Experimental hypothyroidism increases apoptosis in dimethylbenzanthracene-induced mammary tumors

CONSTANZA MATILDE LÓPEZ-FONTANA[1], CORINA VERÓNICA SASSO[1], MARÍA EUGENIA MASELLI[1], FLAVIA ELIANA SANTIANO[1], SILVANA NOEMÍ SEMINO[2], FERNANDO DARÍO CUELLO CARRIÓN[1], GRACIELA ALMA JAHN[1] and RUBÉN WALTER CARÓN[1]

[1]Institute of Medicine and Experimental Biology of Cuyo, CONICET, CCT-Mendoza;
[2]Department of Diagnosis and Treatment, Laboratory of Pathological Anatomy,
University Hospital, National University of Cuyo, Mendoza, Argentina

Received April 5, 2013; Accepted May 30, 2013

DOI: 10.3892/or.2013.2648

Abstract. Epidemiological and *in vitro* data have not provided conclusive evidence concerning the involvement of thyroid hormones (THs) on mammary carcinogenesis. We used an *in vivo* model to assess the relationship between THs, adipose tissue and breast cancer development. Female Sprague-Dawley rats were treated with a dose of 7,12-dimethylbenz(a)anthracene (15 mg/rat) at 55 days of age and were then divided into four experimental groups: hypothyroid rats (HypoT, 0.01% 6-N-propyl-2-thiouracil in drinking water), untreated control (EUT); hyperthyroid rats (HyperT, 0.25 mg/kg/day T_4 s.c.) and vehicle-treated control rats. The latency of tumor appearance and the incidence and progression of tumors were determined. At sacrifice, blood samples were collected for hormone determinations and samples of tumor and mammary glands were obtained for immunohistological studies. HypoT rats had retarded growth and an increase in mammary fat. The latency was longer ($p<0.0001$), the incidence rate was lower ($p<0.05$) and tumor growth was slower in HypoT rats compared to EUT and HyperT rats. Mitotic index and PCNA immunostaining were similar in all groups. HypoT rats showed increased apoptosis ($p<0.05$) as evaluated by the apoptotic index and TUNEL staining. No differences in serum prolactin and progesterone were observed. However, circulating estradiol (E_2) was significantly lower in HypoT and HyperT rats. Serum leptin levels were reduced in HypoT rats even though the abdominal fat mass was similar in all groups. To note, the leptin level was higher in HypoT rats that developed mammary tumors than the level in non-tumoral HypoT rats. In conclusion, hypothyroidism altered animal growth, breast morphology, body composition, leptin secretion and serum E_2 enhancing apoptosis and, consequently, retarding mammary carcinogenesis in rats.

Correspondence to: Dr Rubén Walter Carón, Laboratory of Hormones and Cancer Biology, Institute of Medicine and Experimental Biology of Cuyo (IMBECU), CONICET, CCT-Mendoza, Av. Adrián Ruiz-Leal s/n, CC855, Mendoza 5500, Argentina
E-mail: rcaron@mendoza-conicet.gob.ar

Key words: leptin, estradiol, hypothyroidism, hyperthyroidism, apoptosis

Introduction

Mammary development and breast carcinogenesis are usually characterized by their hormonal control (1). Among the hormones more frequently studied, estrogens (E) and progesterone (Pg) regulate several functions of the normal or tumoral mammary epithelium, such as cell proliferation and apoptosis. Approximately one-third of breast cancers maintain E-dependence for growth and the concentration of estrogen receptors (ERs) in malignant breast tissues is an indicator of this hormonal dependence (2). E are the main steroid hormones implicated in the induction of breast cancer, but experimental evidence in rodents suggests that Pg is also important in its induction and progression (3).

Prolactin (PRL) has been associated with breast cancer pathogenesis and progression. Acting at the endocrine and autocrine/paracrine levels, PRL functions to stimulate the growth and motility of human breast cancer cells (4). Epidemiologic studies have demonstrated that hyperprolactinemia is frequently associated with growth, development and a poor prognosis in mammary cancer (5). Related to PRL actions on mammary gland, the growth hormone (GH) has also been shown to affect mammary development and carcinogenesis. In rodents, GH may potentiate breast cancer since GH- or GH receptor-deficiency is associated with reduced incidence and aggressiveness of experimentally induced breast cancer (6).

Other hormonal influences on the mammary gland involves triiodothyronine (T_3) and activation of mammary thyroid hormone receptors (TRs) inducing differentiation and lobular growth in an E-like manner. However, there is controversy concerning the relationship between thyroid disorders and breast cancer incidence (7). Thyroid hormones (THs) could participate directly on mammary carcinogenesis, or indirectly through their action in changes in the patterns of secretion of other hormones such as E, Pg, PRL and/or GH, among others. It has been shown that hypothyroidism may result in reduced

incidence of primary breast carcinoma (8), and *in vitro* studies also suggest that THs affect cell proliferation and gene expression (9).

Moreover, THs have important effects on energy balance, since they influence both energy intake and expenditure. Abnormal TH levels are frequently associated with changes in body weight and modifications in the function of adipose tissue. The adipocyte is a functionally active cell and produces several biologically active adipokines such as leptin, which could contribute to an increased breast cancer risk for obese women (10). Furthermore, estradiol (E_2) is also produced by adipose tissue and it has been suggested as being responsible for the elevated risk of breast cancer in these women (10).

Since the epidemiologic and *in vitro* data have not provided conclusive evidence of the involvement of THs in mammary carcinogenesis, we used an *in vivo* model to assess the relationship between THs, adipose tissue and breast cancer development. In the present study, we investigated the effect of the hormonal milieu induced by hypothyroidism or hyperthyroidism on mammary carcinogenesis induced by 7,12-dimethylbenz(a)anthracene (DMBA) in rats.

Materials and methods

Animals. Virgin Sprague-Dawley female rats (180-200 g) bred in our laboratory were used. The animals were maintained in a light- (lights on from 06.00 to 20.00 h) and temperature-controlled room (22-24˚C); rat chow (Cargill, Córdoba, Argentina) and tap water or 6-N-propyl-2-thiouracil (PTU) solution were available *ad libitum*.

Animal maintenance and handling were performed according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animal Scientific Procedures, Act 1986). All the experimental procedures were approved by the Animal and Ethics Committee of the School of Medicine of the National University of Cuyo, Mendoza, Argentina.

Experimental protocols. In order to study the effect of hypothyroidism or hyperthyroidism on mammary carcinogenesis, the rats were treated *per os* with a single dose of DMBA (15 mg/rat) at 55 days of age and were then divided into two experimental groups and their respective controls: hypothyroid rats (HypoT, n=26) and untreated rats (EUT, n=12); hyperthyroid rats (HyperT, n=23) and vehicle-treated rats (VEH, n=5). The day of DMBA administration was considered as day 0 of the experiment.

Hypothyroidism was induced by the administration of PTU (Sigma-Aldrich, Buenos Aires, Argentina) at a concentration of 0.1 g/l in the drinking water, starting on day 3. The respective control group (EUT) was maintained under standard conditions during the study period.

Hyperthyroidism was induced by daily subcutaneous administration of L-thyroxine (T_4; Sigma-Aldrich). T_4 was dissolved in saline solution (0.42 N of NaOH) and injected s.c. daily at a dose of 0.25 mg/ml/kg starting on day 3. Control age- and weight-matched female rats received VEH s.c.

Since no statistical differences were observed between the EUT and VEH groups in any of the studied variables, we grouped and analyzed them as a single control group.

Body weight was monitored weekly. Food intake was assessed on week 10 of the experiment and expressed as the weight of rat chow consumed per rat and per day. The animals were assessed every 72 h for the appearance of the first palpable tumor. The latency, incidence and progression of tumors were determined in all groups. The rats were sacrificed by decapitation at 10:00 h on the day of diestrus when the tumors reached a volume >1,000 mm^3 or at the end of the experiment on day 200 when they did not develop mammary tumors. All the animals were weighed and their vaginal smears observed before sacrifice. Trunk blood samples were collected and allowed to clot at room temperature. Serum was separated and stored at -20˚C until assayed for hormone determinations. Immediately after decapitation, intra-abdominal fat was removed, weighed and expressed as a percentage of total body weight. A piece of normal mammary gland and the tumors were removed for histopathological and immunohistochemical analysis.

Induction of mammary tumors. DMBA (Sigma-Aldrich) was dissolved in sunflower oil at a concentration of 5 mg/ml and administered at a dose of 15 mg/rat on day 0 by an intragastric probe, 3 h after food and water deprivation to ensure a complete absorption of the drug. Food and water were replaced 2 h after DMBA administration. DMBA has been extensively used at that dose to study the possible mechanisms responsible for the beginning of the transformation of normal breast tissue into a tumor (11).

Hormone determinations. Thyroid stimulating hormone (TSH), PRL and GH concentrations were measured by double antibody radioimmunoassay using materials generously provided by A.F. Parlow and the National Hormone and Pituitary Program (NHPP, Harbor-UCLA Medical Center, Torrance, CA, USA). The hormones were radio-iodinated using the chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of the rat TSH RP-3, rat PRL RP-3 or rat GH RP-2 standard preparations. Assay sensitivity was 0.5 μg/l serum and the inter- and intra-assay coefficients of variation were <10% for all hormones.

E_2, Pg, T_3 and tetraiodothyronine (T_4) concentrations in sera were measured by radioimmunoassay using commercial kits for total hormones (TKE 21 and TKPG1 double antibody radioimmunoassay; Siemens Healthcare Diagnostics, Inc., Los Angeles, CA, USA and RK-6CT1 and RK-1CT1 double antibody radioimmunoassay; Institute of Isotopes Ltd., Budapest, Hungary; respectively). Inter- and intra-assay coefficients of variation were <10%.

Serum leptin concentrations were determined by a specific radioimmunoassay developed in the Instituto Multidisciplinario de Biología Celular (IMBICE) and validated for rat and mouse leptin against a commercial kit (catalog no. RL-83K; Linco Research, Inc., St. Charles, MO, USA) as previously published (12). Briefly, synthetic murine leptin (PrePro Tech, Inc., Rocky Hill, NJ, USA) was used for both labeled peptide and standard and for the development of anti-leptin serum. Leptin was radio-iodinated by the chloramine T method and purified by elution using a Sephacryl S-300 (Sigma-Aldrich) column. The anti-leptin serum was developed by rabbit immunization with murine leptin coupled to BSA. The intra- and

inter-assay coefficients of variation were 5-8 and 10-13%, respectively.

Latency, incidence and progression of tumors. The latency of mammary tumors was considered as the time between DMBA administration and the appearance of the first palpable tumor. Incidence was calculated as the percentage of rats that had tumors within the study period in respect to the total number of rats per group. We used a caliper to measure the major (DM) and minor (dm) diameters every 72 h and calculated the tumor volume (TV = dm^2 x DM/2). Tumor progression was assessed estimating tumor growth rate [GR = TV/(day of sacrifice - day of appearance of first tumor)].

Tumors and mammary gland histology. Small pieces of the tumor and inguinal mammary gland (contralateral to the tumor) from each rat were processed for histopathologic studies by fixing in buffered formalin, dehydrating in ethanol and embedding in paraffin wax. Sections (3-5 μm) were cut with a microtome and stained with hematoxylin and eosin (H&E) to define the histopathological changes in the mammary glands and to classify tumors according to published criteria (13). Images were captured with an Eclipse E200 microscope fitted with a digital still camera Micrometrics SE Premium (both from Nikon Corp., Japan) under magnification of x100, x400 and x600.

The quantification of the percentages of stroma, mostly composed by adipocytes, and epithelial tissue in the mammary gland was performed by measuring the area occupied by the adipose tissue or epithelium in 8-10 fields of each preparation from all rats using the ImageJ 1.42q software available at the NIH site (http://rsb.info.nih.gov/ij). Each area was expressed as a percentage of the whole field as previously published (14).

Apoptotic and mitotic indices. The microscopic analysis was carried out by two independent observers. The apoptotic and mitotic indices were calculated by counting the total number of apoptotic bodies and mitotic figures, respectively, in the histological sections stained with H&E in 10 x400 magnification-fields from each animal. The mitotic/apoptotic ratio (M/A ratio) was calculated by dividing the mitotic index by the apoptotic index.

Immunohistochemistry (IHC). Serial sections (3-5 μm) were mounted onto 3-aminopropyltriethoxysilane (Sigma-Aldrich)-coated slides for subsequent IHC analysis. The primary antibody used in this study was a monoclonal mouse anti-proliferating cell nuclear antigen (PCNA; Dako Cytomation, Glostrup, Denmark) at 1:500. An antigen retrieval protocol using heat was used to unmask the antigens (30 min in citrate buffer, 0.01 M, pH 6.0). Tissue sections were incubated with the primary antibody overnight at 4˚C in humidity chambers. A commercial kit to detect mouse and rabbit antibodies was used (Dako EnVision system, horseradish peroxidase, diaminobenzidine; Dako, Carpinteria, CA, USA). Slides were lightly counterstained with hematoxylin to reveal the nuclei, examined and photographed. The percentage of positive nuclei was obtained based on an average of 700 cells counted per sample, at a x400 magnification. The immunostaining of the tumor cells was semi-quantitatively scored as: 0, no staining; 1, nuclear staining of <10% of tumor cells; 2, staining between 11 and 33% of tumor cells; 3, staining between 34 and 65% of tumor cells; 4, staining of >66% of tumor cells. These scores were obtained by two independent observers blinded regarding the clinical evaluation, and a few conflicting scores were resolved by consensus.

TUNEL. Apoptosis was measured by a modification of the TUNEL assay using the ApopTag Plus Apoptosis In Situ Detection kit (Oncor, Gaitherburg, MD, USA), as reported previously (15). The TUNEL apoptotic index was calculated as the percentage of positive nuclei, based on an average of 700 cells counted per case, at x400 magnification.

Statistical analysis. Values are expressed as means ± SEM of 17-26 animals/group. All statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, Inc., San Diego, CA, USA). Differences in the distribution of variables between the three studied groups (HypoT, HyperT and controls) were assessed using one-way analysis of variance (ANOVA I) or the Kruskal Wallis test depending on the normality of the variables as evaluated by the Kolmogorov-Smirnov test. Two-way analysis of variance (ANOVA II) was used for analysis of the effects of the PTU or T_4 treatments on body weight over time. Post hoc comparisons between means were conducted by Bonferroni's test or Dunn's multiple comparison test. Student's t-test was used when only two groups were compared. When variances were not homogeneous, logarithmic transformation of data was applied. Incidence and percentages of mammary adipose area were analyzed by Chi-square. Survival curves were compared using the log-rank Mantel-Cox test. The relation between selected variables was performed by Pearson's correlation coefficient. Differences were considered significant when the probability was 5% or less.

Results

Induction of hypothyroidism and hyperthyroidism. The chronic thyroid condition of the animals was evaluated by measuring serum T_4, T_3 and TSH concentrations. The rats treated with PTU had low circulating T_4 (20.96±1.10 ng/ml, $p<0.001$) and T_3 (0.42±0.06 ng/ml, $p<0.05$) concentrations and also markedly increased levels of TSH (4.98±0.43 ng/ml, $p<0.01$) compared to the EUT rats (33.29±2.62, 0.61±0.06 and 0.84±0.06, respectively); all of them sensitive indicators for hypothyroidism. In contrast, L-thyroxine administration increased T_4 (55.88±6.93 ng/ml, $p<0.05$) and T_3 (0.91±0.10 ng/ml, $p<0.05$) and decreased circulating TSH (0.52±0.02 ng/ml, $p<0.01$) confirming the state of hyperthyroidism.

Hypothyroidism affects food intake, body weight and growth of the animals. Chronic treatment with PTU significantly retarded the growth of the HypoT animals as reflected in the decreased weight and reduced levels of circulating GH (Table II) in comparison with the HyperT rats and the untreated controls. HypoT rats had an increase in body weight similar to HyperT and EUT rats during the first 4 weeks of treatment with PTU. Afterwards, they stopped gaining weight until the end of the study showing a significant difference in body

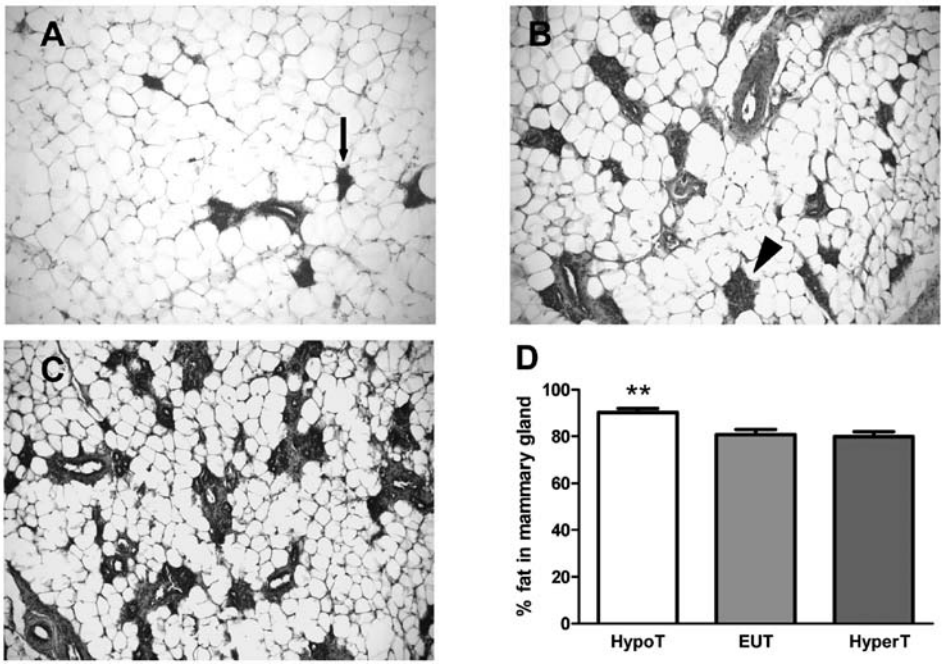

Figure 1. Hypothyroidism modifies the ratio of parenchyma/stroma in the mammary gland. Representative microphotographs (x100) of H&E-stained mammary tissues from (A) HypoT, (B) EUT and (C) HyperT rats. Arrows show lobules type 1 and arrowheads indicate lobules type 2. (D) Quantification of the relative percentages of adipose area. Values represent mean ± SEM of 8-10 fields of each preparation from 17-26 animals/group. $^{**}p<0.001$ compared to HyperT and EUT rats. Comparisons were performed by Chi-square.

weight when compared with the other two groups ($p<0.0001$, data not shown). HyperT rats gained weight steadily similar to the EUT rats. Daily food intake was significantly lower in HypoT rats compared to HyperT and EUT rats (12.6±0.2 vs. 23.2±1.2 and 19.1±0.7 g/rat, respectively; $p<0.0001$).

The mass of abdominal fat expressed as a percentage of the body weight has been previously used as a measure of body composition in rats (16). No statistically significant change was observed in this percentage due to PTU (11.3±1%) or T_4 (9.1±0.6%) treatments compared with controls (11.5±0.7%), although the HyperT rats had a tendency to show lower values.

Effects of hypothyroidism and hyperthyroidism on mammary gland histology. The effect of THs on the development of the mammary gland was evaluated by histological observation and measurement of the areas occupied by parenchyma or stroma. Table I shows that the mammary glands of EUT rats had a normal appearance with few ducts surrounded by a small amount of fibrous connective tissue and abundant adipose tissue. The mammary glands of HyperT and HypoT rats were also normal but in a few cases were associated with mammary benign pathologies such as ductal stasis. Moreover, hyperplasia, intraductal papilloma and adenosis were observed in the HyperT rats (Table I).

Fig. 1A-C show representative microphotographs of H&E-stained mammary tissue from HypoT, EUT and HyperT rats, respectively. Fig. 1D shows the relative percentages of the adipose area. In EUT and HyperT rats, ~80% of the mammary gland was composed of stroma, which consisted of fat and connective tissue. The rest of the gland was represented by the parenchyma including lobules type 1, lobules type 2 and ductal structures (Fig. 1B and C). In HypoT rats, a scarce lobe-alveolar development of the mammary gland (~10%) and an increased percentage of fat in the stroma (~90%, $p<0.001$ compared to HyperT and EUT rats) were observed (Fig. 1D), suggesting that hypothyroidism, but not hyperthyroidism, induced changes in the parenchyma-stroma ratio.

Effects of hypothyroidism and hyperthyroidism on mammary gland carcinogenesis. Table I shows the histopathological characteristics of the tumors in the three groups. On one hand, tumors of EUT and HyperT rats were all of low grade considering the characteristics of the nuclei, the presence of ducts and the number of mitotic figures. They had a predominant *in situ* component with a small invasive component. Necrosis was scarce and they had moderate inflammatory infiltration. Thirty-three percent of EUT and 47% of HyperT rats developed secondary tumors. On the other hand, malignant tumors from HypoT rats were histologically more aggressive with a predominant invasive component and a small *in situ* component. Necrosis was moderate and the host response was lower than in the tumors from HyperT and EUT rats.

The latency of onset of tumors was longer ($p<0.05$) (Fig. 2A) and the incidence was lower ($p<0.0001$) (Fig. 2B) in the HypoT rats when compared to the EUT and HyperT rats. Hypothyroidism retarded tumor growth ($p<0.05$) (Fig. 2C) and increased tumor-free survival ($p<0.001$) (Fig. 2D). No relationship between tumor growth rate and the histological type of the neoplasia was apparent in any of the groups.

Hypothyroidism increases apoptosis without promoting cell proliferation. To assess the effect of THs on tumor progression, we performed a microscopic analysis of mitosis and apoptosis. The mitotic index was similar in the three groups (Fig. 3A) while the apoptotic index tended to increase in

Table I. Summary of mammary gland characteristics and tumors developed in the DMBA-treated rats.

	HypoT rats	EUT rats	HyperT rats
No. of rats	26	17	23
Mammary glands			
Normal	23	16	18
Benign lesions			
Hyperplasia	0	1	1
Intraductal papilloma	0	0	1
Adenosis	0	0	2
Ductal ectasis	3	0	1
Tumors			
Rats with tumors	3	12	17
Tumor weight (g)[a]	1.26±0.57	1.22±0.17	1.32±0.18
Type of tumor			
Ductal carcinoma	3	10	15
Infiltrating lobular carcinoma	0	2	0
Tubular carcinoma	0	0	2
Ductal tumor grade			
I	1	10	13
II	2	0	2
III	0	0	0
Other characteristics			
Predominant component	Invasive	In situ	In situ
Inflammatory response	Low	Moderate	Moderate
Necrosis	Moderate	Scarce	Low
Rats with secondary tumors			
Low-grade ductal carcinoma	1	4	8

[a]Values correspond to the mean ± SEM. The weight of tumors was not statistically different between the groups. DMBA, 7,12-dimethylbenz(a) anthracene.

tumors of the HypoT rats (p=0.07) (Fig. 3B). In order to investigate if the balance between mitosis and apoptosis influences tumor development we calculated the mitotic/apoptotic ratio. This parameter was significantly lower in HypoT rats when compared to EUT and HyperT animals (p<0.05) (Fig. 3C), and was positively correlated with the tumor growth rate (r=0.715; p=0.002) (Fig. 3D) suggesting that the difference in the M/A ratio had biological significance.

We further studied apoptosis by TUNEL assay, a more sensitive technique, and we found an increased number of TUNEL-positive cells in tumors of the HypoT rats compared to the numbers in the HyperT and EUT animals (p<0.05) (Fig. 4A-D). In order to validate the results obtained by counting mitotic figures, we additionally evaluated mitosis

Table II. Hormonal profile of rats with different thyroid states with and without mammary tumors.

	HypoT				EUT				HyperT			
	Grouped	Tumor	No tumor	p-value	Grouped	Tumor	No tumor	p-value	Grouped	Tumor	No tumor	p-value
Serum GH (ng/ml)	6.4±0.3[a]	6.2±0.3	6.4±0.3	0.77	17.0±3.3	16.5±3.8	19.3±7.4	0.76	15.0±3.0	16.1±4.6	13.3±5.9	0.79
Serum E_2 (pg/ml)	10.2±2.0[a]	18.1±9.8	8.7±1.6	0.09	24.0±3.4	22.6±3.6	40.4±8.2	0.06	3.3±0.8[a]	5.6±2.1	0.4±0.0	0.39
Serum PRL (ng/ml)	11.6±1.1	12.3±1.6	11.0±1.2	0.69	13.9±2.3	13.1±2.6	15.8±7.6	0.67	17.6±4.1	26.9±9.6	10.9±3.3	0.38
Serum Pg (ng/ml)	10.9±3.4	10.2±1.4	40.5±15.1	0.38	15.9±2.3	19.6±4.2	15.6±9.1	0.69	12.8±2.0	12.4±2.1	15.2±8.0	0.64
Serum leptin (ng/ml)	1.3±0.2[a]	1.3±0.4	0.6±0.1	0.038	2.2±0.3	1.3±0.7	2.7±0.9	0.015	1.2±0.2	1.3±0.2	0.7±0.1	0.15
Leptin (ng/mg fat)	1.2±0.2	1.4±0.4	0.9±0.2	0.29	1.9±0.2	1.5±0.2	2.1±0.1	0.1	1.9±0.4	2.1±0.7	0.7±0.1	0.3

[a]p<0.0001 HypoT or HyperT rats compared to EUT rats. Each value is the mean ± SEM. The Kruskall Wallis and Dunn's tests were used to compare hormone values between the different grouped values. Circulating hormone levels were not affected by the treatments except for E_2. The Student's t-test was used for comparisons regarding the presence of mammary tumors within each group. p-values show the level of significance of those comparisons. GH, growth hormone; E_2, circulating estradiol; PRL, prolactin; Pg, progesterone.

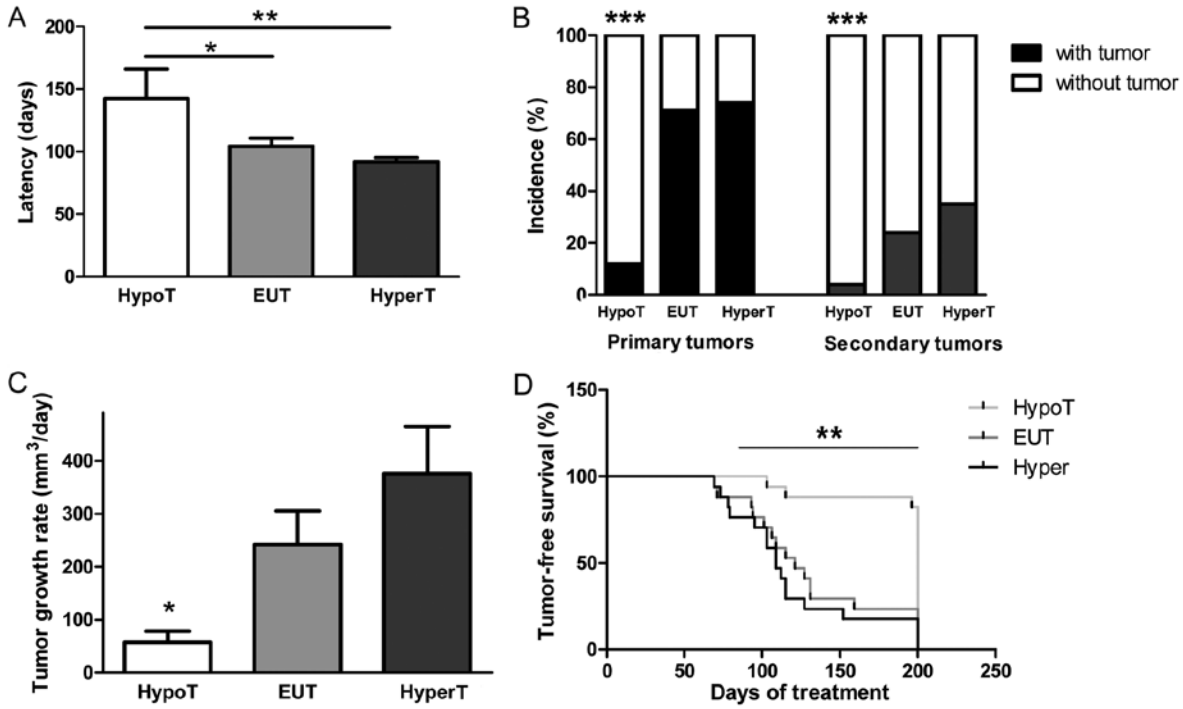

Figure 2. Hypothyroidism affects mammary carcinogenesis. (A) Latency of onset of mammary tumors, (B) percentage of rats developing primary and secondary tumors, (C) tumor growth rate and (D) the percentage of rats surviving without breast tumors. Values represent mean ± SEM of each group. Comparisons in latency and tumor growth were performed by ANOVA I and Bonferroni's test as post hoc. Incidence was expressed as percentages and compared by Chi-square test. Survival curves were compared using the log-rank Mantel-Cox test. $^{*}p<0.05$; $^{**}p<0.001$; $^{***}p<0.0001$, HypoT compared to HyperT and/or EUT rats.

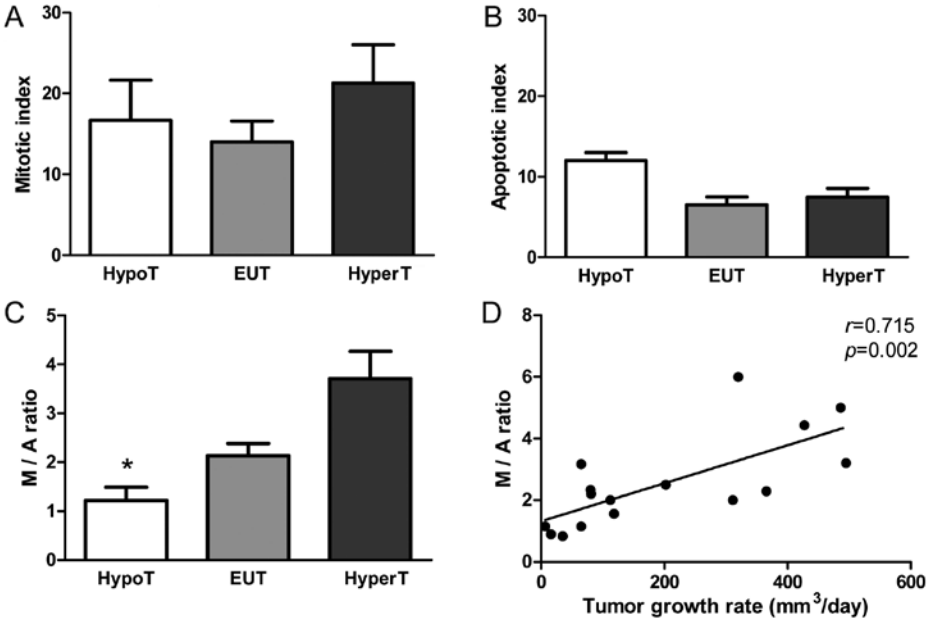

Figure 3. Mitotic/apoptotic (M/A) ratio correlates with the tumor growth rate. (A) Mitosis and (B) apoptosis were evaluated by counting the total number of mitotic figures and apoptotic bodies, respectively, in histological sections stained with H&E of 10 microscopic fields (x400) from each animal. (C) The M/A ratio was calculated by dividing the mitotic index by the apoptotic index. Values represent the mean ± SEM of each group. Comparisons were performed by ANOVA I and Bonferroni's test as post hoc. $^{*}p<0.05$, HypoT compared to HyperT and/or EUT rats. (D) Correlation between M/A ratio and tumor growth including all three treatment groups as evaluated by the Pearson's correlation coefficient.

by IHC of PCNA in the tumors. No statistically significant differences between the treatments were observed (Fig. 4E-H).

Hypothyroidism and hyperthyroidism alter hormone patterns. The influence of PTU and T_4 treatments on circulating hormone concentrations is shown in Table II. Both treatments decreased circulating levels of E_2 compared to EUT rats ($p<0.0001$).

Serum PRL and Pg concentrations were similar between the three studied groups. No significant differences were observed in circulating values of GH, PRL, E_2 and Pg between

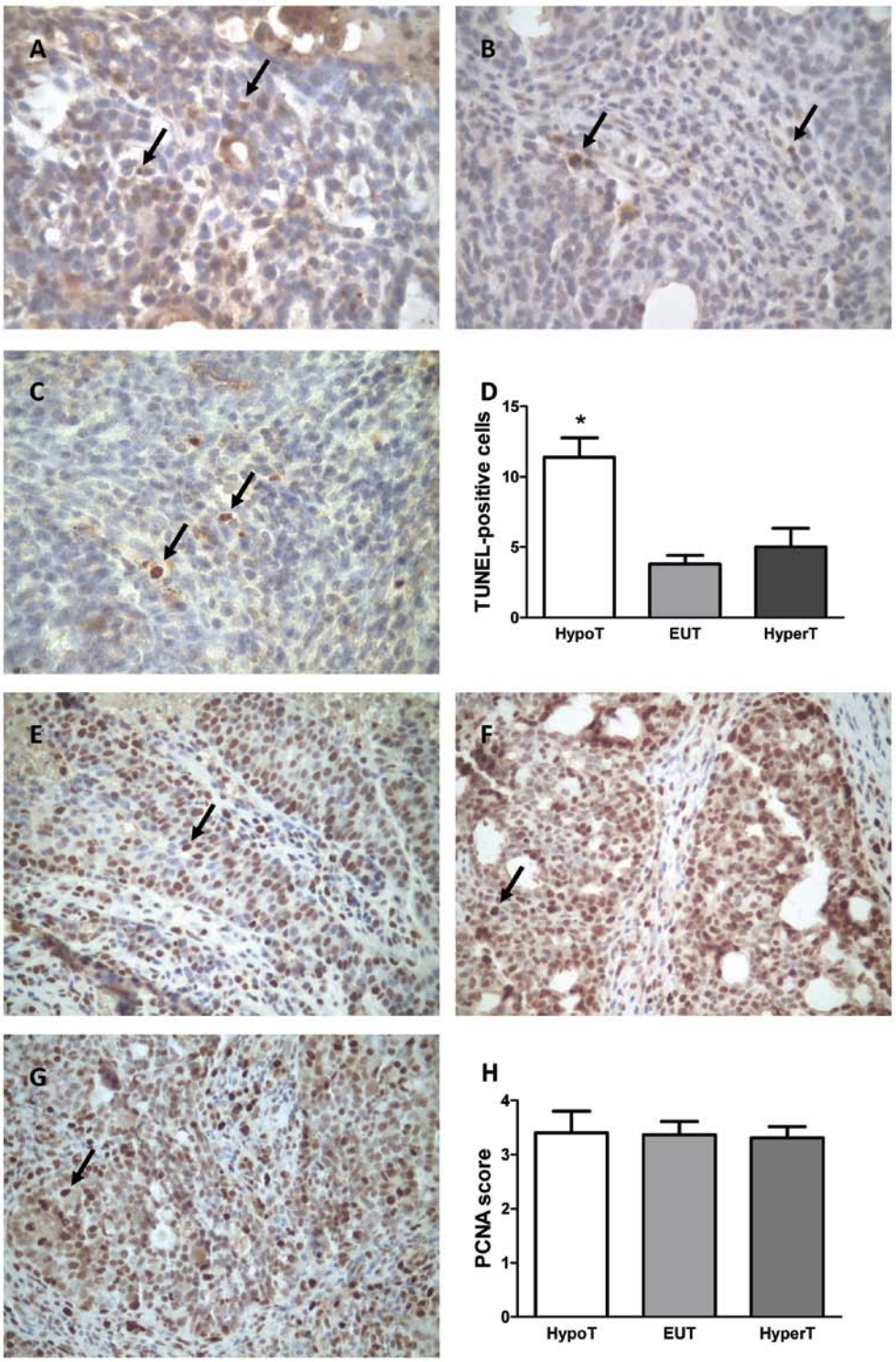

Figure 4. Hypothyroidism increases apoptosis. Representative microphotographs (x600) of tumors immunostained to reveal TUNEL-positive cells from (A) HypoT, (B) EUT and (C) HyperT rats. Arrows show apoptotic cells revealed by TUNEL assay. (D) Number of TUNEL-positive cells/field. Values represent mean ± SEM of 8-10 fields of each preparation from 17-26 animals/group. *p<0.05 compared to HyperT and EUT. Comparisons were performed by ANOVA I and Bonferroni's test as post hoc. Hypothyroidism did not affect cell proliferation. Representative microphotographs (x400) of tumors immunostained to reveal PCNA from (E) HypoT, (F) EUT and (G) HyperT rats. Arrows show mitotic cells. (H) PCNA-inmunostaining score. Values represent mean ± SEM of 8-10 fields of each preparation from 17-26 animals/group. Comparisons were performed by Kruskal-Wallis' test and Dunn's test.

tumor-bearing and tumor-free rats in the HypoT, HyperT and EUT groups.

Effect of adipose tissue and leptin on mammary gland carcinogenesis. Leptin concentrations were significantly decreased in HypoT rats whether they were calculated per milliliter of serum or per gram of abdominal fat mass ($p<0.05$, Table II). This suggested that adipocyte secretion activity was altered in HypoT rats since the body fat mass was similar in all groups. Interestingly, serum leptin levels were higher in HypoT rats with

tumors than in those without tumors (1.3±0.4 vs. 0.6±0.1 ng/ml, respectively; $p<0.05$). No significant differences were observed in EUT or HyperT rats, regarding tumor presence.

Discussion

The possible association between thyroid diseases and breast carcinoma has been debated for decades and remains controversial (8,13,17,18). Epidemiological and prospective studies have not been able to demonstrate a correlation between benign thyroid conditions and the risk of mammary cancer (13,18,19). However, other studies have supported a significant association between primary hypothyroidism and breast cancer (8,20). In the present study, hypothyroidism prolonged the latency of tumor appearance, reduced tumor incidence and retarded tumor growth in rats.

Martinez-Iglesias *et al* (21) injected MDA-MB-468 cells in hypothyroid mice and evaluated the growth of the tumors, thus avoiding the effect of THs on cancer initiation. They found that hypothyroidism had a dual effect on mammary tumorigenesis, since tumor growth was slower in hypothyroid mice, but the tumors were more aggressive and invasive, and the formation of metastasis was strongly enhanced. Contrarily, in humans, Cristofanilli *et al* (8) showed that spontaneous clinical hypothyroidism was a strong protective factor against mammary cancer and decreased tumor incidence and aggressiveness. These authors also found that women with primary hypothyroidism had a 61% reduction in the risk of invasive breast cancer and they were more likely to have localized disease and no lymph node involvement when compared with euthyroid women. Moreover, hypothyroid patients who developed breast cancer progressed with a more indolent disease and smaller tumors. On the other hand, Shering *et al* (18) and Ito and Maruchi (17) observed that Japanese women with Hashimoto's thyroiditis had a higher incidence of mammary cancer than women without thyroid disease. Two recent meta-analyses showed no significant association, except for autoimmune thyroiditis which increased breast cancer risk; however, the heterogeneity of the studies analyzed precludes firm conclusions (7,22).

Our study failed to show a statistically significant association between hyperthyroidism and breast carcinogenesis. In contrast, two studies found a correlation between the administration of THs and increased risk of mammary cancer (23,24). The results of epidemiological studies concerning hyperthyroidism in relation to breast cancer are unclear and seem to depend on the inclusion of pre- or post-menopausal patients (25). The authors speculated that subclinical hyperthyroidism in postmenopausal women contributed to mammary tumor growth as a result of an E_2-like effect through the interactions between T_3/T_4 and ER. *In vitro* interactions between T_3 and ER have been reported in breast cancer cell lines (19). Notably, in our present study, circulating E_2 was significantly reduced in HyperT rats even when mammary carcinogenesis similar to EUT rats was observed. This result supports the role of TH as an E_2-like factor in mammary tumor growth.

The mechanism whereby the thyroid gland influences mammary tumorigenesis is unclear. However, our results suggest that different thyroid states affect breast tumorigenesis by altering body growth, breast morphology, body composition and adipocytoquine release, and patterns of secretion of other hormones such as E, Pg, PRL and/or GH, among others.

Body growth. GH is required for mammary development since it stimulates ductal growth, proliferation and secretion (26). The effect of GH on mammary development is in part mediated by insulin-like growth factor 1 (IGF-1), and the action of E_2 and Pg are dependent upon IGF-1 (27). GH stimulates IGF-1 secretion in the liver and in the mammary stroma, therefore a paracrine role of GH modulating the effects of other hormones on proliferation and differentiation of mammary epithelium must not be ruled out (28). The present results demonstrated that hypothyroidism diminishes the secretion of GH in the rat, supporting previous studies (29).

Clinical evidence suggests that high circulating levels of GH/IGF-1 are associated with an increased risk of subsequently developing breast cancer (30). In studies *in vitro*, human GH was found to promote mammary carcinoma cell proliferation in an autocrine/paracrine manner (31) and resulted in a phenotypic conversion of human mammary carcinoma cells into a more invasive phenotype (32). In our *in vivo* study, we observed retardation in body growth associated with a significant decrease in serum GH levels in HypoT rats, which also had a lower incidence of mammary cancer.

Breast morphology. Breast development occurs through a process of ductal elongation, branching and sprouting of ductules or alveoli, a process that requires extensive cell proliferation and penetration of the ductal epithelium into the stroma (33). Ductal elongation is directed by E_2, GH, IGF-1 and epidermal growth factor (EGF), whereas ductal branching and alveolar budding are influenced by additional factors such as Pg, PRL and THs. The stroma of the mammary gland is a complex structure composed of an extracellular matrix and a variety of cell types including endothelial cells, inflammatory cells, fibroblasts, fibroblast-like cells and adipocytes (34). This compartment can also be referred to as the mammary fat pad (34) since it mainly contains adipose tissue (35).

The response by the ductal epithelium to various hormones and growth factors is modulated by epithelial-stromal interactions, which seem to be bidirectional (36). It is expected that the marked variations in the epithelial-stroma ratio influence the bidirectional connection leading to specific modifications in gene expression that may account for the different susceptibility or risk to develop breast cancer (33).

In the present study, we observed that hypothyroidism retarded the growth of the ductal system and induced an increase in mammary gland fat deposit. In accordance, Vonderhaar and Greco (37,38) noticed that the gland of primiparous hypothyroid adult mice retained a primitive ductal appearance, i.e. that the epithelial component consisted of a sparse ductal system with few branches filling about one-fourth of the fat pad. Coincident with our results, they also described that while glands from hyperthyroid and euthyroid virgin animals preserved a small degree of ductal branching with primitive alveoli, the glands from hypothyroid animals showed less ductal branching and were devoid of alveoli (37). Thus, the decrease in mammary tumor incidence in our HypoT rats may be due to the reduced development of the mammary gland and the decrease in the parenchyma-stroma ratio.

Body composition and adipocytokine secretion. THs have important effects on energy balance, since they influence both energy intake and expenditure. Severe hypothyroidism in the rat causes a sharp drop in food intake and metabolism, and this consequently suppresses the growth of DMBA-induced mammary cancer (11). Accordingly, in the present study, we observed a decreased food intake and body weight in HypoT rats in comparison with the controls and the HyperT animals. Calorie restriction was found to decrease proliferation and angiogenesis and increases apoptosis in mammary tumors (39).

Many changes related to energy homeostasis, body weight and food intake are associated with alterations in the function of adipose tissue. Leptin is the obese gene product secreted exclusively by adipocytes. Accumulating evidence shows that leptin is an important pro-angiogenic, pro-inflammatory and mitogenic factor, whose actions are reinforced through crosstalk with cytokines/growth factors (40). Increasing leptin levels activate the thyroid, GH, and gonadal axes (41). Thus, it is conceivable that leptin may have a role in different states of thyroidal disease. This relationship has been the subject of several studies, but no uniform picture has yet emerged from the results. Valcavi *et al* (42) reported significantly reduced serum leptin concentrations before and during replacement therapy in patients with hypothyroidism. Other authors did not observe alterations in serum leptin in different hypothyroid conditions (43) and Leonhardt *et al* (44) described increased leptin concentrations in patients with primary hypothyroidism or thyroid carcinoma compared with euthyroid controls.

In hypothyroid Wistar rats, a decrease in metabolizable energy intake and energy expenditure together with a shift in lipid and protein partitioning was found after 7 and 15 days of treatment with PTU (11). Consequently, body lipid percentage significantly increased compared to euthyroid rats. Our results in Sprague-Dawley rats showed that, even though the animals treated with PTU had a lower body weight than the controls, the percentage of body fat was similar in the three studied groups. However, adipose tissue of the HypoT rats appeared to be dysfunctional, attending to the lower amount of leptin expressed per gram of fat and the diminished serum levels of leptin in these animals. This fact can be one of the possible mechanisms related to the increased latency in HypoT rats since leptin has been shown to inhibit apoptosis and to stimulate growth (45) and also angiogenesis in breast carcinogenesis (46). However, in our *in vivo* study, we did not observe changes in tumor cell proliferation as evaluated by the mitotic index and by a more sensitive method, PCNA immunostaining. In turn, in cancer cells of our HypoT rats apoptosis was augmented in an inverse relation to serum leptin levels. In support of our results, it has been described that leptin inhibits apoptosis and this action is correlated with increased expression of anti-apoptotic protein Bcl-2 (47).

To summarize, dysfunctional body fat secreting lower levels of leptin may be another factor responsible for the delayed development of mammary tumors in HypoT rats. We cannot rule out the possible role of the stroma in the production of adipokines as tumor growth factors. Ongoing studies in our laboratory aim to evaluate the involvement of leptin locally produced by the peritumoral fat pad in the autocrine/paracrine regulation of tumor growth.

Hormones targeting the mammary gland. In the present study, we demonstrated that chronic hypothyroidism induced by PTU treatment modifies certain hormone patterns. E_2, which plays an essential role in mammary gland development and carcinogenesis, was diminished in HypoT rats. E_2 and Pg are known to promote proliferation and differentiation in the normal breast. Most breast cancers are initially hormone dependent and it is well accepted that E_2 plays a crucial role in their development and progression (48). E_2 exerts carcinogenic effects by simultaneously increasing the number of DNA replication errors by stimulating cell proliferation and gene expression; and through its oxidative metabolism that forms DNA damaging species (49).

There is evidence that Pg and its related signaling pathways are important players in the induction, progression and maintenance of the neoplastic phenotype in the mammary gland (50). However, in our *in vivo* study we did not find any relationship between circulating Pg and mammary carcinogenesis in rats with different thyroid disorders.

On the other hand, PRL secretion during diestrus was not affected in our experimental thyroid conditions. It has been previously shown that hypothyroidism can increase serum levels of PRL during estrous without changing basal secretion on diestrous day (29). In the present study, based on the similar values of circulating PRL between the treatments, we cannot attribute a relevant role of this hormone on mammary carcinogenesis. Moreover, we did not observe any significant differences in serum PRL in rats with and without tumors; further supporting the minor role of PRL in breast cancer development in HypoT rats.

In conclusion, our results to date show that hypothyroidism alters animal growth, breast morphology, body composition and adipocytoquine secretion and serum E_2 enhancing apoptosis, consequently retarding mammary carcinogenesis in rats. Additional studies by us are currently underway to investigate the involved molecular mechanisms.

Acknowledgements

The present study was partially supported by grants from Instituto Nacional del Cáncer and from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina. The authors are deeply indebted to Mrs. Elina Guiñazú de Di Nasso, Miss Paola Estalles and Mr. Juan Rosales for their excellent technical assistance and to Ms. María G. Zubiría and Dr Eduardo Spinedi for the RIA of leptin.

References

1. Neville MC, McFadden TB and Forsyth I: Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia 7: 49-66, 2002.
2. Jensen EV, Cheng G, Palmieri C, Saji S, Mäkelä S, Van Noorden S, Wahlström T, Warner M, Coombes RC and Gustafsson JA: Estrogen receptors and proliferation markers in primary and recurrent breast cancer. Proc Natl Acad Sci USA 98: 15197-15202, 2001.
3. Lanari C and Molinolo AA: Progesterone receptors - animal models and cell signalling in breast cancer. Diverse activation pathways for the progesterone receptor: possible implications for breast biology and cancer. Breast Cancer Res 4: 240-243, 2002.
4. Clevenger CV, Furth PA, Hankinson SE and Schuler LA: The role of prolactin in mammary carcinoma. Endocr Rev 24: 1-27, 2003.

5. Frontini L, Lissoni P, Vaghi M, Perego MS, Pescia S, Ardizzoia A and Gardani G: Enhancement of the efficacy of weekly low-dose taxotere by the long acting anti-prolactinemic drug cabergoline in pretreated metastatic breast cancer. Anticancer Res 24: 4223-4226, 2004.
6. Shen Q, Lantvit DD, Lin Q, Li Y, Christov K, Wang Z, Unterman TG, Mehta RG and Swanson SM: Advanced rat mammary cancers are growth hormone dependent. Endocrinology 148: 4536-4544, 2007.
7. Angelousi AG, Anagnostou VK, Stamatakos MK, Georgiopoulos GA and Kontzoglou KC: Mechanisms in endocrinology: primary HT and risk for breast cancer: a systematic review and meta-analysis. Eur J Endocrinol 166: 373-381, 2012.
8. Cristofanilli M, Yamamura Y, Kau SW, Bevers T, Strom S, Patangan M, Hsu L, Krishnamurthy S, Theriault RL and Hortobagyi GN: Thyroid hormone and breast carcinoma. Primary hypothyroidism is associated with a reduced incidence of primary breast carcinoma. Cancer 103: 1122-1128, 2005.
9. Barrera-Hernandez G, Park KS, Dace A, Zhan Q and Cheng SY: Thyroid hormone-induced cell proliferation in GC cells is mediated by changes in G1 cyclin/cyclin-dependent kinase levels and activity. Endocrinology 140: 5267-5274, 1999.
10. Jeong YJ, Bong JG, Park SH, Choi JH and Oh HK: Expression of leptin, leptin receptor, adiponectin, and adiponectin receptor in ductal carcinoma in situ and invasive breast cancer. J Breast Cancer 14: 96-103, 2011.
11. Goodman AD, Hoekstra SJ and Marsh PS: Effects of hypothyroidism on the induction and growth of mammary cancer induced by 7,12-dimethylbenz(a)anthracene in the rat. Cancer Res 40: 2336-2342, 1980.
12. Giovambattista A, Piermaria J, Suescun MO, Calandra RS, Gaillard RC and Spinedi E: Direct effect of ghrelin on leptin production by cultured rat white adipocytes. Obesity 14: 19-27, 2006.
13. Russo J and Russo IH: Atlas and histologic classification of tumors of the rat mammary gland. J Mammary Gland Biol Neoplasia 5: 187-200, 2000.
14. López-Fontana CM, Maselli ME, Salicioni AM and Carón RW: The inhibitory effect of progesterone on lactogenesis during pregnancy is already evident by mid- to late gestation in rodents. Reprod Fertil Dev 24: 704-714, 2012.
15. Cuello-Carrión FD and Ciocca DR: Improved detection of apoptotic cells using a modified in situ TUNEL technique. J Histochem Cytochem 47: 837-839, 1999.
16. Vazquez-Prieto MA, Renna NF, Diez ER, Cacciamani V, Lembo C and Miatello RM: Effect of red wine on adipocytokine expression and vascular alterations in fructose-fed rats. Am J Hypertens 24: 234-240, 2011.
17. Ito K and Maruchi N: Breast cancer in patients with Hashimoto's thyroiditis. Lancet 2: 1119-1121, 1975.
18. Shering SG, Zbar AP, Moriarty M, McDermott EW, O'Higgins NJ and Smyth PP: Thyroid disorders and breast cancer. Eur J Cancer Prev 5: 504-506, 1996.
19. Nogueira CR and Brentani MM: Triiodothyronine mimics the effects of estrogen in breast cancer cell lines. J Steroid Biochem Mol Biol 59: 271-279, 1996.
20. Brinton LA, Hoffman DA, Hoover R and Fraumeni JF Jr: Relationship of thyroid disease and use of thyroid supplements to breast cancer risk. J Chronic Dis 37: 877-893, 1984.
21. Martinez-Iglesias O, Garcia-Silva S, Regadera J and Aranda A: Hypothyroidism enhances tumor invasiveness and metastasis development. PLoS One 4: e6428, 2009.
22. Hardefeldt PJ, Eslick GD and Edirimanne S: Benign thyroid disease is associated with breast cancer: a meta-analysis. Breast Cancer Res Treat 133: 1169-1177, 2012.
23. Kapdi CC and Wolfe JN: Breast cancer. Relationship to thyroid supplements for hypothyroidism. JAMA 236: 1124-1127, 1976.
24. Mustacchi P and Greenspan F: Thyroid supplementation for hypothyroidism. An latrogenic cause of breast cancer? JAMA 237: 1446-1447, 1977.
25. Saraiva PP, Figueiredo NB, Padovani CR, Brentani MM and Nogueira CR: Profile of thyroid hormones in breast cancer patients. Braz J Med Biol Res 38: 761-765, 2005.
26. Raccurt M, Lobie PE, Moudilou E, Garcia-Caballero T, Frappart L, Morel G and Mertani HC: High stromal and epithelial human gh gene expression is associated with proliferative disorders of the mammary gland. J Endocrinol 175: 307-318, 2002.
27. Kleinberg DL, Wood TL, Furth PA and Lee AV: Growth hormone and insulin-like growth factor-I in the transition from normal mammary development to preneoplastic mammary lesions. Endocr Rev 30: 51-74, 2009.
28. Thijssen JH: On the possible role of mammary-derived growth hormone in human breast cancer. Maturitas 65 (Suppl 1): S13-S16, 2009.
29. Hapon MB, Gamarra-Luques C and Jahn GA: Short term hypothyroidism affects ovarian function in the cycling rat. Reprod Biol Endocrinol 8: 14, 2010.
30. Rinaldi S, Toniolo P, Muti P, Lundin E, Zeleniuch-Jacquotte A, Arslan A, Micheli A, Lenner P, Dossus L, Krogh V, Shore RE, Koenig KL, Riboli E, Stattin P, Berrino F, Hallmans G, Lukanova A and Kaaks R: IGF-I, IGFBP-3 and breast cancer in young women: a pooled re-analysis of three prospective studies. Eur J Cancer Prev 14: 493-496, 2005.
31. Kaulsay KK, Mertani HC, Törnell J, Morel G, Lee KO and Lobie PE: Autocrine stimulation of human mammary carcinoma cell proliferation by human growth hormone. Exp Cell Res 250: 35-50, 1999.
32. Mukhina S, Mertani HC, Guo K, Lee KO, Gluckman PD and Lobie PE: Phenotypic conversion of human mammary carcinoma cells by autocrine human growth hormone. Proc Natl Acad Sci USA 101: 15166-15171, 2004.
33. Russo J, Lynch H and Russo IH: Mammary gland architecture as a determining factor in the susceptibility of the human breast to cancer. Breast J 7: 278-291, 2001.
34. Tan J, Buache E, Chenard MP, Dali-Youcef N and Rio MC: Adipocyte is a non-trivial, dynamic partner of breast cancer cells. Int J Dev Biol 55: 851-859, 2011.
35. Neville MC, Medina D, Monks J and Hovey RC: The mammary fat pad. J Mammary Gland Biol Neoplasia 3: 109-116, 1998.
36. Petersen OW, Rønnov-Jessen L, Weaver VM and Bissell MJ: Differentiation and cancer in the mammary gland: shedding light on an old dichotomy. Adv Cancer Res 75: 135-161, 1998.
37. Vonderhaar BK and Greco AE: Effect of thyroid status on development of spontaneous mammary tumors in primiparous C3H mice. Cancer Res 42: 4553-4561, 1982.
38. Vonderhaar BK and Greco AE: Lobulo-alveolar development of mouse mammary glands is regulated by thyroid hormones. Endocrinology 104: 409-418, 1979.
39. Knight BB, Oprea-Ilies GM, Nagalingam A, Yang L, Cohen C, Saxena NK and Sharma D: Survivin upregulation, dependent on leptin-EGFR-Notch1 axis, is essential for leptin-induced migration of breast carcinoma cells. Endocr Relat Cancer 18: 413-428, 2011.
40. Zhou W, Guo S and Gonzalez-Perez RR: Leptin pro-angiogenic signature in breast cancer is linked to IL-1 signalling. Br J Cancer 104: 128-137, 2011.
41. Mantzoros CS: The role of leptin in human obesity and disease: a review of current evidence. Ann Intern Med 130: 671-680, 1999.
42. Valcavi R, Zini M, Peino R, Casanueva FF and Dieguez C: Influence of thyroid status on serum immunoreactive leptin levels. J Clin Endocrinol Metab 82: 1632-1634, 1997.
43. Sreenan S, Caro JF and Refetoff S: Thyroid dysfunction is not associated with alterations in serum leptin levels. Thyroid 7: 407-409, 1997.
44. Leonhardt U, Ritzel U, Schäfer G, Becker W and Ramadori G: Serum leptin levels in hypo- and hyperthyroidism. J Endocrinol 157: 75-79, 1998.
45. Housa D, Housová J, Vernerová Z and Haluzik M: Adipocytokines and cancer. Physiol Res 55: 233-244, 2006.
46. Rose DP, Gilhooly EM and Nixon DW: Adverse effects of obesity on breast cancer prognosis, and the biological actions of leptin (Review). Int J Oncol 21: 1285-1292, 2002.
47. Artwohl M, Roden M, Hölzenbein T, Freudenthaler A, Waldhäusl W and Baumgartner-Parzer SM: Modulation by leptin of proliferation and apoptosis in vascular endothelial cells. Int J Obes Relat Metab Disord 26: 577-580, 2002.
48. Pasqualini JR: Breast cancer and steroid metabolizing enzymes: the role of progestogens. Maturitas 65 (Suppl 1): S17-S21, 2009.
49. Bolton JL and Thatcher GR: Potential mechanisms of estrogen quinone carcinogenesis. Chem Res Toxicol 21: 93-101, 2008.
50. Goepfert TM, McCarthy M, Kittrell FS, Stephens C, Ullrich RL, Brinkley BR and Medina D: Progesterone facilitates chromosome instability (aneuploidy) in p53 null normal mammary epithelial cells. FASEB J 14: 2221-2229, 2000.