at −20 °C until assay. Determinations were performed in one assay, and the samples were analyzed in duplicate. Plasma total T₃ (TT₃) and free thyroxine (FT₄) were measured by RIA (ICN Pharmaceuticals, Inc., Orangeburg, NY, USA). Plasma TSH was measured by specific RIA, using a rat TSH kit supplied by the National Institute of Health (NIH, St Louis, MO, USA) and expressed in terms of the reference preparation provided (RP-3). Limits of detection were 25 ng/dl for T₃, 0.3 ng/dl for T₄ and 0.18 ng/ml for TSH and intra-assay variations were 3.6, 7.5 and 3.1% respectively.
Deiodinase activities measurement

Tissues were dissected, kept in liquid nitrogen and immediately processed for enzymatic activity. D1 and D2 activities were measured in WAT, BAT, liver, hypothalamus, pituitary, thyroid, skeletal muscle, heart and testis homogenates (Tris-HCl 50 mM, pH 6.8), using the previously reported method based on the release of $^{125}$I from $^{125}$I-reverse T$_3$ (PerkinElmer/NEN, Boston, MA, USA) (Bates et al. 1999, Lisboa et al. 2008). Assays were performed in phosphate buffer containing 1 mM EDTA at pH 6.9. The D1 assay was performed in the presence of 1.5 μM of rT$_3$, 10 mM of DTT and 100 nM of T$_4$ (to inhibit D2). The D2 assay was performed with 2 nM rT$_3$, 40 mM DTT and 1 mM 6-n-propylthiouracil (PTU) (to inhibit D1). Equal aliquots of $^{125}$I-rT$_3$ were purified by paper electrophoresis and placed into each tube assay; the reaction was started by the addition of tissue samples. A blank tube was run in parallel with each assay and contained 50 μl of the substrate solution and 50 μl of buffer, which had its values subtracted from the enzyme samples. Reactions were performed in a shaking-bath at 37°C and stopped after 30 min (D1 in liver and thyroid), 60 min (D2 in hypothalamus, pituitary and thyroid) or 120 min (D1 in soleus; D2 in WAT, BAT, heart and testis). These reactions were stopped by the addition of a mixture of 8% BSA and 10 mM PTU, followed by cold 20% trichloroacetic acid. Samples were centrifuged (1500 g, 4°C, 5 min) and 200 μl of the supernatants were applied to Dowex 50 W-X2 columns (100–200 mesh hydrogen form BioRad). Free $^{125}$I eluted from columns with 10% acetic acid was measured in a γ-counter. The percent of deiodination in the presence of the enzyme was ~10–20%, and the non-specific free $^{125}$I represented <1–2% of the total radioactivity in the mixture. The specific enzyme activity was expressed by femtomoles, picomoles or nanomoles of rT$_3$ deiodinated/h mg of protein.

mGPD activity measurement

Because liver GPD reflects the TH action and is being considered a marker of thyroid status, we measured GPD activity in mitochondrial fraction using phenazine methosulfate (PMS) as an electron transporter between the reduced enzyme and iodonitrotetrazolium chloride violet (INT) (Oliveira et al. 2007). The assay was done with 0.1 M DL-α-glycerophosphate diluted in KCN/KPB and a solution of 7.9 mM INT–0.12 mM PMS. Samples were analyzed at 500 nm and the values were expressed as absorbance (O.D)/mg of protein.

Pituitary explants: secretion of basal TSH and post-TRH stimulation

The in vitro TSH secretion was performed as previously reported (Lisboa et al. 2008). Six pituitaries of SL and NL rats were quickly dissected out and the anterior pituitary was separated from posterior pituitary and transected with a longitudinal midline cut (12 hemi-pituitary per group). Each anterior hemi-pituitary was immediately transferred to a tube that contained 1 ml of minimum essential medium (MEM, Sigma–Aldrich), and was incubated at 37°C in an atmosphere of 95% O$_2$ and 5% CO$_2$ in a Dubnoff metabolic shaker (50 cycles/min). After a 20-min preincubation period, the medium was removed and hemi-pituitaries were suspended in 1 ml of fresh medium. After 60 min, aliquots were collected to determine the basal TSH release. Next, TRH (Sigma–Aldrich) was added at a final concentration of 50 nM; after a 30-min incubation, aliquots were collected to determine the TRH-stimulated TSH release. At the end of the incubation period, each hemipituitary was homogenized in PBS, at pH 7.6, for intra-pituitary TSH content measurement. The TSH levels were measured by RIA as described above. The results are expressed as ng of TSH at basal conditions, as the difference between TSH release before and after TRH incubation and as μg of TSH per mg protein content of pituitary gland.

TRH, UCP1 and TRβ1 protein content measurement

The expression of TRH was detected in the hypothalamus, UCP1 was determined in BAT and TRβ1 was performed in the liver and WAT by western blotting, using β-actin as an internal control. The hypothalamus, BAT and liver were homogenized in ice-cold lysis buffer (50 mM HEPES, 1 mM MgCl$_2$, 10 mM EDTA, Triton X-100 1%, pH 6.4) and WAT was homogenized in ice-cold lysis buffer (20 mM Tris-HCl, 10 mM NaF, 150 mM, 5 mM and 0,1% SDS, pH 7.4), both of which contained Complete Protease Inhibitor Tablets (Roche Diagnostics Corporation). After centrifugation (7500 g for 5 min), homogenates were stored at −20°C. Protein concentrations were determined using the BCA protein kit assay (Thermo Scientific, Rockford, IL, USA). Samples (30 μg total protein) were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes (Hybond P ECL membrane; Amersham Pharmacia Biotech). Membranes were blocked with 5% albumin in Tween-TBS (0.1% Tween-20 and 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 1 h. Next, membranes were washed with TBS and incubated overnight with the following
primary antibodies used: anti-TRH diluted 1:100 (Life-Spam Biosciences, Seattle, WA, USA, catalog number LS-C76393); anti-UCP1 diluted 1:500 (Sigma–Aldrich, catalog number U6382); and anti-TRβ1 diluted 1:1000 (Santa Cruz Biotechnology, Inc. catalog number sc-10822). Subsequently, membranes were washed and incubated with proper secondary antibodies for 1 h at room temperature. Then, immunoreactive proteins were visualized by exposure to X-ray film. The density of the TRH, UCP-1 and TRβ1 bands was also quantified using the Image J 1.34s Software (Wayne Rasband NIH, Boston, MA, USA).

Statistical analysis

The data were analyzed using the GraphPad Prism 5 program (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean ± S.E.M. The results were analyzed using unpaired Student’s t-tests, except for the TSH (Mann–Whitney U test). Significance was assumed at the level of $P<0.05$.

Results

As expected, early overfeeding was associated with higher visceral adiposity during adulthood (+65% retroperitoneal; +51% epididymal; $P<0.05$; +61% mesenteric; $P<0.01$; data not shown). At PN180, the SL rats presented lower plasma T₃ (−25%) and T₄ (−28%), but unchanged TSH levels (Table 1) compared with NL rats. These results are in agreement with the results obtained in a previous study (Rodrigues et al. 2009).

In the hypothalamus, the adult SL group showed a lower TRH content (−22%; $P<0.05$; Fig. 1). As depicted in Table 1, pituitaries from the SL group presented a decrease in basal TSH release (−72%; $P<0.05$), a reduction of TSH release in response to TRH (−30%; $P<0.05$) and lower TSH tissue content (−62%; $P<0.05$) compared with controls.

In PN180, the SL group showed no change in D1 and mGPD activities or in TRβ1 protein expression in the liver (Fig. 2).

Concerning other deiodinase activities (Table 2), SL rats presented lower D1 activity in the thyroid (−13%), WAT (−64%) and skeletal muscle (−59%), and higher D2 activity in the hypothalamus (−76%), pituitary (+17%), BAT (+70%) and WAT (+62%). Postnatal EO did not change D2 activity in the thyroid, heart and testis at adulthood (Table 2).

The protein expression of TRβ1 in WAT and UCP1 in BAT was lower in SL rats than in NL rats (−53% and −41%; $P<0.05$ – Fig. 3).

Discussion

Nutritional factors influence the HPT axis, which regulates energetic metabolism and, consequently, body weight (Hollenberg 2008, Reinehr 2008, Pearce 2012). Previously, we showed that rats raised in small litters had secondary hyperthyroidism (higher serum TSH, T₄ and T₃) at weaning, but that during adulthood, rats developed...
lower serum total T₃ and free T₄ despite normal TSH (Rodrigues et al. 2009). In fact, some studies have shown that hyperthyroidism during lactation is associated with hypothyroidism in adult life (Walker & Courtin 1985, Rodrigues et al. 2009, 2011). These intriguing findings in the adult life of SL rats raised two main questions regarding the present study: i) does the programmed hypothyroidism functional? and ii) what is the cause for inappropriately low TSH? The current work shows the impact of postnatal EO and transient hyperthyroidism on late TH metabolism, action and function. We found that SL rats were programmed for central hypothyroidism. Here, the lower D1 activity in the thyroid, muscle and WAT and higher D2 activity in the hypothalamus, pituitary, BAT and WAT are in accordance with the hypothyroid status of the adult offspring. Because TH are important regulators of WAT storage through the regulation of adipocyte differentiation, lipogenic/lipolytic metabolism and mitochondrial activity (Pearce 2012), the lower TRβ1 content in WAT indicates that some of these processes were compromised, including adipogenesis, lipogenesis and lipolysis, and adipocytokine secretion. Additionally, the lower UCP1 content in BAT suggests a decrease in thermogenesis corroborating based on observed hypothyroid status.

Leptin directly stimulates TRH in the hypothalamic PVN nucleus as well as the production and secretion of TSH in the pituitary (Itoh et al. 2003). In PN180, although SL rats present normal TSH and leptin levels (Rodrigues et al. 2009, Conceição et al. 2011), they exhibited leptin resistance at the level of the hypothalamus and pituitary level (Rodrigues et al. 2009, Rodrigues et al. 2011). This finding is in agreement with the decrease observed in TRH protein expression in the hypothalamus and the decreased intra-pituitary TSH with lower basal and TRH-stimulated TSH secretion. Thus, we demonstrated that SL rats were programmed for a central hypothyroidism (low TRH).

Although the plasma TSH can only be immunologically measured, its bioactivity could be lower, which would explain the lower plasma T₄ and T₃ levels and the possible decrease in TSH clearance. In fact, a high sialic acid content of TSH can reduce the biological activity of plasma TSH and increase its half-life (Miura et al. 1989, Horimoto et al. 1989). In PN180, although SL rats present normal TSH and leptin levels (Rodrigues et al. 2009, Conceição et al. 2011), they exhibited leptin resistance at the level of the hypothalamus and pituitary level (Rodrigues et al. 2009, Rodrigues et al. 2011). This finding is in agreement with the decrease observed in TRH protein expression in the hypothalamus and the decreased intra-pituitary TSH with lower basal and TRH-stimulated TSH secretion. Thus, we demonstrated that SL rats were programmed for a central hypothyroidism (low TRH).

### Table 2  Deiodinase activities of adult rats (PN180) in the programming model of postnatal early overnutrition. Results expressed as mean ± S.E.M. of 12 rats per group. *P<0.05

<table>
<thead>
<tr>
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<th>NL</th>
<th>SL</th>
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<tbody>
<tr>
<td>Hypothalamic D2 (fmol 125I/h mg ptn)</td>
<td>91.3 ± 3.9</td>
<td>160.8 ± 17.0*</td>
</tr>
<tr>
<td>Pituitary D2 (fmol 125I/h mg ptn)</td>
<td>96.1 ± 2.8</td>
<td>112.2 ± 5.0*</td>
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<tr>
<td>Thyroid D1 (nmol 125I/h mg ptn)</td>
<td>6.2 ± 0.2</td>
<td>5.4 ± 1.2*</td>
</tr>
<tr>
<td>Thyroid D2 (pmol 125I/h mg ptn)</td>
<td>2.2 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>BAT D2 (fmol 125I/h mg ptn)</td>
<td>16.5 ± 3.1</td>
<td>28.1 ± 3.2*</td>
</tr>
<tr>
<td>WAT D1 (nmol 125I/h mg ptn)</td>
<td>63.8 ± 14.9</td>
<td>22.9 ± 3.7*</td>
</tr>
<tr>
<td>WAT D2 (fmol 125I/h mg ptn)</td>
<td>52.4 ± 7.6</td>
<td>84.8 ± 9.3*</td>
</tr>
<tr>
<td>Muscle D1 (pmol 125I/h mg ptn)</td>
<td>25.5 ± 1.5</td>
<td>10.5 ± 0.9*</td>
</tr>
<tr>
<td>Heart D2 (fmol 125I/h mg ptn)</td>
<td>14.9 ± 0.8</td>
<td>13.6 ± 1.6</td>
</tr>
<tr>
<td>Testis D2 (fmol 125I/h mg ptn)</td>
<td>28.1 ± 1.7</td>
<td>26.7 ± 4.2</td>
</tr>
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NL, normal litter; SL, small litter; D1, type 1 deiodinase; D2, type 2 deiodinase; BAT, brown adipose tissue; WAT, white adipose tissue.
et al. 1995, Persani et al. 1998, Garcia et al. 2014). In addition, the increase in pituitary D2 activity detected in SL rats may be partially responsible for the normal serum TSH levels.

The D2 activity in the thyroid, heart and testis were unchanged, despite the lower plasma TH, which suggests that other factors that were programmed by postnatal EO are more important regulators of D2 than TH is, at least in these three tissues. Moreover, no study has been specifically designed to analyze the long-term consequences of postnatal EO on iodothyronine deiodinase activities in the adult progeny. In another programming model, we showed that nutritional and hormonal factors during lactation, such as protein malnutrition (Dutra et al. 2003, Lisboa et al. 2008), hyperleptinemia (Toste et al. 2006) and hypoprolactinemia (Bonomo et al. 2008) program changes in deiodinase activity during adult life, could be caused by either hyper- or hypothyroidism. In these experimental models, hyperleptinemia at weaning was also found in neonate SL rats (Rodrigues et al. 2009) and could be responsible for programming the T4 to T3 conversion in different tissues.

Interestingly, we found no change in liver D1, mGP1 and TRβ1 in SL rats at PN180, which are well known positive TH-markers that should be decreased. This finding indicates that the intracellular conversion of T4 to T3 and the TH action were preserved in the liver in this programming model, even with lower plasma TH levels. Moreover, serum cholesterol, which must be higher in hypothyroidism, was unchanged in adult SL rats (Rodrigues et al. 2011).

The transport of TH into the liver across the cell membrane, which is required for its metabolism and action, has been studied in both humans and rats. In humans, fasting is associated with relatively normal serum TSH and low T3 levels and thus reflects a reduced peripheral T4 to T3 conversion as well as lower liver TH uptake (van der Heyden et al. 1986). In rodents, fasting decreases TH uptake in the liver and other tissues (Hennemann et al. 2001). The monocarboxylate transporter (MCT)-8, MCT10, and organic anion transporting polypeptide 1C1 (OATP1C1) are the best-characterized specific TH transporters (Alkemade et al. 2011). In fact, immunohistochemistry studies have shown the high expression of MCT8 in some tissues such as the liver, and MCT8 is thus considered an active and specific TH transporter (Friesema et al. 2003). To the best of our knowledge, there are few reports on the regulation of transporters by TH. Two studies suggested that the hypothyroidism caused by low iodine and low T3 syndrome leads to a compensatory increase in MCT8 (Hu et al. 2014), MCT10 and OATP1C (Mebis et al. 2009). Thus, in our programming model, it is possible that the intracellular TH was preserved, at least in the liver, by the higher TH uptake by transporters. Therefore, our data suggest that the SL rats developed an adaptive mechanism that ensured an adequate intracellular source of TH in the liver, which could explain the almost unchanged glucose and lipid homeostasis in SL animals. Thus, the current...
data invite further study concerning the regulation of the TH-transporters by T₃ in different tissues.

It is well known that BAT thermogenesis through UCP1 expression is the most important non-shivering process to dissipate energy and generate heat in rodents. The ablation of UCP1 gene is able to induce obesity without hyperphagia (Lowell et al. 1993). The UCP1 mRNA expression is stimulated by the sympathetic nervous system (SNS) and leptin via the SNS (Commins et al. 1999, Cettour-Rose et al. 2002). It was also shown that central leptin administration, likely due to the influence of SNS, increased D2 mRNA expression and activity in BAT (Cettour-Rose et al. 2002). The thermogenesis reduction in young adult SL rats (8-week-old) has been reported (Xiao et al. 2007), which had decreased BAT UCP1 mRNA and lower β3 adrenergic receptor expression and activation at room temperature and under cold exposition (adrenergic stimulus). Our current findings concerning BAT D2 activity and UCP1 protein content in PN180 SL rats support these previous data.

In BAT, both the D2 activity (+70%) and UCP1 content (−41%) of SL rats were consistent with their hypothyroid status. The higher D2 activity must be responsible for the local conversion of T₄ to T₃ in this tissue and, consequently, must increase UCP1 content. However, SL rats have lower T₄ (−28%), which may not generate a sufficient amount of T₃ to correct UCP1 to the control level. Although the D2 activity was almost doubled, this activity was evaluated in an in vitro assay, in which the optimal conditions for the enzyme activity were provided.

In summary, it is known that epigenetic mechanisms, such as DNA methylation, histone acetylation and deacetylation, or microRNA, induced by pre- and postnatal factors such as nutrition and hormones, may lead to a higher risk of metabolic diseases in the adult life of the offspring (de Moura et al. 2008). This explanation may help us understand the permanent changes of TRH, TSH, deiodinases, UCP-1 and TRβ1 that are caused by EO. Therefore, it is possible that overfed children can be more susceptible to thyroid disorders in adult life, an issue that deserves further epidemiological and clinical investigation.

In fact, although the mechanisms involved remain unclear, overnutrition during lactation induces long-term effects on the regulation of the HPT axis and TH metabolism and action. There is a lack of clinical, epidemiological or even experimental studies regarding hypothyroidism in adulthood caused by postnatal and childhood overnutrition. For the first time, we have shown that the decreased TH levels observed in SL rats, although mild, are functional, thus contributing to a hypometabolic profile of these programmed overweight animals. Taken together, the present data reinforce the concept of the ‘developmental origins of health and disease,’ mainly concerning the programming of the thyroid function.

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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