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# Long-term impact of hypothyroidism during gestation and lactation on the mammary gland

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**Original Article**

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## Abstract

The functional differentiation of the mammary gland (MG) is fundamental for the prevention of mammary pathologies. This process occurs throughout pregnancy and lactation, making these stages key events for the study of pathologies associated with development and differentiation. Many studies have investigated the link between mammary pathologies and thyroid diseases, but most have ignored the role of thyroid hormone (TH) in the functional differentiation of the MG. In this work, we show the long-term impact of hypothyroidism in an animal model whose lactogenic differentiation occurred at low TH levels. We evaluated the ability of the MG to respond to hormonal control and regulate cell cycle progression. We found that a deficit in TH throughout pregnancy and lactation induces a long-term decrease in Rb phosphorylation, increases p53, p21, Cyclin D1 and Ki67 expression, reduces progesterone receptor expression, and induces nonmalignant lesions in mammary tissue. This paper shows the importance of TH level control during mammary differentiation and its long-term impact on mammary function.

## Introduction

The mammary gland (MG) is one of the few organs whose functional differentiation occurs in adulthood, specifically during pregnancy and lactation.[1] This makes the MG a unique model for the study of organ development and differentiation. The MG's histological development occurs in defined stages associated with sexual development and reproduction. Lactation and the post-weaning involution are key stages in achieving terminal differentiation.[2-4]

The cellular events governing lactogenic differentiation of mammary epithelial cells have been studied for decades. Hormonal regulation is vital for achieving correct milk synthesis and ejection.[2] According to some authors, the MG's functional differentiation is achieved when the mammary histology has characteristics of a secretory epithelium with clear signs of milk production.[5] However, from the cellular point of view, the final mammary differentiation is accomplished when the epithelium is renewed after mammary involution.[1,6,7] This process involves programmed cell death of epithelial cells followed by the proliferation of alveolar stem cells, which maintain the differentiated imprinting and originate an epithelium with histological characteristics of the virgin MG.[1]

Many authors have provided evidence that MG differentiation is crucial for the prevention of mammary pathologies, such as breast cancer.[4,8-13] We have previously shown that thyroid hormone (TH) deficit throughout pregnancy and lactation impairs key molecular events of lactation, such as hormonal receptor expression and Jak/stat signaling pathway, which are key to lactogenic differentiation.[14] Regarding mammary involution, in our previous work we showed that hypothyroidism (hypoT) causes a premature onset of mammary involution, with the mammary tissue losing its capacity to preserve the histological characteristics of differentiated epithelium even though the suckling stimulus is maintained.[15]

The role of TH in the differentiation of many tissues as well as in the metamorphosis of amphibian species has already been described.[16-18] In humans, TH is essential for fetal nervous system differentiation.[19-21] Thyroid pathologies in animals have been also linked to lactation deficits that could be explained by their impact on milk synthesis and ejection signaling pathways.[22] The long-term impact of a TH deficiency throughout gestation and lactation remains, however, unknown. No consensus exists about normal TSH and TH values of pregnant women, which may depend on the specific tests used and geographic/population differences.[23-28] Furthermore, their levels fluctuate throughout pregnancy and postpartum,[29] which hinders the diagnosis of a thyroid pathology and the establishment of an adequate treatment.

Antithyroid drugs to treat hyperthyroidism or T4 replacement for hypothyroidism may not achieve normal TH values and, as a consequence, TH may not be adequately supplied to all differentiating tissues. Furthermore, mammary metabolic activity during lactation is regulated by the alveolar emptying cycles, which in turn depend entirely on the mother–child dyad. This implies that the MG's TH demand may be difficult to supply exogenously, leading to the above-mentioned lactation deficits observed in hypoT mothers.[22,30,31]

Research on the link between thyroid pathologies and mammary pathologies have yielded controversial results,[32-36] possibly because none of these studies have considered that TH deficit during lactogenic differentiation may alter mammary sensitivity to pathological development. In this work, we demonstrate that TH deficiency throughout mammary lactogenic differentiation has a long-term impact that compromises the mammary cellular response even several ovarian cycles after weaning.

## Materials and methods

### Animals

Female Sprague-Dawley rats bred in our laboratory, 60 days old and weighing 150–180 g at the onset of the experiment, were used. The rats were kept in a light (lights on 06.00–20.00 h) and temperature (22–24 °C) – controlled room. Rat chow (Cargill, Cordoba, Argentina) and tap water or 6-n-propyl-2-thiouracil (PTU, P3755, Sigma-Aldrich) were available *ad libitum*. Animal maintenance and handling was performed according to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication N8 86-23, revised 1985 and 1991), the UK requirements for ethics of animal experimentation (Animals Scientific Procedures Act 1986), and the FRAME guidelines of 1999.

Four experimental groups of eight animals each were used: Young Nulliparous (YN), Mature Nulliparous (MN), Control Primiparous (CP), and Hypothyroid Primiparous (HP). To form the YN group, vaginal smears were taken daily and eight animals of approximately 65 days of age, with at least three regular four-day estrous cycles, were sacrificed by decapitation on the morning of estrus day. In the eight animals of Group HP, hypoT was induced by administering 6-propyl-2-thiouracil (PTU) at a concentration of 0.1 g/L in drinking water. PTU treatment was initiated at 72 d of age and 12 d before the onset of pregnancy. Group CP received water instead of PTU. Vaginal smears were taken daily of all animals of CP and HP groups, and the rats were caged with a fertile male on the night of proestrus. The presence of spermatozoa in the vaginal smear the following morning was indicative of successful mating, and this day was counted as day 0 of pregnancy. On day 1 of lactation, the number of pups in each litter was standardized to eight, and mothers and litters were weighed weekly. To prevent alveolar inflammation associated with milk stasis arising after abrupt weaning,[37,38] progressive weaning was performed from day 20 of lactation by removing two pups per day. On day 23, the last pair of pups was removed, which was considered day 1 of weaning. Finally, eight animals did not receive treatment or undergo mating during the experiment and were sacrificed at the same approximate age as the CP and HP groups, forming the group MN. Animals of groups CP, HP, and MN were sacrificed by decapitation on day 28 post-weaning at 09.00–12.00 h.

Trunk blood was collected of all sacrificed animals, and serum was separated by centrifugation and stored at –20 °C until used. Thyroid condition was assessed by measuring total serum T4 concentration by chemiluminescent immunoassays (ECLIA, electrochemiluminescence immunoassay, Creative Biolabs, NY, USA). The inguinal MGs were removed, snap-frozen in liquid nitrogen, and stored at –70 °C until they were analyzed. One whole, frozen MG was cracked with a hammer. Pieces of several regions of the MG were used for RNA and protein extraction. All animal experimental procedures were approved by the Animal and Ethics Committee (CICUAL) of Universidad Nacional de Cuyo, Mendoza, Argentina (Res. N° 84/2016).

### RNA isolation and RT real-time PCR analysis

For total RNA isolation, 150–200 mg of mammary tissue was extracted using TriReagent reagent according to the manufacturer's protocol (Sigma-Aldrich, Inc. San Louis, MO, USA). Ten µg of total RNA were reverse transcribed at 37 °C using random hexamer primers and Moloney murine leukemia virus retrotranscriptase in a 20 µL reaction mixture. The RNA was first denatured at 70 °C for 5 min in the presence of 2.5 µg of random hexamer primers (Thermo Fisher Scientific, Carlsbad, CA, USA). For the subsequent RT reaction, the following mixture was added: RT buffer [50 mm Tris-HCl (pH 8.4), 75 mm KCl, 3 mm $MgCl_2$], 0.5 mm dNTPs, 5 mm DTT, 200 units M-MLV Reverse Transcriptase (Thermo Fisher Scientific). The reaction was incubated at 37 °C for 50 min; then, the reaction was inactivated by heating at 70 °C for 15 min. The cDNA was stored at –20 °C.

The mRNA content of receptors for PRL (RPRL), Cyclin D1 (CD1), and BAX (BAX) was estimated by RT Real-Time PCR using rat-specific primers and reaction conditions described in Table 1. PCR reactions were performed using a Corbett Rotor Gene 6000 Real-Time Thermocycler (Corbett Research Pty Ltd Sydney, Australia) and Eva-Green™ (Biotium Hayward, CA, USA) in a final volume of 10 µl. The reaction mixture consisted of 1 µL of 10x PCR Buffer, 0.5 µL of 50 mm $MgCl_2$, 0.2 µL of 10 mm dNTP Mix (Thermo Fisher Scientific), 0.5 µL of 20x Eva Green, 0.125 µL of 5 U/µL Taq DNA Polymerase (Thermo Fisher Scientific) 0.05 µL of each 2.5 mm primer (forward and reverse primers), and 5 µL of diluted cDNA. The PCR reactions were initiated with 5 min incubation at 95 °C, followed by 40 cycles at 95 °C for 30 s, 30 s at the annealing temperatures shown in Table 1 and 72 °C for 30 s. Melt curve analysis was used to check that a single specific amplified product was generated. Real-time quantification was monitored by measuring the increase in fluorescence caused by the binding of EvaGreen dye to double-strand DNA at the end of each amplification cycle. Relative expression was determined using the Comparative Quantitation method of normalized samples in relation to the expression of a calibrator sample, according to the manufacturer's protocol.[39] Each PCR run included a no-template control and a sample without reverse transcriptase. All measurements were performed in duplicate. The reaction conditions and quantities of cDNA added were calibrated so that the assay response was linear with respect to the amount of input cDNA for each pair of primers. RNA samples were assayed for DNA contamination by performing the different PCR reactions without prior reverse transcription. To select the reference gene, we estimated the expression stability of three candidate reference genes, β-Actin, GAPDH, and HPRT1 using the online software BestKeeper version 1 [http://gene-quantification.com/bestkeeper.html]. This approach allowed us to select HPRT1 as the reference gene since it showed the lowest coefficient variation between treatment groups compared to β-actin and GAPDH. Relative levels of mRNA were normalized to the HPRT reference gene. The

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**Table 1.** Sequences and conditions for the PCR reactions

| Primer | | Sequence | Gen Bank accession N° | Annealing T° | Reference |
|---|---|---|---|---|---|
| Bax | Forward | GCGATGAACTGGACAACAAC | NM_017059.2 | 59.9 | * |
| | Reverse | CACACGGAAGAAGACCTCTC | | | |
| PRLR | Forward | AAAGTATCTTGTCCAGACTCGCTG | NM_001034111.1 | 60 | *[1] |
| | Reverse | AGCAGTTCTTCAGACTTGCCCTT | | | |
| Cyclin D1 | Forward | AAGGGCTTCAATCTGTTCCTG | NM_171992.4 | 61 | * |
| | Reverse | CCGGACTGCCTCCGTGCCT | | | |
| HPRT | Forward | GACCGGTTCTGTCATGTCG | NM_012583.2 | 60 | * |
| | Reverse | ACCTGGTTCATCATCACTAA | | | |

The real-time PCR reactions were carried out for 40 cycles with an initial step of 5 min at 95 °C followed by a three-step scheme: 30 s at 95 °C, 30 s at the annealing temperature shown above for each primer pair, and a final step at 72 °C for 30 s.
*The primers were designed with Beacon Design software 7.92 (Premier Biosoft International, Palo Alto, CA).
*[1] Campo Verde Arbocco, F.; Persia, F. A.; Hapon, M. B.; Jahn, G. A. Hypothyroidism decreases JAK/STAT signaling pathway in lactating rat mammary gland. *Molecular and cellular endocrinology* **2017**, *450*, 14-23.

real-time PCR products were analyzed on 2% agarose gels containing 1% of Sybr Gold using Chemidoc Imaging System (Bio-Rad Laboratories, Inc. Hercules, CA, USA) and a unique band of the approximately correct molecular weight corresponded with a unique peak in the melt curve analysis.

### Protein isolation and Western blot analysis

For total protein isolation, 300–500 mg of mammary tissue was homogenized in an ice bath in two volumes of homogenization buffer (50 mm Tris, pH 7.5, 250 mm sucrose, 10 mm benzamidine, 10 mm NaF, 5 mm sodium pyrophosphate, 20 mm glycerophosphate, 1 mm sodium orthovanadate, 1 mm PMSF, 10 mm p-nitrophenylphosphate, and aprotinin, leupeptin, and pepstatin at 2 mg/L). The homogenate was centrifuged at 12,500 g for 30 min and the supernatant was separated and frozen in several aliquots at −80 °C until used. Proteins were quantified using the BCA method[40] and boiled 3–5 min in loading buffer. 40 µg of protein were separated by SDS-PAGE and transferred to Nitrocellulose Hybond membranes as previously described.[14] After rinsing and blocking with BSA 0.5%, the membranes were probed with anti-TRβ (sc738, Santa Cruz Biotechnology, Dallas, TX, USA), anti-ERβ (ab3577, Abcam, Cambridge UK), anti-PR A + B (PR130, Instituto de Salud y Ambiente del Litoral [ISAL], Universidad Nacional del Litoral, Santa Fe, Argentina), anti-Ki67 (ab15580, Abcam), anti-p53 (ab28, Abcam), anti-Rb (D20, Cell Signaling Technology Inc. Danvers, MA, USA), anti-PhosphoRb (P-Ser807/811, D20B12 Cell Signaling Technology), anti-p21 (sc397, Santa Cruz Biotechnology), anti-p27 (sc528, Santa Cruz Biotechnology), anti-Bcl-2 (sc7382, Santa Cruz Biotechnology) and anti-Survivin (ab24479, Abcam), using Horseradish peroxidase-conjugated secondary antisera (polyclonal goat anti-rabbit, rabbit anti-goat and goat anti-mouse immunoglobulins, Dako Cytomation, Glostrup, Denmark) and chemiluminescence (Amersham ECLTM, GE Healthcare, Buenos Aires, Argentina) to detect specific bands in a Chemidoc Imaging System (Bio-Rad Laboratories). The intensity of each band was quantified by densitometry using FIJI Image processing package.[41] Since the expression of commonly used proteins for quantitation was affected by HypoT induction,[14,15] we used densitometrical analysis of the intensity of the Ponceau staining as loading control.

### MG histology

Mammary tissue was fixed in buffered formalin, dehydrated in ethanol, and embedded in paraffin wax. Sections of 3–5 µm thickness were cut with a Carl Zeiss HYRAX M 25 Rotary microtome and stained with hematoxylin-eosin. Images were taken with a Nikon Eclipse 80i microscope fitted with a digital still camera Nikon DS Fil (Nikon Instruments INC. Japan) under 100x, 400x, and 600x magnifications. Only the inguinal MGs were used for morphological analyses. Sections were evaluated histologically for changes in the extent of lobuloalveolar development and supporting adipose tissue. The morphological state of the alveoli was determined by analyzing two sections from six different animals per group.

### Immunohistochemistry

Serial sections of paraffin embedded tissues (3–5 µm) were cut in a Carl Zeiss HYRAX M 25 Rotary Microtome and mounted onto 3-aminopropyltriethoxysilane (Sigma-Aldrich)-coated slides for subsequent IHC analysis. The primary antibodies used in this study were anti-Ki67 (ab15580, Abcam) and anti-p53 (ab28, Abcam) at 1:500 dilution. An antigen retrieval protocol using heat was used to unmask the antigens (40 min in 0.01 M citrate buffer, 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. Images were taken with a Nikon Eclipse E200 Microscope (Nikon Corp., Japan) fitted with a digital still camera Micrometric SE Premium (Accu-Scope, Commak, NY 11725) under 400x and 1000x magnifications.

### Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software. One-way ANOVA followed by the Bonferroni or Fisher LSD post hoc test was used to compare any two individual means. Log transformation of the data was performed when variances were not homogeneous. Differences between means were considered significant at the $p < 0.05$ level.

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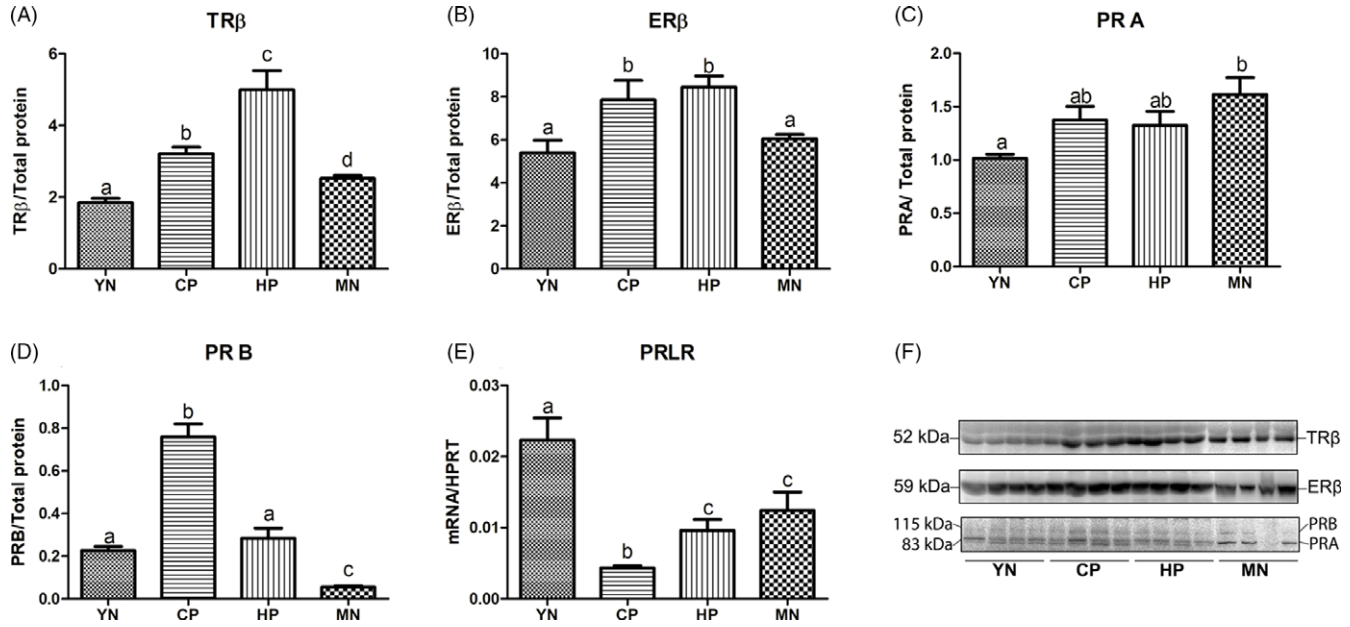


**Fig. 1.** Long-term effect of thyroid hormone deficit on mammary mRNA and protein levels of hormonal receptors relative to HPRT and total protein, respectively, in Young Nulliparous (YN), Control Primiparous (CP), Hypothyroid Primiparous (HP), and Mature Nulliparous (MN) Sprague-Dawley rats. (A) TRβ, (B) Erβ, (C) PRA, and (D) PRB protein levels were measured by Western blot (WB) and normalized to total protein. © PRLR mRNA levels were measured by real-time RT-PCR. (F) Representative TRβ, ERβ, and PRA and PRB WB membranes photographs are shown under the respective panels; the line at the left of the photograph marks the molecular weight. Values are means ± SEM for groups of 6 rats for PCR and 4/5 rats for WB. *p < 0.05 compared with the respective control group. Different superscript letters represent significant differences at p < 0.05 between different groups.

## Results

The thyroid condition of the animals was evaluated. Serum total T4 concentration of rats of the YN, MN, and CP groups were 5.02 ± 0.2 ug/dl, 3.86 ± 0.06 ug/dl, and 3.5 ± 0.1 ug/dl, respectively, while it was 0.50 ± 0.01 ug/dl in HP rats $p < 0.0001$. As in our previous studies, the hypoT state reduced animal weight gain both in pre-gestational state and throughout gestation, as well as the pups' weight gain during lactation (Supplementary Fig. S1).

### TH deficiency during differentiation alters long-term expression of hormone receptors

Even in quiescent stages, the MG is regulated by hormonal fluctuations associated to the estrous cycle. The role of ovarian hormones in mammary regulation as well as the cross-regulation mechanisms between ovarian hormones and TH during lactation have already been described.[42-44] It is, however, not known whether a TH deficit during gestation and lactation may affect the ability of the MG to respond to hormones in the long-term, especially in periods of life with high risk for the development of mammary pathologies. We therefore evaluated hormonal receptor expression in post-lactating and quiescent MG whose lactogenic differentiation occurred in a hypoT state. As shown in Fig. 1A, TRβ expression increased progressively with age (YN vs MN). This increase was greater after pregnancy, full lactation, and involution (YN vs MN vs CP), and a TH deficit led to an even higher expression (YN-MN-CP vs HP). In contrast to this, ERβ expression did not change significantly by the complete reproductive cycle or by aging (Fig. 1B). In the same way the expression level of the short progesterone receptor isoform (PRA) did not change with age or reproductive cycle. The expression level of the long progesterone receptor isoform (PRB) showed an opposite behavior (Fig. 1D): its protein level decreased with age (YN vs MN). However, the cycle of gestation, lactation, and involution increased PRB levels significantly in the long term (YN vs CP); an increase that depended on TH as hypoT dampened its expression (CP vs HP). PRLR mRNA expression also decreased with reproductive cycle (YN vs CP; Fig. 1E). This effect was partially blocked in the hypoT rats, where expression levels were slightly higher and similar to the MN group (YN vs MN vs HP).

### TH deficiency during pregnancy and lactation affects the ability of the MG to control the cell cycle in the long term

In non-lactogenic stages, hormonal control of the MG is mainly oriented toward cell cycle control. Given that there is a link between TH and mammary differentiation, and the latter with the risk of developing mammary pathologies, we evaluated the long-term impact of TH deficiency throughout lactogenic differentiation on the expression level of cell cycle regulators.

CD1 mRNA expression levels were high in YN, but as expected, they decreased with age (YN vs MN; Fig. 2A). This reduction was greater when the MG had gone through a differentiation cycle (MN vs CP), but this was blocked when the differentiation was with TH deficiency (MN vs CP vs HP). The decrease with age was similar in the Ki67 protein level, as shown in Fig. 2B (YN vs MN), however, in this case, the decrease was independent of lactogenic differentiation (MN vs CP) but strongly inhibited by TH since when the differentiation occurs with low TH levels, the Ki67 protein level was even higher than in the YN group (Fig. 2B). The increase in the HP group was corroborated by immunohistochemistry, where also cells with nuclear localization of Ki67 were observed in the HP, but not in the CP group (Fig. 2G). Figure 2C shows that p53 protein levels decreased with age (YN vs MN) but, surprisingly, they increased with the reproductive cycle (YN vs MN vs CP) and this increase was enhanced in the hypoT rats (YN vs MN vs CP vs HP). Rb phosphorylation levels increased with age regardless of the

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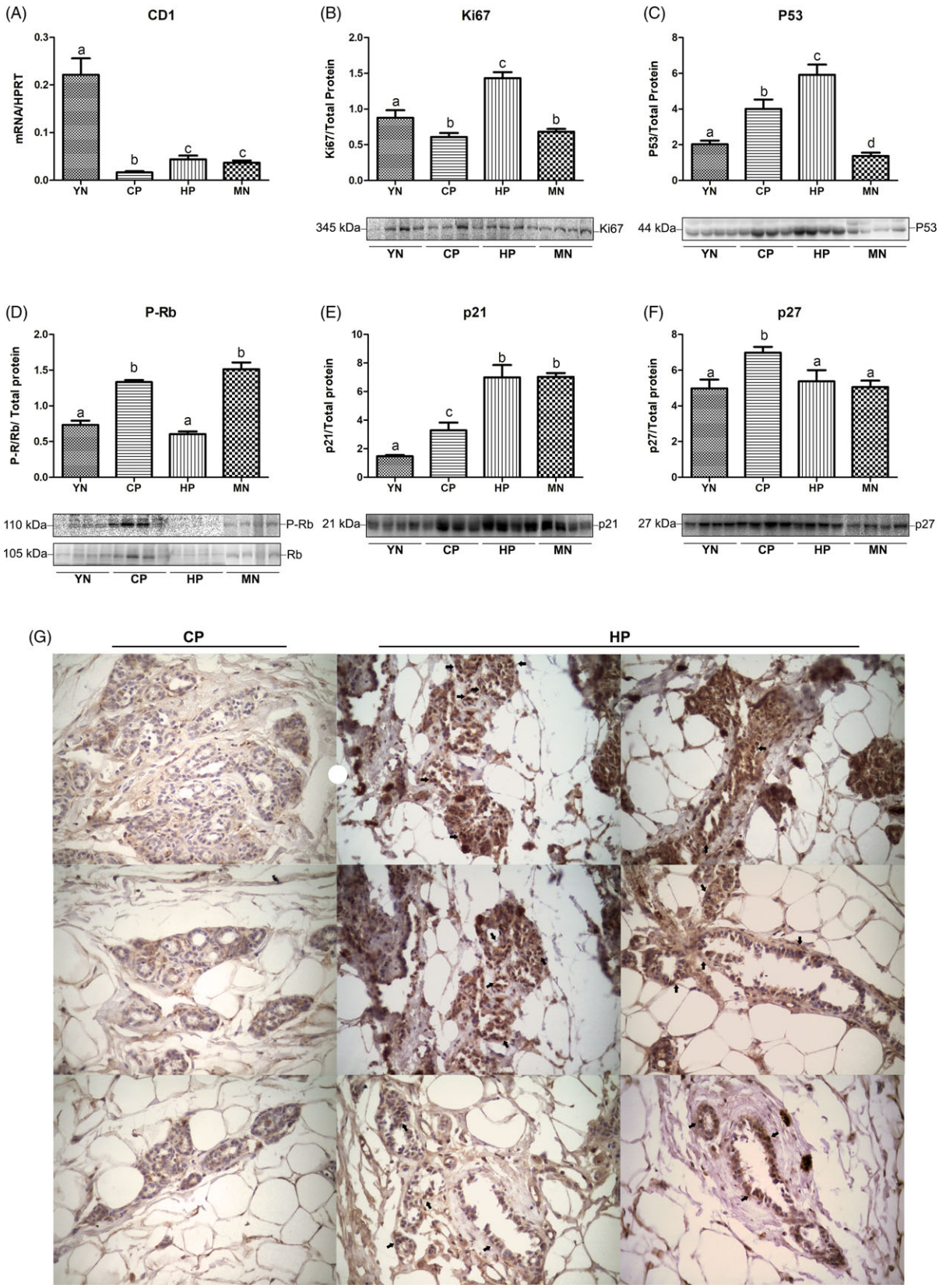


**Fig. 2.** Long-term effect of thyroid hormone deficit on mammary protein levels of cell cycle regulator proteins in Young Nulliparous (YN), Control Primiparous (CP), Hypothyroid Primiparous (HP), and Mature Nulliparous (MN) Sprague-Dawley rats. (A) CD1 mRNA levels were measured by real-time RT-PCR. (B) Ki67, (C) p53, (D) P-R©, (E) p21, and (F) p27 protein levels were measured by Western blot (WB) and normalized to total protein. A representative membrane of WB is shown below the protein level graphs. The line at the left of the WB photograph indicates the molecular weight. Values are means ± SEM for groups of 6 rats for PCR and 4/5 rats for WB. *$p < 0.05$ compared with the respective control group. Different superscript letters represent significant differences at $p < 0.05$ between different groups. (G) Long-term effect of thyroid hormone deficit on mammary localization of Ki67 measured by immunohistochemistry (IHC) in CP and HP groups. Black arrows show the nuclear translocation of Ki67 in the HP group.

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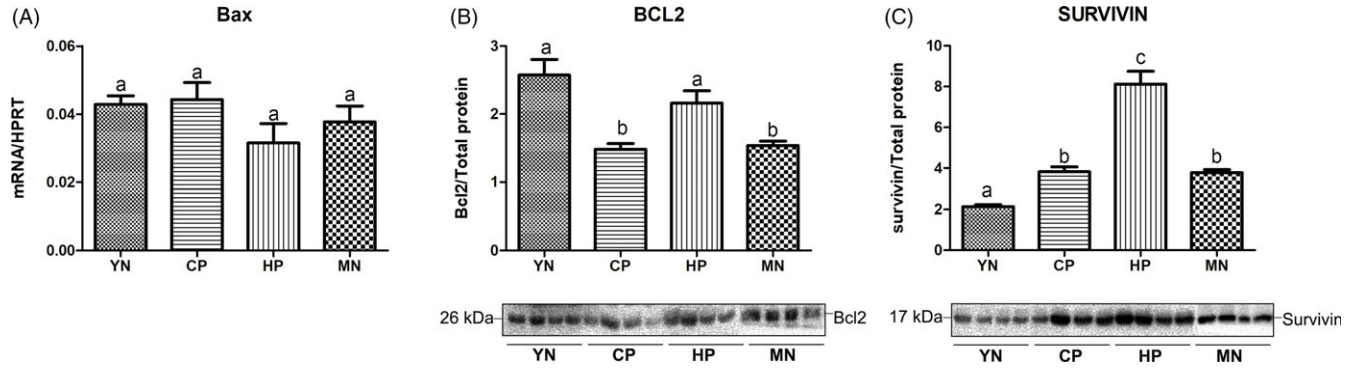


**Fig. 3.** Long-term effect of thyroid hormone deficit on mammary mRNA and protein levels of apoptosis-related proteins in Young Nulliparous (YN), Control Primiparous (CP), Hypothyroid Primiparous (HP), and Mature Nulliparous (MN) Sprague-Dawley rats. (A) Bax mRNA levels were measured by real-time RT-PCR. (B) Bcl-2 and (C) Survivin protein levels were measured by Western blot (WB) and normalized to total protein. A representative membrane of WB is shown below the Bcl-2 and Survivin protein graphs. The line at the left of the WB membrane indicates the molecular weight. Values are means ± SEM for groups of 6 rats for PCR and 4/5 rats for WB. *$p < 0.05$ compared with the respective control group. Different superscript letters represent significant differences at $p < 0.05$ between different groups.

lactogenic differentiation cycle (YN vs MN-CP; Fig. 2D), but this increase was blocked in the TH deficient rats (YN vs MN-CP vs HP). In addition, Fig. 2E shows that the expression level of the cell cycle inhibitor protein p21 increased with age (YN vs MN) and that it was partially blocked by the lactogenic differentiation cycle (YN vs MN vs CP). However, after the reproductive cycle the p21 protein level of hypoT rats returned to levels similar to those of mature virgin rats (YN vs MN vs CP vs HP). Strikingly, p27, another cell cycle inhibitor, showed opposite trends. As evidenced in Fig. 2F, p27 expression levels did not change with age or reproductive cycle (YN vs CP vs HP vs MN).

### *TH deficiency during lactogenic differentiation induces long-term expression of apoptosis inhibitors*

Some factors altered in mammary pathologies include both the ability of the MG to respond to hormonal stimuli and the adequate control of the cell cycle and programmed cell death. In quiescent stages, when the MG is not under hormonal stimulation of pregnancy and lactation, the imbalance of these events could increase the risk of pathological behaviors. For this reason, we evaluated the long-term impact of TH deficit on the expression level of Bax, Bcl-2, and Survivin.

Neither age (YN vs MN), lactogenic differentiation (YN vs MN vs CP), nor TH deficiency (CP vs HP) modified mRNA Bax levels (Fig. 3A). On the other hand, as shown in Fig. 3B, Bcl-2 protein levels decreased significantly with age (YN vs MN), independent of lactogenic differentiation (YN vs MN vs CP), while they increased to YN-like values in post-lactating hypoT rats (YN vs MN-CP vs HP). Survivin protein levels increased in post-lactating rats and mature virgins (YN vs MN vs CP; Fig. 3C), but in hypoT rats, the levels of this protein were several times higher than in the other groups (YN-MN-CP vs HP).

### *TH deficit throughout gestation and lactation induces long-term histological anomalies in the MG*

The relationship between a complete cycle of mammary differentiation and involution and the reduced risk of developing mammary pathologies is associated with the capacity of the MG to maintain the cellular characteristics of the differentiated gland. The results presented above demonstrate that TH deficiency during pregnancy and lactation induces long-term changes in the ability of the MG to respond to ovarian hormones and control the cell cycle, but they do not provide evidence of a specific cellular behavior. For this reason, we carried out a histological evaluation of the MG in search of clues that may help us determine the phenotypic expression of our results.

The MG of the YN group had classic characteristics of virgin mammary tissue, with normal ducts and alveoli and a normal adipose/epithelial relationship, abundant adipose tissue, and epithelial cells forming rudimentary type 1 alveoli (Fig. 4-A). The MG of the MN group were similar to those of YN. Animals of the CP group showed a similar histology, but with a greater number of alveoli type 1/2 and bigger alveolar development than those in the YN group. In contrast to these findings, three of the six analyzed animals of the HP group evidenced histological abnormalities. The histological sections of hypoT rats showed benign lesions consistent with epithelial hyperplasia (*), mammary adenosis ([a]), and mammary fibrosis (o) (Fig. 4-B). Although none of the sections showed cellular atypia consistent with a malignant pathology, the cells of the hypoT MG had striking characteristics, such as hyperchromatic nuclei, and formed a reactive endothelium with alveolar buds, although without secretory characteristics.

## Discussion

Studies performed more than three decades ago showed that full lactogenic differentiation reduced the risk of developing mammary pathologies.[4,45-47] There is evidence linking both the number of gestation-lactation cycles and the duration of lactation with a low risk of developing mammary pathologies[48-50] but no reports exist of a relationship between thyroid deficiencies throughout lactation and the risk of mammary pathologies. In the past decades, some authors have shown controversial results regarding the role of TH in the development and proliferation of mammary pathologies.[32-34] More recently, the search for clear criteria has deepened and it has been shown that thyroid pathologies are related to the risk of developing mammary malignancies.[17,33,35,36,51-53] None of these studies, however, considered the reproductive history and circulating TH levels throughout the reproductive cycle. In pregnancy, the thyroid stimulating actions of hCG, as well as the changes in iodine and TH metabolism and clearance, hinder thyroid disease treatment (T4 for hypoT and antithyroid drugs for hyperthyroidism). This has led to the suggestion that in these patients, treatments do not achieve an optimal TH level.[30,31,54]

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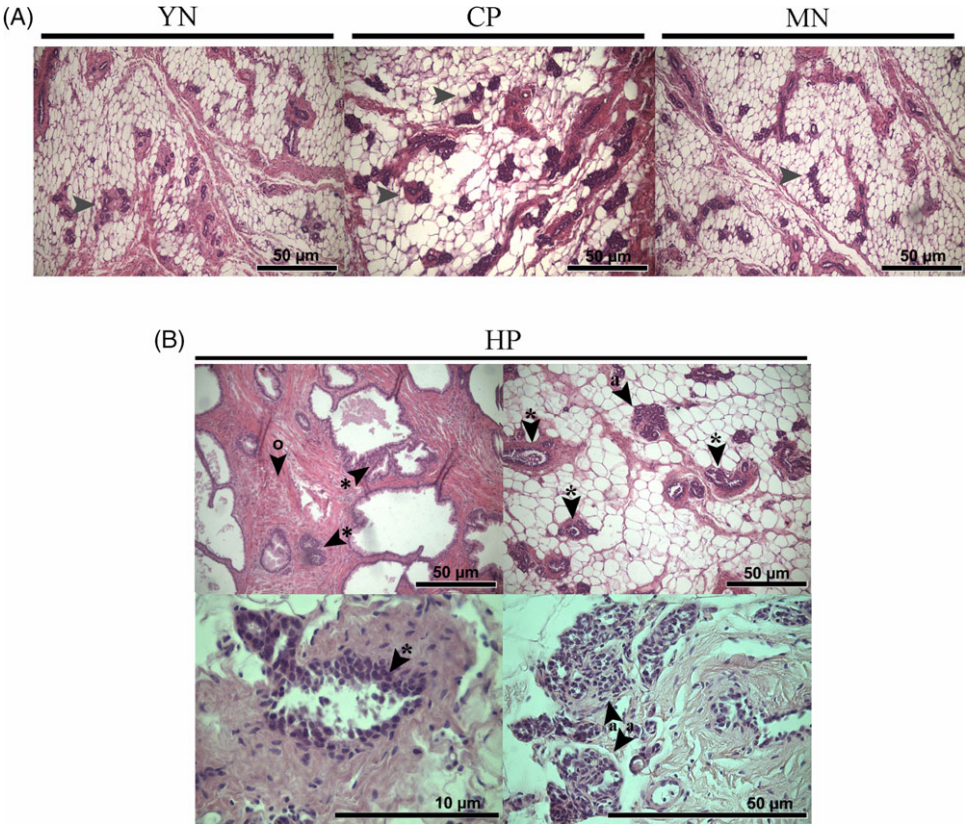


**Fig. 4.** Long-term effect of thyroid hormone deficit on mammary gland histology. (YN) Young Nulliparous, (MN) Mature Nulliparous, (CP) Control Primiparous, and (HP) Hypothyroid Primiparous rats. Mammary glands were fixed and stained with hematoxylin-eosin. (A) Representative images obtained at 100x of mammary tissue of YN, MN, and CP rats showing preponderance of adipose tissue, inactive alveoli, and epithelial integrity (Gray arrow). (B) Representative images obtained at 100x, 400x, and 600x of mammary tissue of HP rats showing epithelial hyperplasia (*), mammary adenosis (a), and mammary fibrosis (o). See Materials and Methods section for further details.

The present work shows that hypoT throughout gestation and lactation modifies mammary tissue response even many ovarian cycles after weaning. Although contradictory, our results are striking in terms of cell cycle control. According to the canonical conception of the cell cycle,[55] p53 and p21 expression levels, as well as Rb phosphorylation levels, suggest that most mammary cells of the HP group are in the arrested stages of the cell cycle before DNA replication. However, CD1 and Ki67 transcription levels as well as tissue histology indicate the opposite, suggesting a hyperplastic, proliferative state.

This is not the first time that heterogeneity in mammary cell cycle markers has been described. In fact, different mammary cell populations maintain a differential behavior in response to hormonal stimulation. Some studies have shown that in the hours following mitosis, and in the absence of mitotic stimulation, mammary cells enter a point of cellular pre-restriction, which is characterized by Rb hypo-phosphorylation and high p21 expression; this pre-restriction stage can be overcome if mitotic stimuli are reestablished.[55,56]

Spencer et al. have demonstrated that after a mitotic cycle, MG cells divide into two populations depending on their ability to overcome the pre-restriction point.[56] Our results indicate that in the HP group, both types of cell populations coexist in the heterogeneous mammary tissue and both show the cellular response described by Spencer et al.[56] This is supported by the increase in Cyclin D1 transcription, which is a signal of overcoming the initial G1 restriction, whose next steps include its translation, kinase binding, and Rb phosphorylation.[57] Added to this, the Ki67 nuclear localization in HP group indicates that at least one mammary cell population has overcome the initial restriction and has continued the cell cycle. The presence of mammary epithelial cells with nuclear localization of Ki67 could also explain the histological abnormalities observed in the mammary tissues. Our results are also in accordance with those of Moser et al[55] who described that MGs contain two cell populations that do not surpass the restriction point after mitosis in a synchronized manner.

Ki67 is a cell proliferation marker whose expression is not restricted to discrete stages of the cell cycle.[58] This may explain why it was increased in the HP group, as its expression is not limited to cells that are in the same stage of the cellular cycle but are still proliferating. Taking into account the roles of Ki67,[59,60] its high levels may be an indicator of the general cellular state that explains the histology of the mammary tissue of the HP group.

In the analyzed stages, the MG is still under the control of ovarian hormones, and estrogen and ERβ play a central role in promoting the cell cycle.[58,59] Our results show that hypoT during pregnancy and lactation does not alter the long-term level of ERβ expression. Using Knockout animal models, Gustafsson and collaborators demonstrated that Ki67 expression levels are inversely related to those of ERβ, but their results did not account for mammary differentiation.[59] TH deficiency altered this pattern by increasing Ki67 levels in the presence of constants ERβ levels. These findings suggest that the cells have acquired independence from ovarian control of the cell cycle.

Similarly, although PR expression has been shown to depend on estrogen and its receptor, PRB decreased in the HP group compared to CP in the presence of normal ERβ levels. Recent reviews have highlighted the role of both PR isoforms in mammary cell differentiation and linked them to the transcriptional activity of the STAT protein family.[61] Accordingly, PRB, which is considered the central mediator of hormone activity, increases with the differentiation cycle under normal conditions. Both PRs promote mammary cell proliferation, and their roles partially overlap.[62] A decrease in PRB isoform expression relative to PRA isoform

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expression could be a marker of early events of pathological mammary development.[61] Although we did not observe pathological development, the exclusive reduction of the PRB isoform in the HP group, added to the previous results, may suggest the possibility of a long-term pathological cellular behavior. This is not the first time that a non-gestating and lactating MG shows abnormal proliferating epithelial cells upon PTU treatment. In accordance with our results, Hapon et al. reported that hypoT rats had greater lobuloalveolar mammary development that euthyroid rats, thus indicating that the mammary cell depends on TH for its cell cycle regulation.[69]

The role of PRLR in promoting mammary tissue proliferation and differentiation has already been described. During lactation, a high level of PRLR is a marker of functional differentiation.[63,64] This process involves demethylation of milk synthesis genes, which improve cellular response in a second gestation and lactation cycle.[1,6] Although in the post-lactating quiescent stages a normal differentiation cycle led to a reduction in transcription levels of PRLR in the CP group, the increase in the HP group was in agreement with a stage of high cellular activity in which PRLR expression could be facilitated by epigenetic changes induced during lactation.[1,6]

The programmed cell death mechanism involved in mammary involution is Anoikis.[65,66] We have previously shown that hypoT during lactation induces premature tissue involution without altering Bax and Bcl-2 expression.[15] At first sight, these results may seem contrary to those presented here, in which hypoT led to an increase in Bcl-2. It is, however, important to note that in the previous work the rats were still lactating and the mammary cells actively producing milk, while the present analyses were performed 28 d after weaning, when the mammary tissue was in a late stage of involution. Moreover, several investigations have shown that the mechanism that mediates cell death in non-lactogenic stages involves Bax and Bcl-2.[60,67] We can thus assume that in quiescent stages and a different hormonal environment, hypoT can induce long-term effects, promoting cell survival by inducing Bcl-2 and Survivin and maintaining Bax transcription levels.

Recent research has shown that hypoT reduces the progression of established mammary pathologies.[35,36] Those studies are very robust, considering the metabolic role of TH.[68] It is, however, important to highlight that hypoT in pregnant and lactating women has deleterious effects on pregnancy and lactation; all women with hypoT therefore receive TH supplementation. Current evidence indicates that in pregnant and lactating woman, TH balance does not always achieve the necessary level of TH to ensure optimal mammary differentiation, even if lactation is adequate.[30,31,54] The results of this work thus suggest that TH deficiency caused by either insufficient medication of hypoT or excessive medication of hyperthyroid pregnant and lactating women could generate a MG with an altered cellular imprint. The deregulated cell cycle, overexpression of survival factors, inability to adequately respond to the hormonal stimulus, and TRβ overexpression could be risk factors that may promote development of mammary pathologies in later life stages with a normal supply of TH.

**Supplementary material.** For supplementary material for this article, please visit https://doi.org/10.1017/S2040174422000320

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**Conflict of interest.** The authors declare that they have no conflict of interest.

**Ethical standards.** All animal experimental procedures were approved by the Animal and Ethics Committee (CICUAL) of Universidad Nacional de Cuyo, Mendoza, Argentina (Res. Nº 84/2016).

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