DOES THYROID PEROXIDASE PROVIDE AN ANTIGENIC LINK BETWEEN THYROID AUTOIMMUNITY AND BREAST CANCER?

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Short Title: Thyroid peroxidase expression in breast cancer

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Abbreviations used (alphabetical order):

AD= Adipose Tissue; ATD= Autoimmune Thyroid Disorders; BC= Breast Cancer; BSA= Bovine Serum Albumine; BTD= Benign Thyroid Diseases; IF= Immunofluorescence; IHC= Immunohistochemistry; KC= Kidney Cancer; LPO= Lactoperoxidase; MKC= Hepatic Metastasis of Kidney Carcinoma; MPO= Myeloperoxidase; MW= Molecular Weight; NIS= Sodium/Iodide Symporter; PC= Pancreatic Adenocarcinoma; PCR= Polymerase Chain Reaction; PEG= Polyethylene Glycol; PKC= Primary Kidney Carcinoma; PT= Peri-Tumoral Breast Tissue; QPCR= Absolute Real-time Quantitative PCR; RT= Reverse Transcription; TgAb= Autoantibodies to thyroglobulin; TPO= Thyroid Peroxidase; TPOAb= Autoantibodies to TPO; TT= Thyroid Tissue; WB= Western Blot.

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Novelty and Impact of the work:

This work shows that thyroid peroxidase (TPO) is not a thyroid-specific enzyme since it is expressed, even if at lower levels, in other tissues. In particular TPO expression in neoplastic breast tissue could explain the increased prevalence and positive prognostic effect of serum autoantibodies to TPO in patients with breast cancer, with implications in future studies searching for potential tumor-specific immunotherapies for breast cancer. The identification of tissue-specific TPO-isoforms could also be useful in diagnosis.

ABSTRACT

Women with breast cancer (BC) and anti thyroid peroxidase (TPO) autoantibodies (TPOAb) have a better prognosis than women lacking TPOAb. Sera from women with TPOAb displayed immunoreactivity to BC tissue by immunofluorescence that was not apparent in women without TPOAb. We hypothesize a BC/thyroid shared antigen that provides a target for humoral or cell-mediated immune activity; candidates include the sodium/iodide symporter (NIS, expressed in thyroid and BC), cross-reacting epitopes in TPO and lactoperoxidase (LPO) or TPO itself. Since the association is with TPOAb, we investigated TPO expression in BC, breast peri-tumoral tissue (PT), other tissues (tumoral and not) and thyroid as positive control.

Transcripts for known and novel TPO isoforms were detected in BC (n=8) and PT (n=8) but at approximately 10^4 fold lower than in thyroid while in non-BC tumours (n=5) they were at the limit of detection. TPO was expressed also in adipose tissue (n=17), 10^3 fold lower than in thyroid. Full length TPO (Mr 105-110 kDa) was detected in western blots in the majority of examined tissues; pre-absorption of the TPO antibody with recombinant TPO (but not LPO) reduced the signal, indicating specificity. The same occurred with some lower molecular weight bands, which could correspond to smaller TPO transcript isoforms, present in all samples.

In conclusion TPO is weakly expressed in BC and other tissues; this could partly explain the high frequency and protective role of TPOAb in BC patients. Further studies will investigate tissue specificity, function and immunogenicity of the novel TPO variants (some BC-specific) identified.

INTRODUCTION

The association between benign thyroid diseases (BTD) and breast cancer (BC) has long been known[1, 2], even if the topic has been a subject of debate for many years, with not all authors agreeing[3-5]. However the majority of the studies support this association, and in particular they provide evidence for the relationship between BC and autoimmune thyroid disorders (ATD)[6-8]. A high prevalence of anti thyroid peroxidase (TPO) autoantibodies (TPOAb) has been found in both treated[9,10] and untreated[11] BC patients and the positive predictive value of serum TPOAb in BC patients with aggressive disease has been reported[12,13]. Only Jiskra et al.[14], despite confirming a higher prevalence of TPOAb in BC patients, found no impact on relapse-free and overall survival; this discrepancy could be due to the relatively small and highly heterogeneous BC patients group. Recently Farahati et al. have found a significantly lower frequency of distant metastases in a large cohort of BC patients with serum TPOAb positivity[15].

BC and follicular thyroid cells share similar functional properties such as the expression of the sodium/iodide symporter (NIS) and a peroxidase activity: TPO in thyroid cells and lactoperoxidase (LPO) in breast cells. Therefore we hypothesize the presence of a shared antigen between BC and thyroid cells able to trigger a common immunoreactivity; this could explain firstly the association between BC and ATD and secondly the influence of ATD on BC prognosis. The shared antigen could be NIS, cross reactive epitopes in TPO and LPO or, since serum TPOAb are frequent and abundant in BC patients, TPO itself.

Human TPO and LPO are heme-containing enzyme members of the family of mammalian peroxidases, which utilize hydrogen peroxide to oxidize a number of inorganic and organic substrates and they are active in a variety of anatomic sites[16]; other members of this group include myeloperoxidase (MPO) and eosinophil peroxidase. Human TPO is the only characterised component of the "microsomal" antigen involved in ATD and the measurement of serum TPOAb is generally considered a marker of thyroid autoimmunity[17]. The human TPO gene is about 150 kbp in size, located on chromosome 2, locus 2p25 and consists of 17 exons and 16 introns[18]. The full-length 3048-bp transcript (TPO1) encodes a protein consisting of 933 amino acids, which has a short intracytoplasmatic tail, a transmembrane domain encoded by exon 15 and a large extracellular domain which contains the catalytic site. TPO gene undergoes alternative splicing to generate different TPO isoforms lacking one or more exons. To date 8 isoforms have been described in thyroid cells, TPO 1, TPO 2[19,20], TPO 3 or TPO Zanelli[21], TPO 4, TPO 5, TPO 6, TPO 2/3 and TPO 2/4[22], as illustrated in table 1. While TPO 3[23] and TPO 4[22] are able to reach the cell surface and show enzymatic activity, TPO 2 and TPO 5 lack enzymatic activity[21,22] but we are unaware of functional studies performed on the other TPO isoforms. In addition TPO isoforms' sequences have been only partially characterized because of the difficulty in obtaining full length mRNA/cDNA[22].

The apparent molecular weight (MW) of TPO in denaturing western blot (WB) is a doublet at 105-110 kDa (table 1), which may be due to variants of TPO[19,22] or differing degrees of glycosylation[24,25].

The aim of this study is to explore the hypothesis that TPO is a thyroid/BC shared antigen by analyzing TPO gene expression (mRNA and protein) in relevant ex vivo samples.

MATERIALS AND METHODS

Tissues

Human surgical tissues (from scheduled operations and obtained with informed consent) were immediately frozen. Eight BC (7 infiltrating ductal adenocarcinoma and 1 mucinous carcinoma) and their 8 corresponding peri-tumoral breast tissues (PT) were collected. Seventeen adipose tissues (AD) from various depots including abdominal (n=4), subcutaneous (n=8), knee (n=1) and orbital fat (n=4) were studied and also malignant tissues, including pancreatic adenocarcinoma (PC; n=3), primary kidney carcinoma (PKC; n=1) and hepatic metastasis of kidney carcinoma (MKC; n=1) were obtained for controls. Human thyroid tissue (TT) from thyroidectomy for multinodular goiter was used as a positive control. Were also collected two additional fresh frozen BC and also 9 paraffin embedded BC sections (all infiltrating ductal adenocarcinoma), for a total of 19 different BC samples analyzed with various techniques (supplemental table 1). Thyroid autoimmunity status was available only in 8/19 BC patients and 1/8 had serum TPOAb positivity (supplemental table 1).

Indirect Immunofluorescence (IF)

Indirect IF was performed on 4 deparaffinized and rehydrated human BC tissue samples and also on human TT as positive control. The primary antibodies comprised pooled sera from women with high titers of TPOAb (n=4) or free of TPOAb (n=3); TPOAb determination was performed by immune enzymometric assay (Tosoh Bioscience, Tessenderlo, Belgium). Tissue sections were incubated in 1:10 dilution of the pooled sera at room temperature for one hour, followed by 1:70 dilution of polyclonal rabbit anti-human-IgG FITC-coniugated (Dako, DK-2600 Glostrup, Denmark) for 1 hour at room temperature. The same tissue sections were also incubated in 1:100 dilution of mouse monoclonal to TPO ab76935 (abcam, Cambridge, UK) at room temperature for one hour followed by 1:50 dilution of Alexa Fluor 488 goat anti-mouse IgG (Life Technologies Ltd, Paisley, UK) at room temperature for one hour. A further 5 paraffin embedded BC samples were analysed in a modified protocol in which slides were initially blocked for two hours at room temperature with goat serum 10% and all primary and secondary antibodies were also diluted in goat serum 10% and incubated with tissue slides for one hour at room temperature. Primary antibodies were pooled human sera from women with high titres of TPOAb or free of TPOAb as above 1:10 dilution and mouse TPO monoclonal ab12500 (abcam, Cambridge, UK) 1:25 dilution. Secondary antibodies were respectively goat polyclonal to human IgG-H&L (FITC) ab97164 (abcam, Cambridge, UK) 1:500 dilution and Alexa Fluor 488 goat polyclonal anti-mouse IgG H + L (Life Technologies Ltd, Paisley, UK) 1:1000 dilution. In all cases images were analysed using Olympus BX51 microscope, trinocular brightfield / fluorescence (Olympus America Inc., Center Valley, PA, USA).

Analysis of TPO mRNA expression

RNA isolation and Reverse Transcription (RT) – Polymerase Chain Reaction (PCR). Total RNA was extracted from tissues and cells using TRIzol Reagent (Ambion, Life Technologies Ltd, Paisley, UK) as described in the manufacturer's protocol; reverse transcription used 1 µg total RNA, M-MLV reverse transcriptase (Promega, Madison, USA) and was primed with oligo dT for standard PCR or random hexamers for Absolute Real-time Quantitative PCR (QPCR) in a standard protocol.
The resulting cDNA was amplified in standard PCR using Ampli Taq Gold DNA Polymerase (Applied Biosystems, Life Technologies Ltd, Paisley, UK) and primers designed using Primer 3 Plus software. The principal primer pairs used for PCR were forward in exon n° 13 (F13: 5'-ACGGGTATGAGCTCCAAGG-3') and reverse in exon n° 17 (R17: 5'-TCTCGGCAGCCTGTGAGTAT-3'). PCR products obtained were electrophoresed on 2% agarose gel and stained with ethidium bromide. PCR single products were purified using Polyethylene Glycol (PEG) solution (26% PEG, 6.6 µM $MgCl_2$ and 0.6 M Na acetate), or extracted from cut gel fragments using the Qiaquick gel extraction kit (Qiagen, Manchester, UK) according to the manufacturer's instructions for sequence analysis performed using the BigDye Terminator Sequencing Kit [Applied Biosystems (ABI), Life Technologies Ltd, Paisley, UK] and an ABI Prism 377 DNA automatic sequencer (as previously described[26]).

QPCR. QPCR was conducted using SYBR Green incorporation measured on a Stratagene (La Jolla, CA, USA) MX 3000. The TPO primers used for QPCR were forward in exon n° 2 (F2: 5'-GCTGTCTGTCACGCTGGTTA-3') and reverse in the junction region between exons n° 3 and 4 (R3-4: 5'-TGAGGTTTCTCTGCATCGTG-3'). Comparison with standard curves comprised of serial dilutions of the relevant PCR amplicon with known copy number were included in each experiment and permitted calculation of absolute values for each sample (transcripts/µg input RNA). In addition, transcripts for a housekeeping gene, APRT, were measured so that values could be expressed relative to this (transcripts/1000 APRT). In a single QPCR experiment all measurements were made in triplicate; the standard curve was also run in at least duplicate in each reaction (as previously described[27]).

Whole TPO mRNA expression according to the different TPO isoforms (LongRange RT-PCR)

To characterize more fully previously reported TPO variants and those identified in this study by PCR, we generated cDNA using the Qiagen (Manchester, UK) LongRange 2Step RT-PCR kit, according to the manufacturer's protocol, and TPO primers F2 and R17. Several tissues were analysed including TT (n=1), BC (n=2) and subcutaneous AD (n=1). The PCR amplification step was conducted in the presence and absence of Q solution; resulting products were electrophoresed on

1.5% agarose gels in Tris-acetate-EDTA (TAE) buffer, stained, extracted, purified and sequenced as described above.

TPO protein expression analysis using WB

Protein extraction. Frozen tissue (200 mg) was diced using a razor blade and thawed in 600 µL of RIPA buffer (phosphate buffered saline PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 10 µL/ml proteases inhibitor [phenylmethanesulfonylfluoride (PMSF) in isopropanol] and homogenized with a Dounce homogenizer at 4°C. Samples were then centrifuged at 15000xg for 20 minutes at 4°C and the protein extract (supernatant) stored at -20°C.

Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-Page) and WB. Extracted proteins (5-15 ug of TT and 50 ug for all other tissues) were boiled for 5 minutes in loading buffer (40% SDS 10%, 20% glycerol, 20% 0.5M Tris pH 6.8, 16% pure water, 2% pyronin Y 0.2%, 2% β-mercaptoethanol, 10 µl/ml PMSF 100 mM) and separated on 8% Tris-glycine SDS-acrylamide gels. After electrophoresis proteins were transferred onto a Polyvinylidine Fluoride (PVDF) membrane Amersham Hybond-P (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) and blocked with 5% non-fat dry milk powder (Marvel, Long Sutton, Spalding, UK) for 2 hours at room temperature followed by overnight incubation at 4°C with mouse monoclonal TPO ab76935 (Abcam, Cambridge, UK) raised to TPO amino acids 672-780, diluted (1:1000) with 5% bovine serum albumin [BSA (Sigma-Aldrich, Gillingham, Dorset, UK)]. After 3 washes, membranes were incubated with peroxidase-labelled anti-mouse IgG (1:5000) for 1 hour at room temperature and then developed with Amersham ECL Plus western blotting detection system (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) according to manufacturer's instructions. Membranes were then stripped for 30 min at 60°C in buffer and re-probed with rabbit monoclonal anti-human â-actin IgG and peroxidase-labelled anti-rabbit IgG, following the same procedure as previously described[28].

Primary antibody absorption test by antigen peptide. Primary TPO antibody ab76935 (8 µg) was pre-incubated overnight at 4° C with TPO recombinant fragments (8 µg) produced in bacteria either TPO 3 (Glu 471 - Ser 720) or TPO 4 (Phe 709 - Leu 993)[29] in 5% BSA. The same primary antibody absorption test was also performed using 10 µg bovine LPO (Sigma-Aldrich, Gillingham, Dorset, UK).

Indirect Immunohistochemistry (IHC)

Indirect IHC was performed on 2 frozen human BC tissue samples and frozen human TT as positive control, using Vectastain ABC-AP Kit (Vector Laboratories Inc, Burlingame, CA, USA) with Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories Inc, Burlingame, CA, USA), as described in the manufacturer's protocol. The primary antibody was the mouse monoclonal to TPO ab12500 (abcam, Cambridge, UK) diluted 1:25 in specific buffer and incubated with tissue sections at

room temperature for one hour. Images were analysed using Olympus BX51 microscope, trinocular brightfield / fluorescence (Olympus America Inc., Center Valley, PA, USA).

RESULTS

TPO antibodies display autoreactivity to a BC antigen

Figure 1 shows representative results obtained by indirect IF, in which pooled human sera from women with high levels of TPOAb were used as the primary antibody. In TT, used as a positive control, the IF signal was strongest on the apical surface of the thyroid cells and most likely reflects binding to TPO. In BC tissues, immunoreactivity was apparent in 9/9 tested, while the signals were absent from TT and BC when using pooled TPOAb free human sera. Similar results were obtained using the mouse monoclonal to TPO antibody (supplemental figure 1). The results indicate immunoreactivity to BC in women with TPO antibodies, thus we conducted experiments to investigate TPO expression in BC.

TPO transcripts expression in ex vivo tissues

Figure 2 panel A shows results obtained in standard RT-PCR reactions in representative TT, BC and PT samples after 50 PCR cycles. After 40 PCR cycles the expected amplicons for F13 and R17 (485 bp and 353/355 bp, as shown in table 1) were obtained only in TT, 8/8 BC, 5/8 PT and 9/9 AD (Figure 2 panel B). These four tissue types also expressed unexpected products of 220 bp and 130 bp; sequencing of the 220 bp amplicon revealed that it is a novel TPO isoform lacking exons 14 and 16 (most abundant in BC and AD) but we were unable to identify the 130 bp product. Sequencing of additional bands from 300 bp to 500 bp revealed they were all nicked products of the 485 bp or 353/355 bp bands. In other tumours such as PC, PKC or MKC TPO transcripts were detected only after 50 cycles and in particular only the 485 bp product in 2/5 tumours; no other amplicons were obtained.

Primer pair F2/R3-4, which should detect all known TPO isoforms and hence provide a measure of total TPO transcripts, was used in QPCR. In TT (n=2) we obtained an average $6x10^6$ transcripts/µg input RNA. In BC (n=8) the mean was $8.9x10^2$ (± sem) transcripts/µg input RNA, which was not significantly different from the level in PT (n=8) but 10^4 fold less than in TT. Since breast tissue contains fat, we measured TPO transcripts in AD from various depots (n=17); the mean was $9.2x10^3$ (± sem) transcripts/µg input RNA with no depot-specific differences noted but 10^3 fold less than in the TT (and 10-fold higher than in BC). In the other tumors (PC, PKC and MKC) TPO transcripts were at the limit of detection.

In an attempt to characterize the various TPO isoforms detected by standard RT-PCR, we used primers in exons 2 (location of start codon) and 17 (location of stop codon) in LongRange RT-PCR; table 1 and figure 3 summarize the expected products and our results, respectively. We used TT as a positive control, in which we obtained a faint amplicon at 2700 bp only in the presence of Q

solution (figure 3 panel A); sequencing confirmed that it was the full length TPO 1 isoform (figure 3 panel B). The most abundant thyroid transcript was the previously described TPO 5 , which lacks exon 8, and hence enzymatic activity. Additional smaller transcripts were also present in TT but these did not correspond to other TPO isoforms, either previously reported or described in this study.
In non-thyroid samples many different bands were detected; a 2000 bp variant was present in BC n°1 (with Q solution) lacking exons 8-9 or in BC n°2 lacking exon 8 (TPO 5); a 1500-1700 bp variant lacking exons 8-10 was present in BC n°1 (with Q solution) and AD; whilst a 1000 bp transcript in which exon 7 then skipped to exon 15 was abundant in the two BC (without Q); a short transcript comprising exons 2 linked to 15-17 was present in AD, BC n°2 and TT. As in the case of TT, we did not find other known TPO isoforms or the new variant lacking exons 14 and 16; figure 3 panel B summarizes all TPO isoforms found with LongRange RT-PCR using primer pair F2/R17.

TPO protein expression in ex vivo tissues

In WB using extracts from human TT probed with the TPO monoclonal antibody ab76935 we observed proteins of apparent molecular mass of 105-110 kDa, corresponding to TPO 1 (table 1). When exposing the blots for extended times (up to 40 minutes) combined with loading 10 times more protein extract than for TT, we were able to detect similar bands in 7/8 BC, 4/8 PT, 2/4 AD, 1/1 PKC, 1/1 MKC and 1/3 PC (figure 4, panel A). To test the specificity we absorbed the antibody using recombinant fragments of TPO spanning the region of the epitope recognized by the antibody. As shown in figure 4 panels B and C, the 105-110 kD signal in TT was greatly reduced and that in the other tissues completely eliminated by the treatment. In contrast, absorption with LPO did not reduce the signal (data not shown).

Figure 4 (panels D and E) illustrates an entire WB of TT probed with the TPO monoclonal antibody ab76935 and demonstrates proteins of apparent MW 70, 60 and 30-35 kDa, in addition to the doublet at 105-110 kDa. Absorption with recombinant TPO strongly reduced not only the 105-110 band corresponding to complete TPO protein (TPO 1) but also the signals at 70 and 60 kDa, indicating that they could be smaller isoforms of TPO or degraded fragments of TPO 1; the 30-35 kD band was not affected by absorption. Absorption with LPO did not reduce the signal (data not shown). The 70 and 60 kDa bands were also present in breast tissues BC/PT and PKC in addition to proteins of 50 and 150 kDa, all of which were reduced by absorption of TPO monoclonal antibody ab76935 with recombinant TPO (supplemental figure 2).

The WB results indicate that breast tissues contain low levels of TPO protein, including TPO 1, but in view of the various PCR data which demonstrate that AD also expresses TPO transcripts, we sought to identify the cellular location of the TPO protein using indirect IHC. Figure 5 demonstrates weak TPO immunoreactivity in a small proportion of breast epithelium, obtained in 2/2 BC tissues analyzed.

DISCUSSION

Our experiments demonstrate that TPO mRNA and protein are not confined to thyroid cells but are also present in other tissues, albeit it at low levels. Our results confirm those of Lai et al.[30] who demonstrated TPO expression in orbital tissue.

TPO expression in BC tissue could explain both the known association between BC and ATD and the protective role of serum TPOAb in patients with aggressive BC. We hypothesize that TPO could be a common antigen between thyroid and BC tissues; since even trace amounts could suffice to trigger B lymphocyte activity although further studies of T lymphocyte immunoreactivity in both thyroid and BC tissues are necessary to validate this hypothesis.

Further investigation is also needed to determine whether TPO expression is confined to BC, especially since we found TPO in PT tissue, although neoplastic features may have been missed. TPO is expressed in several fat depots, and since fat is abundant in the breast this could explain PT TPO expression. A recent review has also reported that adipocytes within mammary gland carcinomas are dynamic cells that may contribute to human BC progression[31]. However adipose tissue does not account for all the TPO we detected in breast since IHC indicates TPO protein in neoplastic breast epithelium.

An increased prevalence of serum TPOAb and other thyroid autoantibodies has been found in several other cancers including kidney[32], pancreas[33], lung[34] and gastric[35] and are associated with better (kidney) and worse (pancreas) prognosis, suggesting a role for these antibodies in tumour prognosis.

Our work revealed a new variant, lacking exons 14 and 16 (15 encodes the membrane spanning region) which is most abundant in breast tissue. LongRange PCR revealed numerous other isoforms, in several tissues tested, in which exons 2 to 7 were conserved, but 8 to 16 (encodes enzyme activity) were spliced out. Surprisingly we did not detect previously described isoforms such as TPO 2, TPO 3 etc. There are two possible explanations: the first and simplest being primer selection and the second the existence of additional START codons in some TPO isoforms. Future experiments will investigate whether mRNA transcripts exist which are the equivalent of the various lower MW bands we found using WB. Exons from 5 to 12 encode the MPO-like sequence which contains important catalytic residues; probably TPO isoforms that lack exons in this region are not functional, as already demonstrated for TPO 2 that lacks exon 10[21] and TPO 5 that lacks exon 8[22].The absence of functional activity does not influence the antigenic role: to this purpose TPO protein sequence must just contain those short linear peptides processed and presented to T-lymphocytes by antigen presenting cells via Major Histocompatibility Complex (MHC) molecules. Many authors identified TPO epitopes recognized by T lymphocytes in the MPO-like sequence[36-40]. Furthermore, two T-lymphocytes epitopes have been found also in C-terminal part of TPO protein, in particular in the transmembrane region[38,39] and in the intracellular region[38].

B-lymphocytes epitopes are different, since a few are linear (C21, C2)[41,42] but the majority are conformational[36,43-46], therefore strongly related to tertiary and quaternary protein structures. TPO

isoforms that lack some exons probably fold into a different conformation when compared with the full-length TPO1 and hence may lose their antigenic property for B cells and autoantibodies.

In conclusion TPO no longer seems to be thyroid specific: mRNAs and proteins for known TPO isoforms are weakly but clearly expressed in BC and other tissues. In particular TPO expression in BC could explain at least in part the high frequency and protective role of serum TPOAb in BC patients, hypothesizing an enhancement of specific T and B lymphocytes immunoreactivity, with potential implications in translational medicine. Moreover we have found many novel TPO mRNA isoforms lacking several exons both in thyroid and other tissues examined, tumoral or not. Some of these TPO mRNA isoforms could be translated into the correspondent lower MW proteins: further studies are needed to investigate their function and immunogenicity and if they can be used as tissue specific markers.

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TABLES

Table 1

	mRNA analysis					Protein analysis			
TPO Isoform	**N° exons**	**Exon lacked**	**bp**	**PCR F13R17 ***	**PCR F2R17 ****	**N° AA**	**MW (kDa) calculated**	**MW (kDa) on WB**	**Function**
TPO 1	17	/	3152	485	2776	933	103	105-110	Yes
TPO 2	16	10	2981	485	2605	876	96	?	No
TPO 3	16	16	3022	355	2646	890	98	?	Yes
TPO 4	16	14	2930	353	2644	889	98	?	Yes
TPO 5	16	8	2543	485	2257	760	84	?	No
TPO 6	12	10,12,13, 14,16	2339	No product	1963	662	73	?	?
TPO 2/3	15	10,16	2851	355	2475	832	91	?	?
TPO 2/4	15	10,14	2849	353	2473	832	91	?	?

TABLE AND FIGURES LEGEND

Table 1. Summary of known TPO isoforms' characteristics. bp= number of base pairs. *= size of expected products (bp) with Polymerase Chain Reaction (PCR) using primer pair F13/R17. **= size of expected products (bp) with LongRange Reverse Transcription (RT)-PCR using primer pair F2/R17. N° AA= number of amino acids. MW= Molecular Weight. kDa= kilodalton. WB= Western Blot.

Figure 1. Indirect Immunofluorescence results using pooled human sera (phs) as primary antibody. **Panel A:** breast cancer (BC) tissue incubated with high levels TPOAb positive phs. **Panel B:** thyroid tissue (TT) incubated with high levels TPOAb positive phs (positive control). **Panel C:** BC incubated with TPOAb free phs. **Panel D:** TT incubated with TPOAb free phs (negative control). In panels A and B is present a positive signal (green stain), which is absent in panels C and D, where is visible only a weak aspecific staining (negative).

Figure 2. PCR Products obtained using primer pair F13/R17.
Panel A: Results after 50 cycles (representative samples) in 1= thyroid tissues (positive control); 2,4,7= breast cancer (BC); 3,5,6= peri-tumoral breast tissues (PT); 8= negative control (pure water). L 100 bp = Ladder 100 base pairs. The expected 485 and 353/355 bp bands and other intermediate bands (nicked products) are expressed by all samples, except for 3,8. The 220 bp unexpected band is clearly expressed in 2,4,5,6,7, faint in 1 and absent in 3,8. The 130 bp unexpected band is expressed only in 6.
Panel B: Summary of PCR products after 40 cycles obtained in all samples. BC= breast cancer, PT= peri-tumoral breast tissues; PC= pancreatic adenocarcinoma; PKC= primary kidney carcinoma; MKC= hepatic metastasis of kidney carcinoma; AD= adipose tissue.

Figure 3. LongRange RT-PCR results with primer pair F2/R17.
Panel A shows agarose gel 1.5% bands using PCR master mix with (Q) or without (N) Q solution obtained in 1= breast cancer (BC) n°1 with N, 2= BC n°1 with Q, 3= BC n°2 with N, 4= BC n°2 with Q, 5= subcutaneous adipose tissue (AD) with N, 6= thyroid tissue (TT) with N and 7= TT with Q.
Panel B shows a schematic representation of novel TPO mRNA isoforms found sequencing PCR products obtained with LongRange RT-PCR using primer pair F2/R17. Numbered squares from 2 to 17 represent TPO gene exons. White and grey squares represent respectively expressed and missed exons.

Figure 4. Western Blot (WB) representative results; 40 minutes exposition time.
Panel A: 105-110 kDa band expression using ab76935 obtained in breast cancer tissue (1,2), breast peri-tumoral tissue (3,4), primary kidney cancer (5), pancreatic adenocarcinoma (6,7), abdominal fat

(10), subcutaneous fat (11), knee fat (12) and orbital fat (13); thyroid tissue (9,15) is used as positive control and it is separated from other samples by empty spaces (8,14).

Panel B: 105-110 kDa band expression using normal ab76935 obtained in breast peri-tumoral tissue (1,2), hepatic methastasis of kidney cancer (3), breast cancer (4,5,6) and thyroid tissue (8), separated from other samples by an empty space (7).

Panel C: 105-110 kDa band expression using TPO absorbed ab76935 obtained in the same samples of panel B: the signal is strongly reduced or eliminated.

Panel D: complete WB results in thyroid tissue using normal ab76935; it shows the doublet at 105-110 kDa and 3 additional bands at 70, 60 and 30-35 kDa.

Panel E: complete WB results in the same thyroid tissue of panel D using TPO absorbed ab76935: the 105-110, 70 and 60 kDa bands are strongly reduced, whilst the 30-35 kDa band is not significantly modified.

Figure 5. Indirect immunohistochemistry results using TPO mouse monoclonal ab12500 as primary antibody. **Panels A and B** show two different breast cancer tissues (BC); in the small square is represented thyroid tissue (TT) as positive control. In TT the TPO signal (red) is strongly positive and located into thyreocytes that express TPO, disposed around the colloid follicles; in BC the TPO signal (red) is weaker but present and located in breast epithelium cells.

Figure 1

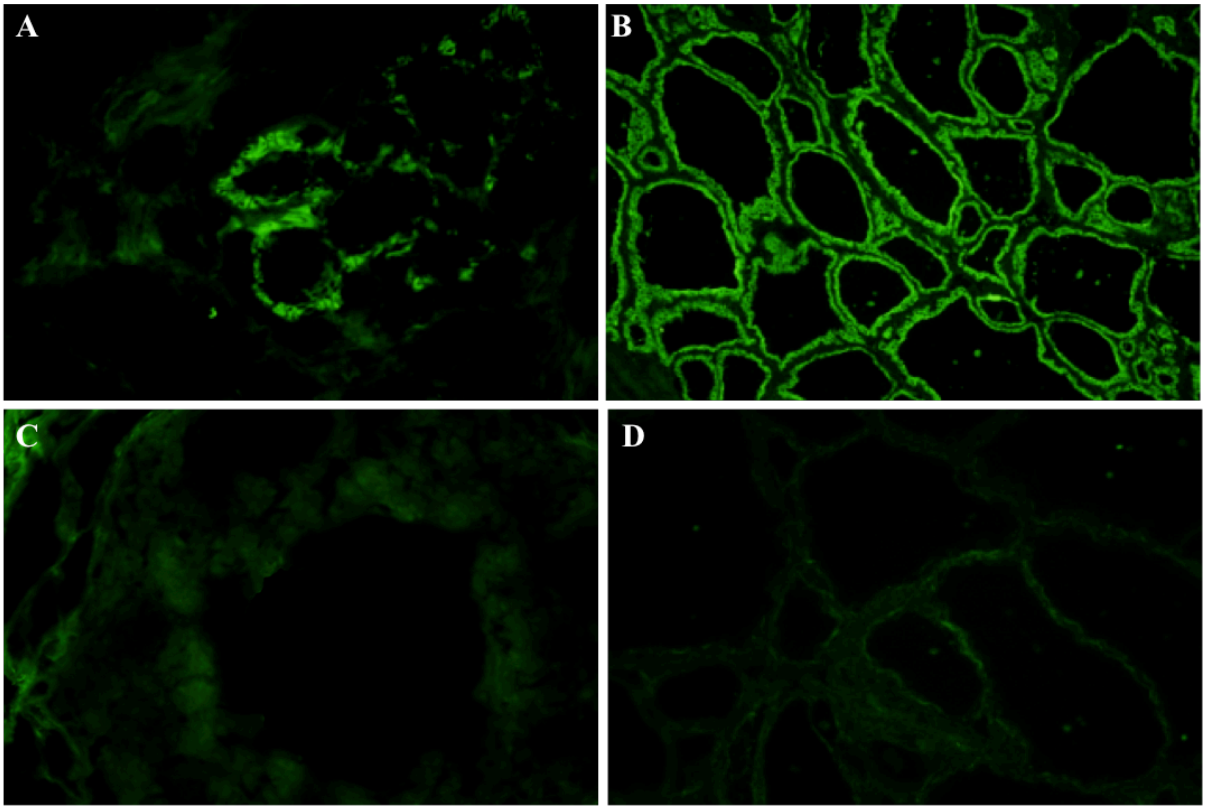
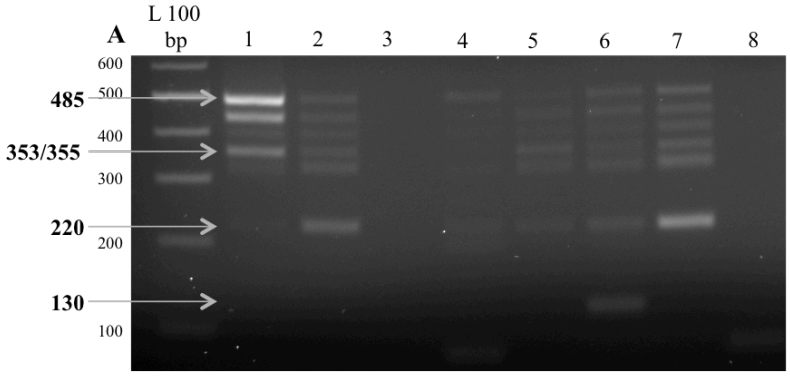


Figure 2

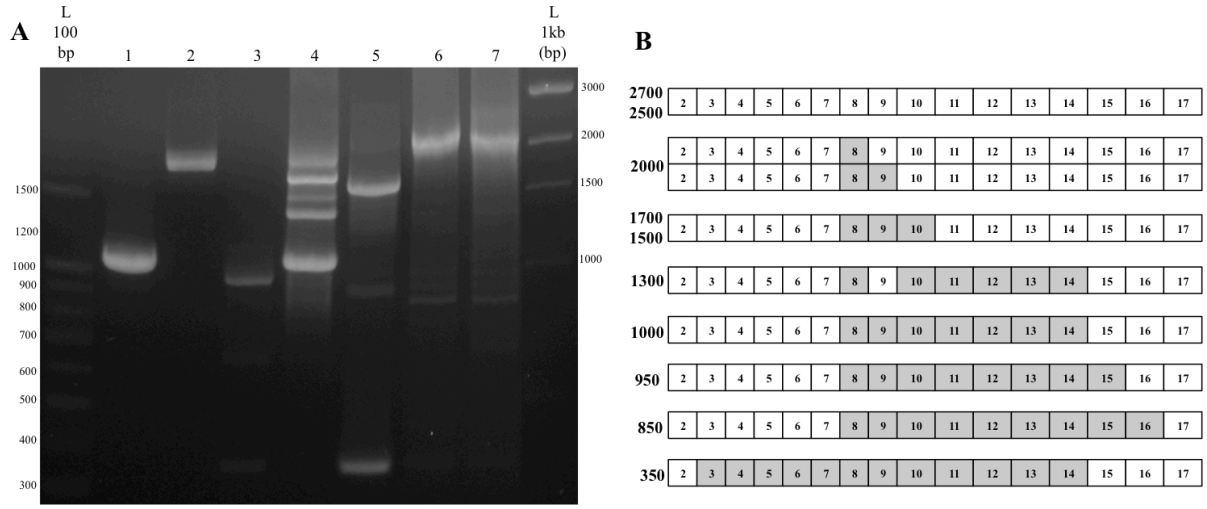
A



B

	485 bp	**353/355 bp**	**220 bp**	**130 bp**
BC	8/8	8/8	6/8	1/8
PT	5/8	5/8	6/8	3/8
PC	0/3	0/3	0/3	0/3
PKC	0/1	0/1	0/1	0/1
MKC	0/1	0/1	0/1	0/1
AD	9/9	9/9	8/9	1/9

Figure 3


A
L
100
bp
1
2
3
4
5
6
7
L
1kb
(bp)
1500
1200
1000
900
800
700
600
500
400
300
3000
2000
1500
1000
B
2700
2500
2000
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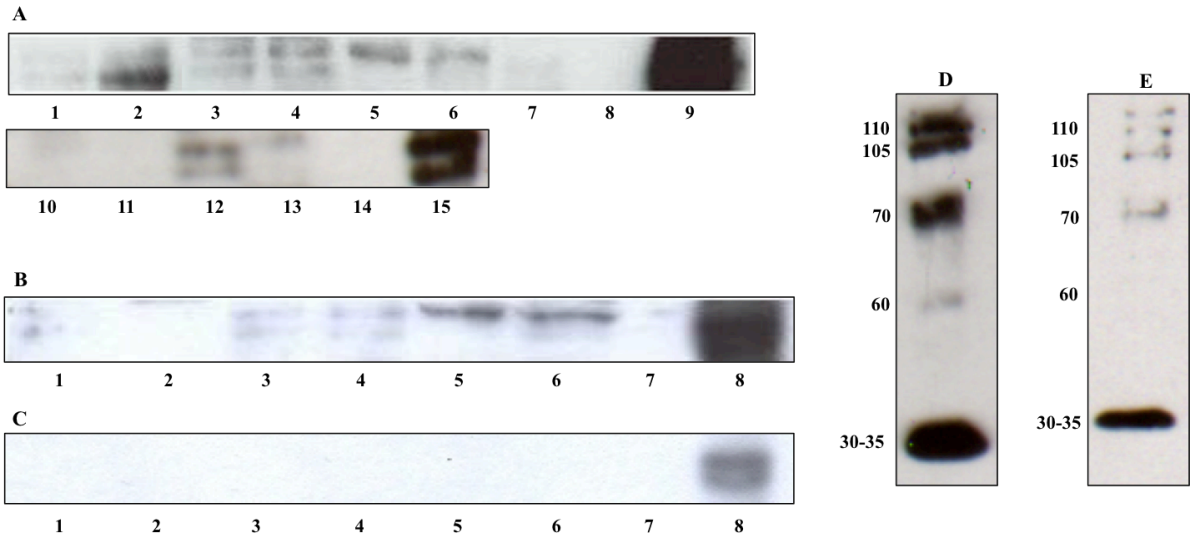
1500
1300
1000
950
850
350
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4


A
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8
D
110
105
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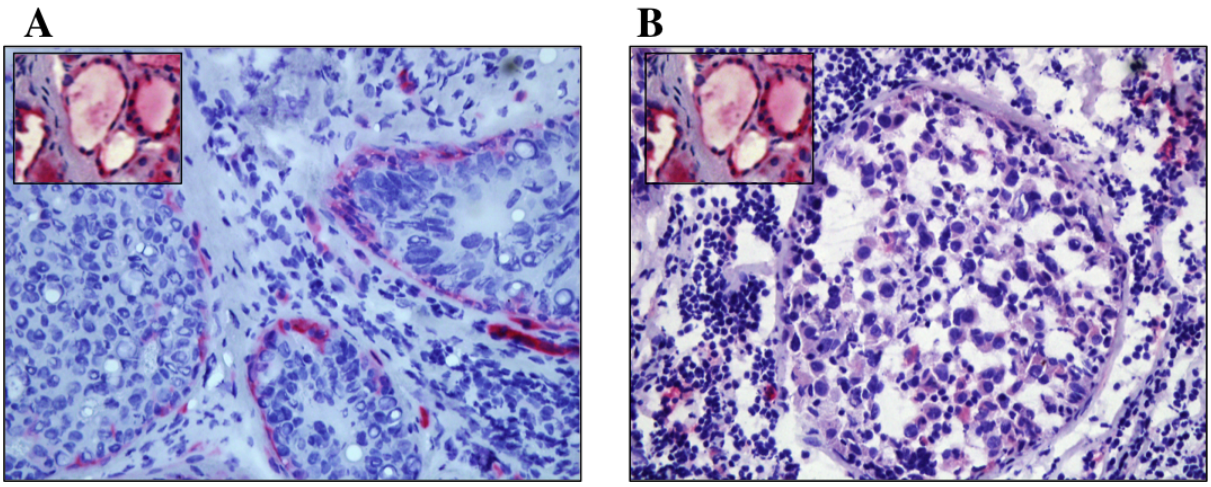
60
30-35
E
110
105
70
60
30-35

Figure 5



SUPPLEMENTAL DATA

Supplemental Table 1

Summary of all the 19 different breast cancer (BC) tissues collected and their related information, when available. f = fresh frozen tissue, p = paraffin embedded sections, PCR= polymerase chain reaction, QPCR= absolute real-time quantitative PCR, WB= western blot, IHC= immunohistochemistry, IF= immunofluorescence, TPOAb= autoantibodies to thyroid peroxidase, TgAb= autoantibodies to thyroglobulin, v.n.= normal value, / = no data available.

Supplemental Figure 1

Indirect Immunofluorescence staining for thyroid peroxidase (TPO), 10x magnification. In the small box for each picture there is the correspondent 20x magnification. In panels B,D,F,H is present a positive signal (green stain), which is absent in panels A,C,E,G.

Panels A,B: thyroid tissue (TT) tested with pooled human sera (phs) from individuals TPO autoantibody negative [A] and positive [B].

Panels C,D: breast cancer tissue (BC) tested with pooled human sera (phs) from individuals TPO autoantibody negative [C] and positive [D].

Panels E,F: TT tested with goat serum 10% lacking primary antibody [E] and mouse monoclonal antibody to TPO [F].

Panels G,H: BC tested with goat serum 10% lacking primary antibody [G] and mouse monoclonal antibody to TPO [H].

Supplemental Figure 2

Panels A,B,C: Entire Western blot representative images of various tissues probed with TPO monoclonal antibody number 76935 and exposed for 40 minutes; the numbers on the left indicate the molecular weight (kDa). In each small box there is the correspondent magnification of the 105-110 kDa band expression (full-length TPO).

Panel A.i and A.ii: Breast cancer (BC) tissue (1,2,16,17), breast peri-tumoral (PT) tissue (3,4,18,19), primary kidney cancer (5), pancreatic adenocarcinoma (6,7), abdominal fat (10), subcutaneous fat (11), knee fat (12), orbital fat (13), thyroid tissue (TT) as positive control (9,15), empty space (8,14).

Panel A.iii: same samples of panel A.ii using TPO ab76935 pre-absorbed with TPO: the signal related to the 105-110 kDa band and to other additional bands (50, 60, 70, 150 kDa) is strongly reduced or eliminated, whilst the 30-35 kDa and 64-70 kDa bands are not significantly modified.

Disclose information: Authors declare that there are not any potential conflicts of interest or affiliations.

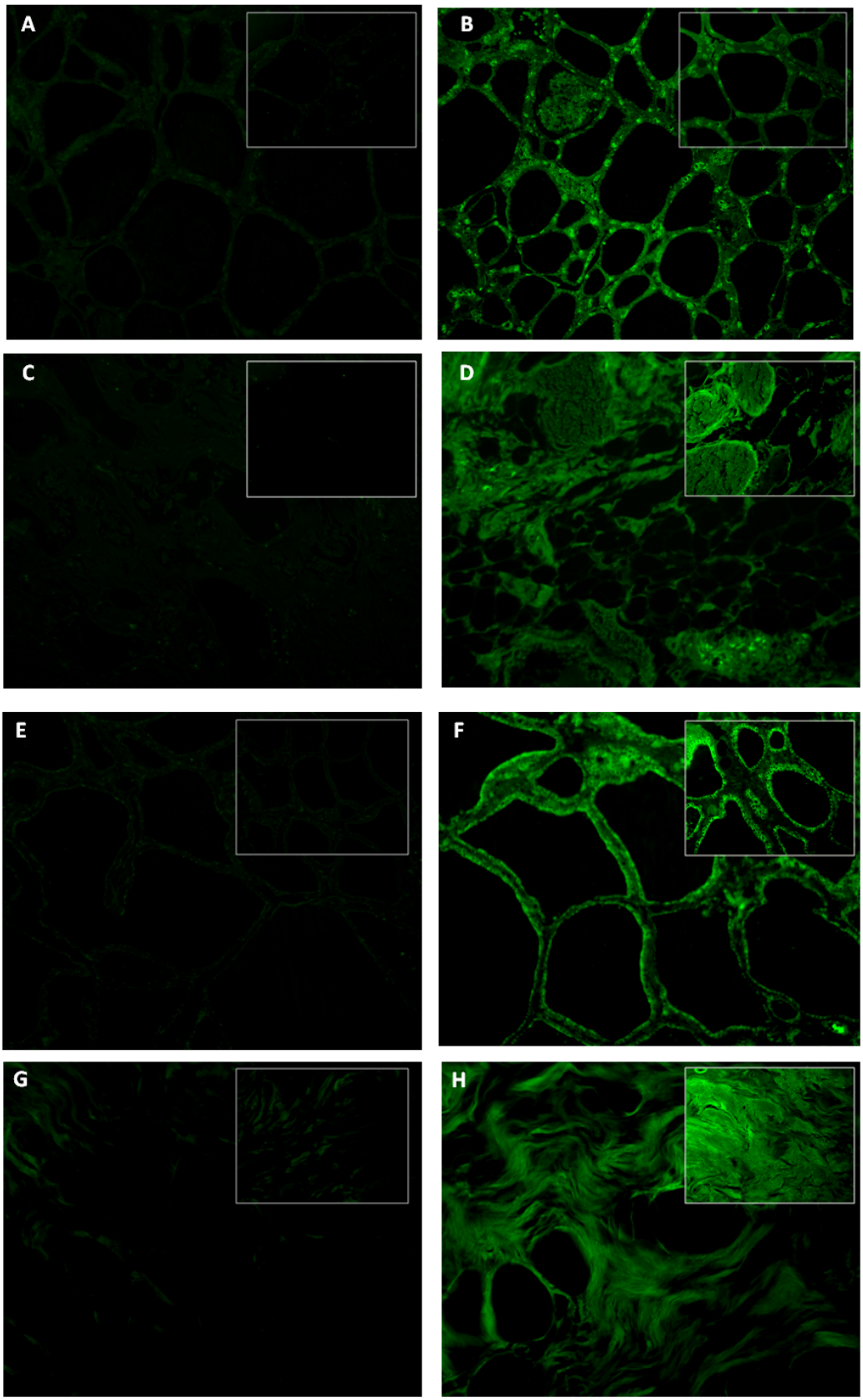
Panel B: PT (1,2), hepatic methastasis of kidney cancer (3), BC (4,5,6), TT as positive control (8), empty space (7).

Panel C: same samples of panel B using TPO ab76935 pre-absorbed with TPO: the signal related to 105-110 kDa band and to other additional bands (50, 60, 70, 150 kDa) is strongly reduced or eliminated, while the aspecific signal of 30-35 kDa band is not significantly modified.

Supplemental Table 1

Sample number	Breast cancer histological type	TPO gene expression (mRNA and protein): Analyses performed	Serum TPOAb (U/ml) v.n. <10	Serum TgAb (U/ml) v.n. <30	Serum TSH (µU/ml) v.n. 0.4-3.4	Patient's age (years)
BC n°1 (f)	Infiltrating ductal adenocarcinoma	mRNA: PCR, QPCR, LongRange PCR Protein: WB	443.87	64.07	2.02	68
BC n°2 (f)	Mucinous adenocarcinoma	mRNA: PCR, QPCR, LongRange PCR Protein: WB	<1	No	0.653	81
BC n°3 (f)	Infiltrating ductal adenocarcinoma	mRNA: PCR, QPCR Protein: WB	<1	5.89	1.03	85
BC n°4 (f)	Infiltrating ductal adenocarcinoma	mRNA: PCR, QPCR Protein: WB	<1	90.42	0.453	72
BC n°5 (f)	Infiltrating ductal adenocarcinoma	mRNA: PCR, QPCR Protein: WB	<1	No	0.911	58
BC n°6 (f)	Infiltrating ductal adenocarcinoma	mRNA: PCR, QPCR Protein: WB	<1	No	1.23	70
BC n°7 (f)	Infiltrating ductal adenocarcinoma	mRNA: PCR, QPCR Protein: WB	<1	No	1.92	72
BC n°8 (f)	Infiltrating ductal adenocarcinoma	mRNA: PCR, QPCR Protein: WB	<1	No	0.809	79
BC n°9 (f)	Infiltrating ductal adenocarcinoma	Protein: IHC	/	/	/	73
BC n°10 (f)	Infiltrating ductal adenocarcinoma	Protein: IHC	/	/	/	70
BC n°11 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°12 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°13 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°14 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°15 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°16 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°17 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°18 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/
BC n°19 (p)	Infiltrating ductal adenocarcinoma	Protein: IF	/	/	/	/

Supplemental Figure 1



Supplemental Figure 2