AUTOIMMUNE RESPONSES TO THYROID/BREAST SHARED ANTIGENS TO DEVELOP NOVEL AND SPECIFIC THERAPIES AND DIAGNOSTICS

By

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ABSTRACTS AND PUBLICATIONS ARISING FROM THIS WORK

PEER-REVIEWED PUBLICATIONS

- Does thyroid peroxidase provide an antigenic link between thyroid autoimmunity and breast cancer? Muller I, Giani C, Zhang L, Grennan-Jones FA, Fiore E, Belardi V, Rosellini V, Funel N, Campani D, Giustarini E, Lewis MD, Bakhsh AD, Roncella M, Ghilli M, Vitti P, Dayan CM, Ludgate ME. *International Journal of Cancer. 2014 Apr 1;134(7):1706-14. Epub 2013 Oct 15*

- The sodium iodide symporter is unlikely to be a thyroid/breast shared antigen. Muller I, Zhang L, Giani C, Dayan CM, Ludgate ME and Grennan-Jones F. *Journal of Endocrinological Investigation. 2015 Aug;* DOI 10.1007/s40618-015-0368-6

- Thyroid autoimmunity as a biomarker of outcome in women with breast cancer: a large scale study using data from the Taxotere as Adjuvant Chemotherapy Trial (TACT CRUK01/001). Muller I, Kilburn LS, Taylor PN, Barrett-Lee P, Bliss JM, Ellis P, Ludgate ME and Dayan CM. *Planned to be submitted to the Journal of the American Medical Association Spring 2016.*

ABSTRACTS PRESENTED AT INTERNATIONAL MEETINGS

- 15th International Thyroid Congress (ITC) 2015 (18th-23rd Oct), Orlando, Florida, USA: Short Oral Communication and Poster Presentation. I have also been awarded as Finalist of the ITC 2015 Trainee Poster Contest. "Thyroid autoimmunity as a biomarker of outcome in women with breast cancer - large scale study using data from the Taxotere as Adjuvant ChemoTherapy (TACT) trial (CRUK01/001)".

- ETA 2014 (6th-10th Sep), Santiago de Compostela, Spain: Poster Presentation. "Investigating the sodium iodide symporter (NIS) as a possible candidate shared antigen in thyroid autoimmunity and breast cancer".

ABSTRACTS PRESENTED AT NATIONAL MEETINGS

- 15th November 2013, Postgraduate Research Day, Cardiff University: I planned a Poster Presentation but family illness prevented me from attending. "Does thyroid peroxidase provide an antigenic link between thyroid autoimmunity and breast cancer?"

- 12th December 2014, Postgraduate Research Day, Cardiff University: I planned a Poster Presentation but family illness prevented me from attending. *"Shared immune response in thyroid autoimmunity and breast cancer: the sodium iodide symporter (NIS) is unlikely to be involved"*.
- 12th November 2015, Infection & Immunity meeting, Cardiff University: Poster Presentation. *"Analysis of links between thyroid autoimmunity and breast cancer"*.
- 27th November 2015, Postgraduate Research Day, Cardiff University: Poster Presentation. *"Thyroid autoimmunity as a biomarker of outcome in women with breast cancer - large scale study using data from the Taxotere as Adjuvant Chemotherapy Trial (TACT) CRUK01/001"*.

SUMMARY

An association between breast cancer (BC) and thyroid autoimmunity (TA) has been frequently observed and several small-scale studies correlated the presence of thyroid peroxidase (TPO) autoantibodies (TPOAb) with an improved BC outcome. The presence of an immune response to shared thyroid/breast antigens has been hypothesized: both tissues express the sodium iodide symporter (NIS) and have a peroxidase activity: TPO in thyroid and lactoperoxidase (LPO) in breast. I have identified 3 possibilities: i) BC patients with TPOAb may also have autoantibodies to NIS (NISAb) ii) BC may express TPO iii) TPOAb may cross-react with LPO in BC. This thesis aimed to identify the TA/BC shared antigen(s) and also investigated the prognostic role of TPOAb in a large cohort of BC patients.

The presence of NISAb was investigated by flow cytometry using CHO cells stably transfected with human NIS. No positive response was obtained in 42 patients with BC and/or TA, therefore NIS is unlikely to be an antigen.

TPO transcripts (RT-PCR, QPCR, LongRange PCR) and protein (western blot, immunohistochemistry, immunofluorescence) were detected in BC tissues but at levels 10^4 times less than in thyroid tissue. TPO was also expressed in adipose tissue and different cancers. Some potentially BC specific TPO isoforms were identified.

The observational large-scale study conducted on 1974 women affected with BC did not reveal any evidence for a significant impact of TPOAb and/or thyroid function on BC prognosis.

In conclusion, BC and thyroid tissues share similar properties and could be common targets of TA, with TPO being the most likely common antigen; further studies are needed to clarify the role of tissue-specific TPO isoforms. The roles of TPOAb and thyroid function on BC prognosis have to be reconsidered, maybe focusing on different TA aspects (e.g. goitre, different autoantibodies).

ABBREVIATIONS

A	adenosine
AA	amino acid
Ab	antibody
AC	adenylate cyclase
AD	adipose tissue
ADP	adenosine diphosphate
AD-A	abdominal adipose tissue
AD-K	adipose tissue from knee
AD-O	orbital adipose tissue
AD-S	subcutaneous adipose tissue
Ag	antigen
AITD	autoimmune thyroiditis
APC	antigen-presenting cell
Apc-Cy7	Allophycocyanin - cyanine Cy-7 tandem conjugate
APRT	adenine phosphoribosyltransferase
APS	ammonium persulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
$A_{260/280}$	absorbance at 260 and 280 nm
BC	breast cancer
BC/TA	(patients affected with) breast cancer and thyroid autoimmunity
bp	base pairs
BSA	bovine serum albumin
BTD	benign thyroid disorders
C	cytosine
cAMP	3',5'-cyclic adenosine monophosphate
CA15.3	cancer antigen 15.3
Ca^{2+}	calcium ion
CCP	complement control glycoprotein
cDNA	complementary deoxyribonucleic acid
CD25	cluster of differentiation 25
CD40	cluster of differentiation 40
CEA	carcinoembryonic antigen
CHO	Chinese hamster ovary
CHO-NIS	CHO cells transfected with pcDNA3-hNIS
CHO-NIS-1	CHO-NIS clone 1

CHO-NIS-6	CHO-NIS clone 6
CHO-Empty	CHO cells transfected with empty pcDNA3 vector
cm^2	squared centimeter
CO	controls (patients affected with non-autoimmune thyroid disorders)
CTCs	circulating tumour cells
ctDNA	circulating tumour DNA
CTLA-4	cytotoxic T lymphocyte-associated molecule-4
C cells	calcitonin-producing parafollicular cells
dH_2O	distilled water
DIT	3,5-diiodo-L-tyrosine
DM	diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleoside triphosphate
DUOX1	dual oxidase 1
DUOX2	dual oxidase 2
D1	deiodinase 1
D2	deiodinase 2
D3	deiodinase 3
E	culture medium supplemented with 1nm β-estradiol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
EI	culture medium supplemented with 1nm β-estradiol/100ng/uL IGF-II
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
ER	oestrogen receptor
ER+	oestrogen receptor positive
ER-	oestrogen receptor negative
ESR	erythrocyte sedimentation rate
Fab	variable region of immunoglobulins
Fc	constant region of immunoglobulins
FCS	foetal calf serum
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate

Flow buffer	PBS/0.5% BSA/2mM EDTA/20 mM Hepes
FOXP3	forkhead box P3
FRTL-5	Fisher rat thyroid line
FSC	forward scatter
FSH	follicle-stimulating hormone
FT3	free-Triiodothyronine(T3)
FT4	free-Thyroxine(T4)
g	times gravity
G	guanosine
GD	Graves' disease
GH	growth hormone
GnRH	gonadotropin-releasing hormone
GO	Graves' orbitopathy
Group M	6 young human males
G418	one aminoglycoside antibiotic
G-I	gastro-intestinal
hCG	human chorionic gonadotropin
H&E stain	hematoxylin and eosin stain
HER2	human epidermal growth factor receptor type 2
HER2+	human epidermal growth factor receptor type 2 positive
HER2-	human epidermal growth factor receptor type 2 negative
HLA	human leukocyte antigen
hNIS	human sodium iodide symporter (NIS)
hPL	human placental lactogen
HR	Hazard Ratio
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
I	culture medium supplemented with 100ng/uL IGF-II
ICR-CTSU	Institute of Cancer Research - Clinical Trials & Statistics Unit
IF	immunofluorescence
IFN-α	interferon alpha
Ig(s)	immunoglobulin(s)
IgG(s)	immunoglobulin(s) class G
IGF(s)	Insulin-like growth factor(s)
IGF-I	Insulin-like growth factor type I
IGF-II	Insulin-like growth factor type II
IHC	immunohistochemistry
IL-2	interleukin-2

IYD	iodotyrosine dehalogenase
I^-	Iodide
I_2	Iodine
kb	kilobases
kbp	kilo-base pairs
kDa	kilodaltons
K^+	potassium ion
LB	loading buffer
LDL	low density lipoproteins
LH	luteinizing hormone
log	logarithm
LPO	lactoperoxidase
LYP	lymphoid tyrosine phosphatase
L 100	Ladder 100 base pairs (bp)
MCT8	monocarboxylate transporter 8
$MgCl_2$	magnesium chloride
mgNIS	sodium iodide symporter express in mammary gland
Mg^{2+}	magnesium ion
MHC	major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
miRNA	microRNA
MIT	3-monoiodo-L-tyrosine
MKC	hepatic metastasis of kidney carcinoma
mL	milliliter
mM	millimolar
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MTC	medullary thyroid carcinoma
MW	molecular weight
M-MLV	Moloney Murine Leukemia Virus
N	normal culture medium
NaOAc	sodium acetate
Na^+	sodium ion
Na^+/K^+-ATPase	Na^+/K^+-adenosine triphosphatase
ng	nanogram
NIS	sodium iodide symporter
NISAb	autoantibodies to NIS

nm	nanometer
odT	oligo-deoxy-thymine
PAX-8	paired box gene 8
PBS	phosphate buffered saline solution
PC	pancreatic adenocarcinoma
pcDNA3-hNIS	pcDNA3 vector containing hNIS gene
PCR	polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	polyethylene glycol
P_i	inorganic phosphate group
PI3K	phosphoinositide-3 Kinase
PKC	primary kidney carcinoma
pmol	picomolar
PMSF	phenylmethanesulfonylfluoride (proteases inhibitor)
PPAR-γ1	peroxisome proliferator-activated receptor γ
PR	progesterone receptor
PRL	prolactin
PR+	progesterone receptor positive
PR-	progesterone receptor negative
PS	penicillin/streptomycin
PT	peri-tumoural breast tissue
PTC	papillary thyroid carcinoma
PTPN22	protein tyrosine phosphatase non-receptor type 22
PVDF	polyvinylidine fluoride
RDf-dH_2O	RNase – DNase free distilled water
RET	rearranged during transfection
RNA	ribonucleic acid
rNIS	rat sodium iodide symporter (NIS)
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcription
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SHBG	sex-hormone-binding globulin
SNPs	simple nucleotide polymorphisms
SSC	side scatter
T	thymidine
TA	thyroid autoimmunity

TAb	anti-thyroid autoantibodies
TAE	Tris-acetate-EDTA
TBG	thyroxine-binding globulin
TBS-T	Tris-buffered saline, 0.1% Tween 20
TCR	T-lymphocyte's receptor
TEMED	tetramethylethylenediamine
Tg	thyroglobulin
TgAb	anti-thyroglobulin (Tg) autoantibodies
TGFß	transforming growth factor beta
Th1	CD4 T-lymphocytes type I
Th2	CD4 T-lymphocytes type II
tNIS	sodium iodide symporter express in thyroid
TNM	primary tumour (T) regional lymph nodes (N) distant metastases (M)
TPO	thyroid peroxidase
TPOAb	anti-thyroid peroxidase (TPO) autoantibodies
TRAb	autoantibodies to TSHR
Treg(s)	regulatory T-lymphocyte(s)/cell(s)
TR	thyroid hormone receptor
TREs	thyroid hormone response elements
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
TSHR	thyroid-stimulating hormone (TSH) receptor
TT	thyroid tissue
T3	triiodothyronine
T4	thyroxine
u	units
US	ultrasonography
UV	ultraviolet
Vis	visible
VJ2	mouse monoclonal antibody to an extracellular domain of hNIS
VPO	vascular peroxidase
WB	Western Blot
WHO	World Health Organization
X3	three times
µg	microgram
µl	microliter
1st Ab	first antibody
°C	degree Celsius

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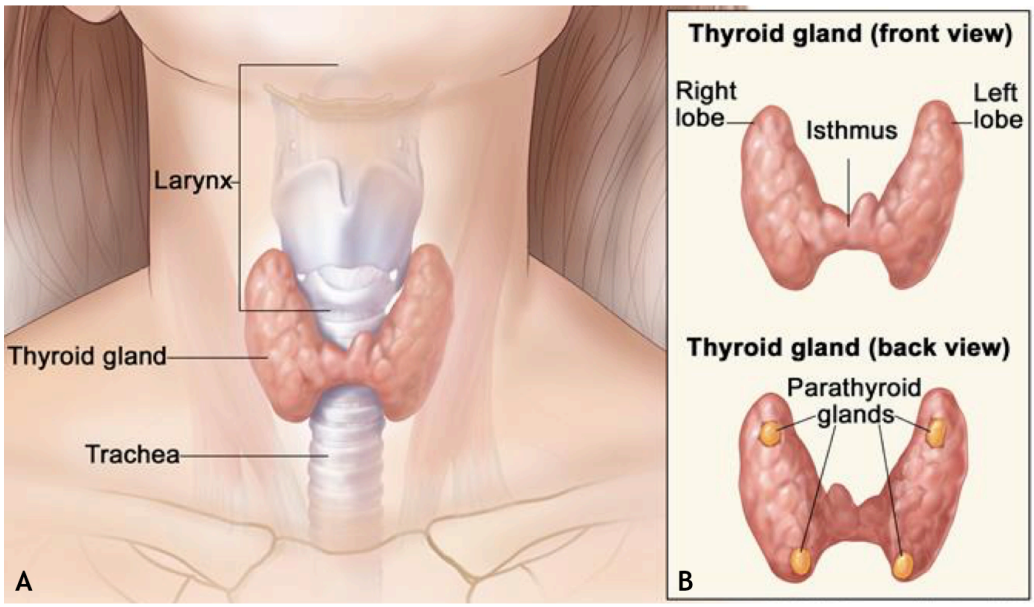
1 GENERAL INTRODUCTION

1.1 THE THYROID GLAND

1.1.1 Anatomy and histology

The thyroid is one of the largest endocrine glands and is located in the neck, just below the larynx. It is made of two lateral lobes, right and left, connected by a central isthmus, with occasionally an accessory pyramidal lobe discernible as a finger-like projection directed upward from the isthmus. Behind the thyroid are located the parathyroid glands, different endocrine glands involved in calcium metabolism (1) (Figure 1-1).

Figure 1-1. Thyroid anatomy

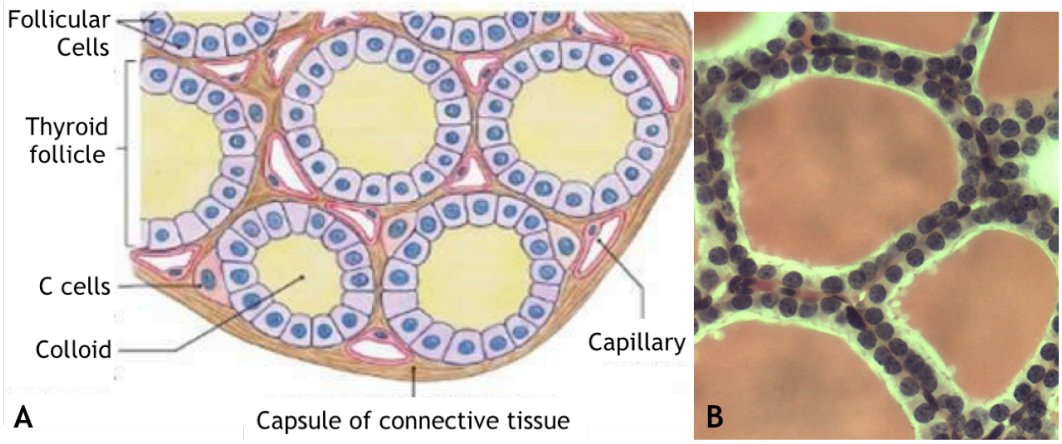


Adapted from © 2012 Terese Winslow LLC. Panel A: localization of the thyroid in the neck. Panel B: details of thyroid gland

The structure of the gland is composed of closely packed follicles, spherical units characterized by a lumen containing proteinaceous colloid surrounded by a continuous single layer of thyroid epithelial cells, named thyrocytes or follicular cells (Figure 1-2). The diameter of the follicles varies considerably, even within a single gland, but averages about 200 nm. The follicular cells vary in height with the degree of glandular stimulation, becoming columnar when active and cuboidal when inactive. The epithelium rests on a basement membrane that is rich in glycoproteins and separates the follicular cells from the surrounding capillaries. Between 20 and 40 follicles are demarcated by

connective tissue septa to form a lobule supplied by a single artery. The functional activity of a given lobule may differ from that of its neighbors. The thyroid gland contains also calcitonin-producing parafollicular cells (C cells), usually localized along the basement membrane of the thyroid epithelium (1).

Figure 1-2: Thyroid histology

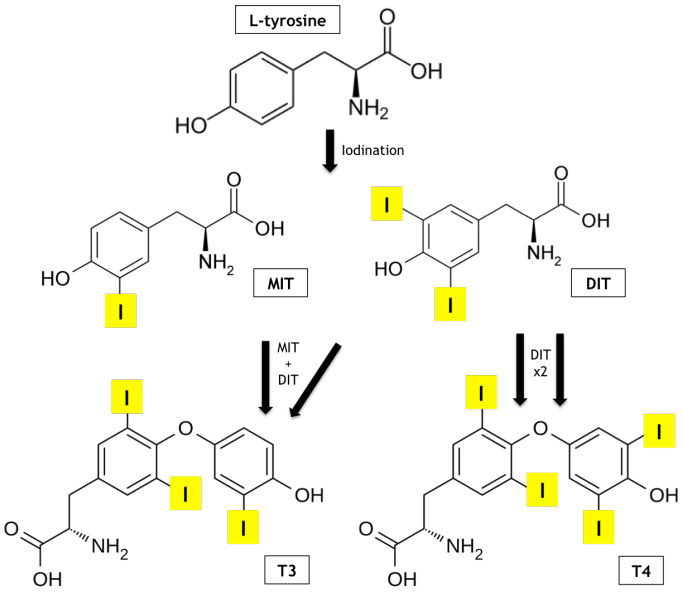


Panel A, adapted from (2): schematic representation of thyroid histology. Panel B, adapted from (3): cross-section of thyroid tissue with hematoxylin and eosin stain (H&E stain).

1.1.2 Physiology and synthesis of thyroid hormones

The thyrocytes' main function is producing thyroid hormones thyroxine (T4) and triiodothyronine (T3), homodimer peptides deriving from the iodination and coupling of the amino acid L-tyrosine (Figure 1-3).

Figure 1-3: Structures of thyroid hormones and their precursors



Adapted from NEUROtiker, Brenton, Hoffmeier (https://commons.wikimedia.org). MIT= 3-monoiodo-L-tyrosine; DIT= 3,5-diiodo-L-tyrosine; T3= triiodothyronine; T4= thyroxine.

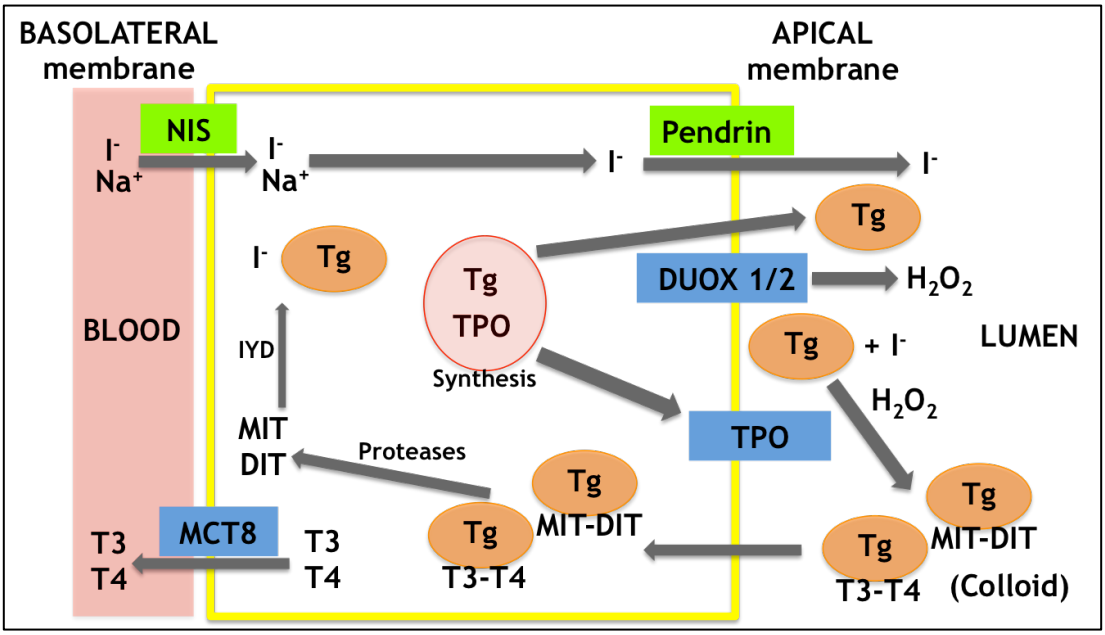
The synthesis of thyroid hormones is a complex process involving different and polarized activities of the thyrocytes (1) (Figure 1-4):

- The basolateral cell membrane is in contact with the blood circulation, from where it takes up the necessary elements to synthetize T3 and T4: amino acids and iodide ion (I^-) by the sodium iodide symporter (NIS), a transmembrane glycoprotein.
- Inside the thyrocytes two of the main thyroid proteins implicated in thyroid hormone production are thyroglobulin (Tg) and thyroid peroxidase (TPO). Tg is secreted through the apical membrane inside the follicular lumen and it represents the major component of colloid. TPO is a transmembrane protein and is located on the apical membrane of thyrocytes.
- The apical border is the key site where T3 and T4 are synthetized. I^- is transported into the follicular lumen by a transporter called Pendrin situated on the apical membrane. In the follicular lumen specific tyrosine residues of Tg homodimers are iodinated to form 3-monoiodo-L-tyrosine (MIT) and 3,5-diiodo-L-tyrosine (DIT), the precursors of thyroid hormones. TPO catalyzes the oxidation of I^- and its transfer to tyrosine; this process requires the formation of hydrogen peroxide (H_2O_2) that is produced by the transmembrane enzymes dual oxidase 1 (DUOX1) and DUOX2. TPO also catalyzes the fusion of two DIT molecules in order to synthetize T4 and of one MIT and one DIT to produce T3. At this stage MIT, DIT, T3 and T4 are still attached to Tg protein and stored in the colloid.

When the thyroid has to release T3 and T4, a multi-step process is activated:

- Apical membrane of thyrocytes: endocytosis of colloid from the follicular lumen through both macropinocytosis (pseudopods) and micropinocytosis (small coated vescicles).
- Inside the thyrocytes: endocytic vescicles fuse with lysosomes and proteolysis determines the release of iodotyrosines (MIT, DIT, T3, T4) from Tg. MIT and DIT are then deiodinated by the iodotyrosine dehalogenase (IYD),encoded by the *IYD* gene, to allow recycling of the iodide together with Tg.
- On the basolateral side of thyrocytes, T3 and T4 are secreted into the blood stream through transmembrane protein transporters, including the monocarboxylate transporter 8 (MCT8), specific for the transport of thyroid hormones (4).

Figure 1-4: Thyrocyte structure and thyroid hormone synthesis



DIT= 3,5-diiodo-L-tyrosine; DUOX1= dual oxidase 1; DUOX2= dual oxidase 2; H_2O_2= hydrogen peroxide; IYD= iodotyrosine dehalogenase; I^-= iodide; MCT8= monocarboxylate transporter 8; MIT= 3-monoiodo-L-tyrosine; Na^+= sodium; NIS= sodium iodide symporter; Tg= thyroglobulin; TPO= thyroid peroxidase; T3 = triiodothyronine; T4= thyroxine.

1.1.3 Hypothalamic - Pituitary -Thyroid Axis

The thyroidal secretion of T3 and T4 is controlled by the thyroid-stimulating hormone or thyrotropin (TSH), a peptide produced by endocrine cells in the anterior pituitary named thyrotrophs (5). TSH production is regulated by two interacting systems (Figure 1-5):

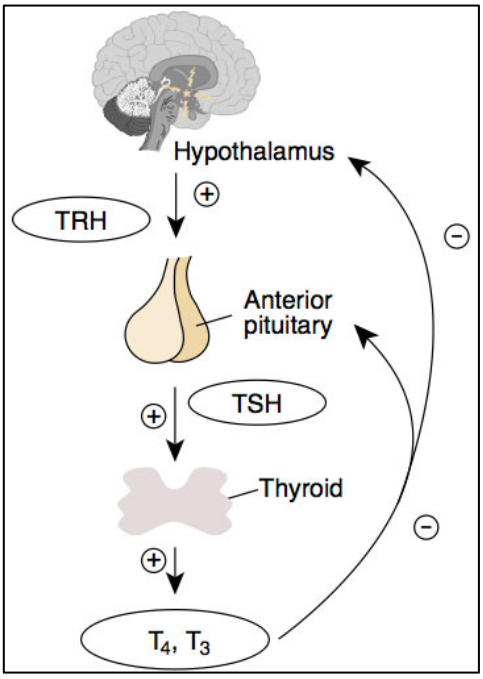
1) open-loop neural control by hypothalamic hypophysiotropic factors

2) negative feedback by thyroid hormones

The neural control is mainly exerted by the thyrotropin-releasing hormone (TRH), a peptide produced by the paraventricular neurons of the hypothalamus that binds to specific receptors on the plasma membrane of the thyrotrophs, stimulating their production of TSH (5). TSH secretion is also influenced by other hormones including oestrogens, glucocorticoids and possibly growth hormone (GH), while it is inhibited by cytokines in the pituitary and hypothalamus (6).

Thyroid hormones, both T3 and T4, exert their negative feedback control on both the pituitary and the hypothalamus, determining a reduction in TRH and TSH release. Circulating T3 and T4 are transported inside the brain, where T4 is converted into T3 by type II deiodinase and then T3 interacts with thyroid hormone receptors (7).

Figure 1-5: Hypothalamic - Pituitary - Thyroid Axis



Modified from Hiller-Sturmhöfel S et al. (8). TSH = thyroid-stimulating hormone; TRH = thyrotropin-releasing hormone; T3 = triiodothyronine; T4 = thyroxine; += activation; -= inhibition.

TSH binds to its specific receptor (TSHR) located on the basolateral membrane of thyrocytes and exerts several functions acting via different intracellular signaling cascades. TSH main actions include: 1) induction of thyroid hormone synthesis 2) stimulation of endocytosis on the apical membrane, causing internalization and digestion of colloid with consequent thyroid hormone release into the blood from the basolateral membrane 3) maintenance of trophic thyroid cell integrity and tropism (9). In particular, two main aspects of thyroid hormone synthesis induction are: A) Tg/TPO/NIS genes transcription induction via adenylate cyclase (AC), an enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic adenosine monophosphate (cAMP) and pyrophosphate B) H_2O_2 generation via Gq-phospholipase C cascade leading to increased intracellular levels of diacylglycerol and calcium ion (Ca^{2+}) (10).

TSH is a glycoprotein hormone composed by two subunits: α and ß. The ß subunit is unique to TSH and therefore determines its receptor specificity. The α subunit contains the effector region and it is nearly identical to other hormones, such as the human chorionic gonadotropin (hCG), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (5).

1.1.4 Transport and metabolism of thyroid hormones

T4 and T3 are released by the thyroid gland with a 10:1 ratio. They are both poorly soluble in water and therefore bind reversibly to plasma proteins, principally thyroxine-binding globulin (TBG) and less to transthyretin and albumin. The small amount of hormones that is not bound to such proteins, respectively 0.02% for T4 and 0.3% for T3, is that available for uptake by peripheral tissues. This fraction is called free-T4 (FT4) and free-T3 (FT3) and is determined by the concentration and degree of saturation of these proteins, especially TBG (1).

For this reason in clinical practice the thyroid status correlates with FT4 and FT3, not with T4 and T3. The normal serum concentrations are usually within the range of respectively 9 to 30 pmol/L (0.7 to 2.5 ng/dL) for FT4 and 3 to 8 pmol/L (0.2 to 0.5 ng/dL) for FT3 (1). There is a linear inverse relationship between the serum FT4 concentration and the logarithm (log) of the TSH, making the serum TSH concentration an exquisitely sensitive indicator of the thyroid state of patients with an intact hypothalamic-pituitary axis (1). As will be better explained in paragraph 1.2.4, the term “euthyroidism” defines the situation when FT4, FT3 and TSH are within the normal range, while the terms “hypothyroidism” and “hyperthyroidism” define a situation of reduced FT4,FT3/increased TSH and increased FT4,FT3/decreased TSH levels, respectively.

The transport of T3 and T4 across the cell membrane of peripheral target organs is mainly mediated by transporter proteins, including MCT8 (4).

Despite T4 being the most abundant hormone produced by the thyroid gland, the principal effector in the target cells is T3, 80% of which is derived from T4 by deiodinases in the peripheral tissues that catalyze the removal of a single 5’ iodine atom from T4 (11). In particular there are 3 types of deiodinases: deiodinase 1 (D1), deiodinase 2 (D2) and deiodinase 3 (D3). Both D1 and D2 produce T3 from T4 but they differ in terms of tissue location and regulation. In particular D1 is mainly present in liver and kidney, while D2 is mainly located in the central nervous system, pituitary, skeletal muscle, brown adipose tissue, placenta and heart. D3 has a different function in deactivating both T3 and T4 and it is mainly located in the central nervous system, placenta, liver and skeletal muscle (1).

T3 and T4 are partly degraded intracellularly before exit by peripheral deiodinases (12). Another pathway involved in T4 metabolism is the hepatic glucuronidation of the phenolic hydroxyl group by the uridine diphosphate glucuronyl transferases (UDPGT) in the liver, but only minimal amounts of T3 undergo this process (13).

1.1.5 Actions of thyroid hormones

Thyroid hormones act by binding to a specific nuclear thyroid hormone receptor (TR); T3 has a 15-fold higher binding affinity for TRs than does T4, explaining its function as the active thyroid hormone. When bound to T3, TR binds to DNA usually as heterodimer with retinoid X receptor at specific sequences called thyroid hormone response elements (TREs) (1). There are two TR genes, α and ß, that are subjected to alternative splicing forming both active and inactive gene products. The active proteins are $TR\alpha_1$, $TR\beta_1$, $TR\beta_2$ and $TR\beta_3$ (14) and there are tissue-specific preferences in expression of the various TRs, suggesting that they serve different functions in different tissues (15). In general, TRα, particularly $TR\alpha_2$, is thought to be important in the hypothalamus and pituitary where regulation of thyroid function occurs (16).

T3 and T4 exert several physiological actions on many different target organs and systems, including the control of metabolic rate, regulation of growth, positive chronotropic and inotropic cardiac effects and development of the central nervous system (17). Thyroid hormones are essential during organogenesis and after birth during infancy and childhood. However thyroid hormones are very important also in adult life and the principal effects of thyroid hormones on target organs are summarized in Table 1-1 (1, 17).

Table 1-1: Actions of thyroid hormones on target organs

CARDIOVASCULAR SYSTEM	▪ ↓ peripheral vascular resistance ▪ Positive chronotropic and inotropic cardiac effects
METABOLISM	▪ ↑ basal metabolic rate ▪ ↑ heat production ▪ ↑ both protein synthesis/degradation ▪ ↑ both lipogenesis and lipolysis
CENTRAL AND PERIPHERAL NERVOUS SYSTEM	▪ ↑ sensitivity to catecholamines ▪ Cortical and cerebellar development ▪ Myelination
SKELETAL SYSTEM	▪ Regulation of bone turnover
KIDNEY	▪ ↑ renal blood flow, glomerular filtration and tubular reabsorptive and secretory maxima
HEMATOPOIETIC SYSTEM	▪ ↑ erythropoiesis
REPRODUCTIVE SYSTEM	▪ Regulation of amplitude and frequency of LH/FSH pulses ▪ ↑ liver production of sex-hormone-binding globulin (SHBG) ▪ ↑ conversion of androgens to oestrogenic products
PITUITARY AND HYPOTHALAMUS	▪ ↓ TSH and TRH secretion

FSH= follicle-stimulating hormone; LH= luteinizing hormone; SHBG= sex-hormone-binding globulin; TSH= thyroid-stimulating hormone; TRH= thyrotropin-releasing hormone; ↑= increase; ↓= decrease.

1.2 THYROID DISORDERS

Benign thyroid disorders are very common and have a clear female preponderance (1). In this thesis they have been subdivided into distinct topics such as thyroiditis, thyroid autoimmunity, goitre and alterations of thyroid function, and they will be discussed separately in the next paragraphs. In a final paragraph a brief overview of thyroid cancer will be also described.

1.2.1 Thyroiditis

The term thyroiditis indicates the presence of thyroid inflammation and comprises a very large group of inflammatory conditions summarized in Table 1-2, including autoimmune, infectious (viral, bacterial, fungal), sclerosing (Riedel's thyroiditis) or granulomatous (sarcoidosis) etiopathogenesis.

Table 1-2: Classification of thyroiditis

ACUTE	Suppurative thyroiditis	Bacterial, fungal
SUBACUTE	De Quervain's	Viral
	Postpartum (silent)	Autoimmune
CHRONIC	Autoimmune thyroiditis (AITD)	Autoimmune
	Post irradiation	Iatrogenic (radioiodine, radiations)
	Riedel's thyroiditis	Sclerosis
	Sarcoidosis	Granulomatous

Thyroiditis does not affect thyroid function directly but may cause hypothyroidism by the damage to the thyroid gland. In the early stages of thyroiditis, a transient excessive release of thyroid hormones by the damaged thyroid gland can result in a condition of excessive serum levels of thyroid hormones, called "thyrotoxicosis" (1).

Acute thyroiditis is very rare and usually caused by bacterial or fungal infections. It is characterized by local pain and tenderness and difficulty in swallowing is often present. It is usually accompanied by general malaise and fever and increased erythrocyte sedimentation rate (ESR) (18).

Subacute thyroiditis has different clinical manifestations according to its pathogenesis. If it has an infectious (viral) origin it is called De Quervain's thyroiditis and usually is characterized by clinical and biochemical features similar to acute thyroiditis. However De Quervain's thyroiditis is usually diffuse, while acute thyroiditis is usually more localized as a painful isolated nodular enlargement of the thyroid. Postpartum thyroiditis is a different form of subacute thyroiditis, with a classical onset during the first months after delivery. In contrast to De Quervain's, postpartum thyroiditis is not usually associated with

general symptoms or ESR alterations but with positivity for anti-thyroid autoantibodies (TAb), as will be better explained in the next paragraph 1.2.2 (18).

The most common form of chronic thyroiditis has autoimmune origins and it will be better discussed in the next paragraph 1.2.2. A second important group is related to iatrogenic chronic thyroiditis induced by radioiodine treatment or external radiation therapy. Finally, rare forms of chronic thyroiditis include Riedel's thyroiditis, characterized by fibrosis of the thyroid gland and adjacent structures but its origin remains uncertain, (19) or granulomatous diseases such as sarcoidosis (1).

1.2.2 Thyroid autoimmunity

Thyroid autoimmunity (TA) is the most common autoimmune disease in humans and accounts for two main types of disease: autoimmune thyroiditis (AITD) and Graves' disease (GD) (20). Early post-mortem studies confirmed histological evidence of chronic AITD in 27% of adult women and 7% of adult men with an increase in frequency in the past 50 years. GD is also the principal cause of hyperthyroidism (21).

1.2.2.1 Brief overview of immunology and immunological synapse

Our immune system consists of innate and adaptive immune responses against a wide range of pathogens (bacteria, viruses, etc.). In the present thesis a brief overview of the adaptive immune responses, mediated mainly by B and T-lymphocytes, will be provided, based on (22).

Both B and T-lymphocytes, through their surface receptors, recognize a specific and short sequence (usually amino acidic; called "epitope") of a molecule (usually a protein; called "antigen") that triggers the immune reaction. Both B and T-lymphocytes' receptors consist of variable and constant regions; the variable region is the part recognizing the antigen, while the constant region has other functional activities. The variable regions are generated through a process of intense gene rearrangements leading to a great variability, therefore generating receptors recognizing a multitude of different antigens.

Both B and T-lymphocytes originate from precursors in the bone marrow and then undergo a process called "central tolerance", where B and T-lymphocytes strongly recognizing self-antigens (antigens expressed by our own body), are inactivated and eliminated; this process happens in bone marrow and thymus respectively for B and T-lymphocytes.

B-lymphocytes' antigen-receptor consists of surface immunoglobulins (Igs) directed versus specific antigens. Igs are complex proteins characterized by a

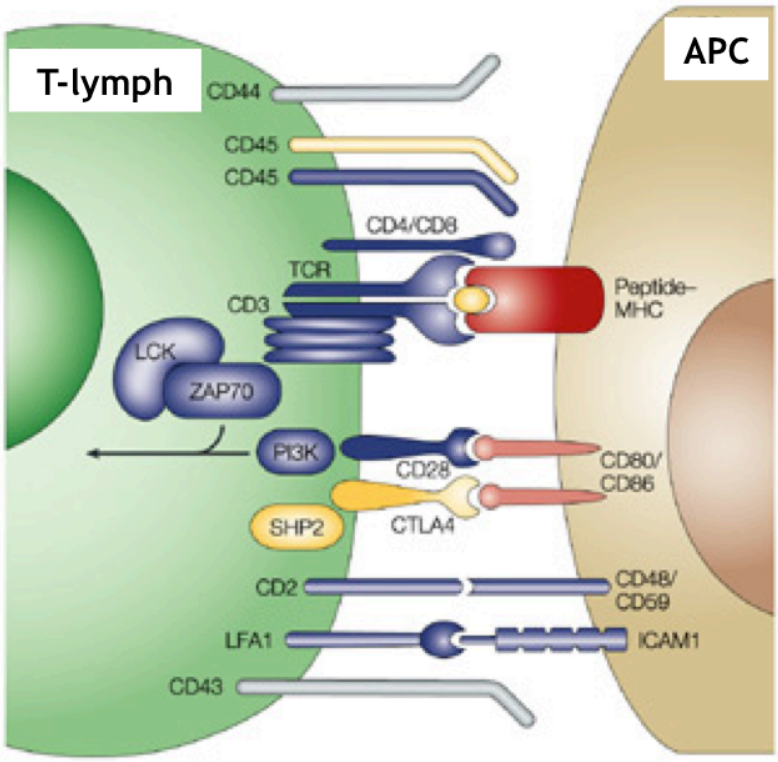
total of 4 different amino acidic chains, two heavy and two light, both types containing constant (Fc) as well as variable (Fab) regions.

T-lymphocytes, in addition to their antigen-receptor (TCR), express surface molecules with crucial roles for the adaptive immune response, as shown in Figure 1-6. In particular, CD4 and CD8 are co-receptor molecules identifying 2 subtypes of effector T-lymphocytes with different tasks, as explained later.

In order to be recognized by T-lymphocytes, antigens need to be presented by antigen-presenting cells (APC) through particular transmembrane glycoproteins belonging to the mammalian major histocompatibility complex (MHC) family, and referred to also as the human leukocyte antigen (HLA) system in humans. Briefly, intracellular antigens (e.g. virus) are expressed on APC's surface through MHC class I (MHC-I), while extracellular antigens (e.g. secreted bacterial toxins) are internalized by APCs and then expressed on their surface through MHC class II (MHC-II). There are different MHC proteins in humans: the major subtypes of MHC-I and MHC-II molecules are respectively HLA-A, HLA-B, HLA-C (type I) and HLA-DP, HLA-DQ, HLA-DR (type II).

T-lymphocytes require two different signals from APC to be activated. The first signal is antigen-specific and provided by the interaction of TCR with antigen-MHC complex expressed on APC's membrane. The second signal is not antigen specific and called "co-stimulatory signal"; it is provided by the interaction between additional co-stimulatory molecules expressed on the membrane of both T-lymphocytes and APC. The interface between APCs and T-lymphocytes is called the immunological synapse and comprises the interaction between surface molecules expressed by APC and T-lymphocytes leading to activation of both cells and is the basis of the adaptive immune response (Figure 1-6). The description of such molecules' function is beyond the aim of the present thesis. Briefly, in addition to TCR and CD4/CD8, T-lymphocytes' surface proteins may be adhesion molecules such as CD44, important activating/co-stimulatory molecules such as CD3 or inhibitory molecules such as cytotoxic T lymphocyte-associated molecule-4 (CTLA4). T-lymphocytes depend from co-stimulatory signals for their differentiation, proliferation and survival; absence of co-stimulation may lead to T-lymphocytes' anergy (impossibility to respond to their specific antigen) and depletion or the development of immune tolerance. APC use the same co-stimulatory molecules to be activated as well; e.g. APC express the CD40, a co-stimulatory protein that, after binding with the correspondent ligand on T-lymphocytes surface (CD40L), activates APCs. Both stimulating and inhibitory molecules, as well as HLAs, are likely to be involved in the pathogenesis of thyroid autoimmunity (TA), as explained in the next paragraph.

Figure 1-6: Immunological synapse



Adapted from (23). Profile view of a T-lymphocyte (T-lymph) and an antigen-presenting cell (APC) and some of the key signaling molecules and ligand pairs involved in the T-lymph/APC interaction. The antigen(peptide)-MHC complex is shown in red; in blue activating/co-stimulatory molecules; in yellow inhibitory molecules; in grey molecules not contributing to signaling. The arrow indicates converging signals leading to T-lymphocyte activation.

As a consequence of the interaction between APCs and T-lymphocytes, two main kinds of adaptive immunity may arise: humoral or cell-mediated, more effective against extracellular and intracellular pathogens respectively. In summary, the humoral immunity consists in antigen-MHC-II APC's complex triggering CD4 T-lymphocytes type II (Th2) that activate B-lymphocytes. Activated B-cells differentiate into "plasma cells" that secrete Igs against a specific antigen, called antibodies (Abs), in order to eliminate the extracellular bacteria or toxins. The cell-mediated immunity is composed by 2 main mechanisms: 1) antigen-MHC-II APC's complex triggers CD4 T-lymphocytes type I (Th1) with consequent activation of the bactericidal activity of macrophages; 2) APC's antigen-MHC-I complex triggers CD8 T-lymphocytes that directly kill the APC (and therefore also the intracellular pathogen infecting the cell).

Another important class of T-lymphocytes are the regulatory T-cells (Treg), involved in the "peripheral tolerance". As previously described, the process of central tolerance in bone marrow (B-lymphocytes) and thymus (T-lymphocytes) is

essential in order to suppress self-reactive immune cells. However, some of them may escape this process and the peripheral tolerance operated by Tregs, which act to eliminate self-reactive peripheral auto-reactive lymphocytes, is essential in order to maintain the immunological self-tolerance (24, 25).

1.2.2.2 Etiopathogenesis of thyroid autoimmunity (TA)

TA results from the breaking of tolerance to self-antigens of the thyroid and different mechanisms are involved: mainly cell-mediated for AITD and antibody-mediated for GD (26).

Thyroid autoimmune diseases are characterized by increased lymphocytic infiltration into the thyroid (27, 28) and thyrocytes have been described expressing CD40 under stressful conditions, therefore, as briefly explained in the previous paragraph, potentially acting as APCs and participating in the immune activation (29).

Several possible mechanisms have been postulated to explain the rise of TA, from genetic predisposition (30) to environmental factors including infections, iodine excess, selenium deficiency, stress, allergy, female hormones, foetal microchimerism, smoking, irradiation and drugs (31, 32).

- **Genetic predisposition**.

  1) HLA system is crucial for the antigen presentation process and therefore very often involved in genetic predisposition for autoimmunity in general. Both HLA class I and II genes have been consistently associated with TA, especially with GD (33, 34). In particular, HLA-DR is a class II HLA gene that plays a critical role in antigen presentation and HLA-DR3 allele has a well-established association with GD but it may be also involved in AITD (35).

  2) Signaling molecules involved in the immunological synapse between T-lymphocytes and APCs may represent another important source of genetic predisposition to autoimmunity (36, 37). In particular, CTLA-4 is associated with both GD and AITD, while CD40 is GD specific (30). The protein tyrosine phosphatase non-receptor type 22 (PTPN22) is a gene encoding the cell signaling molecule lymphoid tyrosine phosphatase (LYP), which inhibits the activation of T cells by interacting with other signal transduction molecules. Initial studies demonstrated an association between certain PTPN22 alleles and TA susceptibility, in particular GD (38).

  3) Treg and peripheral tolerance are logically another important possible cause of general predisposition to autoimmunity. The forkhead box P3 (FOXP3) is a known crucial regulator of the differentiation and function of Treg and various FOXP3 polymorphisms have been reported to be associated with AITD

(30). Similarly, CD25 is the α-chain of interleukin-2 (IL-2) receptor and is involved in the regulation of T cell function and mediates IL-2 signaling, indispensable for Treg survival and growth (39). Genetic variants of CD25 gene have been found to predispose mainly to GD (40, 41).

4) Finally, thyroid specific genes may be involved in loss of central tolerance leading specifically to TA. The target antigens for autoreactive lymphocytes in TA are Tg and TPO proteins for AITD and the TSHR for GD. The role of these antigens and their correspondent genes will be described in more detail in the paragraphs below (1.2.2.3 and 1.2.2.4).

- **Epigenetic mechanisms.** There are different controversial definitions for epigenetic mechanisms and effects; they generally indicate non-coding effects on gene expression and function that are mitotically stable, thus long lasting. They include DNA methylation, histone modifications and ribonucleic acid (RNA) interference by microRNA (miRNA) (42). This is a relatively recent research field and an increasing number of studies suggest a crucial role of genetic-epigenetic interactions in the cases where one of the above mentioned genetic susceptibility variants is present. This would explain why the genetic risk factors alone cannot trigger the disease without other non-genetic modifier and epigenetics is one of the major ones. Epigenetics could also represent the mechanism of interaction between an environmental factor and genetic susceptibility variants (43).
- **Environmental factors**

  1) Infections. The precise mechanisms involved in this association are unknown and the two main hypothesis are I) molecular mimicry: antigen sharing homology between bacterial pathogens and self-antigens, with consequent cross-reactive immune response towards bacterial antigens on one side and autoimmunity on the other side II) the local infection and inflammation against the bacteria create tissue damage and consequent release of sequestered antigens triggering an autoimmune response (31). An additional theory of "bystander effects" proposes that viral infection of a certain tissue can induce local inflammation, resulting in activation of autoreactive T-cells that were dormant or suppressed by peripheral regulatory mechanisms (44).

  Several infections are known to be associated with thyroid autoimmunity: A) Bacteria, including Yersinia enterocolitica, Bifidobacterium and lactobacillis species, Helicobacter pylori and Clostridium botulinum neurotoxin A (26); B) Viruses: Coxsackie B virus, retroviruses, enterovirus, rubella, mumps virus, herpes simplex virus (HSV), EBV, Parvovirus, HCV (45, 46).

2) Excessive iodine intake is a risk factor for thyroid autoimmunity and the incidence of TA has risen concomitantly with increased iodine consumption (47). The etiopathogenic hypothesis is that an excess of iodine may lead to some thyroid destruction and hence presentation of thyroidal antigens to the immune system leading to an autoimmune reaction (31). An alternative explanation involves Tg iodination and Tg iodine-induced conformational changes affecting its uptake and processing by antigen presenting cells (48).

3) Sex differences. Organ specific autoimmune diseases and thyroid disorders in general have a strong female preponderance. The reasons for this phenomenon are unknown, but the influence of the X chromosome seems to be limited, considering that AITD is very prevalent among girls with Turner's syndrome (X0 karyotype), but not in men with Klinefelter's syndrome (XXY karyotype) (49). Therefore hormonal influences are more likely to be operative in the induction of TA (31), as suggested by the presence of oestrogen receptor (ER) in both neoplastic and non-neoplastic thyroid tissue, where 17ßestradiol exerts a growth-promoting effect on thyroid cells (50). Recently Merril et al. proposed a crucial role of leptin-induced inflammation to explain the female preponderance of TA (26), considering that leptin is involved in inflammation (51) and rises in both young girls and boys as they approach puberty but in boys the leptin declines along with testosterone levels (52).

4) Pregnancy. During pregnancy the immune system is suppressed with a fall in the T-helper/suppressor-cell ratio, whereas in the first post-partum months T-cell activation occurs and autoantibody production rises (53). As consequence several types of autoimmune disorders abate during pregnancy but often worsen or arise de novo after the delivery when T-cells reactivate (31).

The transfer of foetal cells into the maternal circulation is called foetal microchimerism and these foetal cells can persist for a long time. Some authors proposed that foetal microchimerism could be one possible mechanism for the etiopathogenesis of postpartum thyroiditis (54-56).

5) Irradiation. Both external and internal irradiation by radioactive iodine expose thyroidal antigens to the immune system and thus induce TA. Indeed, an increased prevalence of TA has been clearly demonstrated after nuclear disasters (e.g. Chernobyl nuclear plant accident) (31).

6) Cigarette smoking has an influence on the immune system, inducing a polyclonal activation of both B and T cells and cytokines production; it also causes cell damage and therefore may also increase the presentation of antigens. Smoking is linked to several autoimmune diseases including

rheumatoid arthritis and GD, in particular with Graves' orbitopathy (GO); the reasons for this strong association are still largely unknown (31).

7) Several drugs acting on the immune system towards different mechanisms are associated with an increased incidence of TA, including antiretroviral therapy, interferon-alpha, IL-2 and alemtuzumab (31). In particular, alemtuzumab (Campath) is a humanized anti-CD52 monoclonal antibody used for the treatment of multiple sclerosis and it triggers TA, in particular GD, in about one third of treated patients (57).

8) Stress has been found driving the immune system towards a Th2 response, therefore suppressing cellular immunity and enhancing humoral immunity (31). This differential action on T-lymphocytes would explain why stress has been repeatedly associated to GD (humoral immunity), while only very few reports support an association between stress and AITD (cellular immunity) (58).

9) Allergic conditions are Th2 disorders, therefore they have been found to be associated with Th2-driven autoimmune diseases such as GD and lupus erythematosus (59).

10) Selenium is a trace mineral and exerts a marked influence on the immune system, acts as anti-oxidant reducing free radical formation and plays an essential role in thyroid hormone synthesis. Low selenium blood levels are associated with increased thyroid volume and thyroid hypoechogenicity, a marker for lymphocytic infiltration (31).

1.2.2.3 Autoimmune thyroiditis (AITD) and role of thyroid autoantibodies

The term AITD refers to the presence of thyroid gland damage, inflammation or lymphocytic infiltration, usually detectable as a hypoechoic and non-homogeneous pattern at the ultrasonography (US) scan examination of the thyroid gland. The definition of AITD covers different clinical entities, such as: chronic thyroiditis, painless sporadic thyroiditis or postpartum thyroiditis with a typical onset in the early months after delivery. Chronic thyroiditis is called Hashimoto's thyroiditis when characterized by the presence of both goitre and hypothyroidism. Alternatively, when the volume of the thyroid gland is greatly reduced, it is named atrophic thyroiditis (60).

The first event in AITD etiopathogenesis is the activation of thyroid antigen-specific helper T cells (60). Tg has been recognized as the potential earliest trigger of AITD (61) and linkage studies indicated Tg as a susceptibility gene for TA (43). The role of TPO as autoimmune antigen in TA will be extensively explained in chapter 3. There are different theories explaining the possible causes

of T-cell activation: 1) result from a viral infection with common properties between viral and thyroid proteins (62); 2) thyrocytes present their own intracellular proteins to T cells (29); 3) foetal microchimerism (56).

Once helper T cells are activated, they induce B cells to secrete TAb. The main two antigens involved in AITD are Tg and TPO, therefore anti-Tg autoantibodies (TgAb) and anti-TPO autoantibodies (TPOAb) are the main thyroid autoantibodies (TAb) and they are both considered typical serum biomarkers of TA, AITD in particular. Autoantibodies to colloid antigen, thyroid hormones and NIS have also been detected in patients with AITD, even if they are rare and therefore much less important compared with TgAb and TPOAb (60). However both TgAb and TPOAb are also largely present in the general population, without being associated with the presence of thyroiditis, thyroidal damage or thyroid dysfunction. In particular Hollowell et al. estimated a TPOAb and TgAb prevalence among general population of respectively 13% (8.7% males, 17% females) and 11.5% (7.6% males, 15.2% females) (63). Vanderpump et al. described an even higher TAb prevalence of 26.4%, considering together TPOAb and TgAb (64). The great majority of authors in this field agree that TAb prevalence increases with age (63-67) and varies with ethnicity, noticing a major TAb prevalence among whites compared with blacks or Mexican Americans (63). An increase of TAb prevalence has also been registered after iodization programs and the generation of autoantibodies seems to be related to a sudden increase of iodine intake, more than to exposure to a constant but high iodine level (68). Possible mechanisms may be 1) free radical-mediated damage of thyroid tissue induced by the sudden shift from low to high iodine intake (69) and 2) major changes in Tg stereochemical configuration caused by its increased iodization with consequent changes in its immunoreactivity and induction of TgAb production (48, 70). TPOAb are the most valid clinical biomarker for the detection of early TA, considering that they are present in 90% of patients affected with autoimmune thyroiditis (AITD) (71). However, it is currently unknown why such a high percentage of individuals from the general population develop TPOAb; it is also unclear why many individuals positive for TAb do not develop clinical thyroid disease (63, 72).

The mechanisms for autoimmune destruction of thyroid tissue probably involves both cellular and humoral immunity, in fact in AITD the lymphocytic infiltration of the thyroid gland is characterized by equal numbers of B cells and cytotoxic T cells (60). Furthermore, thyrocytes of AITD patients express the Fas gene, a member of the closely linked group of tumour necrosis factor genes, therefore apoptosis could represent an additional cause of thyrocyte destruction (73). In particular, the proposal for a causative role of TPOAb in the pathogenesis

of Hashimoto's thyroiditis remains controversial (74-76). Some authors consider TPOAb as secondary to the thyroid damage inflicted by T cells (71), while others proposed a role of TPOAb in AITD antibody-dependent cell cytotoxicity (77), considering that TPOAb are complement-fixing and thus directly cytotoxic to thyrocytes (78). It has also been repeatedly noticed that TPOAb positive women have an increased risk of developing pregnancy complications such as miscarriage and pre-term delivery (79, 80).

The obvious consequence of thyroid tissue destruction is a reduced thyroid function (hypothyroidism). However an alternative cause of hypothyroidism could be the presence of autoantibodies blocking the TSHR, that has been reported in up to 10% of patients with AITD and they could have a role in both development and severity of hypothyroidism (81).

The principal clinical manifestation of AITD is hypothyroidism, however the majority of patients with measurable TAb have normal thyroid function (60). A goitre may be present and this is typical of Hashimoto's thyroiditis. Epidemiological studies estimated that 10% and 0.5% of postmenopausal women with high serum TAb had respectively subclinical hypothyroidism and overt hypothyroidism, although euthyroid individuals with high serum TAb titre had a progression to overt hypothyroidism at a rate of 2 to 4 percent a year (64, 82).

1.2.2.4 Graves' disease (GD)

GD has an estimated incidence of 80/100'000/year in women and 8/100'000/year in Western countries (83)

GD is a Th2-driven disorder and therefore involves humoral autoimmunity. GD etiopathogenesis involves the presence of stimulating TRAb causing a persistent activation of the TSHR and therefore hyperthyroidism (84).

A popular hypothesis regarding the pathogenesis of GD involves a defect in negative selection of autoreactive T cells to the TSHR, either in the thymus or the peripheral immune system, supported by the existence of autoantibodies to TSHR (TRAb) (30).

Many different single nucleotide polymorphisms (SNPs) of TSHR have been found to be associated with GD (30, 38), with the 10 principal SNPs spread across a 340 kb region encompassing the gene upstream of the TSHR (85). Interestingly, although TPOAb are not involved in GD etiopathogenesis, TPOAb positive persons have an increased risk of developing GD (86). A likely explanation is that the genetic susceptibility of AITD and GD involves shared genes, as well as unique genes (87).

The main clinical manifestations of GD include hyperthyroidism and goitre. In rare cases it can be associated with Graves' orbitopathy (GO) and very rarely with an infiltrative dermopathy of the legs called pretibial myxedema. The pathogenesis of GO is complex and involves several mechanisms, including the accumulation in the extracellular matrix of glycosaminoglycans that are secreted by fibroblasts under the influence of cytokines, with consequent functional disruption and impairment of ocular muscles. Extraocular muscles have also been found to be infiltrated with T cells, mainly TSHR-reactive. Less is known about the etiopathogenesis of the rare pretibial mixedema, characterized by the accumulation of hyaluronic acid and chondroitin sulfates in the dermis, probably secondary to cytokine activation of fibroblasts, with consequent compression of the dermal lymphatics and edema (1). However, the expression of immunoreactive TSHR mRNA and protein has been found in orbital pre-adipocytes, fibroblasts and other cells, therefore it is probable that an immune response to the TSHR plays a direct role in the development of all 3 these manifestations: GD, GO and pretibial mixedema (88).

1.2.3 Goitre and iodine deficiency

The term goitre means the enlargement of the thyroid gland and it can be diffuse or nodular, endemic or sporadic. Endemic goitre affects more than 12% of the world's population; the World Health Organisation (WHO) defined the goitre "endemic" when present in 5% or more of schoolchildren aged 6-12 years (89). Considering that iodine is an essential component of thyroid hormones, one of the major causes of goitre is iodine deficiency and the prevalence of goitre varies widely depending on the iodine intake of the referred population (1). Formation of normal quantities of thyroid hormone requires a daily thyroidal uptake of approximately 60 to 75 µg of iodine. Considering a fecal loss of about 10 to 20 µg, at least 100 µg of iodine per day is required in order to ensure the availability of adequate quantities of exogenous iodide for thyroid hormone synthesis (90). The daily dietary intake of iodine varies widely throughout the world, depending on the iodine content of soil and water and on dietary practice; in particular, iodine deficiency is common especially in mountainous and formerly glaciated regions of the earth (91). The level of iodine availability in a population is usually established measuring the urinary iodine excretion and a level of 100 to 150 µg as urinary iodine is considered for iodine-sufficient populations (90).

Similarly to the rest of thyroid diseases, the goitre has a female preponderance and its overall prevalence among the general population according to the Framingham survey and the Wickham study is estimated respectively as

4.6% (6.4% in women, 1.5% in men) and 3.2% (female to male ratio = 6.6:1) (82, 92). When ultrasonography (US) scan is applied as the screening method instead of palpation, the prevalence of goitre increases to 30% to 50% in an unselected adult population and even higher in iodine-deficient areas and among older people (82, 92-95). Thyroid nodules, associated or not with goitre, are also very common and a prevalence of up to 50% has been described in autopsy series (93) and greater than 60% in healthy adults screened with US scan of the thyroid gland (94).

The pathophysiological mechanisms of goitre involve an adaptive response of the thyroid follicular cell to any factor that impairs the synthesis of thyroid hormones. As previously introduced, iodine deficiency is one of the major causes of goitre, but considering that not all the inhabitants of iodine deficient geographic areas develop the goitre, other factors, both genetic and environmental, are likely to be involved. By studying families affected with goitre, several gene abnormalities involving proteins related to thyroid hormone synthesis have been detected, including Tg, NIS, TPO, DUOX2, pendrin and TSHR (1). Environmental factors include cigarette smoking, infections, drugs and goitrogens (95) and a role for oestrogens in goitre formation has been proposed, considering its female preponderance (21, 96).

The hyperplasia of thyroid follicular cells leading to the goitre is mainly driven by increased TSH levels as response to decreased thyroid hormone synthesis (97). However, the serum TSH concentration is normal in most patients with nontoxic goitre, therefore any factor that impairs thyroid hormone synthesis, especially iodine deficiency, may lead to gradual development of goitre in response to normal concentrations of TSH (95). Indeed, a complex network of both TSH–dependent and TSH–independent pathways plays a role in the goitrogenic process, including a variety of autocrine or paracrine growth factors (97).

As clinical manifestation, the nontoxic goitre is usually palpable and sometimes may be seen as a visible lump in the neck in correspondence of the thyroid gland. Large goitres, which may displace or compress the trachea, esophagus and neck vessels, can be associated with symptoms and signs including inspiratory stridor, dysphagia and a choking sensation. Vocal cord paralysis can occasionally result from benign nodular goitres, even if the compression of the recurrent laryngeal nerve, with resulting hoarseness, suggests carcinoma rather than nontoxic goitre. Finally, an acute local painful enlargement with enhancement or induction of obstructive symptoms can suddenly manifest in case of hemorrhage into a nodule or cyst (95). In case of toxic goitre, symptoms of thyrotoxicosis are also present and they will be discussed in the next paragraph 1.2.4.

1.2.4 Hypothyroidism and hyperthyroidism

As previously introduced in paragraph 1.1.4, a reduced level of thyroid hormones is termed hypothyroidism and biochemically defined by FT4 levels below the lower normal limit accompanied by raised TSH. On the contrary, an increased production of thyroid hormone is termed hyperthyroidism and biochemically corresponds to FT4 levels above the normal limit, accompanied by decreased or undetectable TSH. The condition of abnormality of TSH levels with FT4 levels still within the normal range is called subclinical hypothyroidism or subclinical hyperthyroidism when TSH levels are respectively raised or reduced. The term thyrotoxicosis means elevated plasma levels of thyroid hormones and includes not only hyperthyroidism but also different conditions where the excess of thyroid hormone is not related to an increased thyroid production.

Table 1-3 summarizes the possible different causes of hypothyroidism: the principal main distinction of hypothyroidism is between primary, when the thyroid dysfunction is the consequence of a disease primarily affecting the thyroid gland, or central, when the thyroid dysfunction is secondary to an altered pituitary secretion of TSH (secondary) or an altered hypothalamic secretion of TRH (tertiary). The prevalence of hypothyroidism is 10 times higher among women, the elderly and in some racial and ethnic groups, with a prevalence in iodine-replete communities between 1 and 2% (21, 63). The two most common causes of hypothyroidism are iodine deficiency and, in iodine sufficient areas, AITD (83). Congenital hypothyroidism affects about one newborn in 3500–4000 births and is the most treatable cause of mental retardation (98).

Table 1-3: Causes of hypothyroidism

PRIMARY	**CONGENITAL** ▪ Reduced thyroid mass (agenesis or dysplasia) ▪ Reduced thyroid function: iodide transport or utilization defect (NIS or pendrin mutations), iodotyrosine dehalogenase deficiency, organification disorders (TPO deficiency or disfunction), defectes in Tg synthesis or processing, TSHR defects, thyroidal G_s protein abnormalities, idiopathic TSH unresponsiveness
	ACQUIRED ▪ Iodine deficiency ▪ Autoimmune ▪ Inflammatory/Degenerative: De Quervain thyroiditis, Riedel's thyroiditis, amyloidosis, hemochromatosis, sarcoidosis, cystinosis, sclerodermia ▪ Iatrogenic: thyroidectomy, radioiodine treatment ▪ Drugs blocking synthesis or release of thyroid hormones: carbimazole, methimazole, propylthiouracil, potassium perchlorate, lithium, ethionamide, sulfonamides, amiodarone, interferon α, iodine
CENTRAL	▪ Secondary: isolate TSH deficit, panhypopituitarism, resistance of pituitary receptor for TRH ▪ Tertiary: reduced TRSH biological activity
▪ Refetoff syndrome (generalized resistance to thyroid hormones)	

Tg= thyroglobulin; TPO= thyroid peroxidase; TRH= thyrotropin-releasing hormone; TSH= thyroid-stimulating hormone.

The possible causes of thyrotoxicosis are summarized Table 1-4: in this classification it is important to distinguish between true hyperthyroidism caused by an increased thyroid activity or thyrotoxicosis associated with a decreased thyroid activity. The most common causes of hyperthyroidism are GD, followed by toxic multinodular goitre, whilst rarer causes include an autonomously functioning thyroid adenoma or thyroiditis. The prevalence of hyperthyroidism in women is between 0.5 and 2%, and is 10 times more common in women than in men in iodine-replete communities (21).

Table 1-4: Causes of hyperthyroidism and thyrotoxicosis

HYPERTHYROIDISM	**AUTOIMMUNE** ▪ Graves' disease (GD) **NOT AUTOIMMUNE** ▪ Toxic multinodular goitre ▪ Toxic adenoma ▪ Gestational hyperthyroidism ▪ Trophoblastic tumours secerning excess of serum human chorionic gonadotropin hormone ▪ Struma ovarii (monodermal teratoma containing mostly thyroid tissue) ▪ Excess of iodine (e.g. amiodarone) ▪ Metastatic functioning thyroid carcinoma ▪ TSH receptor or G protein mutations ▪ TSH-secreting pituitary tumours ▪ Thyroid hormone resistance with pituitary predominance
THYROTOXICOSIS	**ENDOGENOUS** ▪ Transient thyroid hormones release due to thyroid gland damaging in Hashimoto thyroiditis (Hashitoxicosis) or subacute thyroiditis ▪ Drug induced: amiodarone, lithium, IFN- α, IL-2 **EXOGENOUS** ▪ Iatrogenic overreplacement ▪ Thyroxicosis factitia ▪ Ingestion of natural products containing thyroid hormone (e.g. "hamburger thyrotoxicosis, natural foodstuffs)

IFN-α = interferon alpha ; IL-2 = interleukin-2 ; TSH= thyroid-stimulating hormone.

Hypothyroidism and thyrotoxicosis/hyperthyroidism have many clinical manifestations involving different organs and systems and they are summarized in Table 1-5. The severity of the clinical manifestations depends from the grade of thyroid hormone deficiency/excess and from the duration of the disease. Hyperthyroidism caused by GD is usually more severe compared to that of toxic goitre or adenoma and usually symptoms are more evident in young patients

compared to elderly (1). One of the clinical features of full-blown hypothyroidism is a mucinous edema (myxedema) caused by an accumulation of hyaluronic acid that alters the composition of the ground substance in the dermis and other tissues (99).

Table 1-5: Clinical manifestations of hypo- and hyper-thyroidism in adults

	HTPOTHYROIDISM	HYPERTHYROIDISM
CARDIOVASCULAR system	▪ Bradycardia ▪ Peripheral vasoconstriction ➔ Pallor and intolerance to cold	▪ Tachycardia, palpitations ▪ Blood pressure: ↓ diastolic and ↑ systolic ▪ Cardiac arrhythmias, e.g. atrial fibrillation
METABOLISM	▪ ↓ appetite ▪ Weight gain ▪ ↑ LDL cholesterol	▪ ↑ appetite ▪ Heat intolerance ▪ Weight loss ▪ ↑ plasma free fatty acids ▪ ↓ cholesterol levels ▪ Slight ↓ triglyceride levels
CENTRAL AND PERIPHERAL NERVOUS SYSTEM	▪ Slowing of all intellectual functions ▪ Loss of initiative ▪ Memory defects ▪ Lethargy and somnolence ▪ Paranoid and depression	▪ Nervousness ▪ Emotional lability ▪ Hyperkinesia ▪ Insomnia
MUSCLE	▪ Weakness and fatigue ▪ ↑ creatine kinase levels	▪ Weakness and fatigue ▪ Muscle wasting
EYES		▪ Retraction of upper lower eyelids ➔ typical “stare”
SKIN AND HAIR	▪ Dry skin ▪ Mucinous edema (myxedema) especially around the eyes, on supraclavicular fossa, hands, feet, tongue, pharyngeal and laryngeal mucous membranes ▪ Slow wounds healing ▪ Hair dry and brittle, ↑ hair loss	▪ Excessive sweating ▪ Palmar erythema ▪ Hair fine and friable and ↑ hair loss ▪ Nails soft and friable ▪ Plummer’s nails (onycholysis)
RESPIRATORY SYSTEM	▪ Dyspnea: myxedematous involvement of respiratory muscles; depression of hypoxic/hypercapnic ventilator drives ▪ Obstructive sleep apnea	▪ Dyspnea: weakness of respiratory muscles
BONE, CALCIUM METABOLISM	▪ Bone frailty and osteoporosis	▪ Osteoporosis ▪ Hypercalcemia
RENAL FUNCTION	▪ Edema and fluid retention	▪ Mild polyuria
HEMATOPOIETIC SYSTEM	▪ Mild normocytic normochromic anemia	▪ Anemia
G-I SYSTEM	▪ Constipation	▪ Diarrhea
REPRODUCTIVE FUNCTION	▪ Anovulatory cycles ▪ ↓ libido	▪ Anovulatory cycles ▪ Gynecomastia

G-I = gastro-intestinal; LDL = low density lipoproteins.

1.2.5 Thyroid neoplasms

Thyroid tumours are the most common endocrine neoplasms and they are for the great majority (about 95%) benign, presenting as thyroid nodules. The follicular adenoma results from a genetic mutation in a single precursor cell, usually affecting the TSHR and causing its continuous activation. Thyroid adenomas can be both functioning and not functioning and in the first case they usually determine a clinical condition of hyperthyroidism. The thyroid adenoma is typically solitary and it has to be distinguished from nodules in the multinodular goitre, where they are the result of a hyperplastic response to a certain stimulus, e.g. iodine deficiency, as previously discussed (100).

Thyroid malignant neoplasms account for about 0.5% of cancers in men and 1.5% in women and may be secondary (thyroid metastasis from different primary carcinoma) or primary (101). Primary thyroid cancers are classified according to the cell of origin, as summarized in Table 1-6: rare mixed follicular and medullary carcinoma have also been described (1).

Table 1-6: Classification of thyroid primary malignant neoplasms

CELL OF ORIGIN	NAME OF CANCER	FREQUENCY
THYROCYTES	**Differentiated** ▪ Papillary (PTC) ▪ Follicular (FTC)	90%
	Poorly differentiated	1%
	Undifferentiated (anaplastic)	2%
C CELLS	**Medullary carcinoma (MTC)**	5%
NON EPITHELIAL	▪ Malignant lymphomas ▪ Sarcomas ▪ Others	2%

Papillary thyroid carcinoma (PTC) is the most common, accounting for the 80-85% of total thyroid cancers in iodine-sufficient areas and has a very good prognosis, with a 10-year survival rate of 93-94%, versus 84-85% of follicular variant (FTC) (102). Medullary thyroid carcinoma (MTC) has an intermediate prognosis with a 10-year survival of 50%-75%, while anaplastic carcinoma has the absolute worse prognosis, with a disease-specific mortality of almost 100% (101).

Thyroid cancer may be sporadic and familial. In particular, differentiated thyroid cancer may be present in (102):

- Cowden syndrome (hamartomas, breast, colon and lung tumours)
- Gardner syndrome (intestinal polyps, osteomas, fibromas and lipomas)
- Carney complex (pigmented adrenal nodules, pituitary tumours, skin pigmentation and myxomas)
- Turcot syndrome (large intestinal polyps and brain tumours)

MTC may be associated with hereditary multiple endocrine neoplasias (MEN) type 2A and 2B, associated respectively with parathyroid hyperplasia/adenomas and pheochromocytoma (MEN2A) and with pheochromocytoma, marfanoid body habitus and multiple intestinal and mucosal ganglioneuromas (MEN2B) (103).

Thyroid cell tumourigenesis can develop along several paths, sometimes specific and sometimes common to other solid tumours. The RET (abbreviation for "rearranged during transfection") proto-oncogene encodes for a receptor tyrosine kinase and its activating mutations are involved in the development of various types of cancer, especially MTC, MEN2A and MEN2B (104). RET/PTC mutations leading to unregulated tyrosine kinase activation, together with B-RAF and RAS genes mutations, have been found in about 70% of patients affected with PTC. Finally, translocations of the DNA-binding domain of paired box gene 8 (PAX-8) to the peroxisome proliferator-activated receptor γ (PPAR-γ1) gene have been identified in some FTC (102).

1.3 BREAST CANCER

Breast cancer (BC) is the most frequent malignant disease and the leading cause of cancer death among women in both economically developed and developing countries. Worldwide, 1.4 million new BC cases are diagnosed each year, with a related mortality in about one third (105).

BC mortality rates have declined in recent years thanks to several factors, including increased awareness leading to early detection and the availability of better treatment options (106). However, despite current efforts to prevent the disease, BC incidence is rising in most countries mainly as a consequence of increasing numbers of women with major risk factors for breast cancer, especially obesity, sedentary life and alcohol consumption but also cultural and reproductive changes in women's lives (107).

1.3.1 Breast anatomy and physiology

Both males and females have breasts, but normally they are well developed only in women. Mammary glands in females are accessory to reproduction, while in men they are functionless, consisting of only a few small ducts or cords (108).

1.3.1.1 Breast anatomy

The breasts or mammary glands are located in the thorax region and are modified sweat glands and therefore have no special capsule or sheath (Figure 1-7). The contour and volume of the breasts are produced by subcutaneous fat except

during pregnancy, when the mammary glands enlarge and new glandular tissue forms. Breast size and shape result from genetic, racial and dietary factors (108).

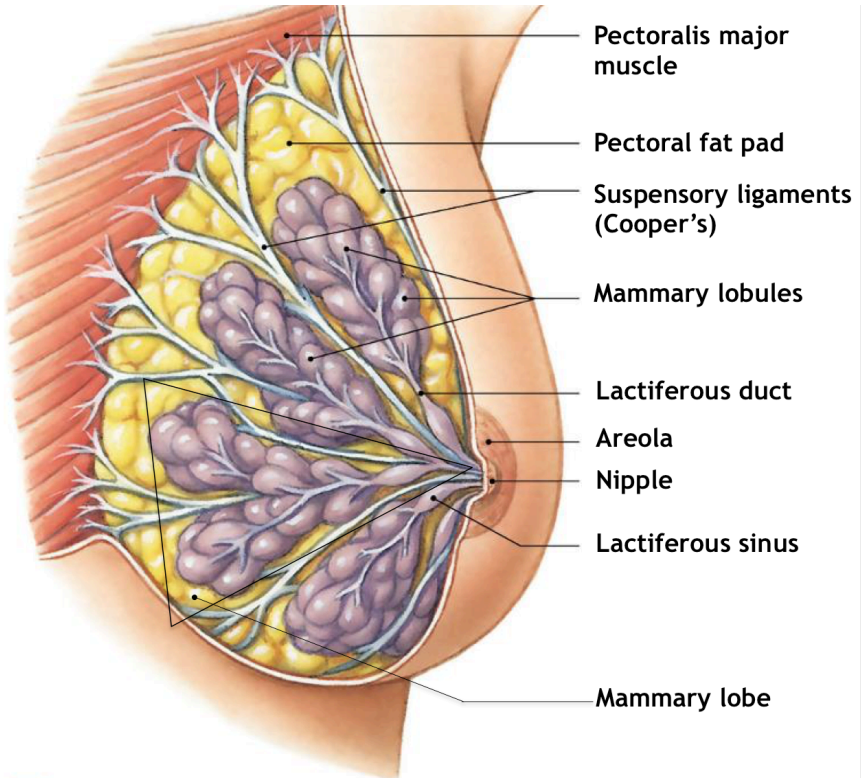
Between the breast and the deep pectoral fascia is a loose connective tissue plane or potential space, called “retromammary space” or “bursa”; it contains a small amount of fat, that allows the breast some degree of movement on the deep pectoral fascia. At the greatest prominence of the breast is the nipple, surrounded by a circular pigmented area (the areola) (108).

The lymphatic drainage of the breast is important because of its role in the metastasis of cancer cells. The lymph passes from lobules of the gland, nipple and areola to the subareolar lymphatic plessus and from it to the axillary lymph nodes (>75%), pectoral (anterior) nodes, parasternal lymph nodes, abdominal lymph nodes (inferior phrenic nodes) (108).

The breast contains 15 to 20 lobes of glandular tissue, which constitute the parenchyma of the mammary gland (108):

- Each mammary lobe consists of a system of draining ever-branching ducts that penetrate deep into the breast fibro-adipose tissue. The duct system finally converges into a unique lactiferous duct that opens independently on the nipple; just deep to the areola, each lactiferous duct has a dilated portion, the lactiferous sinus (108). Each duct is lined by columnar or cuboidal epithelium, with a continuous surface layer of epithelial cells and an outer discontinuous layer of myoepithelial cells. Each duct is surrounded by loose fibrocollagenous support tissue containing rich capillary network (109).
- The branching duct system ends in a cluster of blind-ending terminal ductules, each cluster and its feeding duct comprising a mammary lobule. The terminal ducts and lobules are embedded in a loose fibrous support tissue, which is rich in capillaries and also contains a few lymphocytes, macrophages and mast cells. The tissue is surrounded by a more dense fibrocollagenous support tissue intermingled with adipose tissue (109).
- Each lobule is composed by alveolar cells, secretory units responsible for milk production that during lactation become columnar and look like exocrine cells. Myoepithelial cells are located in the basement membrane of the lobule and along the intralobular ducts (110).

Figure 1-7: Mammary gland and its associated structures



Adapted from © Pearson Education, Inc.

1.3.1.2 Breast physiology

The structure, size, form and function of breast tissue result from an intricate combination of hormone signals and ratios. Lactation is indeed the primary function of the breast and it serves two equally important functions: it supplies the newborn with adequate nutrients and it lengthens the interval between births. The postpuberal breast is mature but inactive; a proliferative phase occurs during pregnancy, followed by regression after lactation ceases (110).

The maternal breast milk contains all the nutrients that an infant needs in the first 6 months of life, including fat, carbohydrates, proteins, vitamins, minerals, growth factors and water. In particular, the main carbohydrate is lactose, a disaccharide and the main proteins are casein and alpha-lactalbumin, together with a balance of various amino acids. One important function of the maternal milk is to protect the newborn from infections, therefore it contains immunoglobulins (Ig), white blood cells, special proteins that can kill bacteria, viruses and fungi (lysozyme and lactoferrin) and oligosaccharides which prevent bacteria from attaching the mucosal surfaces (111).

The breast function is regulated by many different hormones and growth factors (110). In this thesis only a brief overview of their actions on the mammary

gland will be provided, without mentioning their complex mechanisms of regulation and secretion that are beyond the scope of the present thesis.

- **Pregnancy-related hormones,** oxytocin, prolactin (PRL), human placental lactogen (hPL), have the major role of stimulating milk production and release during lactation (Figure 1-8).

  1) Oxytocin is synthetized in the neurons of the supraoptic and paraventricular nucleus of the hypothalamus, transported into the posterior pituitary gland and then released into the circulation. Oxytocin determines the contraction of the myoepithelial cells of the mammary lobules, promoting milk ejection.

  2) PRL is synthetized and secreted by the lactotrophs cells of the anterior pituitary gland. PRL is an obligate regulator of mammary organogenesis, lobular and alveolar growth and functional differentiation. It is also the hormone primarily responsible for the induction and maintenance of milk protein, lactose and lipid production.

  3) hPL is synthetized by the syncytiotrophoblasts of the placenta and its main role is related to breast growth and differentiation during pregnancy. A role similar to PRL in lactation has also been described.

- **Steroid hormones:** oestrogens, progesterone, glucorticoids.

  1) Oestrogens are secreted by ovaries and act via nuclear oestrogen receptor (ER). They are responsible for the proliferation of mammary epithelium, especially the ductal portions of the gland. They have complex actions on lactation, since they directly inhibit milk secretion but at the same time they promote PRL secretion by the anterior pituitary gland.

  2) Progesterone is mainly synthetized in the ovaries and placenta and acts via a nuclear receptor whose expression is induced by oestrogens. Progesterone synergizes with oestrogen and prolactin to produce full lobuloalveolar development of the mammary gland. Similarly to oestrogens, it prevents milk protein synthesis and inhibits milk secretion during pregnancy.

  3) Glucocorticoids are mainly secreted by the adrenal glands and act via nuclear receptors. They are essential for the process of lactation and act in synergism with the other hormones involved.

- **Other hormones:** GH, insulin, thyroid hormones

  1) GH is produced by the anterior pituitary gland and it is important for the function and development of the breast in concert with the other hormones, where it has a substantial lactogenic activity.

  2) Insulin is produced by ß-cells of pancreatic isles and its main function on the breast is regulating the transport of glucose into the mammary acinar cells, essential requisite for synthesis of lipids for milk fat.

3) Thyroid hormones are involved in mammary growth and lactation and have a permissive role, more than regulatory. The interactions between thyroid hormones and breast cell will be further described in paragraph 1.4.2.

- **Growth factors:** several growth factors are involved in normal growth and development of the breast and with its function:

  1) The fibroblast growth factor (FGF) family is composed of several heparin-binding structurally related polypeptides acting via tyrosine kinase receptors and they are important in mammary development during sexual maturity.

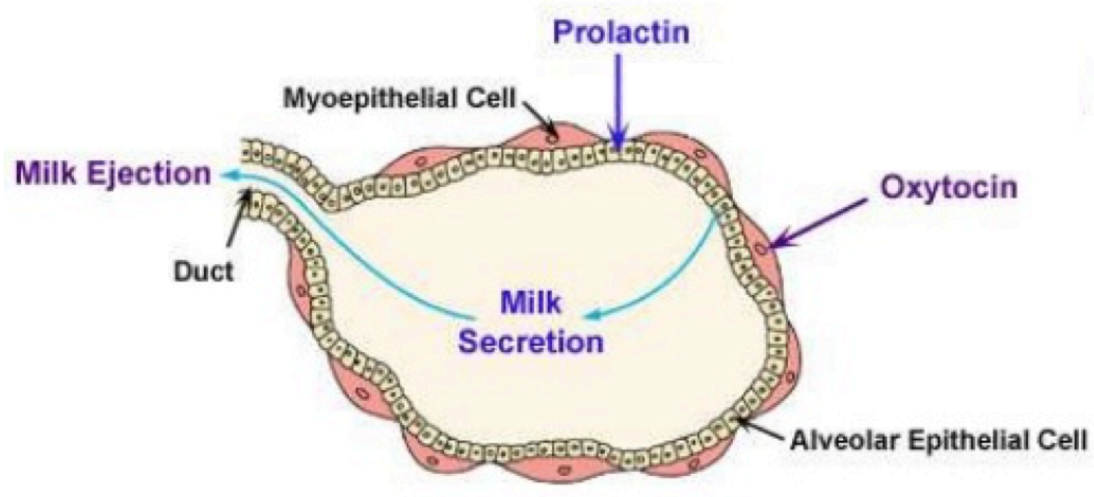
  2) The epidermal growth factor (EGF) family comprises several different proteins that act via a set of transmembrane receptors and are mitogens for mammary epithelial cells.

  3) The transforming growth factor beta family (TGFß) is composed of several related multifunctional proteins that modulate a wide variety of cellular functions. In the mammary gland they are important for ductal morphogenesis and regulation of the onset of lactation.

  4) Insulin-like growth factors (IGFs) are structurally homologous peptides involved in many biological processes and they primarily have a proliferative effect.

  5) Other growth factors. Platelet-derived growth factor (PDGF) is a potent mitogen stimulating the proliferation of mesenchymal cells, thought to act principally via mammary stromal cells. Another growth factor that may act indirectly on the breast is colony stimulating factor-1 (CSF-1).

Figure 1-8: Breast lobule and maternal milk production



Mammary lobule and main hormones involved in lactation. *Adapted from (112).*

1.3.2 Risk factors

Many different risk factors for BC have been identified and they can be classified as genetic, environmental/lifestyle and medical conditions.

1.3.2.1 Genetic factors

It is currently estimated that 5-10% of all BC are hereditary and due to mutations in high-penetrance susceptibility genes. Only two of these genes have been identified so far: BRCA1, associated with families having BC and ovarian cancer, and BRCA2, associated with families having male breast tumours (113). BRCA1 and BRCA2 genes are classified as tumour suppressors and linked with key metabolic processes, including deoxyribonucleic acid (DNA)-damage repair, regulation of gene expression and cell-cycle control (114, 115). About 85% of all alterations in BRCA1 and BRCA2 tumours are frameshift or nonsense mutations and yield a truncated protein product (113).

Several other less known mutations related to BC exist and they include high penetrance lower frequency mutations leading to neoplasias in multiple organs, such as p53 (Li-Fraumeni syndrome), PTEN (Cowden syndrome) and STK11 (Peutz-Jeghers syndrome). Other genetic mutations have only more recently been identified and are characterized as high frequency but lesser penetrance mutations, such as CHEK2, ATM, BRIP1 and PALB2 (116).

1.3.2.2 Environmental and lifestyle factors

The fraction of BC cases attributable to lifestyle and environmental factors in the UK was estimated to be 26.8% in 2010 (117). The major lifestyle and environmental risk factors for BC are:

- **Hormonal factors.** Hormones, oestrogens in particular, are believed to be the most important risk factor for BC. Oestrogens are considered weak carcinogens, as a consequence of their stimulatory activity on breast epithelium by increasing the rate of cell division and proliferation, thereby allowing for an increase in the accumulation of random genetic errors (118). In particular, a high exposure to oestrogens and therefore an increased risk of BC is a consequence of the following situations (119):

  1) Endogenous oestrogens: early menarche, late menopause.

  2) Exogenous oestrogens. The two main sources of exogenous oestrogens are oral contraception in pre-menopausal women and hormonal replacement therapy in post-menopausal women. Numerous studies examining the association between exogenous hormones and BC risk have been published but they report contrasting results and must be interpreted cautiously as a consequence of changes in hormonal dose over time and methods of delivery

(e.g. sequential versus combination) (119). As an example, hormonal birth control has been associated to an increased development of premenopausal BC (120) but other meta-analyses and studies did not confirm these findings among general population (121, 122) or even among high-risk women BRCA1 and BRCA2 mutation carriers (123).

- **Other reproductive factors.** Breast cells that have never undergone differentiation to produce milk divide 20 times more often than cells that have acquired the terminal phenotype (124). Therefore the following situation are considered risk factors for BC:

  1) Low parity and late pregnancy

  2) Abbreviated breast feeding

- **Obesity**. Strong observational data indicate that weight gain in the premenopausal period and being overweight or obese after menopause increase the risk of BC. Obesity is in general related to an increased risk of different types of cancer and the involved mechanisms are not yet fully understood, but likely to involve three different hormonal systems: 1) the insulin and insulin-like growth factor (IGF) axis, 2) sex steroids, and 3) adipokines. In particular, the specific link between increased body weight and postmenopausal BC could be explained by an increased aromatase activity (present in the adipose tissue, therefore overexpressed in obese people), with consequent increased oestrogen production from androgen precursors (125).
- **Diet and iodine**. The impact of dietary components such as the amount of saturated fat, fruit and vegetables on the risk of BC has been analyzed in different studies, mainly leading to contrasting or inconclusive results (107). However, a recent systematic review of dietary patterns and BC concluded that a Mediterranean dietary pattern and diets composed largely of vegetables, fruit, fish and soy are associated with a decreased risk of BC (126). Iodine plays an important role in the mammary gland and iodine deficiency has been associated with an increased risk of BC. Milk, seafood and seaweeds are very rich in iodine; Japanese women, having a diet rich in seafood/seaweeds, have one of the lowest incidence of both benign and malignant breast diseases (127). The topic of iodine and BC will be further discussed in paragraph 1.4.4.
- **Alcohol**. It is estimated that BC cancer risk is increased by 7% to 10% for each one-unit increase in intake of alcohol per day (a unit is half pint of 4% strength beer or cider or 25 milliliters (mL) of 40% strength spirits; one small 125 mL glass of 12% strength wine is 1.5 unit). Several studies suggest that women who want to minimize their BC risk should not be drinking more than one alcohol

unit daily and they are recommended to include a period of at least 2 alcohol-free days weekly (107).

- **Physical activity**. A recent review of 73 observational studies indicated that moderate to vigorous physical activity reduces the risk of BC by an average of 25% in pre- and post-menopausal women compared with inactive women. However, the optimal level of physical activity for BC cancer risk reduction is unclear and may be greater than current recommendations (128). Furthermore, these kind of observational studies conducted so far have major limitations, such as the heterogeneity of self-reported questionnaires used to measure the amount of physical activity (107).
- **Radiations**. The increased neoplastic risk, including BC, after exposure to ionizing radiations is well known and dose dependent (129). Although the radiation from mammography is a low dose, it is estimated that yearly screening from 40 to 80 years of age can cause approximately 225 cases of fatal BC per million of screened women (130).
- **Chemicals**. The exposure to several chemicals has been associated with an increased risk of BC, including polychlorinated biphenyls, polycyclic aromatic hydrocarbons, organic solvents (131) and a number of pesticides (132).

1.3.2.3 Medical conditions

Type II diabetes mellitus (DM) has been associated with an increased risk of developing many different types of cancer. A recent extensive review of existing meta-analysis and observational studies confirmed a significant association of type II DM only with 4 different types of cancer and BC was one of them (133).

The precise mechanisms involved are not fully known, but several hypotheses have been postulated, considering that DM and cancer share several common mechanisms, including increased insulin and IGF signaling, dysregulation of ovarian steroid hormones and chronic inflammation (134).

1.3.3 Classification

BC cannot be viewed as a single clinical-pathological entity and it must be necessarily dissected into a number of more homogeneous entities, therefore its classification is complex and taking into account histological, biological and molecular factors. Despite all the efforts, the “perfect” classification of BC has not been written yet (135).

1.3.3.1 Histopathological classification

The histopathological classification of breast tumours is based on the different morphological features of mammary lesions and the World Health Organization

(WHO) classification, recently updated in 2012 and published by the International Agency for Research on Cancer (IARC), is adopted worldwide. The whole WHO classification is very complex and takes into account all the existing benign and malignant breast tumours; its integral report is beyond the scope of the present thesis. Malignant breast lesions may be primary, originating from breast epithelium, mesenchymal cells or breast lymphocytes, and secondary (metastatic tumours). In particular, malignant mesenchymal tumours include liposarcoma, angiosarcoma, rhabdomyosarcoma, osteosarcoma, leiomyoma and leiomyosarcoma (136).

In the present thesis the term BC refers to invasive malignant lesions derived from the breast epithelium, summarized in Table 1-7. The most common form of BC is adenocarcinoma arising from the epithelial cells of the lactiferous ducts in the mammary gland lobes (108) and traditionally BC takes a different name according to the different part of the mammary gland it originates from (109):

- Ductal carcinomas: originating from the epithelial components of the mammary ducts, both extralobular or intralobular
- Lobular carcinomas: originating from the epithelial components of the terminal ductules

The last version of WHO classification replaced the term “ductal carcinoma” (adopted until the previous 2003 version), with the more general “carcinoma of no special type”, referring to all tumours without the specific differentiating features that characterize the other categories of BC (exclusion diagnosis based on histopathological criteria). The rationale for this is that the term “ductal” conveys unproven histogenetic assumptions (derivation of the tumours from the ductal system) and also invasive ductal carcinoma does not comprise a uniform group of carcinomas (137). Invasive carcinoma of no special type (ex “ductal”) is the most common form of invasive BC, comprising between 40% and 75% of cases, followed by invasive lobular carcinoma (5-15%) (136).

Table 1-7: BC WHO histopathological classification of malignant lesions of the breast epithelium

INVASIVE BREAST CARCINOMA	Invasive carcinoma of no special type (ex invasive ductal carcinoma) ▪ Several subtypes	40-75 %
	Invasive lobular carcinoma ▪ Several subtypes	5-15 %
	Tubular carcinoma	2 %
	Cribriform carcinoma	0.3-0.8 %
	Mucinous carcinoma	2 %
	Carcinoma with medullary features ▪ Several subtypes	<1 %
	Carcinoma with apocrine differentiation	4 %
	Carcinoma with signet-ring-cell differentiation	NA (rare)
	Invasive micropapillary carcinoma	0.9-2 %
	Metaplastic carcinoma of no special type ▪ Several subtypes	0.2-5 %
	Carcinoma with neuroendocrine features ▪ Several subtypes	<1 %
	Other rare types ▪ Several subtypes	<1 %
TUMOURS EPITHELIAL-MYOEPITHELIAL	Adenomyoepithelioma with carcinoma	NA (rare)
	Adenoid cystic carcinoma	<0.1 %
PRECUSOR LESIONS	Ductal carcinoma in situ	20-25 %
	Lobular carcinoma in situ ▪ Several subtypes	NA
PAPILLARY LESIONS	Intraductal papillary carcinoma	NA (rare)
	Solid papillary carcinoma	<1 %

NA = Not available. *Adapted from (136).*

1.3.3.2 Molecular classification

Molecular classification of BC is essential for both BC prognostic assessment and choice of therapies, therefore mandatory in all BC cases. It is based on the following molecular biomarkers: oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type 2 (HER2) and proliferation index Ki67. (135).

- **ER and PR** are nuclear transcription factors that stimulate the growth of both normal and neoplastic breast cells (136, 138). They are routinely detected by immunohistochemistry (IHC) on primarily formalin-fixed paraffin-embedded (FFPE) tissue sections of BC and their expression is defined as a continuum ranging from 0% to 100% of positive cells (136). Both ER and PR are considered positive in the presence of ≥1% positive-staining tumour cells (139) and ER and PR positive expression is reported in respectively 80% and 60-70% of invasive BC, with PR expression highly correlated with ER expression (136). In fact PR expression is regulated by ER, therefore PR positivity indicates that the oestrogen-ER pathway is intact and functional. However, ER-PR correlation is

imperfect and 4 possible phenotypes of combined expression are possible, although it is debatable whether ER-negative/PR-positive tumours exist (136).

- **HER2 oncogene and oncoprotein**. The HER2 gene encodes for a growth factor receptor on the surface of normal breast epithelial cells. This gene is amplified in approximately 15% of BC and its amplification is highly correlated with elevated protein expression. HER2 gene amplification and protein expression status are mainly determined on FFPE samples respectively by fluorescence in situ hybridization (FISH) and IHC (136). In addition, other chromogenic methods that can accurately determine gene copy number using routine bright-field microscopy are becoming popular (140). HER2 expression is considered positive when an amplification of HER2 gene is detectable by FISH and/or >30% of tumoural cells show IHC staining of HER2 protein that can be classified as strong (3+) or moderately strong (2+). When tumours show little (1+ staining) or no (0 staining) protein expression by IHC, usually FISH reports a normal number of gene copies and the tumours are classified as HER2-negative (136, 141).
- **Proliferative fraction (Ki67)**. Tumour proliferation is an important BC prognostic factor and in clinical practice is usually determined by the immunohistochemical staining of Ki67 antigen. In particular, the Ki67 mouse monoclonal antibody recognizes a nuclear antigen present only in proliferating cells and not in resting cells (142). Proliferative fraction, by Ki67 detection, can be classified as low or high and is essential for a complete molecular classification of BC (135).

Several different approaches have been attempted to unveil the molecular basis for heterogeneity of BC. Perou et al. used a hierarchical clustering analysis of gene expression profiling, including keratins, to identify molecularly defined classes of BC (luminal, HER2-enriched, basal-like, normal-like). They found that basal and luminal cells of breast epithelium were respectively ER-negative and ER-positive and since then the terms “basal” and “luminal” have been introduced in BC molecular classification to describe ER-expression state (143). This classification has been shown to have prognostic value and predictive of the response to chemotherapy (135). However, it was derived from investigations on frozen tissue and therefore not applicable to formalin-fixed and paraffin-embedded (FFPE) material routinely used.

Consequently, new classifications based on surrogate protein markers detectable using the more familiar IHC have been applied and they are based on the combined evaluation of HER2, ER, PR and Ki-67 (144). The different BC

molecular categories are frequently updated and modified and one of the latest versions is reported in Table 1-8, according to the 2011 St. Gallen International Consensus. Despite the lack of complete overlap among BC molecular classes determined by gene expression profiling and protein expression by IHC, the molecular classification of BC based on IHC staining of proteinaceous markers has been endorsed to determine the choice of systemic treatments for BC (145).

Table 1-8: BC molecular classification based on IHC staining

	ER and/or PR	HER2	Ki67
Luminal A	Pos	Neg	Low
Luminal B – HER2 negative	Pos	Neg	High
Luminal B – HER2 positive	Pos	Pos	Any
HER-2 positive	Neg	Pos	NA
Basal-like (Triple negative)	Neg	Neg	NA

BC molecular classification according to the 2011 St. Gallen International Consensus, *modified from (145)*. ER= oestrogen receptor; HER2= human epidermal growth factor receptor type 2; low Ki67 = <14%; NA = not assessed; Neg = negative; Pos = positive; PR= progesterone receptor.

1.3.4 Clinical manifestations

Usually BC presents as a firm lump in the breast, but can have additional local manifestations, including lymphedema (excess fluid in the subcutaneous tissue), nipple retraction or deviation or a thickened, leather-like appearance of the skin and abnormal contours of the breast because of traction on the suspensory ligaments (108).

The BC cell can spread outside the breast and this process is called metastasization; the spread BC cells then produce nests of tumoural cells called metastases. There are two types of metastasization: via lymphatic system to the lymph nodes (lymphogenic metastasis) or via veins to different organs (distant metastasis). The most common site of BC metastases are axillary lymph nodes, but other different nodes may be involved, including cervical, parasternal, infraclavicular, supraclavicular and abdominal and both sides of the body may be involved (e.g. presence of contralateral lymph node metastases). The major sites of distant metastases of BC through the hematic route include bone, brain, lung and liver (108).

1.3.5 Blood biomarkers

In contrast to markers detected in the primary tumour tissue, blood tumour markers reflect a dynamic situation and their measurement can be repeated as required. A large number of blood tumour markers have been proposed for BC and

the most widely used in clinical practice are carcinoembryonic antigen (CEA) and cancer antigen 15.3 (CA15.3) (146).

- CEA is an oncofoetal protein and its increased levels have been detected not only in BC but in several other different types of cancers, especially primary colorectal cancer (146).
- The mucin MUC1 is a glycoprotein expressed by most "wet" epithelia, including mammary epithelial cells, and both aberrant and up-regulated expression of MUC1 are features of malignancy. MUC1 is highly heterogeneous and a large number of distinct monoclonal antibodies have been raised against different epitopes; a specific monoclonal antibody recognizing the antigen CA15.3 is the gold standard for MUC1 assessment in BC (146).

CA15.3 has a higher sensitivity compared with CEA, considering that they are positive in respectively 54-80% and 30-50% of metastatic BC. Unfortunately, they have low specificity, since they may both be positive in many different malignant and benign (especially inflammatory) conditions (146).

Although CA15.3 is more sensitive than CEA, CA15.3 is positive only in less than 10% of early stage BC, therefore it is not recommended for screening or diagnosis of BC onset (147). Therefore, both CEA and CA15.3 have clinical utility only in advanced and metastatic BC, especially to monitor the response to treatments (146). Additional clinical tools include the assessment of distant metastasis at BC diagnosis and screening or early diagnosis of BC recurrence (148).

Circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) released by tumoural cells have been identified as very promising blood tumoural markers with great and important potentialities in clinical practice. In particular, they may be applied to assess cancer diagnosis, prognosis, treatment choice and prediction of tumoural response to it. In fact solid tumours exhibit both spatial and temporal heterogeneity; tumoural cells are affected with clonal and subclonal diversity, therefore two different cells are likely to be genetically different with a different behavior, especially when comparing primary tumour and its distant metastases. Furthermore, tumoural cells evolve over time under selection pressure from cancer specific treatments. CTCs and ctDNA therefore have the great potentiality of a non-invasive real-time assessment of tumour molecular profiles during the course of disease, considered as "liquid biopsy technologies". However, further studies are still needed in order to validate and routinely use them in clinical practice (149, 150).

MicroRNAs (miRNAs) are a group of small non-coding RNAs able to regulate gene expression at the post-transcriptional level by binding to the 3' un-translated region of target messenger RNAs (mRNAs) (151). They have been proposed as well

as potential prognostic and predictive biomarkers in BC management, however they are still in a premature phase of investigation and further studies are needed in order to propose their assessment in clinical practice (152).

1.4 THYROID DISEASES AND BREAST CANCER

1.4.1 Benign thyroid diseases and breast cancer: an overview

The relationship between benign thyroid disorders (BTD) and breast pathology has been studied since 1896, when Beatson reported that the use of oophorectomy and thyroid extract was useful in the treatment of metastatic BC (153). Subsequently, many studies addressed the connection between BC and thyroid diseases, in particular BTD and so far, after more than one century, this topic is still debated and rich in conflicting and contradictory results. Some authors support the presence of an association between BTD and BC (154-158) but others do not (159-163). The possible causes of this discrepancy are many, including the use of different diagnostic criteria for the existing BTD, the predominant retrospective or cross-sectional nature of existing studies, the high heterogeneity of BC and its subtypes and the fact that both BTD and breast pathology are two of the most common diseases among women, therefore a high number of studied subjects is required in order to obtain significant results.

The association between BTD and BC seems to be bilateral, in the sense that a higher prevalence of BTD has been found among patients with BC, as well as a higher risk of BC among patients with BTD. In fact in 2003 Turken found a higher prevalence of both autoimmune and non-autoimmune BTD in 150 patients affected with BC compared with 100 controls (156). Many other authors confirmed a higher prevalence of BTD, in particular thyroid autoimmunity (TA), among patients with BC compared to controls, even if not confirmed by other studies. This will be described in more detail in the following paragraph 1.4.3.

Regarding the opposite relationship, 2 cross-sectional studies from the same research group and 1 independent retrospective study showed about three-fold increased risk of BC in patients affected with any kind of BTD, both autoimmune or not, compared with the expected BC prevalence among age-matched general population calculated from national cancer registries (158, 164, 165). Interestingly, in studies from Prinzi et al. patients negative for anti-thyroid autoantibodies (TAb) had a higher BC prevalence compared with TAb positive, suggesting a protective role of TAb (158, 164). The impact on BC prognosis exerted by TAb, TPOAb in particular, will be introduced in the next paragraph 1.4.3 and further discussed in paragraph 4.1.1.

1.4.2 Thyroid function and breast cancer

The relationship between thyroid function and BC has been debated for a long time and contrasting results have been generated, with some authors claiming hypothyroidism associated with an increased (166) or reduced (167, 168) risk of developing BC, while others did not find any correlation at all (155, 159, 160, 169).

In particular, an increased risk of BC among hyperthyroid patients is a recurrent finding and Hellevik et al. in a prospective population study involving almost 30'000 participants found that individuals with a TSH below the normal range had a generalized increased risk to develop different types of cancers, with a Hazard Ratio (HR) for BC of 1.20 (170). Similarly, other population-based case-control studies confirmed that hyperthyroidism seems to be associated with an increased risk of different types of cancer (171).

The epidemiological finding of increased risk of BC among hyperthyroid patients and protective effect exerted by hypothyroidism is supported by *in vitro* experiments showing a tumour-promoting effect of thyroid hormones. In fact normal mammary epithelial cells express thyroid hormone receptors, therefore mammary gland development and gene expression are responsive to T3 (172, 173). Furthermore, in contrast to other hormone receptors, BC cells also express thyroid hormone receptors (174, 175). The activation of thyroid hormone receptors of the mammary gland induces differentiation and lobular growth in an oestrogen-like manner (175-177). In tumour transplant rodent models, thyroid hormones appear to stimulate tumour growth and metastasis, whereas treatment-induced hypothyroidism has opposite effects with favorable outcome not just in BC but in several other cancer types (171).

During recent decades it has been debated whether an increased risk of cancer among hyperthyroid patients could be possibly due to the treatment with radioiodine. A recent Finnish long-term follow-up study conducted on a total of 6148 patients affected with hyperthyroidism and treated with thyroidectomy or radioiodine seems to have definitively solved this debate, concluding that the type of treatment (thyroid surgery versus radioiodine) did not affect the overall risk of cancer and therefore radioiodine can be considered a safe treatment (178).

1.4.3 Thyroid autoimmunity and breast cancer

A relatively recent extensive meta-analysis published in 2012 confirmed the presence of a strong association between thyroid autoimmunity (TA) and BC (157), in total contrast with a previous meta-analysis published in 2002, that failed in confirming such association (161). A possible explanation is the use in the 2012

meta-analysis of more specific and appropriate definition of TA and the inclusion of additional studies that were not available in 2002.

Several authors found an increased prevalence of anti-thyroid autoantibodies (TAb), especially anti-thyroid peroxidase (TPO) autoantibodies (TPOAb), among patients with BC compared with healthy controls (166, 179-181). In particular TPOAb prevalence in BC patients compared with healthy controls was respectively 36% and 19% according to Shering et al. (179), 23.5% and 8% according to Giani et al. (180) and 15% and 9.8% according to Kuijpens et al. (166). However, these findings have not been confirmed by other authors (163).

According to two recent extensive cross-sectional studies, patients affected with non-autoimmune BTD had a higher prevalence of BC compared with TAb positive patients, suggesting a protective role of TAb (158, 164). In particular, several small-scale studies described a better BC outcome among women positive for serum TPOAb compared with TPOAb negative (182-184), even if not confirmed by all authors (185); this topic will be further discussed in paragraph 4.1.1.

1.4.4 Iodine deficiency, goitre and breast cancer

The mammary gland during pregnancy and lactation is highly effective in capturing I^-, essential to form iodoproteins (e.g. iodocasein) and provide the neonate with iodine to make his/her own thyroid hormones necessary to normal neural development (127). This is possible through the expression by the mammary gland of NIS and lactoperoxidase (LPO), that will be better discussed in the next paragraph 1.5.1 and in chapters 2 (NIS) and 3 (LPO).

Iodine also exerts an anti-oxidant effect in iodide-concentrating cells, e.g. thyroid and breast cells, therefore iodine deficiency may cause a decrease in antioxidant defenses and therefore an increase of oxidative stress and cell damage (186-188). Accordingly, Eskin and co-workers showed that iodine deficiency may cause evident dysplasia and cell atypia in mammary tissue of rats and that iodine replacement was able to reverse these changes (189). The same authors also demonstrated that the block of I^- uptake with perclorate was associated with the presence of mammary gland atypia in rats (190).

A possible role of iodine in human breast pathology is provided by the study of Kilbane et al., showing a reduced content of tissue iodine in BC tissue compared to breast benign tumours, e.g. fibroadenomas (187).

Iodine deficiency as a common risk factor for BC and BTD is further supported by the evidence of an increased prevalence of goitre among patients affected with BC, as first reported by Smyth et al. (191) and then confirmed in other independent studies (156).

1.4.5 Thyroid cancer and breast cancer

According to Surveillance, Epidemiology and End Results (SEER) program of U.S. cancer registry data, the cumulative incidence of developing any second cancer after thyroid cancer is 16% at 25 years, with BC being the most common (36%) (192). A reciprocal association between thyroid cancer and BC has been described (193) but still the subject of controversy. Accordingly, some authors observed an increased prevalence of BC among patients with thyroid cancer (192), while others reported an increase of thyroid cancer cases among BC patients (194), not confirmed in a different large retrospective cohort study (195).

Although still a matter for debate, the possible explanations for an association between BC and thyroid cancer are several and involve common genetic, endocrine or environmental risk factors. The increased prevalence of BC after thyroid cancer could be also due to a combination of several elements, including 1) the excellent 10-year overall survival of thyroid cancer (up to 94%) (102); 2) the increased medical surveillance of neoplastic patients and consequent screening bias (196); 3) BC is the most common neoplastic disease among women (197), therefore it is not surprising if also the most common neoplastic disease among patients with thyroid cancer.

Regarding the opposite relationship, an increased prevalence of thyroid cancer after BC could also be due to the involvement of thyroid gland within the radiation field during radiotherapy for BC, however the adult thyroid is much less susceptible to the carcinogenic effects of medical radiation compared with the thyroid of a child. Accordingly, the National Surgical Adjuvant Breast and Bowel Project involving 818 women with BC found that only 2 cases of thyroid cancer occurred after breast irradiation (198). Similarly, no increased risk of thyroid cancer after radiation therapy for BC has been found in a large retrospective cohort study (195).

Regarding the genetic factors, BC and thyroid cancer are surely associated in Cowden's syndrome, one rare autosomal dominant genetic condition characterized by a germline mutation of tumour suppressor *PTEN* gene, determining an increased predisposition to different cancer types. However, Cowden's syndrome is quite rare, therefore responsible for only a small proportion of associated BC and thyroid cancer (193).

Regarding the endocrine factors, sex hormones are likely to be involved, considering that oestrogens are fundamental for BC progression and ER has been found also in both neoplastic and non-neoplastic thyroid tissue (50).

1.5 THESIS AIMS

The present thesis has two main aims:

1) To identify hypothetical common antigens between thyroid and breast cancer (BC) tissue

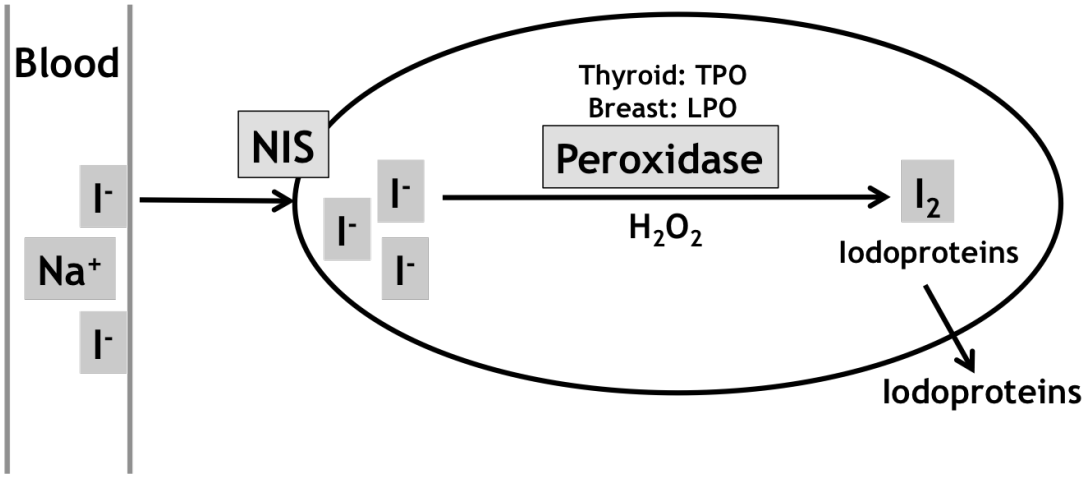
2) To verify the prognostic role of TPOAb in a large cohort of women with BC

1.5.1 Common antigens between thyroid and breast cancer tissues

It is known that thyroid and tumoural mammary glands share similar physiological properties and activities (199), as summarized in Figure 1-9:

- They both express NIS and iodine has a crucial role for both glands
- They both have a peroxidase activity: TPO in thyroid and lactoperoxidase (LPO) in breast

Figure 1-9: Schematic representation of thyroid/tumoural breast cells' similar properties



H_2O_2 = hydrogen peroxide; I^- = iodide; I_2 = iodine; LPO = lactoperoxidase; Na^+ = sodium; NIS = sodium iodide transporter; TPO = thyroid peroxidase.

The epidemiological suggestion of an association between thyroid autoimmunity (TA) and BC and in particular the suggested protective role of TPOAb among patients with BC led us to hypothesize the presence of a common immune response against a shared antigen between thyroid and BC cells, leading to thyroid tissue damaging on one side and BC growth control on the other side.

As previously introduced in paragraph 1.1.2, NIS is a transmembrane glycoprotein involved in active transport of iodide into follicular thyroid cells. Tazebay *et al.* demonstrated by immunohistochemistry that both lactating breast cells and BC cells express NIS, while no NIS activity was shown in normal non-lactating samples from reductive mammoplasties in humans (200). Therefore, considering that NIS is expressed by both thyroid and BC tissues, we hypothesized NIS as putative common antigen between BC and thyroid cells and we searched for

autoantibodies to NIS (NISAb) as possible epiphenomenon of this shared immune reaction. This will be discussed further in chapter 2.

As alternative possible antigen, considering that TPOAb are more prevalent among patients affected with BC, we hypothesized that TPO itself is expressed not only in thyroid tissue but also in BC tissue. As third possibility, since TPO and LPO belong to the same family of proteins (peroxidases) and have considerable homology, we hypothesized that TPOAb could cross-react with shared or similar epitopes present in both TPO and LPO. Both hypotheses will be discussed in chapter 3 (TPO) and chapter 5, paragraph 5.2.1 (TPOAb cross-reactivity).

1.5.2 Prognostic role of TPOAb in a large cohort of women with breast cancer

Considering that so far a protective role for TPOAb among patients with BC has been described only in small-scale studies with not all authors agreeing, the second aim of this thesis was to investigate the impact of TPOAb on BC prognosis in a large-scale cohort of individuals. This will be accurately described in Chapter 4.

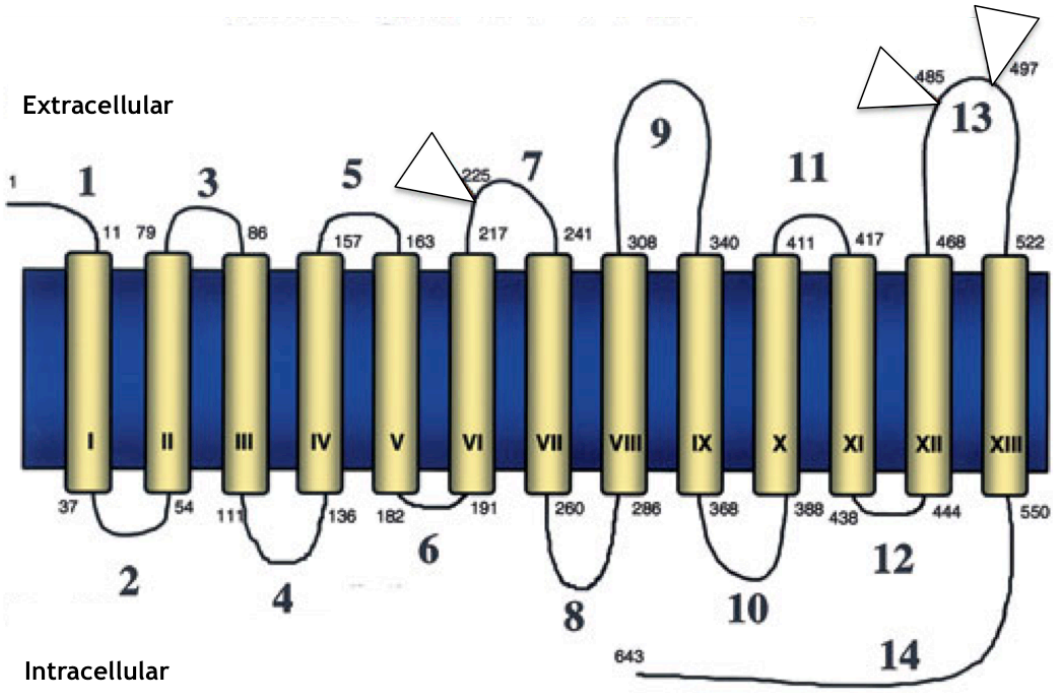
2 NEUTRAL SERUM AUTOANTIBODIES TO THE SODIUM IODIDE SYMPORTER (NIS)

2.1 INTRODUCTION

2.1.1 Human NIS gene and protein: molecular characterization, function, regulation

The Human NIS (hNIS) gene is localized on chromosome 19p12-13·2 and consists of 15 exons interrupted by 14 introns, with an open reading frame of 1929 nucleotides (201). In 1995 Carrasco's group cloned the rat NIS (rNIS) from a Fisher rat thyroid line (FRTL-5)-derived complementary-deoxyribonucleic acid (cDNA) library (202) and in 1996 Smanik et al. cloned hNIS, having 87% identity with rNIS (203). hNIS gene encodes for a 643 amino acid membrane-bound glycoprotein located at the baso-lateral portion of the thyroid follicular cell (202, 204). The protein has a molecular mass of approximately 70-90 kilodaltons (kDa) due to variable levels of glycosylation and presents 13 putative transmembrane domains, 14 extracellular domains, an extracellular amino-terminus and an intracellular carboxyl-terminus (Figure 2-1).

Figure 2-1: Schematic representation of NIS transmembrane glycoprotein

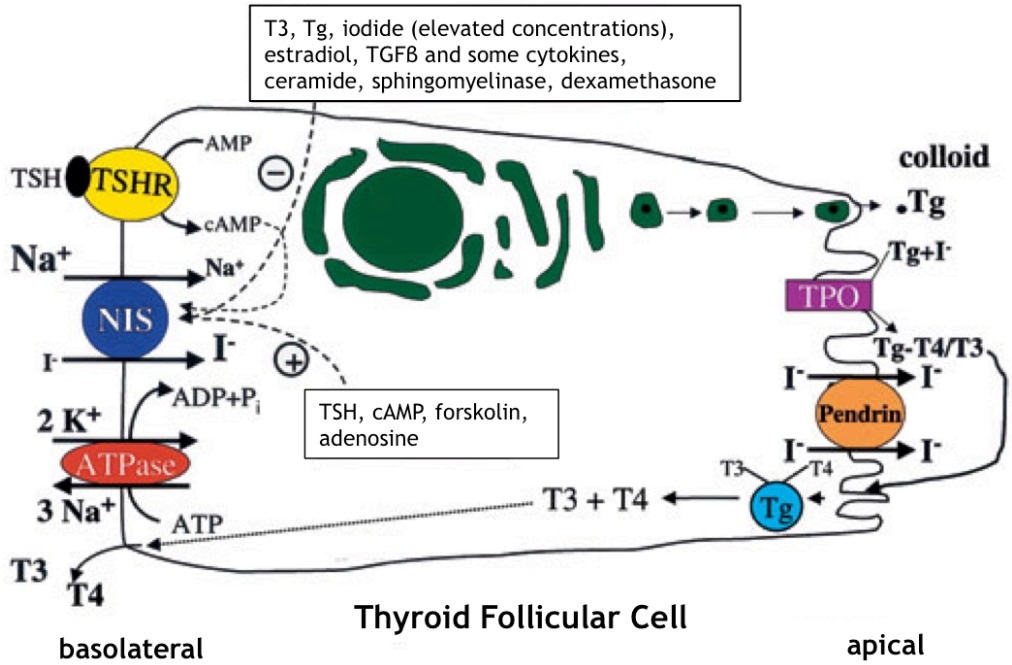


Adapted from (205). 1-643 = amino acid sequence number. I-XIII = transmembrane domains. 1-14 = extra membrane domains. Δ = N-linked glycosylation sites.

NIS carrier co-transports two sodium ions (Na^+) along with one iodide (I^-), with the transmembrane sodium gradient serving as the driving force for iodide uptake (Figure 2-2). The sodium gradient is generated by the ouabain-sensitive Na^+/potassium (K^+)-adenosine triphosphatase (Na^+/K^+-ATPase). NIS-mediated iodide transport is therefore inhibited by the Na^+/K^+-ATPase inhibitor ouabain as well as by the competitive inhibitors thiocyanate and perchlorate (204).

NIS expression in thyroid tissue is extensively regulated, as illustrated in Figure 2-2. Several inhibitors of NIS expression have been identified: triiodothyronine (T3), thyroglobulin (Tg), elevated concentrations of iodide (acute Wolff-Chaikoff effect), estradiol, transforming growth factor-ß (TGFß), various cytokines, ceramide, sphingomyelinase and dexamethasone. The most powerful positive regulator is TSH, stimulating both NIS gene and protein expression and also its iodide transport activity, acting through the cyclic adenosine monophosphate (cAMP) signal transduction pathway. Other positive regulators are forskolin and adenosine. Retinoic acid receptor agonists have shown opposing effects depending on the differentiation state of thyroid cells: they suppress NIS expression and iodide uptake in normal thyroid cells, but in thyroid tumours exert the opposite effect, i.e. enhancement of NIS expression and iodide uptake (206).

Figure 2-2: NIS protein function and regulation



Schematic representation of a thyroid follicular cell and sodium iodide symporter (NIS) function and regulation, *adapted from (205)*. ADP = adenosine diphosphate; ATPase = adenosine triphosphatase; cAMP = cyclic adenosine monophosphate; I^- = iodide ion; K^+ = potassium ion; Na^+ = sodium ion; P_i = inorganic phosphate group, Tg = thyroglobulin, TGFß = transforming growth factor ß, TPO = thyroid peroxidase, TSH = thyroid-stimulating hormone, TSHR = TSH receptor, T3 = triiodothyronine, T4 = thyroxine.

2.1.2 NIS expression in breast tissue and other tissues

Iodide accumulation in breast tissue was first reported in 1957 and it is crucial to supply iodide to the infant for the biosynthesis of thyroid hormones (207). Carrasco's group demonstrated by immunohistochemistry (IHC) that 80% of human BC tissues express NIS, compared with 23% of peri-tumoural breast tissue (PT) and no expression at all in any of the normal non-lactating breast samples obtained from reductive mammoplasties. In the same study, they also analyzed rat NIS (rNIS) expression by Western Blot (WB) and found the presence of immunoreactivity only in mammary glands from lactating rats, while it was absent in non-lactating mammary glands. Authors therefore concluded that NIS protein is expressed in lactating breast tissue and BC tissue but not in normal breast tissue (200).

The earlier promise of a potential application of NIS as a specific indicator of breast malignancy was uncertain, considering that NIS protein expression has been found, even if at a lower extent, also in apparently normal PT (200, 208). This promise has also been further undermined by studies showing hNIS protein or messenger ribonucleic acid (mRNA) expression and iodide accumulation in benign breast tissues (fibroadenoma) (187, 209), with hNIS cDNA expression found to be even higher in fibroadenoma compared with BC (210).

Following Tazebay et al. (200), other authors confirmed the presence of NIS protein expression by IHC in 66-90% of BC tissues tested, reviewed in Beyer et al. However this interesting study underlined how all the previous authors mainly assessed NIS protein by IHC using only one antibody to NIS, often polyclonal (therefore more likely to be aspecific compared with monoclonal antibodies) and predominantly giving intracellular staining, instead of membranous as expected with a transmembrane glycoprotein like NIS. Beyer et al. tested further BC tissues by IHC using different antibodies to NIS (monoclonal, polyclonal and affinity purified polyclonal), all giving discrepant results. They therefore concluded that the previous finding of NIS protein expression by IHC in high percentages of BC tissues are likely to be the result of aspecific staining and stated that NIS protein could be expressed by a much lower number of BC than previously thought (211). Similar conclusions were reached by Peyrottes *et al.*, analyzing by IHC 30 breast cancer tissues using 3 different monoclonal NISAb. They found no significant membrane staining in any of the samples, with only few samples showing a weak and diffuse intracellular anti-NIS staining. By western blot (WB), they did not find the fully glycosylated NIS protein expression but only the partially glycosylated form; moreover, WB results did not correspond with the few cases found positive (intracellular staining) by IHC. They therefore concluded that it could not be

excluded that the BC intracellular staining obtained by IHC with antibodies to NIS (even if monoclonal) was non-specific (212). The hypothesis that NIS is expressed by a minority of BC and usually at a low level (211, 212) is in agreement with the evidence that only 17-25% of apparently NIS protein positive BC demonstrate functional radionuclide uptake by scintigraphic studies (213, 214), even if an impairment of cell surface trafficking in BC cells leading to reduced NIS surface expression cannot be excluded (211).

The nucleotide sequence of hNIS cDNA expressed in thyroid (tNIS) and mammary gland (mgNIS) have been found to be identical (215). Regarding the NIS protein, studies in rats showed a higher level of glycosylation in tNIS compared with mgNIS, having a molecular weight of respectively 100 kDa and 75 kDa (200).

In addition to thyroid and breast, other tissues have long been known to have an active TSH independent process of iodide accumulation (204). Accordingly, NIS mRNA and/or protein expression have been found also in other tissues, either neoplastic or non-neoplastic, including colon, kidney, ovary, endometrium, prostate, lung, gastric mucosa, salivary (parotid) and lacrimal glands (207, 208).

2.1.3 NIS protein as possible autoantigen and autoantibodies to NIS (NISAb)

2.1.3.1 NISAb in autoimmune patients and healthy controls

Thyroid peroxidase (TPO), Tg and thyroid-stimulating hormone (TSH) receptor (TSHR) are well known thyroid autoantigens and Raspè et al. in 1995 suggested NIS as a possible additional thyroid autoantigen, even if rarely. In fact these authors found that 1/147 (0.7%) human sera from patients with several types of autoimmunity was able to reduce the I^- uptake of dog thyrocytes in culture by selective inhibition of NIS carrier. Additional experiments with mouse monoclonal antibodies indicated that autoantibodies to NIS (NISAb) are most likely responsible for this inhibition, therefore NIS can be considered as a potential autoantigen (216).

Subsequent studies further investigated the presence of inhibiting NISAb and reached very contrasting conclusions. Weetman's group found the presence of iodide uptake inhibition in about 30% of sera from patients with Graves' disease and obtained the same results when using purified immunoglobulins (Igs), therefore they concluded that inhibiting NISAb exist and are common (217, 218). On the other hand, two independent research groups (Costagliola's and Pinchera's) found respectively very few and moderate iodide uptake inhibition in sera from patients affected by thyroid autoimmunity (TA) compared to healthy

controls, but in both studies the inhibition disappeared after serum dialysis and was not present when testing purified Igs instead of sera, therefore they concluded that inhibiting NISAb are very rare or barely exist (219, 220). A possible explanation for these discordant results is that many non-specific I^- uptake inhibitors could have created artifacts, such as inorganic salts including I^- itself, perchlorate, azide, disyedenin and growth factors, e.g. epidermal growth factor (EGF) (216).

Additional studies investigated the presence of "neutral" NISAb (bind NIS but do not affect its symporter function), using recombinant hNIS protein produced by an *in vitro* transcription/translation system. Using immunoprecipitation, sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, they reported the presence of neutral NISAb in 24% (218), 20.8% (221) and 14% (222) of autoimmune hypothyroid patients, significantly higher than healthy control subjects in all cases. In particular, Seissler et al. detected neutral NISAb in 4.8% of healthy controls (221), while both authors of Weetman's group found no NISAb in healthy controls (218, 222). In one of these studies NISAb activity was also explored with iodide uptake assays using CHO cells transfected with full-length hNIS; the presence of NIS inhibiting activity was found in both sera and isolated Igs. Interestingly, a poor correlation was observed between neutral and blocking NISAb; in particular, antibodies binding NIS (detected by immunoprecipitation) were absent in 50% sera resulted positive for inhibiting NISAb (investigated by iodide uptake assays) (218).

2.1.3.2 NISAb in patients with breast cancer

Smyth's group reported a more than 3 fold lower iodine (I_2) content in both BC tissue specimens and their correspondent PT tissue, compared with benign breast tumours (fibroadenoma). They therefore suggested a disorder in I^- uptake in patients with BC and postulated the presence of inhibiting NISAb in sera from patients with BC, able to block NIS function in breast cells and consequently reduce I_2 content. Therefore in the same study authors investigated sera from different individuals for the presence of I^- uptake inhibition activity and such activity was found to be significantly more prevalent in BC patients (19%) compared with healthy controls (3%), even if a similar inhibiting activity was present also in sera from patients affected with fibroadenoma (16.3%) and Graves' disease (31.4%). The inhibiting activity was preserved also after Igs purification, therefore authors concluded that the reduced I_2 content observed in BC tissue could arise from the presence in patients' sera of inhibiting NISAb (187).

In order to verify this hypothesis in a different cohort, Pinchera's group used purified Igs from sera of patients with both BC and TA, TA alone and age-matched healthy controls. They investigated Igs interference on I^- uptake by a Chinese hamster ovary (CHO) cell line stably transfected with hNIS and found no inhibiting activity in any of the patients' Igs tested, concluding that the I^- uptake inhibition cannot be the mechanism to explain the lower content of iodine in BC tissue (223). Similarly, these contrasting results may be partially explained by the presence of artifacts due to non-specific I^- uptake inhibitors, as commented in the previous paragraph.

2.1.4 Hypothesis and aims

The main hypothesis of the present thesis is the presence of an immune response to a shared thyroid/breast auto-antigen, which would explain the relationship between TA and both BC prevalence and improved outcome. Considering that NIS glycoprotein is expressed in both thyroid and BC cells, in this chapter we hypothesized NIS as the likely common antigen and we searched for neutral NISAb (binding NIS but not necessarily affecting its function) among sera of patients with BC and/or TA. In fact the presence of these antibodies, especially in BC patients, would support our hypothesis that NIS is a thyroid/breast-tissue shared auto-antigen.

2.2 MATERIALS AND METHODS

2.2.1 Establishment of CHO cell lines expressing hNIS

2.2.1.1 CHO cells transfection with hNIS complementary deoxyribonucleic acid (cDNA)

CHO cells were maintained (+37°C; 5% CO_2) in Ham's F-12 with L-glutamine culture medium (BioWhittaker®, Lonza, Belgium) supplemented with 10% foetal calf serum (FCS), 2% penicillin/streptomycin (PS), 1 millimolar (mM) sodium pyruvate and 0.1% sodium bicarbonate.

We used hNIS cDNA cloned into the eukaryotic expression vector pcDNA3 (pcDNA3-hNIS) encoding amino acid 1 to 612 of hNIS and containing the aminoglycoside antibiotic G418 resistance gene, gift from Dr. S.M. Jhiang (The Ohio State University, Columbus, Ohio) (203, 217).

CHO cells were transfected with pcDNA3-hNIS (CHO-NIS) and empty pcDNA3 vector (CHO-Empty) as control using the transfection reagent TransFast® (Promega) according to the manufacturer's instructions. Briefly, a 90% confluent 25 cm^2 flask was split into 3 60 mm dishes. The day after the culture medium was

removed, dishes were washed with serum-free culture medium and the following were added to each dish: i) 2 mL of serum-free culture medium ii) 5μg of DNA (one dish pcDNA3, one dish pcDNA3-hNIS, one dish left without DNA) iii) TransFast™ Reagent 15 μl (1:1 ratio with DNA: 3 μl TransFast™ Reagent per μg of DNA). After 1 hour of incubation at 37°C, 4 mL of normal culture medium were added. The following day, the selection of transfected cells using 400 micrograms/milliliter (μg/mL) G418 enriched culture medium was started and continued for 4 weeks. A total of 3 different consecutive transfections have been performed.

2.2.1.2 Cloning by serial dilutions

Four weeks after the transfection, CHO-NIS transfected cells were seeded by serial dilution into 96-well plates and nourished with G418 enriched culture medium, in order to isolate positive CHO-NIS expressing clones. After 7-10 days, wells showing only one colony were transferred into 12-well plates, nourished with G418 enriched culture medium and, when confluent, transferred into 25 cm^2 and 75 cm^2 flasks for freezing or NIS expression testing. A total of 26 different CHO-NIS clones were isolated.

2.2.1.3 Testing for hNIS mRNA expression

- **Ribonucleic acid (RNA) was extracted** from CHO-NIS clones, CHO-Empty controls and fresh human thyroid tissue (TT) as positive control (obtained with ethical approval and informed consent from a patient undergoing thyroidectomy for Graves' disease), using TRI Reagent® solution (monophasic solution of phenol and guanidine isothiocyanate from Ambion, Life Technologies, Paisley, UK). Preparation of cells (CHO-NIS and CHO-Empty): 80-90% semi-confluent cells in a 25 squared centimeter (cm^2) flask were washed twice with 5 mL phosphate-buffered saline solution (PBS), aspirated and then 2mL TRI Reagent® solution was added, pipetted several times in order to obtain a homogeneous lysate and transferred to two 1.5 mL Eppendorf tubes. Preparation of fresh tissue (TT): 100 mg fresh frozen TT [fresh tissue snap-frozen in liquid nitrogen and immediately stored at -80 degrees Celsius (°C) until further use] was manually homogenized using a porcelain mortar and pestle containing liquid nitrogen to keep the sample frozen during the whole procedure and homogenized material finally transferred in a 1.5 mL Eppendorf tube containing 500 microliters (μl) of TRI Reagent® solution. Eppendorf tubes containing cells/tissue lysates and TRI Reagent® solution had 100 μl chloroform added for every 500 μl of TRI Reagent® solution used, shaken vigorously for 60 times, left for 5 minutes at room temperature and

centrifuged (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany) at 4°C at 13'000 revolutions per minute (rpm) for 15 minutes. The upper aqueous phase was transferred to a new 1.5 mL Eppendorf tube containing isopropanol 250 µl, gently mixed 6 times, left for 10 minutes at room temperature and centrifuged at 4°C at 13'000 rpm for 10 minutes. The supernatant was discarded, the RNA pellet was washed with 500 µl of 75% ethanol (brief vortex) and centrifuged at 4°C at 8600 rpm for 5 minutes. Finally, the supernatant was discarded, the RNA pellet air-dried and re-suspended in 20 µl distilled water (dH_2O) RNase – DNase free (RDf-dH_2O). Total RNA concentration in nanograms(ng)/µl was determined from spectrophotometric absorption at 260 nanometers (nm) with GeneQuant *pro* ultraviolet-visible (UV/Vis) spectrophotometer (Biochrom Ltd, Cambridge, UK); the RNA sample purity was also tested, verifying a ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) between 1.7 and 2.0 with all the preparations. The total RNA integrity was tested by separating 1 µl total RNA on 2% agarose gel and verifying the presence of ribosomal RNA 28S and 18S bands at approximately 5 kilobases (kb) and 2 kb levels. The RNAs were stored in water at -80°C until further use.

- **Reverse transcription (RT)** procedure was applied to synthesize first-strand cDNA from total RNA. 1 µg total RNA previously denatured in RDf-dH_2O at 65°C for 10 min was added to a RT reaction mixture (Promega, Madison, WI, USA) in a final volume of 20 µl containing 8 mM deoxy-nucleoside triphosphate (dNTP) mix, 200 units (u) Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, 40 u rRNasin RNase inhibitor, 0.4 µg odT (oligo-deoxy-thymine nucleotides short sequence), 4 µl of 5x buffer and RDf-dH_2O for final volume adjustment. The reaction mixture was incubated at 37°C for 1 hour, heat inactivated at 95°C for 5 minutes, finally cooled at 4°C and immediately stored at -20°C.
- **Standard polymerase chain reaction (PCR) and agarose gel analysis.**

  NIS primers were designed from the published sequences of hNIS gene (referred as the member 5 of solute carrier family 5, SLC5A5; GenBank NM_000453) using Primer 3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were then tested for specificity with NCBI Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) and produced by Invitrogen (Carlsbad, USA): forward primer CTCCCTGCTAACGACTCCAG (1842-1861 exon 12) and reverse primer GAGGTCCCACCACAACAATC (2029-2048 exon 14), delimiting a hNIS nucleotide sequence of 207 base pairs (bp).

The RT-resulting cDNA was amplified by PCR using the AmpliTaq Gold™ DNA Polymerase kit (Applied Biosystems, Life Technologies, Paisley, UK) in a 25 µl PCR reaction mix containing 1 µl (approximately 2 ng) cDNA, 2.5 mM magnesium chloride ($MgCl_2$), 2mM dNTP mix, 0.1 µM NIS forward primer, 0.1 µM NIS reverse primer, 2.5 u AmpliTaq Gold™ DNA Polymerase, 2.5 µl 10x buffer II and RDf-dH_2O for final volume adjustment. Reactions always included a negative control with dH_2O in place of cDNA and were performed using the TC-512 gradient thermal cycler (Techne, Minneapolis, USA): 1 cycle, 95°C for 5 minutes; 35 cycles, 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; 72°C for 6 minutes.

Resulting PCR products were electrophoresed on 2% agarose gel in TAE buffer [Tris-acetate-ethylenediaminetetraacetic acid (EDTA); containing ethidium bromide 1 ng/µl] using PowerPac™ 3000 machine (Bio-Rad, Hercules, USA). cDNA samples were loaded on the gel using blue/orange loading dye 6x (Promega, Madison, WI, USA; 1 µl dye for 9 µl cDNA sample) and 100 bp DNA Ladder, ranging from 100 bp to 1000 bp (Promega, Madison, WI, USA), was used to estimate the amplicons' molecular weight. The gel was read with MultiImage II AlphaImager HP (Alpha Innotech, San Leandro, USA).

- **Sequencing of PCR amplicon to confirm identity.**

PCR single products were first precipitated and purified by adding an equal amount of polyethylene glycol (PEG) solution (26% PEG, 6.6 lM magnesium chloride (MgCl2) and 0.6 M Na acetate). The mix was vortexed briefly (Whirlimixer™, Fisons Scientific Equipment, now Fisher Scientific, Loughborough, UK), left at room temperature for 10 min, centrifuged at room temperature at 13'000 rpm for 30 minutes, supernatant discarded, 500 µl of 70% Ethanol was added, vortexed for 30 seconds, spun at room temperature at 13'000 rpm for 10 minutes, supernatant discarded, samples air-dried, pellet re-suspended in 15-40 µl distilled water RNAase – DNAase free, vortexed twice for 10 seconds each. 5 µL of sample with 1.5 µl of loading dye were analysed on a 2% agarose gel in Tris-acetate-EDTA (TAE) buffer in order to verify the presence of PCR products after the PEG-purification steps.

Purified PCR products were sequenced in a 10 µl reaction containing 2 µl BigDye Terminator Sequencing Kit (Applied Biosystems [ABI], Life Technologies, Waltham, USA), 1 µl NIS forward primer (10 pmol/l), approximately 25 ng PEG-precipitated PCR products (5-7 µl) and distilled water to reach the final reaction volume (0-2 µL). Sequencing reactions were developed on TC-512 gradient thermal cycler (Techne, Minneapolis, USA) using the same annealing temperature as for the primer combination.

The products where then sodium acetate (NaOAc)-precipitated applying the following procedure: to the 10 µL sample was added 3 µl of NaOAc pH4.6, 62.5 µl of non-denatured 95% ethanol, 24.5 µl deionized water (final volume reaction = 100 µl), vortexed briefly, left at room temperature for 15 minutes to precipitate the extension products, centrifuged at room temperature at 13'000 rpm for 20 minutes. Then the following steps have been repeated twice: supernatant discarded, 250 µl of 70% ethanol added, mixed briefly, centrifuged at room temperature at 13'000 rpm for 5 minutes, supernatant discarded. Finally, samples were air-dried and analyzed on an ABI Prism 377 DNA automatic sequencer (Applied Biosystem [ABI], Life Technologies, Waltham, USA).

2.2.1.4 Testing for hNIS protein expression

NIS protein expression on the surface of CHO-NIS cells was tested by flow cytometry using a mouse monoclonal antibody to hNIS, as described in the next paragraph 2.2.2.

2.2.2 Flow cytometry

2.2.2.1 Standard protocol

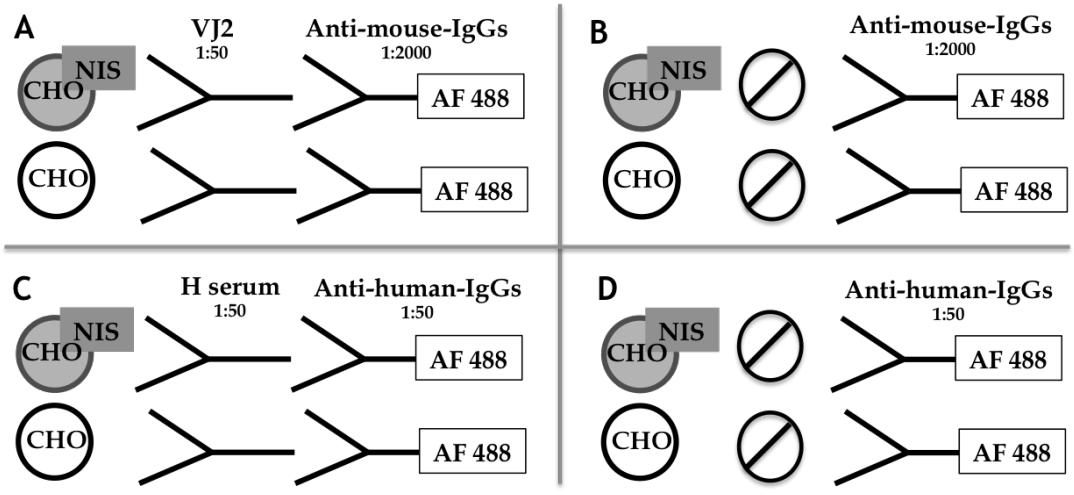
- CHO cells preparation. CHO cells 70-90% confluent were detached from 75-cm^2 flasks (one for CHO-NIS and one for CHO-Empty) with 5 ml PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA)/ethylene glycol tetraacetic acid (EGTA), transferred to universal tubes, and washed in a PBS solution containing 0.5% bovine serum albumin (BSA), 2mM EDTA and 20 mM Hepes (flow buffer). Cells were pelleted at 1200 rpm for 6.5 minutes at 4°C and the supernatant removed by inversion. Cells were counted and transferred to BD Falcon round bottom polystyrene tubes (Scientific laboratories supplies, Nottingham, UK) and adjusted to 50,000 cells/tube.
- Incubation with first antibody (1^{st} Ab). Incubations with flow buffer (100µl) containing the mouse monoclonal antibody VJ2 directed against an extracellular domain of hNIS (AA 272 – 515, 1:50 dilution, kindly donated by Dr S. Costagliola, Free University of Brussels) (224) or heat inactivated human serum (normal or pre-adsorbed, 1:50 dilution) or extracted immunoglobulins (Igs) from various patients (1:50 dilution) were performed for 1 hour at room temperature (Figure 2-3).
- Incubation with second antibody (2^{nd} Ab) and viability dye. Cells were then washed three times (X3) with flow buffer (1ml) and centrifuged as above, followed by a 30 minutes incubation at 4°C in the dark with flow buffer

(100µl) containing goat polyclonal heavy and light chains directed against mouse Igs class G (IgGs) conjugated with Alexa Fluor 488 at 1:2000 dilution (ab150113 goat polyclonal anti-mouse IgG H&L Alexa Fluor 488, abcam, Cambridge, UK) or goat polyclonal heavy (H) and light (L) chains directed against human IgGs, conjugated with green-fluorescent dye Alexa Fluor 488 at 1:50 dilution [goat polyclonal anti-human IgG (H+L) Alexa Fluor 488, Life Technologies, Carlsbad, USA], like shown in Figure 2-3.

50µl of 1:1000 diluted viability dye, LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Carlsbad, USA) were finally added to each tube and incubated at 4°C in the dark for a further 30 minutes, to facilitate detection of damaged cells. Experimental controls omitting 1st Ab (either VJ2 or patients' serum) were also analyzed in the same experiment to investigate nonspecific binding of either mouse or human Alexa Fluor conjugated 2nd Ab.

- Final wash and cells' fluorescence analysis. Following the final incubation cells were washed X3 with flow buffer as before and re-suspended in 250µl Flow Buffer. The fluorescence of 10'000 cells/tube was assayed by BD FACSCanto II flow cytofluorometer, FACSDiva Software (BD Biosciences, San Jose, USA). Channels able to detect fluorescent dyes fluorescein isothiocyanate (FITC) and Allophycocyanin - cyanine Cy-7 tandem conjugate (Apc-Cy7) were used to detect respectively Alexa Fluor 488 and Near-IR dead cell stain signals. No compensation was needed since their excitation-emission peaks had different wavelengths (500-520 nm and 633-750 nm respectively) and as expected their compensation matrix provided values less than 0.0017. Additional two channels were also used to sort cells according to forward scatter (FSC) and side scatter (SSC), respectively detecting the size and the granularity of cells.

Figure 2-3: Schematic representation of a flow cytometry experiment



AF 488 = fluorescent dye Alexa Fluor 488, conjugated to each second antibody (2nd Ab); Anti-mouse-IgGs = goat heavy and light chains directed against mouse immunoglobulins

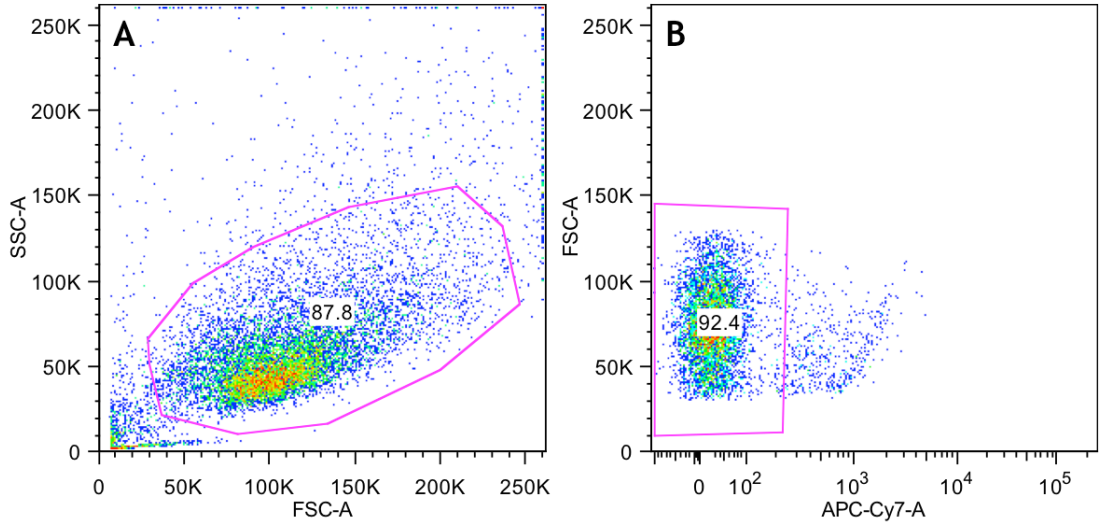
class G (IgGs); Anti-human-IgGs = goat heavy and light chains directed against human immunoglobulins class G (IgGs); CHO = CHO cells transfected with empty PCDNA3 vector (CHO-Empty); CHO-NIS = CHO cells transfected with human NIS (hNIS) gene; VJ2 = mouse monoclonal antibody directed against hNIS. Panel A represent the positive control. Panels B and D represent the negative controls, respectively for anti-mouse and anti-human 2nd Abs. Panel C represents how the presence of NISAb has been investigated: if the human serum tested contained anti-NIS IgGs, they would bind to CHO-NIS cells (but not CHO), therefore the 2nd anti-human Ab would bind as well (not in CHO). Each human serum has been tested separately.

2.2.2.2 Computer data analysis

Flow cytometric data were analyzed using FlowJo 8.8.6 Software (TreeStar Inc., Ashland, USA).

Cells were first gated according to FSC and SSC in order to exclude damaged cells, usually characterized by low signal intensity in both FSC and SSC, as represented in Figure 2-4 (panel A). Then, only cells showing low staining in Apc-Cy7 channel were gated and analyzed, in order to exclude damaged and dead cells, usually positive for Near-IR dead cell stain and therefore characterized by high signal in Apc-Cy7 channel (Figure 2-4 panel B). FITC channel fluorescence intensity values, relative to Alexa Fluor 488 dye, were measured in CHO-NIS cells and compared with control CHO-Empty cells.

Figure 2-4: Gating cells with FlowJo in order to exclude damaged and dead cells



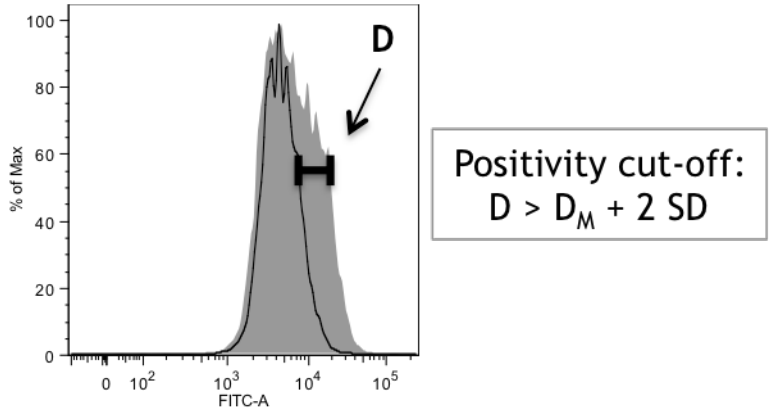
FlowJo 8.8.6 software graphs representing the analyzed cell population. Cells are represented by dots and their colours indicate cells density in decreasing order: red, yellow, green and blue. Panel A shows a side scatter (SSC: ordinate axis) versus forward scatter (FSC: abscissas axis) graph. Panel B shows a FSC (ordinate axis) versus APC-Cy7-A (abscissas axis) graph. In both panels, the gated cells are those included within the pink form and the number indicates the percentage of selected cells compared with the total.

2.2.2.3 Statistical analysis

The geometric mean of FITC-channel fluorescence intensity values detected in CHO-NIS and CHO-Empty cells were compared for all sera and the Kolmogorov Smirnov univariate two-sample test was used to obtain the greatest difference between the two histograms (CHO-NIS and CHO Empty), quoted as D value (D) (225).

Positivity cut-off values were defined based on the mean D + 2 standard deviation (SD) of a population reference (6 young males' sera) defined as group M (further described in next paragraph 2.2.3), with all values higher than this considered positive (Figure 2-5).

Figure 2-5: Kolmogorov Smirnov statistics



Representative flow cytometry histograms of FITC fluorescence intensity obtained using permeabilized CHO-Empty (empty black line) and CHO-NIS (grey tinted figure). D = D value = greatest difference in FITC signal intensity between the two histograms (CHO-NIS and CHO Empty). D_M = mean D value among group M (6 young males' sera).

2.2.2.4 Pre-adsorption of individual human sera using CHO-Empty cells

Seven representative selected human sera were pre-incubated with CHO-Empty cells in a pre-adsorption step before incubation with CHO-NIS and CHO-Empty cells for flow cytometry analysis, in order to pre-adsorb aspecific Igs directed towards antigens different from hNIS protein expressed by CHO cells.

Procedure: CHO-Empty cells (one 75-cm^2 flask 80% confluent) were detached with 5 ml PBS containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM ethylene glycol tetraacetic acid (EGTA) and re-suspended in 1 mL flow buffer. Sera to be tested were diluted 1:10 with CHO-Empty cells in flow buffer (90 µl total volume) and mixed gently for 1 hour at room temperature (each 2µl of serum were adsorbed approximately with $9x10^4$ cells, very similar to the proportion used in flow cytometry assay, assessing the signal obtained with 2µl of serum incubated with $5x10^4$ cells). Cells were removed by centrifugation at 1200

rpm for 6.5 minutes at 4°C, the pre-adsorbed sera were recovered and used for flow cytometry as described in the next paragraph.

2.2.2.5 Permeabilization protocol

Seven representative selected human sera were tested by flow cytometry following a modified protocol in order to expose hypothetical internal NIS antigens through cell membrane permeabilization, which may hypothetically arise during the autoimmune destruction of thyroid tissue.

Modified protocol for flow cytometry (permeabilization): just before the incubation with the 1st Ab, CHO cells were fixed in 2% paraformaldehyde in PBS on ice for 30 minutes, washed once in PBS/0.1% BSA and then incubated with 0.1% BSA, 0.2% saponin PBS for 30 minutes at room temperature. The subsequent steps were similar to the original protocol, except they were all performed in 0.1% BSA, 0.2% saponin PBS instead of flow buffer.

2.2.3 Human sera tested with flow cytometry

Human sera from 42 women and 6 young males (Group M) were heat inactivated at 56°C for 30 minutes before being tested by flow cytometry. Women were divided into 4 groups, according to the disease they were affected with: breast cancer (BC), thyroid autoimmunity (TA), both TA and BC (BC/TA) and non-autoimmune thyroid disorders (e.g. non toxic goitre) (CO), as shown in Table 2-1. CO group was considered as "control group" since patients were not affected neither with TA nor BC.

Anti-TPO autoantibodies (TPOAb) and anti-Tg autoantibodies (TgAb) were measured by standard automated immunoenzymometric assay (Tosoh Bioscience, Tessenderlo, Belgium) and considered positive if respectively ≥ 10 U/mL and ≥ 30 U/mL.

Group M, characterized by young human males, was included in order to identify a control population for statistical analysis with very low probability of having NISAb. In fact NISAb positivity has also been described among healthy adults therefore, considering that TA prevalence is predominant among women and increases with age, young males are the part of general population with less probability to have NISAb positivity.

Table 2-1: Characteristics of individuals providing sera

	Age: Mean and Range	TPOAb positive	TgAb positive
BC (n=12)	66 y ± 12 y SD (range 42-81 y)	0/12	1/12
TA (n=11)	48 y ± 13 y SD (range 31-76 y)	10/11	9/11
BC/TA (n=10)	54 y ± 7 y SD (range 43-63 y)	9/10	8/10
CO (n=9)	51 y ± 21 y SD (range 13-68 y)	0/9	1/9
M (n=6)	10 y, 3.3 m ± 5 m SD (range 9y, 6m – 10y, 6m)	0/6	0/6

BC = women affected with breast cancer. TA = women affected with thyroid autoimmunity, defined as positivity of TPOAb and/or TgAb. BC/TA = women affected with both BC and TA. CO = women affected with non-autoimmune thyroid disorders (e.g. non toxic goitre). M = young human males. y = years. m = months. SD = standard deviation.

2.2.3.1 Flow cytometry with extracted immunoglobulins (Igs)

Total Igs were extracted from four sera, one for each patient group: BC, TA, BC/TA, CO. This has been performed incubating the sera at 4°C for 4 hours with ammonium sulphate 3.75M (1.49 ml for each 2 ml serum), centrifuging at 2000 RPM for 20 minutes, resuspending the pellet in 1 ml dH_2O and finally dialyzing at 4°C against 100 volumes of PBS X3 (respectively 2 hours, 4 hours and overnight) (226).

The same flow cytometry experiment was then performed, as previously described in paragraph 2.2.2, using the same dilution applied for serum (1:50). The normal reference range for serum IgGs concentration among women is 7-18 µg/µl (medium concentration = 12.5 µg/µl) (227). Having used a 1:50 serum dilution in flow experiments, consequently 0.25 µg/µl IgGs (12.5 µg/µl divided by 50) had to be used in the 100 µl sample volume, in order to maintain the same concentration used with serum. IgGs concentration of each sample was determined from spectrophotometric absorption at 280 nanometers (nm) with GeneQuant *pro* ultraviolet–visible (UV/Vis) spectrophotometer (Biochrom Ltd, Cambridge, UK).

2.3 RESULTS

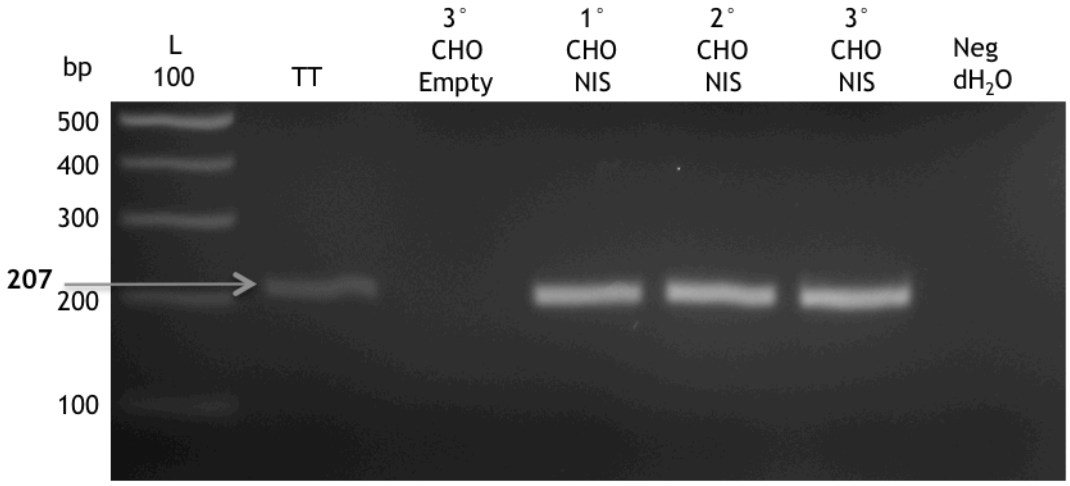
2.3.1 Characterization of CHO-NIS cell lines

2.3.1.1 NIS mRNA expression

Successful CHO-NIS transfections were confirmed by the presence of a 207 bp amplicon in different mixed pool of CHO-NIS transfected cells, while no

amplicons were obtained in CHO-Empty. The 207 bp amplicon was expressed in the positive control (TT) and absent in the negative control with dH_2O (Figure 2-6).

Figure 2-6: 207bp expected PCR amplicon in CHO-NIS cells



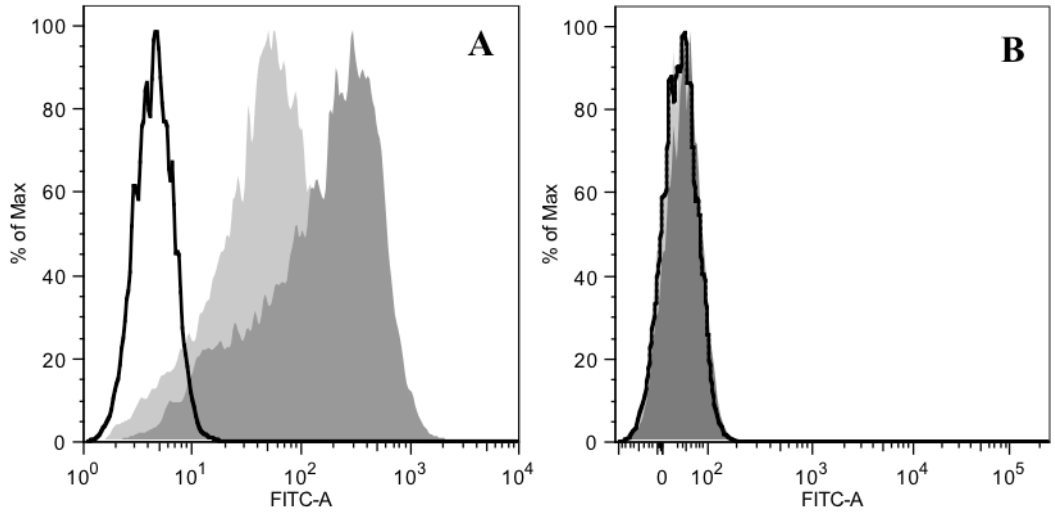
bp = base pairs; CHO-Empty = CHO cells transfected with empty vector pcDNA3; CHO-NIS = CHO cells transfected with pcDNA3 containing hNIS gene; L 100 = ladder 100 bp; Neg dH_2O = negative control with distilled water in place of cDNA; TT = thyroid tissue (positive control); 1°, 2°, 3° = numbers of transfection experiment.

Of the 26 CHO-NIS clones isolated, 6 were tested for NIS mRNA expression and only clones 1 (CHO-NIS-1) and 6 (CHO-NIS-6) expressed NIS mRNA (images not shown).

2.3.1.2 NIS protein expression

Flow cytometric analysis indicated a high specific binding of the mouse monoclonal NISAb VJ2 to the NIS protein expressed on the CHO-NIS cells surface; CHO-NIS-6 expressed the NIS protein at a higher level than CHO-NIS-1, with a mean fluorescent signal intensity of 10^4 and 10^3 respectively. As expected, a negative (10^2) signal was obtained in CHO-Empty cells (Figure 2-7 panel A). The negative control experiment performed using only the 2nd Ab without the 1st Ab step excluded the presence of an aspecific binding of the 2nd Ab on the surface of CHO cells, since the signal intensity was negative (10^2) in all three cell lines tested (Figure 2-7 panel B).

Figure 2-7: Flow cytometry with mouse monoclonal NISAb VJ2 and anti-mouse 2nd Ab



Flow cytometry histograms showing the FITC fluorescence intensity obtained in CHO-NIS-6 (dark grey tinted figure), CHO-NIS 1 (light grey tinted figure) and CHO-Empty (empty black line) cells. Panel A: results obtained using VJ2 as first antibody (1st Ab) and goat polyclonal anti-mouse IgG H&L Alexa Fluor 488 as second antibody (2nd Ab). Panel B: results obtained using only the goat polyclonal anti-mouse IgG H&L Alexa Fluor 488 as 2nd Ab, without the 1st Ab step.

2.3.2 Flow cytometry with human sera

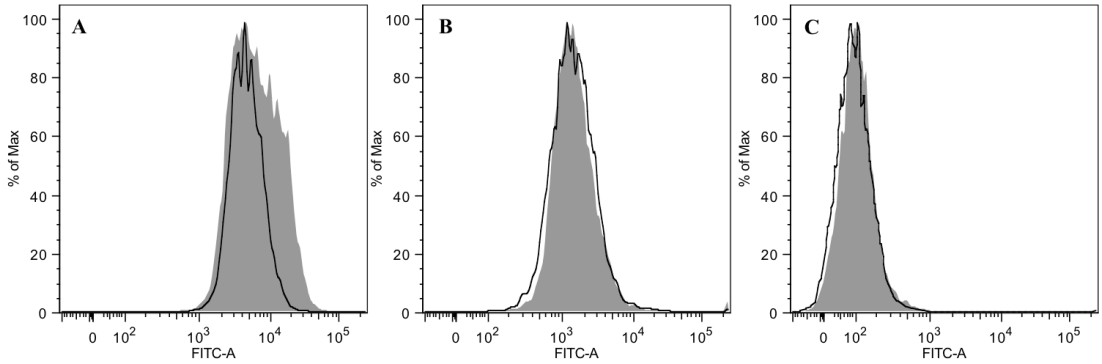
2.3.2.1 Standard flow cytometry protocol with human sera

All 42 heat-inactivated human sera were tested for NISAb in 3 separate experiments: twice using CHO-NIS-6 (experiments 1 and 2) and once with CHO-NIS-1 (experiment 3), always including CHO-Empty as negative control and testing Group M as reference control. For each experiment, the human sera were split in two different sets, each of them analyzing half of them (21 + 21) and the group M with both CHO-Empty and CHO-NIS cells.

In the first experiment using CHO-NIS-6, 8/42 sera (19%) showed a fluorescence intensity signal slightly higher in CHO-NIS compared with CHO-Empty cells and were classified as borderline positive (Figure 2-8 panel A). The other sera were negative, with no difference in signal intensity between CHO-NIS and CHO-Empty (Figure 2-8 panel B). When incubating the cells with the 2nd Ab only, the signal intensity was negative in both CHO-NIS and CHO-Empty, indicating absence of nonspecific binding of the 2nd Ab goat polyclonal anti-human IgG (H+L) Alexa Fluor 488 (Figure 2-8 panel C). When using human sera as 1st Ab, an aspecific background signal (10^3-10^4) was present in both CHO-Empty cells and CHO-NIS (CHO-NIS-6 as well as CHO-NIS-1), while absent when using VJ2 (Figure 2-7 panel A) or when incubating cells with the 2nd Ab only, both the anti-mouse (Figure 2-7 panel B) or the anti-human (Figure 2-8 panel C). These findings

suggested that the nonspecific signal was due to the human sera itself and this will be further evaluated in the next paragraphs 2.3.2.2 and 2.3.2.3.

Figure 2-8: Flow cytometry results using human sera



Representative flow cytometry histograms showing the FITC fluorescence intensity signal obtained in CHO-Empty (empty black line) and CHO-NIS-6 (dark grey tinted figure) after incubation with human sera (1st Ab) and goat polyclonal anti-human IgG (H+L) Alexa Fluor 488 (2nd Ab). Panels A and B show borderline positive and negative results respectively, in presence of a high aspecific background signal (10^3-10^4). Panel C shows incubation with the 2nd Ab only: the signal is negative (10^2) in both cell lines, with no aspecific background.

Using the Kolmogorov Smirnov statistics, the mean D + 2 SD of the 6 Group M sera (positivity cut-off) was 0.290 and 0.650 for set 1 and set 2 respectively. Table 2-2 reports the range of D values found in the 42 human sera tested. The 8 borderline positive sera, similarly distributed in all 4 patient groups, had D values close to the positivity cut-off but not higher, therefore none of them resulted significantly positive for NISAb.

Table 2-2: D values of human sera

	D value Set 1 (range)	D value Set 2 (range)	Borderline human sera
BC	0.110 – 0.180	0.100 – 0.610	2/12 (16.7%)
TA	0.026 – 0.190	0.100 – 0.280	1/11 (9.1%)
BC/TA	0.056 – 0.240	0.210 – 0.350	2/10 (20%)
CO	0.130 – 0.200	0.130 – 0.440	3/9 (33.3%)
			8/42 (19%)

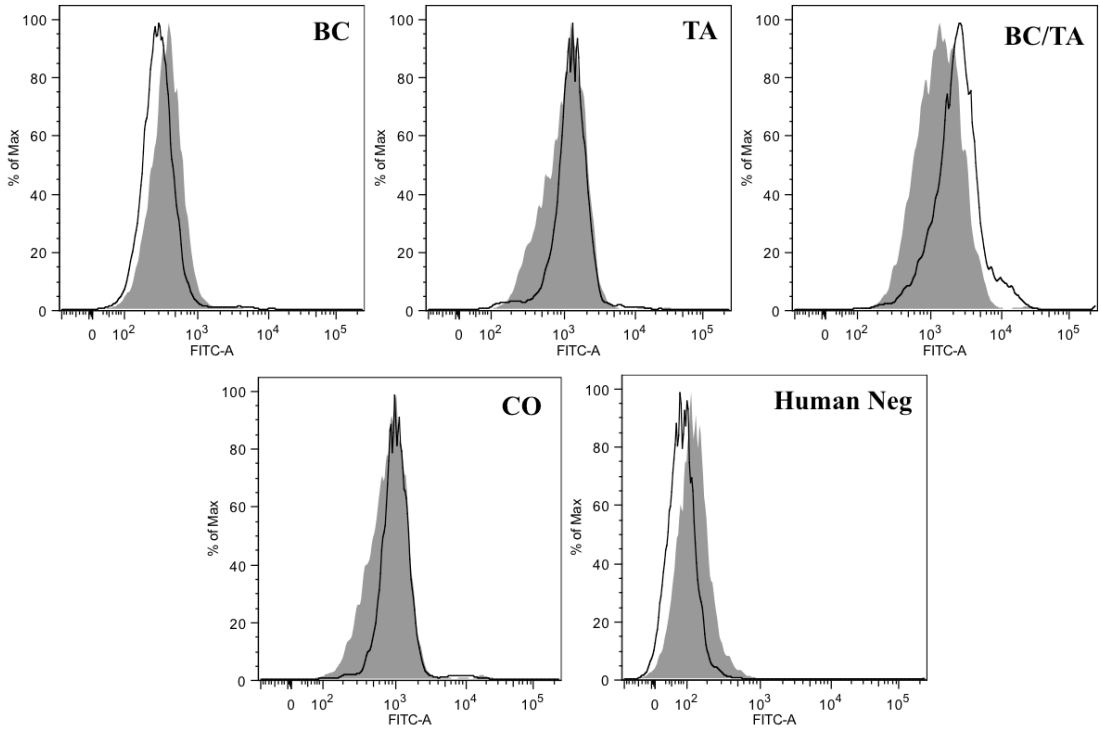
BC = breast cancer; BC/TA = breast cancer and thyroid autoimmunity; Borderline human sera = sera that gave a D value near to the cut-off (0.290 and 0.650 respectively for set 1 and set 2); CO = controls (non-autoimmune thyroid disorders); D = greatest difference between the geometric mean fluorescence intensity values of histograms obtained incubating each sera with CHO-NIS-6 and CHO-Empty; TA = thyroid autoimmunity.

Some borderline positive results of experiment 1 were not reproduced in experiments 2 and 3. On the contrary, in experiments 2 and 3 some different borderline positive sera were identified; they were discordant between experiments 2 and 3 and always resulted negative when analyzed with Kolmogorov Smirnov statistics (results not shown).

2.3.2.2 Standard flow cytometry protocol with extracted Igs

In order to clarify the origin of the aspecific background signal obtained when using human sera, flow cytometry experiments were performed using extracted Igs from 4 representative sera (one for each patient group), showing the same nonspecific background with both CHO-NIS and CHO-Empty cells (Figure 2-9). These results suggested the presence of serum antibodies against unknown antigens expressed on the CHO cells surface.

Figure 2-9: Standard flow cytometry with human sera

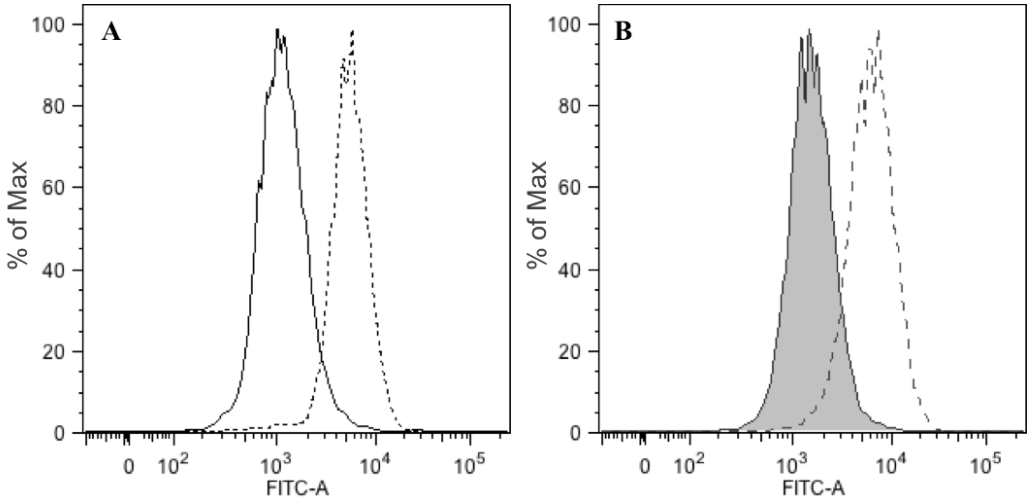


Flow cytometry histograms showing the FITC fluorescence intensity signal obtained in CHO-Empty (empty black line) and CHO-NIS-6 (dark grey tinted figure) after incubation with purified human Igs (1st Ab) and goat polyclonal anti-human IgG (H+L) Alexa Fluor 488 (2nd Ab). Human Igs were purified from patients affected with BC = breast cancer; BC/TA = breast cancer and thyroid autoimmunity; CO = controls (non-autoimmune thyroid disorders); TA = thyroid autoimmunity. Human Neg = incubation of cells with 2nd Ab only and no 1st Ab.

2.3.2.3 Modified flow cytometry protocol: pre-adsorption of human sera (1st Ab)

Once determined that the aspecific background is likely due to human IgGs directed towards CHO cells antigens, a flow cytometry experiment was carried out using 7 human sera pre-adsorbed with CHO-Empty cells (2 BC, 1 TA, 1 BC/TA, 1 CO and 2 M), in order to try and eliminate aspecific anti-CHO human IgGs, hoping to reduce the nonspecific binding and potentially enhance a hypothetical specific NISAb signal. The pre-adsorption step was effective in slightly reducing the nonspecific background in 6/7 (85.7%) human sera for both CHO-Empty and CHO-NIS-6 cells (Figure 2-10).

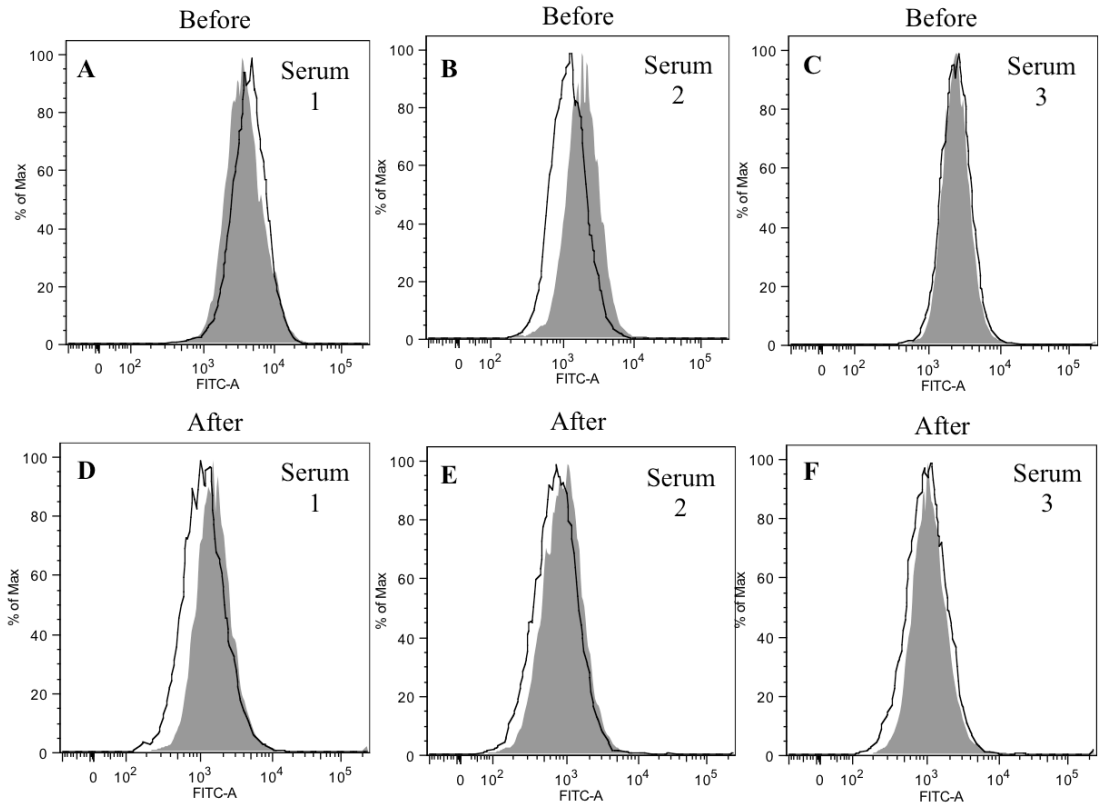
Figure 2-10: Pre-adsorbed flow cytometry: difference in aspecific background



Representative flow cytometry histograms of FITC fluorescence intensity. Panel A: CHO-Empty cells incubated with normal serum (dashed empty black line) or the same serum after pre-adsorption step (continuous empty black line). Panel B: CHO-NIS-6 cells incubated with the same serum of panel A, normal (dashed empty grey line) or pre-adsorbed (dark grey tinted figure).

Contrary to what was expected, the pre-adsorption step was unable to significantly enhance any difference between CHO-Empty and CHO-NIS-6 cells (signal specificity; Figure 2-11), since it was only minimally increased in 4/7 (57%) (panels A and D), minimally decreased in 2/7 (29%) (panels B and E) and unchanged in 1/7 (14%) (panels C and F). As a consequence, the presence of NISAb was investigated in all 42 human sera by flow cytometry without the pre-adsorption step.

Figure 2-11: Pre-adsorbed flow cytometry: effects on signal specificity

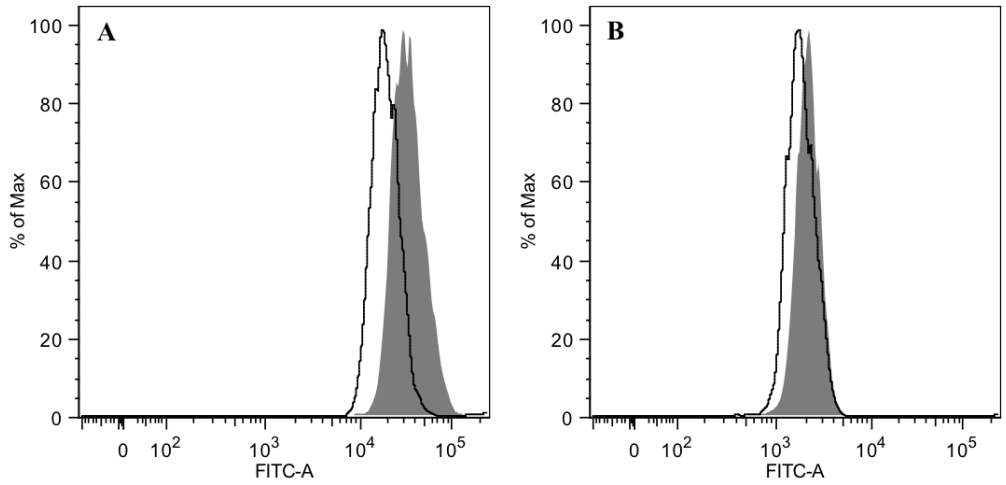


Representative flow cytometry histograms of FITC fluorescence intensity in CHO-Empty (empty black line) and CHO-NIS-6 (dark grey tinted figure) cell lines incubated with 3 human sera before (upper panels A, B, C) and after (lower panels D, E, F) adsorption with CHO-Empty cells. The signal difference between CHO-Empty and CHO-NIS-6 cells (signal specificity) after the pre-adsorption step in respect to before pre-adsorption was slightly increased in serum 1, slightly reduced in serum 2 and unchanged in serum 3.

2.3.2.4 Modified flow cytometry protocol (Cells permeabilization)

The experiment testing 7 different human sera (4 BC and 1 for each of remaining patient groups: TA, BC/TA, CO) with permeabilized CHO-NIS-6 and CHO-Empty cells demonstrated a further increase in nonspecific background signal (up to 10^5) in both CHO-NIS and CHO-Empty cells without any significant difference between cells expressing NIS and controls (Figure 2-12). Furthermore, cell permeabilization also increased the aspecific binding of the 2nd Ab, that was absent when using normal CHO cells (Figure 2-9).

Figure 2-12: Flow cytometry with permeabilized cells



Representative flow cytometry histograms showing the FITC fluorescence intensity signal obtained in CHO-Empty (empty black line) and CHO-NIS-6 (dark grey tinted figure) permeabilized cells. Panel A = cells incubated with human sera (1st Ab) and goat polyclonal anti-human IgG (H+L) Alexa Fluor 488 (2nd Ab). Panel B = cells incubated with 2nd Ab only.

2.4 CONCLUSIONS

2.4.1 NIS as common antigen between thyroid and breast cancer tissues

In this study 42 examined human sera were analyzed, grouped into 4 possible categories: patients affected with breast cancer (BC), thyroid autoimmunity (TA), BC and TA (BC/TA) or non-autoimmune thyroid disorders (CO). None of them was found positive for neutral NISAb (e.g. binding the symporter without affecting its function), as no signals consistently above the positive cut-off value were found. Eight out of 42 (19%) sera, distributed throughout the 4 patient groups, were considered borderline positive, since they fell near to the positive cut-off. These higher D values, even if not statistically significant, could be representative of low titre neutral NISAb and/or demonstrate random variability without any significance. The second interpretation is the most likely, considering that 1) the 8 borderline positive sera were not confirmed in all three experiments performed and 2) a great variability was found between the different experiments, as evident in the different mean D values of group M between set 1 and set 2. There are several possible explanations for this variability, including the expression of different types and quantities of surface antigens depending on cell cycle phase (228). This could partly explain the variations of both aspecific background and signal specificity between CHO-Empty and CHO-NIS cells in the different experiments. Therefore only D values clearly above the cut-off established by Kolmogorov-Smirnov statistics should be considered positive; no sera positive for NISAb were found according to this criterion.

To my knowledge this is the first reported use of flow cytometry to detect neutral serum NISAb binding the symporter. Previously, other groups using a recombinant hNIS protein identified neutral NISAb in sera from patients affected by TA, but which were also present in the general population (218, 221, 222). The results of the present thesis do not confirm these previous reports. The most likely explanation is that expression of hNIS using an *in vitro* transcription/translation system may result in different and/or incomplete folding of the hNIS protein. Similarly, in those assays glycosylation may be absent or, if present, different glycosylation patterns may be involved, compared with the recombinant hNIS produced in the CHO cell system used in the present study. In fact NIS is strongly bound to the cell membrane with 13 transmembrane segments, therefore it needs a cell membrane in order to correctly fold, which is impossible in an *in vitro* transcription/translation system. CHO expression of this complex symporter should allow its complete folding and glycosylation, with expression in its original location as a transmembrane protein. This is supported by the fact the hNIS expressed by CHO-NIS was recognized by the mouse monoclonal Ab VJ2, directed versus a conformational epitope, therefore indicating a correct folding of this transmembrane protein. It is therefore probable that the conformational hNIS epitopes recognized by antibodies are likely to be different between the two recombinant protein expression techniques (*in vitro* transcription/translation system versus protein expression in CHO cell lines). Furthermore, the *in vitro* transcription/translation systems are more likely to detect linear epitopes instead of conformational. For completeness, it is important to comment that to express hNIS we transfected CHO cells with a plasmid encoding for a product of 612 amino acid (AA), while the hNIS encodes for a 643 AA protein. The missing AA encode for a cytoplasmic portion of the protein, therefore it is unlikely that they represent epitope regions recognized by the immune system, or contribute to the final folding and conformation of NIS protein. The presence of functional NIS activity, e.g. the ability to trap iodide, has not been investigated in the present study, although other authors have previously confirmed the function of the same hNIS construct (203, 217).

The finding that serum neutral NISAbs are rare is in agreement with a number of previous studies (216, 219, 220) although not all (217, 218). Similarly, NISAb interfering with the symporter function have been described as rare by several authors who found no iodide uptake inhibitory activity in patients with or without TA and hypothesized that the inhibitory activity observed in some sera was not antibody-mediated, since purified IgGs lacked NIS blocking activity (219, 220, 223). On the contrary, Ajjan et al. found iodide uptake inhibitory activity

mediated by IgGs, even if 50% of those IgGs did not bind NIS, therefore they also postulated that the inhibitory activity could be in part not antibody-mediated or due to antibodies that do not act directly on NIS (217, 218).

In conclusions, despite being expressed in both thyroid and BC cells (200), NIS is unlikely to be a shared antigen responsible for a common immune response between thyroid and BC tissue. However, to definitively address this question, further studies with larger number of sera are needed. In addition, alternative techniques such as the enzyme-linked immunosorbent assay (ELISA) or Western Blot (WB) could be used to detect NISAb (229), as such techniques may expose epitopes of the hNIS protein not accessible when anchored in the cell membrane, that could be exposed as a consequence of tissue damage. Furthermore, differences in protein folding are possible with these techniques, which again may result in a greater diversity of epitope exposure. The presence of NISAb recognizing intracellular epitopes potentially arising only during or after tissue damage cannot be excluded in this study, since they would be inaccessible in the flow cytometry protocol applied. The preliminary experiments using permeabilized cells were hampered by high non-specific background staining which precluded drawing any conclusions. Finally, the absence of NISAb does not exclude that NIS linear epitopes could trigger a hypothetical lymphocytes T reaction and specific studies are needed to explore this possibility.

However, the absence of NISAb in patients affected with both TA and/or BC, together with the consideration that NIS could be expressed by BC more rarely (211, 212) than initially detected (200), strongly reduces the possibility of NIS as potential shared antigen between thyroid and BC tissues. Therefore considering the increased prevalence of anti-thyroid peroxidase (TPO) autoantibodies (TPOAb) among patients with breast cancer (BC) (166, 179-181) and the suggested correlation of TPOAb positivity with a better BC outcome (158, 182-184), the next hypothetical shared thyroid/BC antigen to investigate is TPO and this will be addressed in chapter 3.

2.4.2 Aspecific background: human Abs against CHO cells surface antigens

Flow cytometry results were characterized by the presence of a high aspecific background, also reported by other authors using flow cytometry to detect antibodies to the thyrotropin receptor (TSHR) (230, 231). Since the background remained even when using purified Igs and was only slightly reduced by pre-adsorption, it was clearly due to antibodies to surface CHO proteins. CHO cells are the most commonly used mammalian cell lines for production of

therapeutics (232-234) and some 60-70% of recombinant protein pharmaceuticals and almost all currently approved therapeutic antibodies are produced in mammalian cells (235). This would account for some CHO-reactivity (236, 237) but Xue et al. analyzed 83 normal individuals with no known exposure to therapeutic biologics and found antibodies to CHO proteins in 45/83 (54%) cases, mainly isotype G Igs (IgGs). Therefore they postulated alternative explanations such as blood transfusions, vaccinations or keeping animals as pets (238). Despite this problem I have confidence in the flow cytometry protocol used, which has previously served to demonstrate neutral antibodies to another thyroid autoantigen, the receptor of thyroid-stimulating hormone receptor (TSHR) (230, 231, 239).

3 THYROID PEROXIDASE (TPO) EXPRESSION IN BREAST CANCER

3.1 INTRODUCTION

3.1.1 TPO

Human TPO is one of the principal antigens involved in thyroid autoimmunity (TA) and has been identified as the only component of the "microsomal" antigen. Therefore, the measurement of serum autoantibodies to TPO (TPOAbs) is generally considered a marker of TA (240).

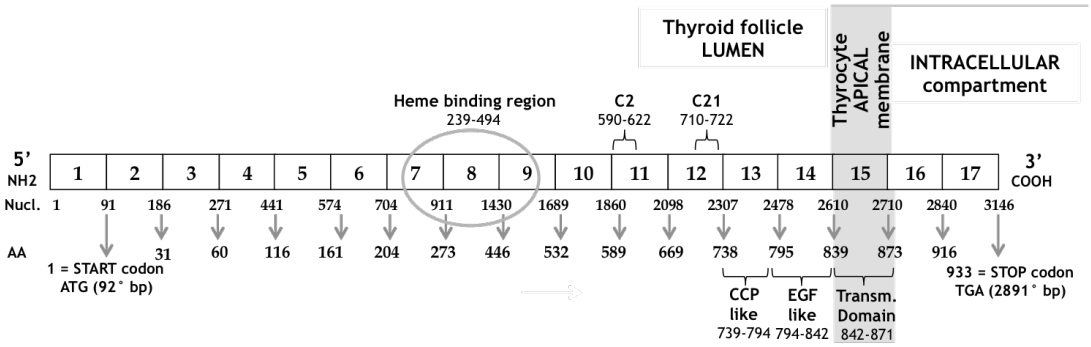
Human TPO is a heme-containing enzyme member of the family of mammalian peroxidases, which utilize hydrogen peroxide to oxidize a number of inorganic and organic substrates and they are active in a variety of anatomic sites (241). The main function of TPO protein in thyroid tissue is the synthesis of thyroid hormones (1), as previously described in paragraph 1.1.2.

The human TPO gene is about 150 kilo-base pairs (kbp) in size, located on chromosome 2, locus 2p25 and consists of 17 exons and 16 introns (242). The full-length 3048-bp transcript (TPO1) encodes a protein consisting of 933 amino acids (AA) (243, 244), which has a short intracytoplasmatic tail, a transmembrane domain encoded by exon 15 and a large extracellular domain which contains the catalytic site (Figure 3-1). TPO protein presents different functional domains (245, 246):

- Catalytic site containing the region binding the heme group (derivative of protoporphyrin IX), included between the distal and proximal histidines, respectively AA 239 and 494 (exons 7-8-9) (247).
- Antigenic site. Serum human TPOAbs mainly recognize conformational epitopes, formed by different parts of the AA chain often far between them in protein primary structure, becoming juxtaposed as a consequence of the final folding of the protein in its tertiary structure. Therefore, it is unusual for TPOAbs to recognize linear antigens, consisting in epitopes formed by consecutive AA on the protein primary sequence. However, using some recombinant TPO polypeptide fragments some linear epitopes recognized by patients with TA have been identified: C2 (AA 590-622: encoded by exon 11 and C21 (AA 710-722: exon 12) (246, 248). TPO conformational and linear epitopes will be further discussed in the conclusions paragraph 3.5 and general discussion (Chapter 5), paragraph 5.2.2.

- Region similar to a complement control protein [CCP (AA 741-795, exon 13)], existing in a wide variety of complement and adhesion proteins (244). This region is also known as the "*sushi domain*" and is formed by 2 disulfide bonds connecting 1st – 3rd cysteines and 2nd – 4th cysteines (249, 250).
- Homologous region to epidermal growth factor (EGF)/low density lipoprotein (LDL) receptor gene families (EGF-like region): AA 794-839 (exon 14) (246). This region contains 6 conserved cysteine residues forming 3 disulfide bonds and also contains 5 AA that constitute a consensus sequence for calcium binding. The functional consequences of calcium binding are not yet known; however a homologous domain has been found in human fibrillin-1, a glycoprotein present in connective tissue and it was suggested that calcium may stabilize its structure and may protect the molecule from proteolytic degradation (250).
- Transmembrane region (AA 847 – 871; exon 15) (244).

Figure 3-1: TPO gene and protein



Schematic representation of the correspondence between TPO gene's nucleotide sequence (Nucl.) and TPO protein's amino acid sequence (AA). bp = base pairs. CCP = complement control protein. C2 and C21 = antigenic sites. EGF = epidermal growth factor. START codon ATG = nucleotide triplet corresponding to the beginning of translation process. STOP codon TGA = nucleotide triplet corresponding to the end of translation process. Transm. Domain = transmembrane domain. 1-17 = exon number. 1-3146 = nucleotide number. 1-933 = amino acid number. 3' COOH = 3-prime-end of the gene, encoding the intracellular carboxy-terminus (or C-terminus) of the protein. 5' NH2 = 5-prime-end of the gene, encoding the extracellular amino-terminus (or N-terminus) of the protein.

3.1.1.1 TPO isoforms

The TPO gene undergoes alternative splicing to generate different TPO messenger ribonucleic acid (mRNA) isoforms lacking one or more exons. To date 8 isoforms have been described in thyroid cells, TPO 1, TPO 2 (244, 251), TPO 3 or TPO Zanelli (252), TPO 4, TPO 5, TPO 6, TPO 2/3 and TPO 2/4 (253), as illustrated in Table 3-1. While TPO 3 (254) and TPO 4 (253) are able to reach the cell surface and show enzymatic activity, TPO 2 and TPO 5 lack enzymatic activity (252, 253).

To my knowledge no functional studies have been performed on other TPO isoforms. It is also important to state that TPO isoforms' mRNA sequences to date have been only partially characterized because of the difficulty in obtaining full length mRNA/cDNA (253); this will be better discussed in the conclusions of the present chapter (paragraph 3.5.3) and in the general discussion (chapter 5, paragraph 5.2.2).

So far only the protein translated from the complete mRNA transcript TPO1 has been characterized and it will be addressed as TPO protein. The apparent MW of TPO protein in denaturing western blot (WB) is a doublet at 105-110 kDa (Table 3-1), which may be due to variants of TPO (251, 253) or differing degrees of glycosylation (255, 256).

Table 3-1: Summary of known TPO isoforms' characteristics

	mRNA analysis				Protein analysis		
TPO Isoform	**N° tot exons**	**Exon lacked**	**bp**	**N° AA**	**MW (kDa) predicted**	**MW (kDa) on WB**	**Function**
TPO 1	17	/	3152	933	103	105-110	Yes
TPO 2	16	10	2981	876	96	?	No
TPO 3	16	16	3022	890	98	?	Yes
TPO 4	16	14	2930	889	98	?	Yes
TPO 5	16	8	2543	760	84	?	No
TPO 6	12	10,12,13,14,16	2339	662	73	?	?
TPO 2/3	15	10,16	2851	832	91	?	?
TPO 2/4	15	10,14	2849	832	91	?	?

bp= number of base pairs. kDa= kilodalton. MW= Molecular Weight. N° AA= number of amino acids. WB= Western Blot.

3.1.1.2 Comparison between TPO and other peroxidases

TPO is a member of the family of mammalian peroxidases, all having similar enzymatic functions (oxidation) and including also lactoperoxidase (LPO), myeloperoxidase (MPO) and eosinophil peroxidase (EPO) (257) and the more recently discovered vascular peroxidase (VPO) (258). MPO, EPO and LPO are all encoded by single genes on human chromosome 17, while TPO gene is located on chromosome 2, suggesting that TPO gene evolved independently from the other peroxidases gene (247). There are two types of VPO, VPO1 and VPO2, approximately 63% identical and encoded by genes located in exons 2 and 8 respectively (258). The main difference between TPO and the other peroxidases is that TPO is the only membrane-bound enzyme, while the other peroxidases are intracellular or extracellular (257). For this reason TPO is bigger, in fact contains similar sequences to MPO, LPO and EPO from AA 1 to 738 (encoded by exons from 2 to 12), while the AA sequences encoded from exons from 13 to 17 (AA 739 -

933), including the transmembrane region (AA 847 – 871), are peculiar of TPO and missing from the other peroxidases (246, 247). Table 3-2 summarizes the main characteristics of the principal peroxidases, with also indicated their molecular weight (MW) in kilodalton (kDa) (244, 245, 247, 257-259).

Table 3-2: Peroxidases

	CELLS	POSITION	N° AA	MW (kDa)
TPO	- Thyrocytes	Membrane-bound (main part is extracellular)	933	105-110 (single chain)
MPO	-Neutrophil granulocytes -Mononuclear phagocytes	Intracellular (lysosomal)	745 (precursor*)	150 (dimeric): 2 x 75 Each part composed by: - 1 heavy chain 57 (467 AA) - 1 light chain 15 (113 AA)
LPO	- Mammary ductal epithelial cell - Secrectory cells of exocrine glands	Extracellular (milk, saliva, tears)	712	80-90 (single chain with different glycosylation grades)
EPO	- Eosinophils	Intracellular (lysosomal)	715	70 Monomer composed by: - 1 heavy chain 58 - 1 light chain 12
VPO	- Vessels endothelium and smooth muscle - Heart - Many other tissues (only mRNA, no protein)	Intracellular	>1200	165

AA = amino acid. kDa = kilodalton. MW = molecular weight.

* MPO precursor (745 AA) is glycosylated and then cleaved by 125 AA to form the mature dimeric protein (580 AA x 2)

3.1.2 Hypothesis

In the general introduction (Chapter 1, paragraph 1.4) the association between TA and breast cancer (BC) has been discussed. Of relevance, serum TPOAbs have been described to be more prevalent among patients with BC compared with controls (166, 179-181) and several studies suggested that BC patients with positivity for serum TPOAb had a better prognosis compared with TPOAb negative patients (182-184, 260), as will be better explained in chapter 4.

The consequent main hypothesis of the present thesis is the presence of a shared immune activity between thyroid and BC tissue, leading to TA on one hand and to a better control of BC tumour growth on the other hand. In chapter 2 we did not find any autoantibodies directed towards the sodium iodide symporter (NIS), known to be expressed by both BC/thyroid tissues, therefore NIS is unlikely to be the hypothesized shared antigen. Since TPOAbs, considered a marker of TA (240), are more prevalent among patients with BC, we hypothesized TPO itself as possible candidate common antigen.

3.1.3 Aims

The aim of the present chapter is to explore TPO gene expression (mRNA and protein) in BC, using relevant *ex vivo* and *in vitro* samples.

First, we investigated TPO gene expression, both messenger RNA (mRNA) and protein, in BC and breast peri-tumoural (PT) *ex vivo* tissues, using thyroid tissue (TT) as positive control. Considering that TPO expression has already been described in orbital adipose tissue (261), other tissues were included in order to investigate whether an hypothetical TPO expression by BC would be exclusive or not: adipose tissue from different depots and other cancers, as detailed below in materials section.

Subsequently, *in vitro* models using 3 different BC cell lines grown with known regulators of breast function were conducted in order to investigate the regulation of TPO gene expression in BC cell lines. In fact in chapter 1 (paragraph 1.3.1.2) it has been explained that breast function is regulated by many different hormones and growth factors, including oestrogens and Insulin-like growth factors (110). Oestrogens exert a stimulatory activity by increasing the rate of cell division and proliferation of breast epithelium (118), while IGF I and II (IGF-I and IGF-II) are potent mitogens for a variety of cell types and considered important regulators of epithelial tumour growth in BC (262, 263). In particular oestrogen receptor (ER) and IGF-I receptor (IGF-IR: able to bind both IGF-I and IGF-II) are co-expressed in the MCF-7 BC cell line and engage in a crosstalk that results in synergistic growth (264).

A possible TPOAb cross-reactivity with LPO expressed in mammary tissue will be also discussed in the conclusions session of the present chapter (paragraph 3.5.2) and in the general discussion (chapter 5, paragraph 5.2.1).

3.2 MATERIALS

3.2.1 Tissues

Fresh human surgical tissues (from scheduled operations and obtained with informed consent) were collected in liquid nitrogen and immediately frozen (-80°C). Eight BC (7 infiltrating ductal adenocarcinoma and 1 mucinous carcinoma) and their 8 corresponding peri-tumoural breast tissues (PT) were collected. Since breast tissue contains fat, 17 adipose tissues (AD) from various depots including abdominal (AD-A; n=4), subcutaneous (AD-S; n=8), knee (AD-K; n=1) and orbital fat (AD-O; 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 goitre was used as a positive control.

Two additional fresh frozen BC and also 9 paraffin embedded BC sections (all infiltrating ductal adenocarcinoma) were also collected, for a total of 19 different BC samples analyzed with various techniques, later explained in the Methods section (paragraph 3.3). Data about thyroid autoimmunity status were available only in 8/19 BC patients and 1/8 had serum TPOAb positivity; age was known only in 10 BC patients (Table 3-3).

Table 3-3: Characteristics of BC tissues analyzed

Sample number	BC histological type	TPO gene expression: analyses performed	TPOAb (U/ml) v.n. <10	TgAb (U/ml) v.n. <30	TSH (μU/ml) v.n. 0.4-3.4	Age (yrs)
BC n°1 (f)	Ductal	mRNA: PCR, QPCR Protein: WB	<1	5.89	1.03	85
BC n°2 (f)	Ductal	mRNA: PCR, QPCR Protein: WB	<1	90.42	0.453	72
BC n°3 (f)	Ductal	mRNA: PCR, QPCR Protein: WB	<1	<1	0.911	58
BC n°4 (f)	Ductal	mRNA: PCR, QPCR Protein: WB	<1	<1	1.23	70
BC n°5 (f)	Ductal	mRNA: PCR, QPCR, LongRange PCR Protein: WB	443.87	64.07	2.02	68
BC n°6 (f)	Mucinous	mRNA: PCR, QPCR, LongRange PCR Protein: WB	<1	<1	0.653	81
BC n°7 (f)	Ductal	mRNA: PCR, QPCR Protein: WB	<1	<1	1.92	72
BC n°8 (f)	Ductal	mRNA: PCR, QPCR Protein: WB	<1	<1	0.809	79
BC n°9 (f)	Ductal	Protein: IHC	/	/	/	73
BC n°10 (f)	Ductal	Protein: IHC	/	/	/	70
BC n°11 (p)	Ductal	Protein: IF	/	/	/	/
BC n°12 (p)	Ductal	Protein: IF	/	/	/	/
BC n°13 (p)	Ductal	Protein: IF	/	/	/	/
BC n°14 (p)	Ductal	Protein: IF	/	/	/	/
BC n°15 (p)	Ductal	Protein: IF	/	/	/	/
BC n°16 (p)	Ductal	Protein: IF	/	/	/	/
BC n°17 (p)	Ductal	Protein: IF	/	/	/	/
BC n°18 (p)	Ductal	Protein: IF	/	/	/	/
BC n°19 (p)	Ductal	Protein: IF	/	/	/	/

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

3.2.2 BC cell lines

3 different BC cell-lines obtained from pleural effusion of breast adenocarcinoma were used: MCF-7 and T-47D, both oestrogen-progesterone receptor positive (ER+/PR+) and MDAMB-231, triple-negative: ER-/PR- and human epidermal growth factor receptor type 2 negative (HER2-). All BC cell lines were obtained from the American Type Culture Collection and stored in liquid nitrogen until use.

3.3 METHODS

3.3.1 Analysis of TPO mRNA expression

3.3.1.1 Total RNA extraction and reverse transcription (RT)

Total RNA was extracted from both fresh tissues and BC cell lines and reverse transcribed in complementary DNA (cDNA) by RT following the same procedure previously described in chapter 2, paragraph 2.2.1.3. The only difference consisted in primers used for RT (0.4 µg in 20 µl reaction); oligo-deoxy-thymine nucleotides short sequence (odT) or random primers (both from Promega, Madison, WI, USA) to obtain cDNA to be analyzed respectively by standard polymerase chain reaction (PCR) and Absolute Real-time Quantitative PCR (QPCR). In some standard PCR reactions, a TPO reverse primer was used instead of odT or random primers, used at the same dilution.

3.3.1.2 TPO primers design

TPO forward (F) and reverse (R) primers were designed from the published sequences of human TPO gene (Genbank: NM_000547) as previously described in chapter 2, paragraph 2.2.1.3; in this case, BLAST alignment was always performed in order to exclude homology with peroxidases other than TPO, especially lactoperoxidase (LPO). As reported in Table 3-4, 6 different primers (3 F and 3 R), were initially designed for standard PCR; two additional primers located in exon 2 (F2) and in the junction region between exons n° 3 and 4 (R 3-4) were subsequently designed to perform QPCR and LongRange RT-PCR as later described.

Table 3-4: TPO F and R primers

PCR technique	Forward (F) or Reverse (R)	TPO Exon	TPO mRNA Nucleotide N°	Primer's nucleotide sequence (5' – 3')	Primer acronym
S-PCR	F	7	859-878	GGCTGACTGCCAGATGACTT	F7
S-PCR	F	9	1527-1546	CCACTGTGTCCAACGTGTTC	F9
S-PCR	F	13	2400-2418	ACGGGTATGAGCTCCAAGG	F13
S-PCR	R	9	1546-1527	GAACACGTTGGACACAGTGG	R9
S-PCR	R	11	1885-1866	GCAGAACTCCCTCCACTCAT	R11
S-PCR, LR-PCR	R	17	2884-2865	TCTCGGCAGCCTGTGAGTAT	R17
QPCR, LR-PCR	F	2	109 - 128	GCTGTCTGTCACGCTGGTTA	F2
QPCR	R	3-4	278 – 259	TGAGGTTTCTCTGCATCGTG	R3-4

LR-PCR = LongRange PCR; QPCR = Absolute Real-time Quantitative PCR; S-PCR = standard polymerase chain reaction.

Primers were combined into different primer pairs in order to distinguish between the different TPO mRNA isoforms. The expected size of PCR amplicons according to each primer pair used and known TPO isoforms are summarized in Table 3-5.

Table 3-5: PCR expected amplicons according to known TPO mRNA isoforms

	QPCR	Standard RT-PCR				LongRange RT-PCR
	F2/R3-4 (bp)	F13/R17 (bp)	F7/R9 (bp)	F7/R11 (bp)	F9/R17 (bp)	F2/R17 (bp)
TPO 1	170	485	688	1027	1357	2776
TPO 2 No exon 10 (171bp)	170	485	688	856	1186	2605
TPO 3 No exon 16 (130bp)	170	355	688	1027	1227	2646
TPO 4 No exon 14 (132bp)	170	353	688	1027	1225	2644
TPO 5 No exon 8 (519bp)	170	485	169	508	1357	2257
TPO 2/3 No exon 10 (171bp) No exon 16 (130bp)	170	355	688	856	1056	2475
TPO 2/4 No exon 10 (171bp) No exon 14 (132bp)	170	353	688	856	1054	2473
TPO 6 No exons 10 (171bp), 12 (209bp), 13 (171bp), 14 (132bp), 16 (130bp)	170	No product	688	856	544	1963

LR-PCR = LongRange PCR; QPCR = Absolute Real-time Quantitative PCR; S-PCR = standard polymerase chain reaction.

3.3.1.3 Standard PCR

Standard PCR was performed on cDNA using the AmpliTaq Gold™ DNA Polymerase kit (Applied Biosystems, Life Technologies, Paisley, UK) in a 25 µl PCR reaction mix, as previously described in chapter 2, paragraph 2.2.1.3. The thermal cycle program used was the following: 1 cycle, 95°C for 5 minutes; 40 cycles, 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute; 72°C for 6 minutes. A small quantity of obtained PCR products were electrophoresed on 2% agarose gel in Tris-acetate-EDTA (TAE) buffer (containing ethidium bromide 1 ng/µl) as previously described in chapter 2, paragraph 2.2.1.3. The rest of the PCR product was further amplified with 10 additional PCR cycles and then sequenced.

In order to verify the quality of amplified cDNA, control PCR was also performed for all samples to test the expression of housekeeping gene adenine phosphoribosyltransferase (APRT). The 25 µl reaction solution was the same as previously described for TPO PCR, but using the following APRT primer pair, GCTGCGTGCTCATCCGAAAG (F) and CCTTAAGCGAGGTCAGCTCC (R), selecting an amplicon of 247 bp. The PCR machine program used was the following: 1 cycle, 95°C for 5 minutes; 35 cycles, 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; 72°C for 6 minutes.

3.3.1.4 LongRange RT-PCR

In order to characterize TPO mRNA variants in their full-length, the LongRange 2Step RT-PCR kit (Qiagen, Manchester, UK) was used to generate cDNA and amplify it using TPO primers F2 and R17. Representative tissues were analyzed, TT (n=1), BC (n=2) and AD-S (n=1), according to the following procedure:

- Total RNA extracted from tissues was initially denatured at 65°C for 5 minutes, in order to reduce RNA secondary structures.
- The percentage of guanosine (G) and cytosine (C) present in total RNA was calculated using "Oligo Calc: Oligonucleotide Properties Calculator" software (http://www.basic.northwestern.edu/biotools/oligocalc.html). If RNA/cDNA sequence is rich in GC regions, then the use of Q solution in the reaction mix is recommended.
- LongRange RT step. The 20 µl reaction solution, containing 4 µl of LongRange RT Buffer 5x, dNTP mix (1 mM each dNTP), 1 µM odT, 0.04 U/µl LongRange RNase inhibitor, 1x (1µl) LongRange Reverse Transcriptase and 1 µg RNA, was incubated at 42°C for 90 minutes and finally heated at 85°C for 5 minutes to inactivate the enzyme, using an unstirred water bath (Clifton, UK).

- LongRange PCR Step. The 50 µl reaction solution contained 5 µl of LongRange PCR Buffer with magnesium ion (Mg^{2+}) 10x (2.5 mM final Mg^{2+} concentration), dNTP mix (500 µM each dNTP), 0.4 µM F2, 0.4 µM R17, 2 total U LongRange PCR Enzyme Mix and 200 ng cDNA. Reactions, always including a negative control with dH_2O in place of cDNA and performed both with or without the Q-solution addiction, were performed using the TC-512 gradient thermal cycler (Techne, Minneapolis, USA): 1 cycle, 93°C for 3 minutes; 45 cycles, 93°C for 15 seconds, 57°C for 30 seconds, 68°C for 3 minutes. Resulting products were electrophoresed on 1.5% agarose gels in TAE buffer (containing ethidium bromide 1 ng/µl) and sized using 2 different ladders, 100 bp (described in chapter 2, paragraph 2.2.1.3) and Quick-Load® 1 kb DNA Ladder (BioLabs), ranging from 0.5 to 10 kilobases (kb).

  Before proceeding to the sequencing of PCR products, PCR was repeated using the first PCR reaction product in place of cDNA, in order to increase the quantity of amplicons and improve the chances to successfully sequence long sequences of nucleotides.

3.3.1.5 Sequencing of PCR products

The PCR amplicons obtained with standard PCR (F9/R11; F13/R17) and LongRange RT-PCR (F2/R17) were sequenced. Single PCR products were purified using Polyethylene Glycol (PEG) solution as previously described (chapter 2, paragraph 2.2.1.3). PCR multiple products were extracted from cut gel fragments using the Qiaquick gel extraction kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Briefly, DNA fragments were excised from the agarose gel with a scalpel, weighed and 3 volumes of Buffer QG were added to each 1 volume gel. After 10 minutes of incubation at 55°C and quick vortex, 1 gel volume of isopropanol was added and mixed. The sample was then applied to a QIAquick column and centrifuged for 1 minute at 13'000 RPM; the flow-through was discarded and the QIAquick column placed back on the same tube. Subsequently, 0.75 ml of Buffer PE were added to the QIAquick column, left to stand for 5 minutes and then centrifuged x2 for 1 minute at 13'000 RPM, discarding the flow-through. Finally, the QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and 30 µl of Buffer EB were added, left to stand for 4 minutes and then centrifuged for 1 minute at 13'000 RPM: the flow-through containing the extracted PCR product was collected and stored at -20°C.

Purified PCR products were then sequenced 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 in chapter 2,

paragraph 2.2.1.3, using the appropriate TPO F or R primer of the relative PCR reactions (F9, F11, R11, R17 for standard PCR and F2, R17 for LongRange RT-PCR). The program used was the same applied in the relative PCR reaction.

3.3.1.6 QPCR

QPCR experiments were conducted using Platinum® SYBR® Green qPCR supermix-UDG (Invitrogen by Life technologies, ThermoFisher scientific, USA) incorporation measured on a Stratagene (La Jolla, CA, USA) MX 3000. TPO primers F2 and R3-4 were used, in order to quantify an amplicon of 170 bp ideally common between the different TPO mRNA isoforms (mainly differing from exon 8 onwards) and hence providing a measure of total TPO transcripts (Table 3-1).

The TPO standard template consisted of a 170 bp amplicon amplified from TT in a PCR reaction conducted as described before (paragraph 3.3.1.3) using F2 and R3-4 as TPO primers and subsequently purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA), according to the manufacturer's instructions. The DNA concentration of the amplicon was measured using a spectrophotometer as described before (paragraph 2.2.1.3) and the correspondent number of DNA copies was assessed using the "URI Genomics & Sequencing Center Calculator for determining the number of copies of a template" created by Andrew Staroscik, 29 January 2004 (http://cels.uri.edu/gsc/cndna.html).

Subsequently, different dilutions (100nM, 300nM, 500nM) of TPO primers F2 and R-4 were tested in a 25 µl reaction solution containing 1µl F, 1µl R, 12.5 µl SYBR and 1µl F2/R3-4 purified amplicon template; 2 separate reaction solutions with different F2/R3-4 template dilutions were performed (10^2 and 10^5 cDNA copies).

A TPO standard curve was generated using 25 µl reaction solutions containing 12.5 µl SYBR, 1 µl F2 300 nM, 1 µl R3-4 300 nM and 1 µl of F2/R3-4 purified amplicon template in different amounts obtained by serial dilutions: 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 cDNA copies number.

The TPO cDNA copies number present in every BC cell line and human tissue sample was assessed using cDNA obtained with RT from 1 µg total RNA as described before (paragraph 2.2.1.3), using random primers instead of odT in order to optimize the transcripts quality. In particular, the 25 µl reaction solution contained 12.5 µl SYBR, 1 µl F2 300 nM, 1 µl R3-4 300 nM and 1 µl (approximately 50 ng) of cDNA template. A complete TPO standard curve was included in each experiment to calculate the absolute values for each sample (transcripts/µg input RNA), as well as a negative control containing dH_20. In a single QPCR experiment

all measurements were made in triplicate and the standard curve was run at least in duplicate.

In addition, transcripts for the housekeeping gene APRT were measured: the applied QPCR methods were the same as TPO, except for using F and R APRT primers previously described for standard PCR (see paragraph 3.3.1.3) at the final concentration of 0.4 μM each. The APRT plasmid used to produce the APRT standard curve was a gift from Alenka Janezic (Cardiff University) and consisted of a 247 bp APRT fragment obtained by the previously described APRT PCR reaction (paragraph 3.3.1.3), subcloned into a pGEM®-T Easy plasmid (Promega, Madison, USA) and purified before use, as described above for TPO standard.

3.3.2 Analysis of TPO protein expression

3.3.2.1 Indirect immunofluorescence (IF)

Indirect IF was initially performed on 4 paraffin embedded human BC tissue samples and human TT as positive control. Tissue sections were dewaxed and rehydrated through 10 consecutive passages of 5 minutes each through graded alcohols, distilled water (dH_20) and phosphate-buffered saline solution (PBS), as summarized in Table 3-6.

Table 3-6: Deparaffination and rehydration procedure

1	2	3	4	5	6	7	8	9	10
Xylene 100%	Xylene 100%	Xylene 50% Ethanol 50%	Ethanol 100%	Ethanol 100%	Ethanol 90%	Ethanol 70%	Ethanol 50%	dH_2O	PBS

dH_2O = distilled water; numbers indicate the order of passages

Tissues sections were then incubated with the first antibodies (1:10 dilution) at room temperature for 1 hour. The primary antibodies comprised pooled sera from 4 women with high titres of TPOAb (range 446 - >1000 U/ml) or free of TPOAb (n=3); TPOAb determination was performed by immune enzymometric assay (Tosoh Bioscience, Tessenderlo, Belgium; positive if >10 U/ml). Tissue sections were then washed 5 minutes x3 in PBS and then incubated with a polyclonal rabbit anti-human-IgG fluorescein isothiocyanate (FITC)-coniugated (Dako, DK-2600 Glostrup, Denmark) 1:70 dilution for 1 hour at room temperature. Tissue sections were then washed again 5 minutes x3 in PBS, rinsed, mounted and left overnight at 4°C to dry and fix.

The same 4 BC and TT paraffin embedded tissue sections were also analyzed in a similar protocol using mouse monoclonal to TPO ab76935 (abcam, Cambridge, UK) as first antibody (1:100 dilution) and Alexa Fluor 488 goat anti-mouse IgG (Life Technologies Ltd, Paisley, UK) as second antibody (1:50 dilution).

A further 5 paraffin embedded BC samples were analyzed with a modified protocol in which slides, after the deparaffination and rehydration procedure, were initially blocked for two hours at room temperature with goat serum 10%; primary and secondary antibodies were also diluted in goat serum 10%, maintaining the same incubation conditions of the first protocol (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 IF experiments images were analysed using Olympus BX51 microscope, trinocular brightfield / fluorescence (Olympus America Inc., Center Valley, PA, USA).

3.3.2.2 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 (mouse IgG; 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.

- Briefly, frozen tissue sections were air dried, fixed with acetone 100% for 10 minutes and then transferred into PBS.
- Tissue sections were incubated for 20 minutes with Vectastain® blocking horse serum (1:67 dilution) and then with mouse monoclonal to TPO ab12500 (abcam, Cambridge, UK) diluted 1:25 in PBS for one hour at room temperature.
- Tissue sections were subsequently washed for 5 minutes in PBS and then incubated for 30 minutes with Vectastain® biotinylated secondary antibody solution (horse anti-mouse IgG).
- After, they were washed for 5 minutes in PBS, incubated for 30 minutes with Vectastain® ABC-AP reagent, washed for 5 minutes in PBS and incubated in 20-30 minutes in alkaline phosphatase substrate solution (Vector Red).
- Tissue sections were then rinsed in tap water and counterstained with hematoxylin: 3 minutes incubation with 20% hematoxylin (diluted in water), 3 minutes wash in tap water, 3 minutes incubation in tap water containing ammonium 2%, 3 minutes in 95% alcohol, 3 minutes in 99% alcohol and 1 minute in 100% xylene. Tissue sections were finally cleared and mounted.

- Images were analysed using Olympus BX51 microscope, trinocular brightfield / fluorescence (Olympus America Inc., Center Valley, PA, USA).

3.3.2.3 Western Blot (WB)

Fresh human tissues samples and BC cell lines were investigated for TPO protein expression with WB, applying the following procedure:

- Protein extraction. From fresh frozen tissues: frozen tissue (200 mg) was diced using a razor blade and thawed in 600 µL of RIPA buffer [1% tergitol-type NP40 detergent, 0.5% sodium deoxycholate and 0.1% Sodium Dodecyl Sulphate (SDS) in phosphate buffered saline (PBS)] 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) was stored at -20°C in loading buffer (LB) containing 40% of 10% SDS, 20% glycerol, 0.5M Tris pH 6.8 and 16% pure water.

  From cells: 6-well plates were washed X2 with 2 ml PBS and cells were scraped with LB and transferred into 1.5 ml Eppendorf tubes, then boiled for 5 minutes in order to break DNA, inactivate proteases and break tertiary and quaternary protein structure. Finally, samples were centrifuged at 13000 rpm for 5 minutes and the protein extract (supernatant) was stored at -20°C.
- Measure of protein concentration. 60% trichloroacetic acid was added (1:1 ratio) to 50 µl of protein sample in LB and the optical density at 630 nm was measured with a microprocessor-controlled photometer (Opsys MR™ Microplate Reader (Dynex Technologies, Chantilly, USA), using different dilutions of bovine serum albumin (BSA) 1mg/ml (in LB) as standard.
- SDS - PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Extracted proteins (5-15 ug of TT and 50 ug for all other tissues and BC cell lines) were added with 2% pyronin Y 0.2% and 2% ß-mercaptoethanol; cells protein only were added also with 10 µl/ml PMSF 100 mM, already present in tissue proteins (contained in previously used RIPA buffer). Protein samples were boiled for 5 minutes and 30 seconds respectively for tissue and cells protein (since cells protein extracts had already been previously boiled for 5 minutes). Samples where then loaded on 8% tris-glycine SDS-acrylamide gels [2.64 ml 30% acrylamide, 3.75 ml 1M tris pH 8.8, 100 µl 10% SDS, 100 µl ammonium persulfate (APS), 5 µl tetramethylethylenediamine (TEMED) and 2.92 ml distilled water (dH_2O)] and separated for half a hour by electrophoresis in running buffer (tris 15g, glycine 72g and SDS 5g in 1L dH_2O). In order to mark the protein size, the Prestained

Protein Marker, Broad Range 7-175 kDa (New England BioLabs®, Ipswich, USA) was diluted 1:1 in LB and loaded on the gel.

- Membrane blotting. After electrophoresis proteins were transferred onto a Polyvinylidine Fluoride (PVDF) membrane Amersham Hybond-P (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) by electrophoresis for 1 hour.
- Membranes blocking and incubation with first and second antibody. Membranes were blocked for 2 hours at room temperature with 5% non-fat dry milk powder (Marvel, Long Sutton, Spalding, UK), 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)] or 5% not-fat dry milk powder, respectively for tissues and cells protein analysis. Membranes were then washed three times for 5 minutes with TBS-T (Tris-buffered saline, 0.1% Tween 20). After washing, membranes were incubated for 1 hour at room temperature with ECL anti-mouse IgG horseradish peroxidase (HRP)-linked whole antibody (from sheep; GE Healthcare UK limited, Little Chalfont Buckinghamshire) diluted (1:5000) with 5% non-fat dry milk powder. Membranes were then washed with TBS-T two times for 30 seconds, 1 time for 15 minutes and three times for 5 minutes.
- Blot developing. The blot was developed in a dark room with Amersham ECL Plus western blotting detection system (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) according to manufacturer's instructions. Films were analysed using the Alpha Imager 1200 digital imaging system (Alpha Innotech Corp., San Leandro, CA, USA).
- Stripping and re-probing for human-actin. Membranes were then stripped for 30 min at 60°C in stripping buffer (6.25 ml tris 0.5M pH 6.8, 10 ml 10%SDS, 0.35 ml mercaptoethanol and 33.5 ml dH_2O), washed 10 minutes X3 with large volumes of TBS-T (50 ml/blot), blocked for 2 hours at room temperature with 5% non-fat dry milk powder and re-probed with rabbit polyclonal anti-human actin IgG (I-19, sc-1616-R, Santa Cruz Biotechnology, Dallas, USA) at 1:000 dilution and ECL anti-rabbit IgG HRP-linked whole antibody (from donkey; GE Healthcare UK limited, Little Chalfont Buckinghamshire) at 1:5000 dilution, following the same procedure described above.

A primary antibody absorption test by antigen peptide (TPO and LPO) was performed in order to verify the specificity of identified bands on WB.

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 933) (265) in 5% BSA. These TPO fragments

were generated by others in Prof. Marian Ludgate's laboratory in Cardiff University and stored frozen until further use.

The same primary antibody absorption test was also performed using 10 µg bovine LPO (Sigma-Aldrich, Gillingham, Dorset, UK).

3.3.3 Cell culture conditions

BC cell lines were cultured (+37°C; 5% CO_2) in 6-well plates until 90% confluent in the following medium:

- MCF-7: Dulbecco's modified Eagle's medium (DMEM; BioWhittaker®, Lonza, Belgium) supplemented with 10% foetal calf serum (FCS), 2% penicillin/streptomycin (PS), 1 millimolar (mM) sodium pyruvate and 10 µg/ml bovine insulin.
- T-47D: Roswell Park Memorial Institute (RPMI) medium supplemented with 2 ml of L-Glutamine 200 mM in 0.85% NaCl, 10% FCS and 2% PS.
- MDAMB-231: DMEM (BioWhittaker®, Lonza, Belgium) supplemented with 10% FCS and 2% PS.

4 different culture conditions were used for all the 3 different BC cell lines: normal culture medium (N) or supplemented with 1nm ß-estradiol (Sigma-Aldrich; E), 100ng/µl IGF-II (Sigma; I) or the two agents combined (EI). Cells were harvested at 6 hours (6), 24 hours (24), 72 hours (72) and 8 days (8) for RNA or protein extraction, for a total of 16 different combinations, as summarized in Table 3-7.

Table 3-7: 16 different combinations for each BC cell line

		Cell collection time			
		6 hours (6)	24 hours (24)	72 hours (72)	8 days (8)
Cell culture conditions	Normal culture medium (N)	6N	24N	72N	8N
	+ 1nm ß-estradiol (E)	6E	24E	72E	8E
	+ 100ng/uL IGF-II (I)	6I	24I	72I	8I
	+ 1nm ß-estradiol and 100ng/uL IGF-II (EI)	6EI	24EI	72EI	8EI

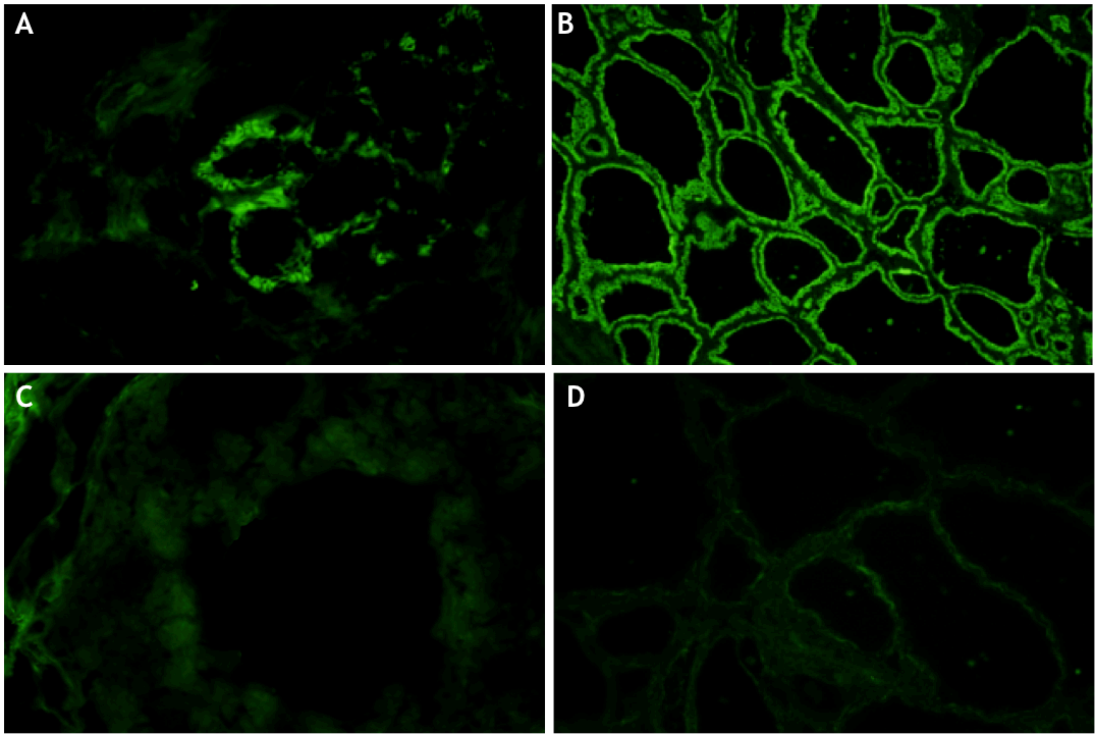
3.4 RESULTS

3.4.1 TPOAb display auto-reactivity to a breast cancer antigen

The hypothesis of TPO expression in BC tissue was first explored by IF performed on 4 deparaffinized and rehydrated BC tissue samples with human TT as positive control using pooled human sera positive for TPOAb at high titre (TPOAb+) or negative for TPOAb (TPOAb-). 5 additional paraffin embedded BC tissues were later tested with a slightly modified indirect IF protocol, as

previously described in paragraph 3.3.2.1. When using TPOAb+ pooled human sera, the immunoreactivity was apparent in 9/9 BC tested (Figure 3-2 panel A) and the IF signal was strong on TT, much more evident on the apical surface of the thyroid cells, most likely reflecting binding to TPO (Figure 3-2 panel B). The immunoreactivity was absent in both BC and TT tissues when using TPOAb- pooled human sera (Figure 3-2 panels C and D respectively).

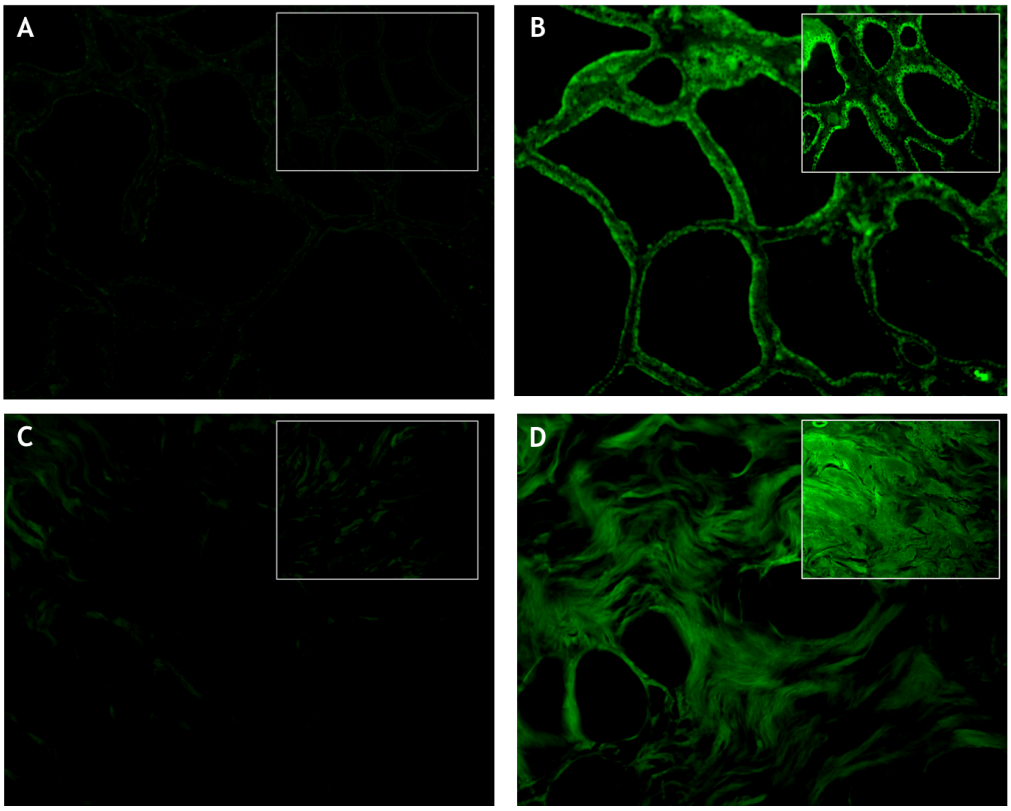
Figure 3-2: Indirect IF results using pooled human sera



Representative results of indirect Immunofluorescence using pooled human sera as primary antibody. Panel A (10x magnification): breast cancer (BC) tissue incubated with pooled human sera strongly positive for TPOAb (TPOAb+). Panel B (10x magnification): thyroid tissue (TT) incubated with TPOAb+ pooled human sera (positive control). Panel C (20x magnification): BC incubated with TPOAb free (TPOAb-) pooled human sera. Panel D (20x magnification): TT incubated with TPOAb- pooled human sera (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 3-3 shows the indirect IF results obtained with the initial 4 paraffin embedded BC tissues and TT as positive control using a monoclonal TPOAb; they confirmed the results obtained with pooled human sera in Figure 3-2.

Figure 3-3: Indirect IF results using monoclonal TPOAb



Representative results of indirect immunofluorescence (IF) staining for thyroid peroxidase (TPO), 20x magnification. In the small box for each picture there is the correspondent 10x magnification. Panels A,B: thyroid tissue (TT) tested with goat serum 10% lacking primary antibody [A] and mouse monoclonal antibody to TPO [B]. Panels C,D: breast cancer tissue (BC) tested with goat serum 10% lacking primary antibody [C] and mouse monoclonal antibody to TPO [D]. Panels B and D show a positive signal (green stain), which is absent in panels A and C.

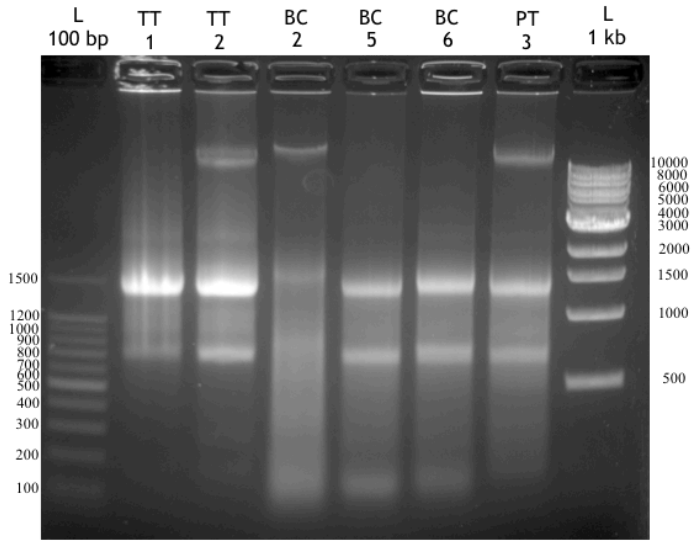
The above reported IF results clearly indicated the presence of immunoreactivity to BC in TPOAb+ women, thus further experiments were conducted to accurately investigate TPO expression, both mRNA and protein, in BC and other tissues.

3.4.2 TPO gene expression (mRNA)

3.4.2.1 Quality controls for RNA extraction and cDNA synthesis by RT

Figure 3-4 shows the results of 2% agarose gel electrophoresis of RNA (1 µl) extracted from representative tissue samples: the presence of 2 bands corresponding to ribosomal RNA 28S and 18S indicated the integrity of extracted RNA. In cases of degraded RNA, e.g. BC2, the RNA was extracted again and used for cDNA synthesis and analyses only if passing the quality control. BC characteristics had been previously summarized in Table 3-3.

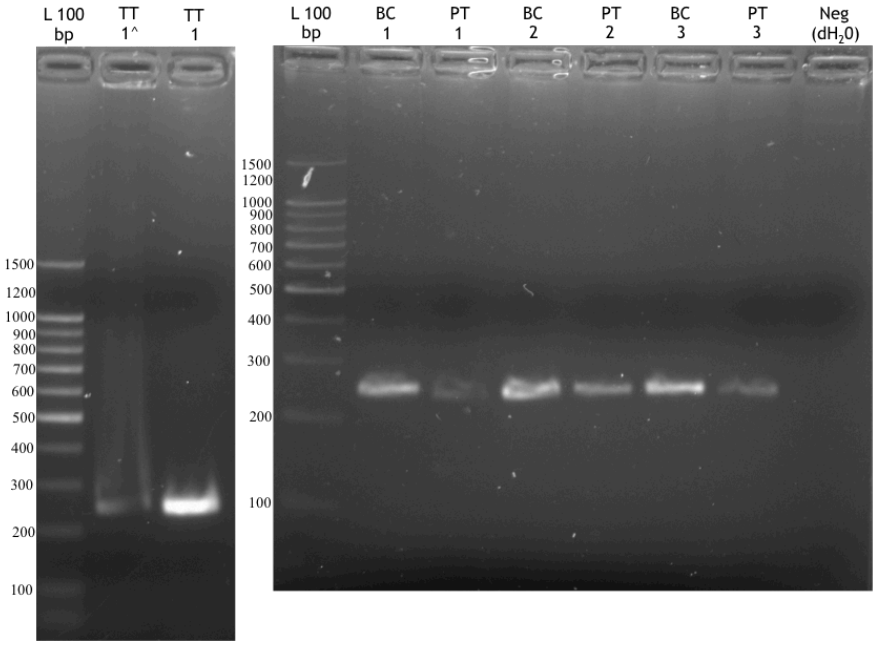
Figure 3-4: RNA quality control (representative samples)



L 1 kb = ladder 1 kilobases. L 100 bp = ladder 100 base pairs. BC = breast cancer. PT = breast peri-tumoural tissue. TT = thyroid tissue. 100-10'000 = number of bp.

Figure 3-5 reports some APRT PCR results in representative tissue samples. All samples analyzed contained the expected 247 bp amplicon and the bands obtained in BC tissues tended to be sharper compared with the correspondent PT, possibly reflecting higher efficiency of RT reaction in some samples. When a sample showed a faint APRT band (e.g. TT1^), the RT reaction was repeated in order to try and increase the quality of obtained cDNA (TT1).

Figure 3-5: Standard PCR for APRT gene (representative samples)



BC = breast cancer. L 100 bp = ladder 100 base pairs. Neg = negative control with distilled water (dH_2O). PT = breast peri-tumoural tissue. TT= thyroid tissue. TT^= cDNA obtained from TT via a poor quality reverse transcription reaction. 100-1500 = number of bp.

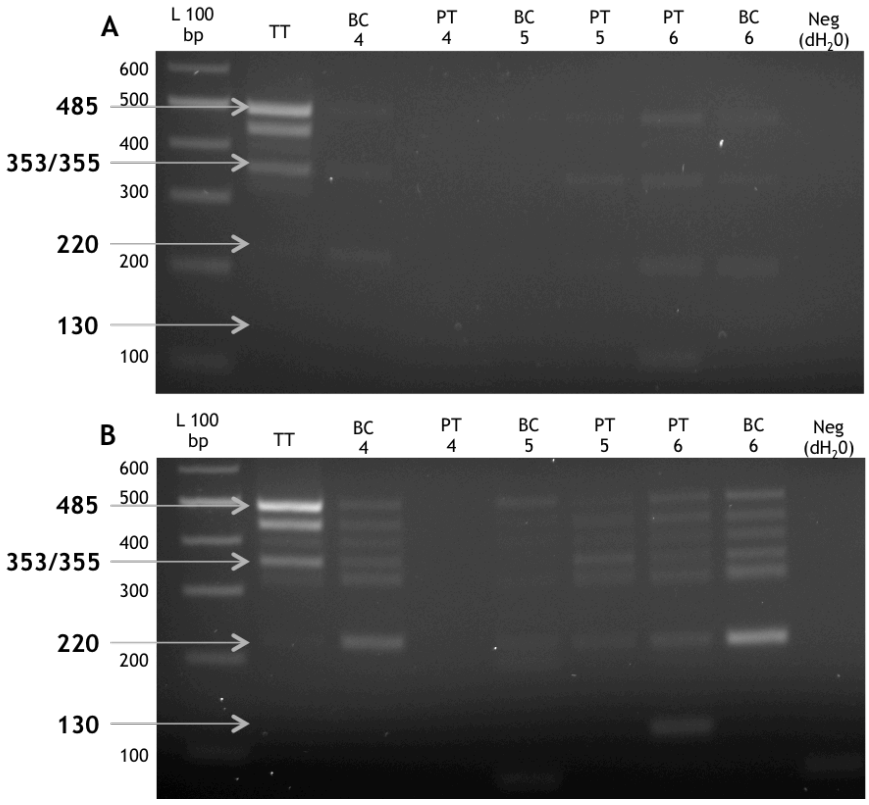
3.4.2.2 Standard PCR and TPO isoforms

Table 3-5 (paragraph 3.3.1.2) reports the expected amplicons using different TPO primer pairs. The best results were obtained with F13/R17 and nearly all samples were tested and produced one or more amplicons. F7/R9 and F9/R11 PCR gave less quality PCR experiments, therefore only few representative samples were investigated.

F13/R17

Figure 3-6 shows results obtained in standard RT-PCR reactions in representative TT, BC and PT samples after 40 (panel A) and 50 (panel B) PCR cycles. As previously summarized in Table 3-5, the expected 485 bp band corresponded to TPO 1, TPO 2 or TPO 5 and 353-355 bp band corresponded to TPO 3, TPO 4, TPO 2/3 or TPO 2/4; this was verified by sequencing. The sequence analysis of 400/450 bp and 320 bp unexpected bands revealed that they were just nicked PCR products corresponding respectively to the 485 bp or 353-355 bp bands.

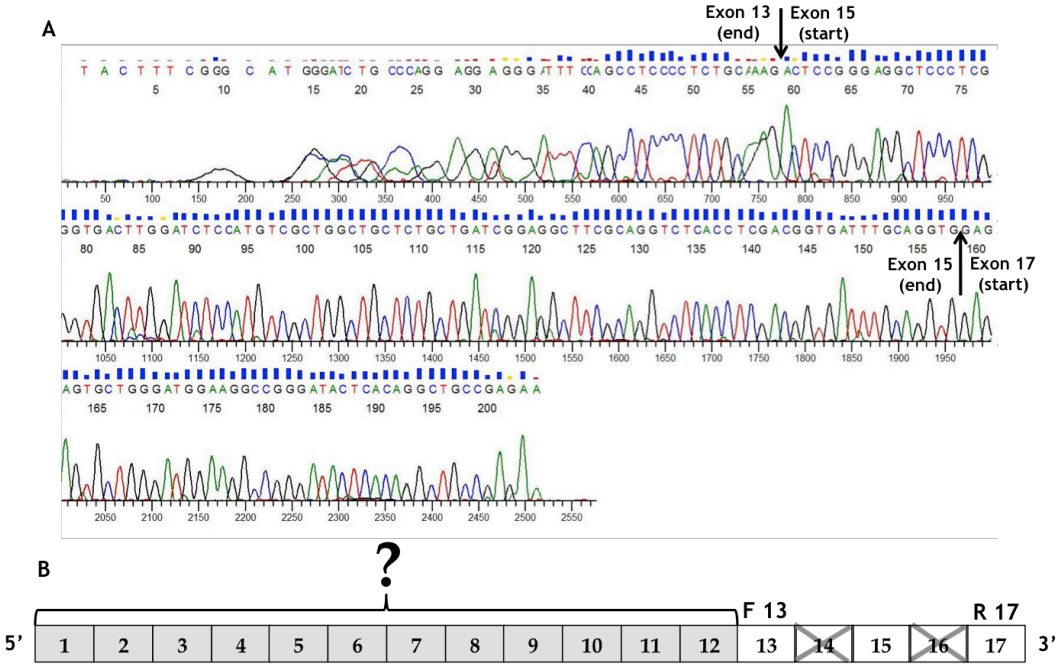
Figure 3-6: F13/R17 PCR results in representative tissue samples



BC = breast cancer; L 100 bp = ladder 100 base pairs; Neg (dH_2O) = negative control with distilled water; PT = breast peri-tumoural tissue; TT= thyroid tissue; 100-600 = number of bp. Panel A = after 40 PCR cycles. Panel B = after 50 PCR cycles.

The sequence analysis of the unexpected 130 bp band was inconclusive, since the sequence analysis performed on BLAST did not show similarities with any existing human gene. The sequence analysis of the 220 bp band revealed a newly described TPO mRNA isoform lacking both exons 14 and 16, therefore still expressing exon 15 corresponding to the transmembrane region, as shown in Figure 3-7.

Figure 3-7: New TPO mRNA isoform lacking exons 14 and 16



Panel A: Electropherogram showing the sequencing result of the 220 bp unexpected amplicon with PCR using forward primer in exon 13 and reverse primer in exon 17 (F13/R17 PCR). A = adenosine; C = cytosine; G = guanosine; T = thymidine. Exons 14 and 16 are missing, therefore exons 13, 15 and 17 are consecutive in the nucleotide sequence. Panel B: schematic representation of the newly identified TPO mRNA isoform. 1-17 = TPO exons; white squares = exons present; white squares containing a grey cross = exons absent; grey squares = exons not investigated with F13/R17 PCR.

All 3 BC cell lines and all tissue samples except for AD were tested with F13/R17 PCR. Only 9/17 (52.9%) AD were tested: 3 AD-A, 4 AD-SC, 1 AD-K and 1 AD-O. Table 3-8 and Table 3-9 summarize the detected amplicons in tissue samples (40 cycles) and BC cell lines (40 and 50 cycles) respectively.

Table 3-8: Summary of F13/R17 PCR (40 cycles) in tissue samples

	485 bp	353/355 bp	220 bp	130 bp
BC	8/8 (100%)	8/8 (100%)	6/8 (75%)	1/8 (12.5%)
PT	5/8 (62.5%)	5/8 (62.5%)	6/8 (75%)	3/8 (37.5%)
AD	9/9 (100%)	9/9 (100%)	8/9* (88.9%)	1/9* (11.1%)
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

Summary of PCR products after 40 cycles obtained in all samples. AD = adipose tissue; BC = breast cancer; MKC = hepatic metastasis of kidney carcinoma; PC = pancreatic adenocarcinoma; PKC = primary kidney carcinoma; PT = peri-tumoural breast tissues.

* The only AD not expressing the 220 bp amplicon was abdominal (AD-A): the same sample was the only AD expressing the 130 bp amplicon.

Table 3-9: Summary of F13/R17 PCR in BC cell lines

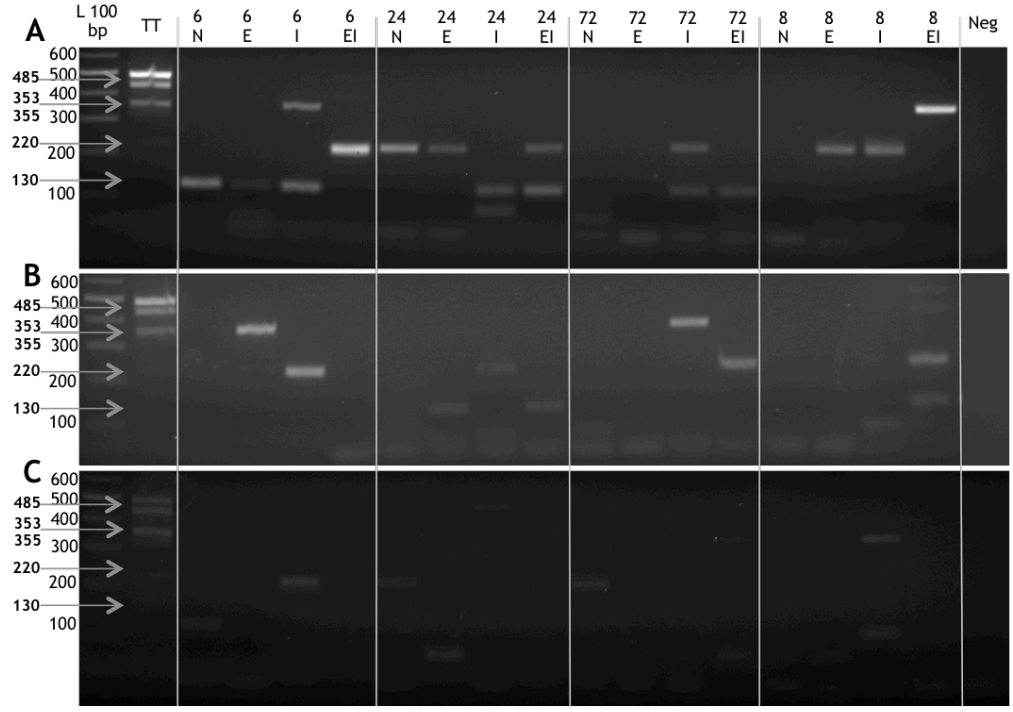
		40 PCR cycles					50 PCR cycles				
Cell line	**Ampl. (bp)**	**6 hours**	**24 hours**	**72 hours**	**8 days**		**6 hours**	**24 hours**	**72 hours**	**8 days**	
MCF-7	**485**										0/16 (0%)
	353-355	6I					6I			8EI	2/16 (12.50%)
	220	6EI	24N, 24EI	72I	8E, 8I		6EI	24N, 24E, 24EI	72I	8E, 8I	**7/16 (43.75%)**
	150	6N, 6E, 6I	24EI	72EI			6N, 6E, 6I	24I, 24EI	72I, 72EI		**7/16 (43.75%)**
T-47D	**485**				8EI					8EI	1/16 (6.25%)
	353-355			72I			6E		72I		2/16 (12.50%)
	220	6I	24I	72 EI	8 EI		6I	24I	72EI	8EI	**4/16 (25%)**
	130							24E, 24EI		8EI	**3/16 (18.75%)**
MDAMB-231	**485**						6 E	24I			2/16 (12.50%)
	353-355			72 EI	8 I				72EI	8I	2/16 (12.50%)
	220	6 I	24 N	72 N			6 I	24 N	72 N		**3/16 (18.75%)**
	150	6 N					6 N				1/16 (6.25%)

Summary of F13/R17 PCR products after 40 and 50 cycles obtained in BC cell lines MCF-7, T-47D and MDAMB-231 cultured in normal medium (N), or supplemented with 1nm ß-estradiol (E), 100ng/uL IGF-II (I) or both (EI), collected after 6 hours (6), 24 hours (24), 72 hours (72) or 8 days (8) from the beginning of treatment. The amplicons (Ampl.) size is expressed in base pairs (bp) in the second column on the left. The last column on the right summarize the number and percentage of cell combinations expressing (calculated after 50 cycles) a particular amplicon.

Known TPO isoforms (amplicons 485 and 353-355 bp) were expressed after 40 PCR cycles by TT (positive control), all BC and AD tested and the great majority of PT, but in none of the other tumours tested (PC, PKC, MKC). Results after 50 PCR cycles were identical, except that the 485 bp amplicon was expressed also in 1/3 PC and MKC. In the 3 BC cell lines, known TPO isoforms were detected only in some culture conditions, notably in the presence of oestrogen and/or IGF1, but this was unrelated to the incubation time (Figure 3-8).

The 220bp amplicon corresponding to the newly identified TPO mRNA variant missing exon 14 and 16 was expressed by the great majority of BC, PT and AD, while it was absent in the other tumours tested and only faintly expressed in TT, as previously shown in Figure 3-6. Importantly, the 220bp amplicon was the most abundant in all the 3 cell lines, especially MCF-7, compared with the amplicons corresponding to the other TPO isoforms (Figure 3-8). TPO expression was present in all the different culture medium combinations, however it seemed to be slightly more prevalent in stimulated cells, especially with IGF-I, alone or in combination with estradiol.

Figure 3-8: F13/R17 PCR results (50 cycles) in all BC cell line combinations

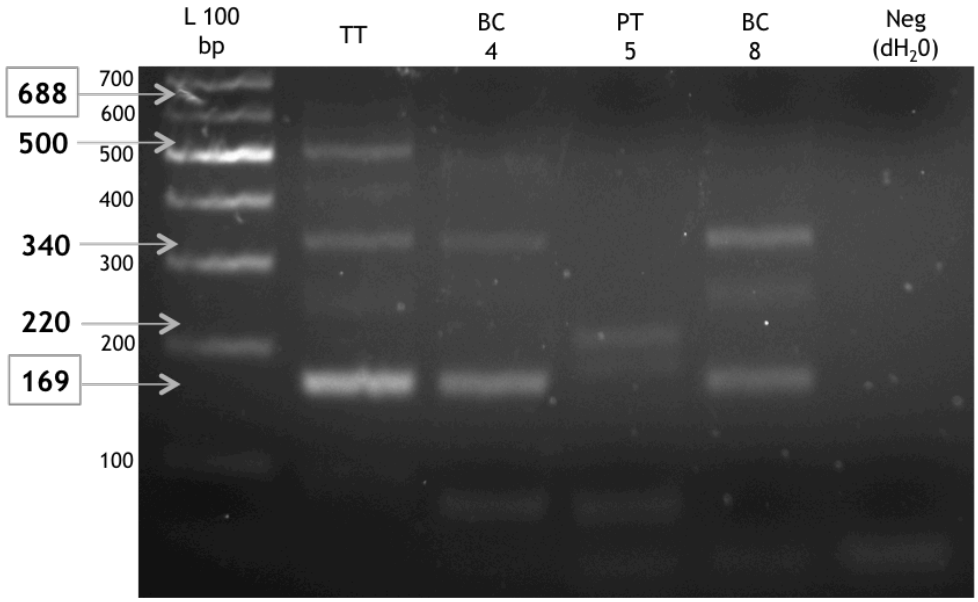


Panel A, B and C: MCF-7, T-47D and MDAMB-231 cell lines respectively. Numbers indicating the collection time of cells: 6 = 6 hours, 24 = 24 hous, 72 = 72 hours, 8 = 8 days. Letters indicating the cells culture medium: N = normal, E = supplemented with 1nm ß-estradiol, I = supplemented with 100ng/uL IGF-II, EI = supplemented with both 1nm ß-estradiol and 100ng/uL IGF-II. The arrows indicate the expected (485 and 353/355 bp) and newly described (220 and 130 bp) amplicons. L 100 bp = ladder 100 base pairs; Neg = negative control (distilled water); TT = thyroid tissue; 100-600 = number of bp.

Other TPO primer combinations in standard PCR

PCR with TPO primer pairs different from F13/R17 gave poor results and were performed mainly on TT and only a few samples of BC and PT; contrary to other standard PCR reactions, they were often performed using cDNA obtained from RT using TPO reverse primers instead of odT (e.g. R11 for F7/R9 PCR), since giving better results. As representative example, Figure 3-9 shows the results obtained with F7/R9 PCR after 50 cycles performed on TT, two BC and one PT: after 40 cycles only very faint bands were obtained (not shown).

Figure 3-9: F7/R9 PCR (50 cycles)



L 100 bp = ladder 100 base pairs; BC = breast cancer; Neg (dH_2O) = negative control (distilled water); PT = peritumoural breast tissues; TT = thyroid tissue. The arrows indicate the expected (169 and 688 bp, corresponding respectively to TPO 5 and all the other TPO isoforms) and newly described (220, 340 and 500 bp) amplicons. 100-700 = number of bp.

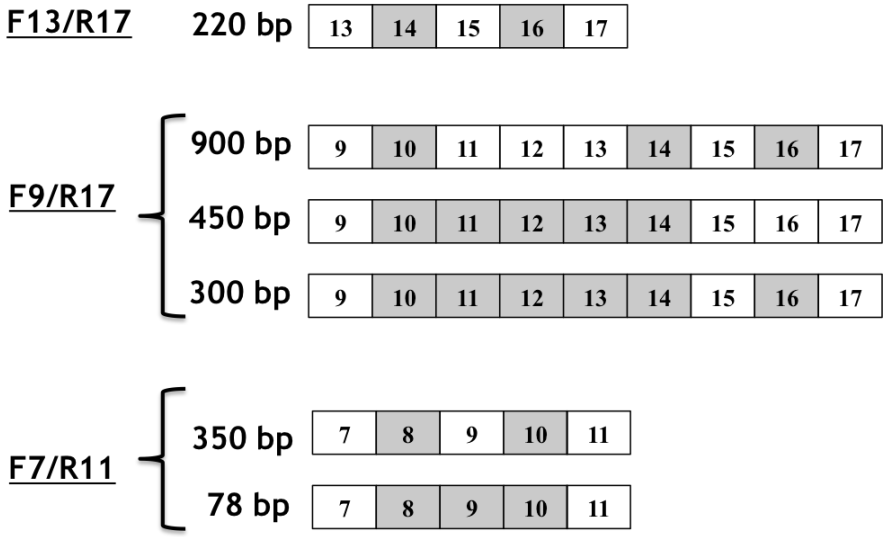
According to Table 3-5, 2 amplicons were expected: 169 bp corresponding to TPO 5 isoform missing exon 8 and a 688 bp corresponding to all the other TPO isoforms. The expected 169 bp amplicon was present, but not the 688 bp, while several intermediate bands were present: 500, 340 and 220 bp.

Similarly to F7/R9 PCR, the other primer pair combinations gave additional unexpected bands, then identified as new TPO mRNA isoforms by gene sequencing (agarose gel results not shown).

TPO mRNA isoforms identified by standard RT-PCR

Figure 3-10 summarizes the newly identified TPO mRNA isoforms identified by standard PCR and gene sequencing.

Figure 3-10: Summary of newly identified TPO mRNA isoforms with standard PCR



Numbered squares from 7 to 17 represent TPO gene exons. White and grey squares represent respectively expressed and missed exons. bp = base pairs (size of the sequenced amplicon).

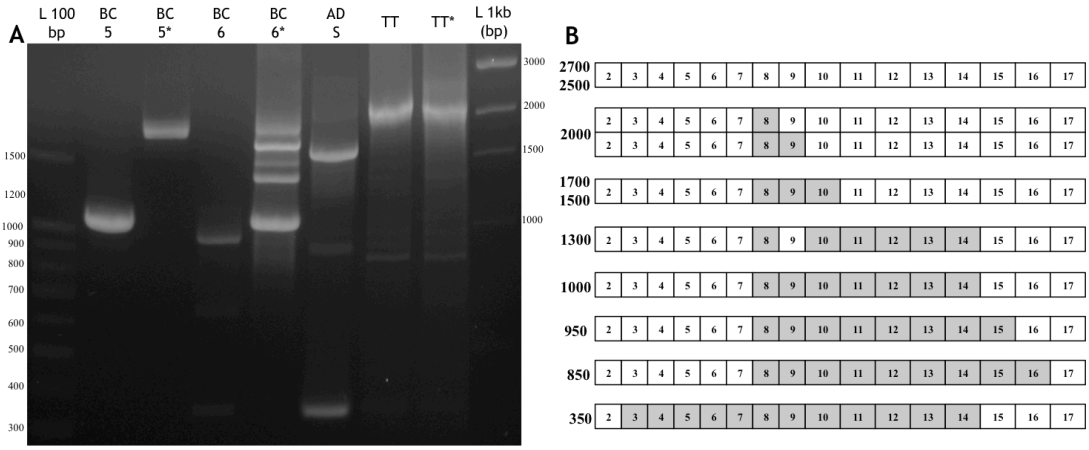
The TPO mRNA isoform corresponding to the 220bp F13/R17 amplicon was demonstrated to be present also in BC (both tissues and cell lines), PT and AD, as described above. The other TPO variants/amplicons were sequenced only in TT due to time restrictions. It is very likely that the 220bp F13/R17 and the 900 bp F9/R17 amplicons identified the same TPO variant.

The discovery of so many TPO mRNA isoforms never identified before lead me to further explore this topic with different techniques, considering that standard RT-PCR gives poor quality results when investigating long amplicons.

3.4.2.3 LongRange RT-PCR and TPO mRNA isoforms

In an attempt to characterize the various TPO isoforms detected by standard RT-PCR, TPO primers in exons 2 (location of start codon) and 17 (location of stop codon) were used in LongRange RT-PCR. Due to time restrictions, only few samples were tested: TT, BC 5, BC 6, PT 3, one AD-S and MCF-7 EI. All samples were tested both with (Q) and without (N) Q solution; amplicons were obtained in all samples except PT 3, MCF-7 EI and AD-S when tested with Q solution (Figure 3-11). Different amplicons were obtained with or without Q solution and this will be further discussed in the conclusions section (paragraph 3.5.3).

Figure 3-11: LongRange RT-PCR (F2/R17) results



Panel A: agarose gel 1.5% bands obtained with LongRange RT-PCR using TPO primers in exon 2 and 17 (F2/R17). AD-S = subcutaneous adipose tissue; BC = breast cancer; TT = thyroid tissue; * = Q solution added to the PCR reaction mix. Panel B: schematic representation of novel TPO mRNA isoforms found sequencing PCR products obtained with LongRange RT-PCR F2/R17. Numbered squares from 2 to 17 represent TPO gene exons. White and grey squares represent respectively expressed and missed exons.

Table 3-5 shows the expected amplicons with PCR F2/R17 according to known TPO isoforms. In particular, TT showed the presence of a faint amplicon at 2700 bp, corresponding to the full length TPO1, only in the presence of Q solution (Figure 3-11 panel A); sequencing confirmed that it was the full length TPO 1 isoform (Figure 3-11 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 known TPO isoforms, either previously reported or already described in this study.

In non-thyroid samples many different bands were detected; a 2000 bp variant was present in BC n°5 (with Q solution) lacking exons 8-9 or in BC n°6 lacking exon 8 (TPO 5); a 1500-1700 bp variant lacking exons 8-10 was present in BC n°5 (with Q solution) and AD-S; whilst a 1000 bp transcript in which exon 7 then skipped to exon 15 was abundant in the two BC (without Q solution); a short transcript comprising exons 2 linked to 15-17 was present in AD-S, BC n°6 and TT. As in the case of TT, we did not find other known TPO isoforms or the new TPO mRNA variant lacking exons 14 and 16 identified with standard RT-PCR F13/R17.
Figure 3-11 panel B summarizes all TPO mRNA isoforms found with LongRange RT-PCR using primer pair F2/R17. Many other transcripts were isolated but they were not successfully identified by gene sequencing due to the presence of overlapping sequences.

3.4.2.4 QPCR

Table 3-10 reports the cycle threshold (Ct) and copy number (N° copy referred to estimated 50 ng of input cDNA) of TPO gene obtained in human tissues. BC and PT expressed TPO mRNA at the same level, around 10^4 fold less than in TT. The AD tissues expressed TPO mRNA 10^2 fold less than in TT and therefore 100-fold higher than in BC/PT, with no depot-specific differences. In the other tumours (PC, PKC and MKC), TPO transcripts were at the limit of detection.

Table 3-10: Absolute QPCR values for TPO copy number obtained in human tissues

Sample	Ct	N° copy	Sample	Ct	N° copy	Sample	Ct	N° copy
BC 1	31,74	4,43E+02	AD-A 1	31.65	1.04E+04	PKC	NO	0
BC 2	30,59	9,58E+02	AD-A 2	29.79	3.40E+04	MKC	NO	0
BC 3	32,56	2,57E+02	AD-A 3	28.72	6.73E+04			
BC 4	29,33	2,21E+03	AD-A 4	30.48	2.19E+04	PC 1	35	5,02E+01
BC 5	31,72	4,50E+02	AD-S 1	29.28	4.72E+04	PC 2	36,94	1,38E+01
BC 6	31,82	1,50E+03	AD-S 2	29.64	3.74E+04	PC 3	33,12	1,77E+02
BC 7	31,7	4,56E+02	AD-S 3	28.45	8.03E+04	*Mean*		*8.02E+01*
BC 8	30,75	8,58E+02	AD-S 4	30.64	1.98E+04			
Mean		*8.92E+02*	AD-S 5	31.42	1.21E+04	TT	18,53	3,01E+06
			AD-S 6	30.71	1.89E+04			
PT 1	32,01	3,71E+02	AD-S 7	32.49	6.07E+03			
PT 2	29,19	2,43E+03	AD-S 8	37.76	2.10E+02			
PT 3	28,97	2,82E+03	AD-K	28.33	8.64E+04			
PT 4	35,55	3,50E+01	AD-O 1	33.72	2.77E+03			
PT 5	31,23	6,25E+02	AD-O 2	28.82	6.32E+04			
PT 6	29,91	4,20E+02	AD-O 3	28.99	5.68E+04			
PT 7	32,53	2,62E+02	AD-O 4	30.04	2.89E+04			
PT 8	36,13	2,36E+01	*Mean*		*3.49E+04*			
Mean		*8.73E+02*						

AD-A = abdominal adipose tissue; AD-K = adipose tissue from knee; AD-O = orbital adipose tissue; AD-S= subcutaneous adipose tissue; BC = breast cancer; Ct = cycle threshold; MKC = hepatic metastasis of kidney carcinoma; N° copy = TPO cDNA copy number (referred to approximately 50 ng of cDNA); PC = pancreatic adenocarcinoma; PKC = primary kidney carcinoma; PT = breast peri-tumoural; TT = thyroid tissue.

Table 3-11 reports the cycle threshold (Ct) referred to input cDNA (estimated 50 ng) obtained in all different combinations of MCF-7 BC cell line with TPO primers and APRT primers. Ct TPO values showed that TPO mRNA was expressed at the limit of detection in all the combinations of MCF-7 BC cell lines. Even if not shown in the present thesis due to space restrictions, both TPO and APRT quantifications, with the relative TPO and APRT standard curves and always including positive control with TT and negative control with dH_2O, have been performed for all tissues and BC cell lines.

Table 3-11: Absolute TPO and APRT QPCR values obtained in MCF-7 BC cell lines

MCF-7	Ct TPO	Ct APRT		Sample	Ct TPO	Ct APRT
6 N	37,41	22,23		TT	27,88	29,16
6 E	36,54	21,71				
6 I	37,70	21,50		TPO/APRT 10^6	25,76	20,52
6 EI	38,14	21,74		TPO/APRT 10^5	31,55	24,21
24 N	36,76	20,80		TPO/APRT 10^4	33,10	27,76
24 E	36,62	21,43		TPO/APRT 10^3	37,38	30,34
24 I	37,60	21,83		TPO/APRT 10^2	35,90	30,79
24 EI	No	22,66		TPO/APRT 10^1	36,84	31,07
72 N	37,95	22,28				
72 E	38,70	22,91				
72 I	36,35	21,70				
72 EI	38,40	23,59				
8 N	36,68	22,53				
8 E	37,39	22,02				
8 I	38,49	21,58				
8 EI	37,33	23,23				

Cells cultured in normal medium (N), or supplemented 1nm ß-estradiol (E), 100ng/uL IGF-II (I) or both (EI), collected after 6 hours (6), 24 hours (24), 72 hours (72) or 8 days (8) from the beginning of treatment. Ct TPO and Ct APRT = cycle threshold to obtain respectively TPO and APRT cDNA detection (referred to approximately 50 ng cDNA); TT = thyroid tissue.

TPO mRNA was expressed at the limit of detection also in all the combinations of T-47D and MDAMB-231 cell lines, as shown in Table 3-12.

Table 3-12: Absolute TPO QPCR results for T-47D and MDAMB-231 BC cell lines

T-47D	Ct TPO		MDAMB-231	Ct TPO		TT Ct TPO
6N	No Ct		6N	35.1		16.82
6E	33.99		6E	33.02		
6I	No Ct		6I	34.66		
6EI	No Ct		6EI	No Ct		
24N	36.31		24N	No Ct		
24E	32.94		24E	37.03		
24I	No Ct		24I	34.15		
24EI	35.14		24EI	No Ct		
72N	No Ct		72N	No Ct		
72E	No Ct		72E	34.4		
72I	No Ct		72I	No Ct		
72EI	No Ct		72EI	34.46		
8N	No Ct		8N	No Ct		
8E	No Ct		8E	No Ct		
8I	37.81		8I	34.31		
8EI	No Ct		8EI	35.23		

Cells cultured in normal medium (N), or supplemented 1nm ß-estradiol (E), 100ng/uL IGF-II (I) or both (EI), collected after 6 hours (6), 24 hours (24), 72 hours (72) or 8 days (8) from the beginning of treatment. Ct TPO = cycle threshold to obtain TPO cDNA detection (referred to approximately 50 ng cDNA); TT = thyroid tissue.

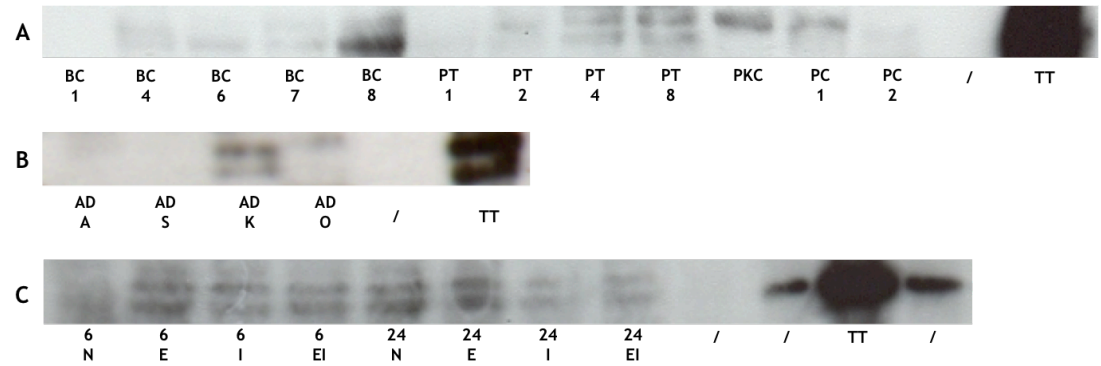
3.4.3 TPO gene expression (protein)

3.4.3.1 Western Blot (WB)

105-110 kDa band

As previously explained in paragraph 3.1.1.1. and summarized in Table 3-1, TPO protein is usually detected by WB as a double band of 105-110 kDa molecular mass. When the blot was exposed for a short time (from 15 seconds to 3 minutes: depending from the strength of antibodies and reagents used), the 105-110 kDa band was present only in TT. However, exposing the blots for extended times (up to 40 minutes), combined with loading 10 times more protein extract than for TT, lead to the detection of similar bands in 7/8 BC, 4/8 PT, 2/4 AD, 1/1 PKC, 1/1 MKC and 1/2 PC (only 2 of the 3 PC were examined because PC3 sample was insufficient for protein analysis), as shown in Figure 3-12 (panel A and B). Similar results were obtained with all the 3 different BC cell lines, where a 105-110 kDa band was present independently from the cell collection time and the addition of growth factors to the cell medium (Figure 3-12 panel C).

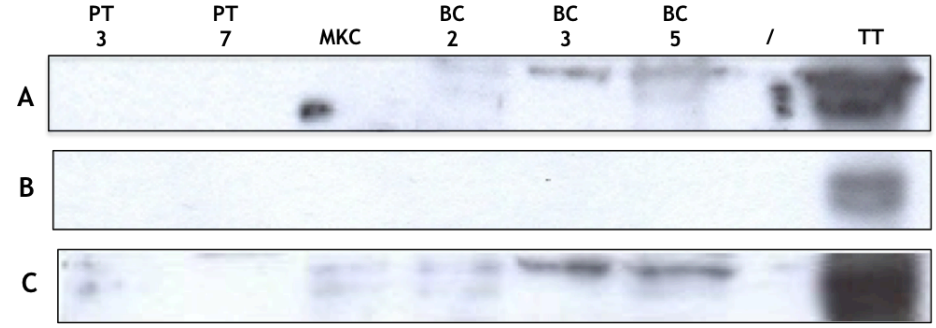
Figure 3-12: 105-110 kDa band observed with WB



Western Blot representative results of 105-110 kDa band expression observed in human tissues (panels A and B: 40 minutes exposition time) and MCF-7 cell line (panel C: 20 minutes exposition time), using mouse monoclonal to TPO ab76935. Tissues: AD-A = abdominal adipose tissue; AD-K = adipose tissue from knee; AD-O = orbital adipose tissue; AD-S = subcutaneous adipose tissue; BC = breast cancer; MKC = metastatic kidney cancer; PC = pancreatic adenocarcinoma; PT = breast peri-tumoural tissue; TT = thyroid tissue. Cells: 6 = cell collection after 6 hours from treatment; 24 = cell collection after 24 hours from treatment; E = culture medium supplemented with 1nm ß-estradiol; EI = culture medium supplemented with 1nm ß-estradiol and 100ng/uL IGF-II; I = culture medium supplemented with 100ng/uL IGF-II; N = normal culture medium. / = no proteins loaded.

In order to test the specificity of obtained signals, the primary antibody was absorbed using recombinant fragments of TPO spanning the region of the epitope recognized by the antibody. As shown in Figure 3-13 (panels A and B), the 105-110 kDa signal in TT was greatly reduced and that in the other tissues completely eliminated by the treatment. Conversely, when the primary antibody was absorbed with bovine LPO, the intensity signal was not reduced and paradoxically enhanced in some cases (Figure 3-13 panel C).

Figure 3-13: Absorption of first antibody with TPO fragments and bovine LPO

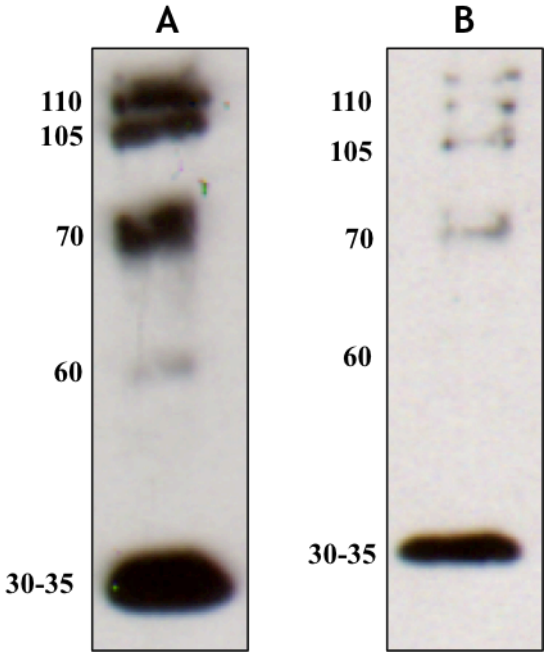


Representative results of 105-110 kDa band expression at Western Blot (5 minutes exposure) in BC = breast cancer, MKC = hepatic metastasis of kidney cancer, PT = breast peri-tumoural tissue, TT = thyroid tissue, / = no protein loaded. Panel A: normal ab76935. Panel B: TPO absorbed ab76935: the signal is strongly reduced or eliminated. Panel C: bovine LPO absorbed ab76935: the signal is unchanged or paradoxically enhanced.

Additional bands

When exposing the blot for longer times (3-40 minutes), additional bands corresponding to proteins of apparent molecular weight (MW) 70, 60 and 30-35 kDa were identified in TT, in addition to the doublet at 105-110 kDa (Figure 3-14 panel A). Absorption with recombinant TPO strongly reduced not only the 105-110 kDa 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 kDa band was not affected by absorption (Figure 3-14 panel B). Absorption with LPO did not reduce the signal (Images not shown).

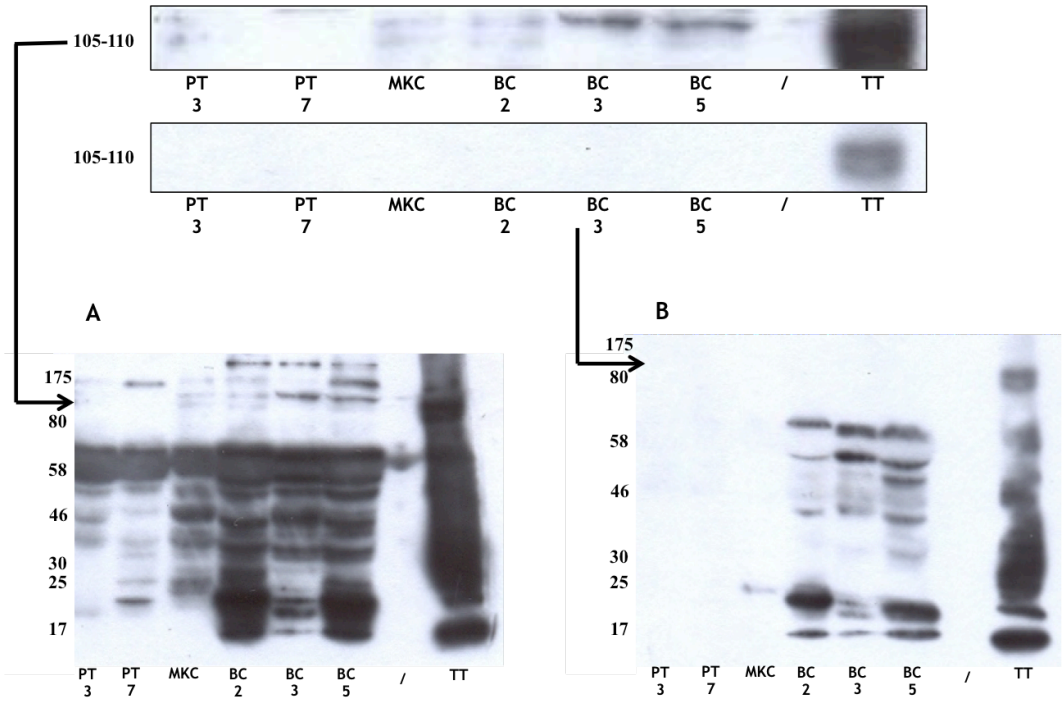
Figure 3-14: Entire WB obtained in thyroid tissue



Panel A: complete WB results in thyroid tissue (TT) using normal ab76935; it shows the doublet at 105-110 kDa and 3 additional bands at 70, 60 and 30-35 kDa. Panel B: complete WB results in the same TT of panel A 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.

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 (Figure 3-15).

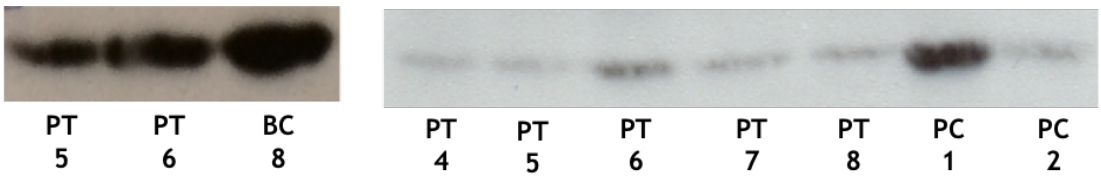
Figure 3-15: Entire WB on human tissues



Entire Western blot representative images (40 minutes exposition time) of various tissues probed with TPO monoclonal antibody ab76935 normal (panel A) or pre-absorbed with TPO fragments (panel B). 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), previously shown in Figure 3-13. BC = breast cancer; MKC = hepatic metastasis of kidney carcinoma; PT = peri-tumoural breast tissue; TT = thyroid tissue; / = no sample loaded. In panel B 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.

After being probed for TPO, the membranes were stripped and re-probed for human actin to reveal the presence of a 42 kDa band as expected, corresponding to ß-actin in these tissues (Figure 3-16).

Figure 3-16: Western Blot for ß-actin

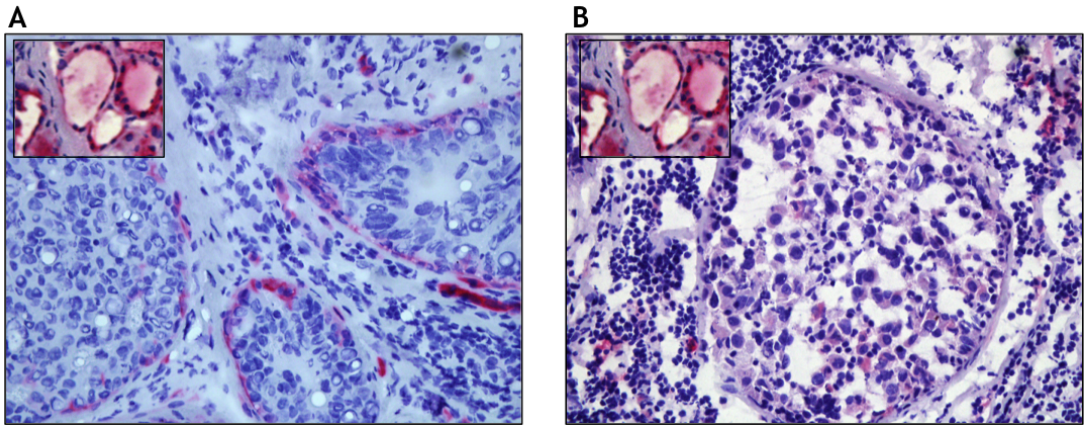


Representative Western Blot images (20 minutes exposition time) of the 42 kDa obtained in various tissues probed with primary antibody to human actin. BC = breast cancer; PT = breast peri-tumoural tissue.

3.4.3.2 Indirect Immunohistochemistry (IHC)

The PCR and WB results indicated that both breast (tumoural and peri-tumoural) and adipose tissues contain low levels of TPO protein. Considering that breast is rich in fat, indirect IHC experiments were performed in order to identify the cellular location of TPO protein in BC tissues. A weak TPO immunoreactivity was found in a small proportion of breast epithelium, and obtained in 2/2 fresh frozen BC tissues analyzed (Figure 3-17).

Figure 3-17: Indirect IHC results in 2 fresh frozen BC



Panels A and B show two different breast cancer tissues (BC) analyzed by indirect IHC using TPO mouse monoclonal ab12500 as primary antibody (20x magnification); in the small square is represented thyroid tissue (TT) as positive control (10x magnification). In TT the TPO signal (red) is strongly positive and located in thyrocytes that express TPO, disposed around the colloid follicles; in BC the TPO signal (red) is weaker but present and located in breast epithelium cells.

3.4.4 TPO gene expression: correspondence between mRNA and protein

Table 3-13 summarizes the results of TPO mRNA and protein expression in BC, PT and other tumours. A similar comparison has not been performed for AD, since only 9 and 4 tissue samples were analyzed respectively for TPO mRNA and protein expression, not paired because of scarcity of available material.

Table 3-13: Correspondence between TPO mRNA and protein expression

Tissue		TPO mRNA		TPO1 Protein WB 105-110 kDa	Tissue	TPO mRNA		TPO1 Protein WB 105-110 kDa
		TPO1	New isoform (no 14/16)			TPO1	New isoform (no 14/16)	
1	BC	1	2	0	PT	0	0	0
2	BC	3	3	1	PT	3	2	1
3	BC	3	0	3	PT	3	3	1
4	BC	3	3	1	PT	0	1	2
5	BC	2	2	3	PT	2	2	0
6	BC	3	3+	1	PT	3	3	0
7	BC	1	0	1	PT	0	0	0
8	BC	3	1	3	PT	3	2	2
PKC		2	0	2				
MKC		3	0	1				
1	PC	0	0	2				
2	PC	0	0	0				
3	PC	2	0	NA				

TPO mRNA expression is referred to PCR F13/R17 (50 cycles). kDa = kilodaltons; NA = not available (not performed because of scarcity of tissutal material); No 14/16 = absence of exons 14 and 16 (variant corresponding to 220 base pairs band); TPO1 = TPO isoform 1 (complete, corresponding to 485 base pairs); WB = western blot. Numbers indicate the intensity of expressed bands in PCR (mRNA) or WB (protein): 0 = absent, 1 = very low, 2 = low, 3 = moderate, 3+ = high (values from 1 to 3+ were considered positive).

3.5 CONCLUSIONS

3.5.1 TPO gene is expressed in breast cancer and other tissues

These experiments demonstrate that thyroid peroxidase (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. (261) who demonstrated TPO expression, both mRNA and protein, in orbital tissue (fat and fibroblasts). In my study, the highest level of TPO mRNA expression, around 100 times less than in thyroid tissue (TT), was found in adipose tissue (AD), independently from its source (abdominal, subcutaneous, orbital or knee-derived). Breast cancer (BC) and its relative peri-tumoural breast tissue (PT) expressed TPO mRNA 50-100 times less than AD and therefore 10^4 fold less than TT. TPO expression in BC tissue could explain both the known association between BC and thyroid autoimmunity (TA) and the protective role of serum thyroid peroxidase (TPOAb) in patients with aggressive BC; this will be further discussed in chapters 4 and 5.

The other cancers investigated as controls did not express substantial levels of TPO mRNA when investigated with QPCR, the same was true for the 3 BC cell lines. However, the tumoural tissues were found to be negative for TPO mRNA expression also with standard PCR, differently from all the 3 BC cell lines that showed TPO relative amplicons after 40 cycles of PCR, even if faint. Considering that a high number of PCR cycles were needed in order to detect such amplicons (up to 50 PCR cycles), initially the hypothesis of "illegitimate transcripts", corresponding to the low level presence of specific transcripts in non-specific cells, was considered (266). However, TPO protein was present in the majority of analysed tissues with western blot (WB), including the other tumoural tissues examined, even in some negative for TPO mRNA expression; therefore TPO gene expression seemed to be real. Specificity tests using pre-absorption of the first antibody with TPO fragments and bovine lactoperoxidase (LPO) confirmed that the obtained signal at WB was TPO. The specificity test is really important, since TPOAb could cross-react with non-TPO components in the examined tissues, especially in immunofluorescence experiments using pooled human sera positive for TPOAb, and therefore led to aspecific signal and false positive conclusions. The discrepancy between mRNA and protein expression may be first of all a consequence of the fact that WB is not a quantitative technique and long exposure times have often been used, therefore a signal present in the other tumours may have been overestimated. To this purpose, it is important to highlight that the monoclonal TPOAb used for WB experiments was obtained new, delivered on ice and immediately stored at -20°C. Despite this, even thawing it just once determined a reduction of the intensity signal and longer exposure times were needed in order to develop a good signal (from 5 to 40 minutes). Moreover, gene expression is a complex process involving several fundamental steps: transcription, mRNA degradation, translation and protein degradation (267). Several authors agreed that there is a poor correlation between mRNA and correspondent protein levels mainly due to uncoupled processes of synthesis and degradation. Levels of mRNA (transcription) explain only around 40% of the variability in protein levels; post-transcriptional, translational and degradation regulation processes contribute at least at the same extent (268). In the study of Schwanhäusser et al. proteins were found to be on average 5 times more stable than mRNA (267) and this could explain the presence of TPO protein expression in samples negative for TPO mRNA expression. Furthermore, tumoural tissue is characterized by a general dis-regulation of many normal cellular functions, leading to a different phenotype compared with normal tissue and to an aberrant gene expression (269). Unfortunately in the present study no normal tissues were

available as controls, while it would have been interesting to test whether normal kidney or pancreatic tissue expressed tiny amount of TPO as found in the correspondent tumours. Similarly to BC, an increased prevalence of serum TPOAb and other thyroid autoantibodies has been found in several other cancers including kidney (270), pancreas (271), lung (272) and stomach (273); TPO expression in these cancers could explain a raise in TPOAb levels and further experiments are needed to verify this hypothesis.

The presence of TPO in BC is even more convincing, since found at a higher level and not confined to the tumoural tissue, but also in the peri-tumoural breast tissue (PT). PT tissue could express neoplastic or pre-neoplastic features, therefore further experiments with normal breast tissue obtained from reductive mammoplasty would be the ideal control in order to assess whether TPO is expressed in breast tumoural cells or also in normal breast cells. Importantly, considering that fat is abundant within the breast and that RNA and protein were extracted from the whole homogenized tissue, the positive TPO expression found in PT could represent the TPO expressed by AD within the breast. This could also explain the discrepancy between BC tissue and cell lines results, with TPO mRNA found to be expressed at a higher level in tissue and at the limit of detection in BC cell lines. However, TPO expression by BC cells has been demonstrated by immunohistochemistry (IHC) experiments that clearly showed the presence of signal in the ductal breast epithelium. Furthermore, a recent review reported that adipocytes within mammary gland carcinomas are dynamic cells that may contribute to human BC progression (274). Therefore I can hypothesize that the tissue micro-environment present within BC may induce or enhance TPO expression in *in vivo* tissues, while in in vitro experiments, where the BC cells are grown alone, TPO expression is reduced.

In BC cell lines no significant differences in terms of TPO expression were found between the different stimulation conditions and collection times. However stimulated cells, especially with Insulin-like growth factor type II (IGF-II), alone or in combination with δ-estradiol, tended to express more TPO compared to the other ones. This is plausible since, as previously introduced in paragraphs 1.3.1.2 and 3.1.3, both IGF-II and oestrogens are considered mitogens for BC cells growth (118, 262, 263) and are engaged in a crosstalk resulting in synergistic growth in MCF-7 BC cell line (264). As an afterthought, a possible link between oestrogens activity and TPO expression it is likely to have been underestimated considering that phenol red, a usual component of culture media (e.g. DMEM and RPMI) in quality of pH indicator, has been demonstrated to partially have an oestrogenic stimulatory activity on MCF-7 cells at concentrations used in tissue culture media

(275). Similarly, foetal calf serum (FCS) could also represent an additional uncontrolled source of oestrogens in the cell culture conditions. It is also important to highlight that MCF-7 cells, the most differentiated BC cell line, expressed more TPO compared with MDAMB-231, the less differentiated one. These data taken together may suggest the possibility that TPO expression in BC could be regulated by hormones and growth factors, in a similar way to the sodium iodide symporter (NIS), whose expression has been found to positively correlate with the oestrogen receptor expression (210). However, experiments with BC cell lines provided equivocal results and did not allow to draw substantial conclusions about the regulation of TPO gene expression. Further studies are needed to investigate the regulatory mechanisms of TPO expression in breast tissues; in particular, using culture medium phenol red free and containing charcoal stripped foetal bovine serum instead of FCS, where hormones are selectively removed.

TPO is not the only thyroidal antigen present in orbital adipose tissue, since thyroid-stimulating hormone (TSH) receptor (TSHR) has been demonstrated to be also expressed and represents one of the major antigen involved in Graves' orbitopathy (1). Interestingly, TSHR transcripts have also been found in different depots of adipose tissue, even if at a lower level compared with orbital fat (276) and adipose tissue is also a key target for thyroid hormones (1, 277). Therefore the discovery of TPO transcripts and protein expression in adipose tissue from different depots further increases the similarities and connections between thyroid and adipose tissue; however, this topic is not related to the present thesis and will be developed in collateral research projects.

Finally, it is important to state that this study verified the quality of RNA and protein analyzed, therefore the results of gene expression experiments presented in this thesis are reliable. In particular, the good quality of RNA was verified by the presence of ribosomal RNA 28S and 18S and an absorbance ratio at 260 and 280 nm ($A_{260/280}$) between 1.7 and 2.0 obtained with UV spectrophotometry. The good quality of cDNA obtained from RNA by reverse transcription and of extracted proteins was guaranteed by the verified expression of housekeeping genes, such as adenine phosphoribosyltransferase (APRT) and ß-actin. APRT protein is a ubiquitous enzyme that catalyzes the formation of AMP and inorganic pyrophosphate from adenine and 5-phosphoribosyl-1-pyrophosphate (278). ß-actin is a non-muscle cytoskeletal actin involved in cell structure and motility, therefore highly conserved and used as housekeeping control protein in WB (279).

Therefore we can conclude that variable levels of TPO seem to be expressed in a wide variety of different tissues, adipose tissue and breast cells in particular; further and larger experiments are needed in order to better investigate in which other human tissues TPO is expressed (and why).

3.5.2 TPOAb cross-reactivity with other peroxidases

Considering that LPO is highly expressed in breast tissue and that the peroxidase family members have many similarities (257), when discussing TPO protein expression results it is important to exclude the presence of TPOAb cross-reactivity with other peroxidases, LPO in particular. In terms of TPO mRNA expression there is no need for such proof, considering that 1) PCR products have been sequenced and they 100% correspond to TPO; 2) PCR experiments have been mainly performed with primers designed in exons 13 and 17, that are specific to TPO and missing in the other peroxidases, as previously explained in paragraph 3.1.1.2.

3.5.2.1 Structural similarities between TPO and other peroxidases

AS previously introduced in paragraph 3.1.1.2, TPO has considerable structural similarity with the other members of the peroxidase family, previously summarized in Table 3-2; in particular, the highest homology is with myeloperoxidase (MPO), as summarized in Table 3-14. The TPO sequence of amino acids (AA) 122-737, encoded from exon 5 and 12 (referred as "MPO like" region), has 71% similarity and 46% identity with MPO (280).

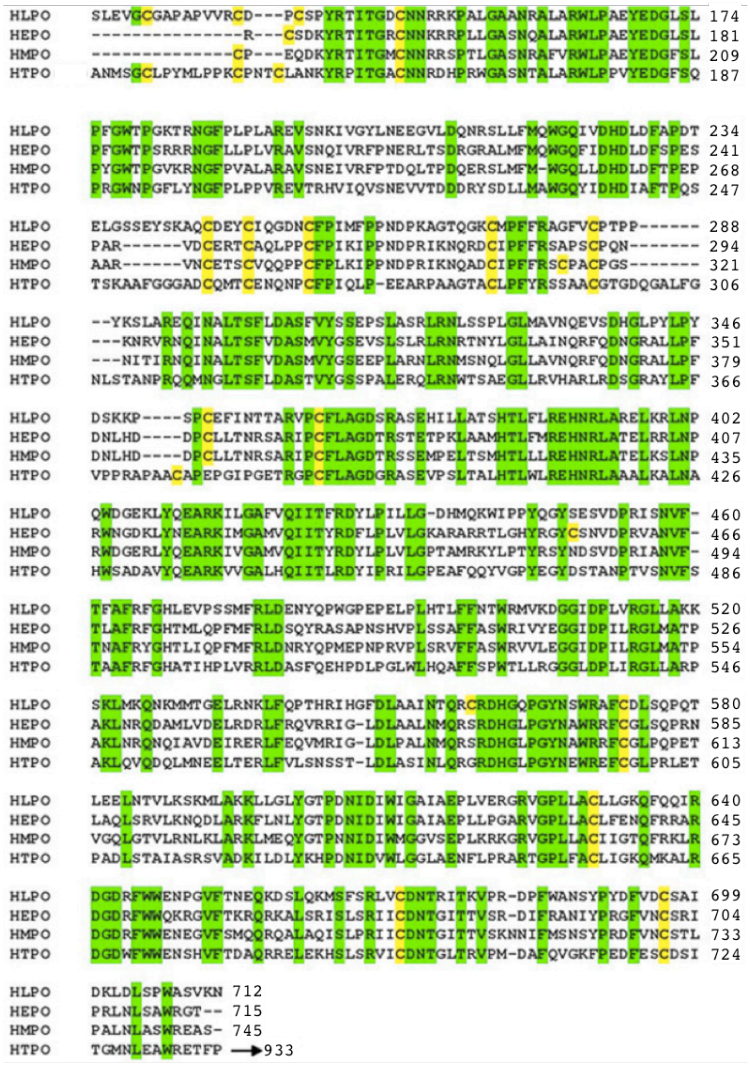
Table 3-14: Summary of TPO regions in relation to MPO

EXONS	AA	REGION NAME	COMPARISON WITH MPO
2-5	1-121	Pro-sequence MPO	Cleaved away in final MPO sequence
5-12	122-737	MPO-like	46% identical, 71% similar
13	741-795	CCP-like	Absent
14	794-842	EGF-like	Absent
15	842-871	Transmembrane	Absent
16-17	872-933	Intracellular	Absent

AA = amino acids. CCP = complement control glycoprotein. EGF = epidermal growth factor. MPO = myeloperoxidase. TPO = thyroid peroxidase.

However, considering that lactoperoxidase (LPO) is highly expressed in breast tissue, as previously reported in paragraph 3.1.1.2, in the present study it is important to compare TPO and LPO structures, in order to investigate for possible cross-reactivity of TPOAb with LPO. Human LPO is a single chain monomeric protein of 712 residues, 46% identical to human TPO and 57% and 58% respectively to human MPO and human eosinophil peroxidase (EPO) (281).

Figure 3-18: Amino acidic sequence of human peroxidases alignment and comparison



Adapted from (281). HEPO = human eosinophil peroxidase; HLPO = human lactoperoxidase; HMPO = human myeloperoxidase; HTPO = human thyroid peroxidase. The identical sequences are highlighted in green. Cysteine (Cys) residues are highlighted in yellow.

Although LPO was first isolated in 1943 (282) and its primary structure has been known since 1991 (283), the crystal structure of caprine LPO was achieved only in 2008 (284). On the contrary, even if human TPO protein sequence was first reported in 1987 (244), despite much effort, TPO crystals suitable for X-ray diffraction have not been obtained yet (246, 285); as a consequence, insights into the structure of TPO come from related proteins. Even if the primary AA sequence is known for both LPO and TPO, the comparison of the relative tertiary and quaternary structures is essential since, as further explained in paragraph 5.2.2, TPOAb usually recognize conformational and not linear epitopes. To this purpose, interesting attempts at TPO structure modeling have been realized (286), based on the known crystal structure of MPO (287), factor H (a typical CCP module) (288) and EGF-like domain of fibrillin-1 (289), for respectively the MPO-like, EGF-

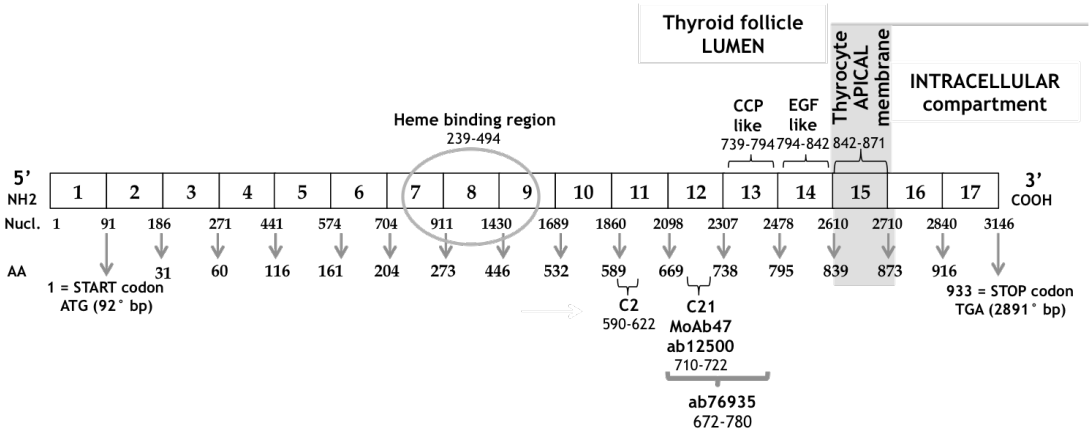
like and CCP-like domains of TPO. In particular, CCP-like TPO region (encoded by exon 13) has 21% identity and 48% similarity with factor H, while EGF-like TPO region (encoded by exon 14) has 36% identity and 62% similarity with EGF-like domain of fibrillin-1 (286). TPO modeling with particular focus on surface epitopes will be further discussed in the general discussion chapter (paragraph 5.2.2).

3.5.2.2 TPOAb cross-reactivity with other peroxidases, in particular LPO

In this study 2 different mouse monoclonal antibodies to TPO were used for TPO protein detection (Figure 3-19):

- ab76935 (abcam, Cambridge, UK), originated using a TPO recombinant fragment corresponding to AA 672-780 (encoded by exons 12 and 13) as immunogen. The ab76935 has been used for western blot (WB) and indirect immunofluorescence (IF).
- ab12500 (abcam, Cambridge, UK), originated using human TPO purified from thyroid microsomes by immunoaffinity chromatography as immunogen. Ab12500 is a mouse monoclonal antibody 47 (MoAb47) clone (290), found to recognize a linear sequence (AA 710-722, encoded by exon 12) identified as one of the major antigenic site in human TA (defined C21), as previously introduced in paragraph 3.1.1 (246). The ab12500 has been used for indirect immunohistochemistry (IHC).

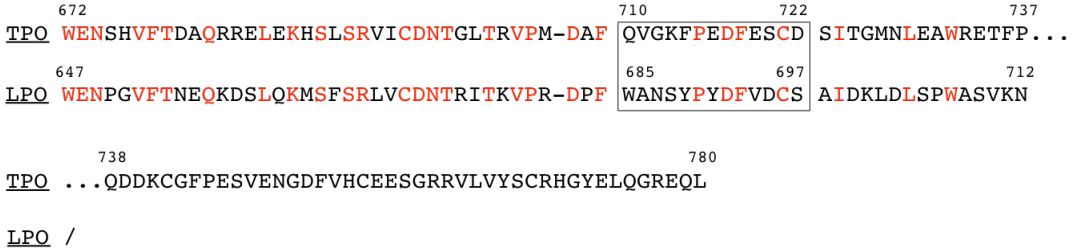
Figure 3-19: ab76935 and ab12500 binding site on human TPO



Schematic representation of the correspondence between TPO nucleotide sequence (Nucl.) and TPO amino acid sequence (AA). bp = base pairs; CCP = complement control glycoprotein; C2, C21 = antigenic sites; EGF = epidermal growth factor; MoAb47/ab12500 and ab76935 = mouse monoclonal antibodies to TPO; START codon ATG = nucleotide triplet corresponding to the beginning of translation process; STOP codon TGA = nucleotide triplet corresponding to the end of translation process; 1-17 = exon number; 1-3146 = nucleotide number; 1-933 = amino acid number; 3' COOH = 3-prime-end of the gene, encoding the intracellular carboxy-terminus (or C-terminus) of the protein; 5' NH2 = 5-prime-end of the gene, encoding the extracellular amino-terminus (or N-terminus) of the protein.

Figure 3-20 summarizes the sequence alignment of TPO and LPO residues corresponding to the two mouse monoclonal antibodies. The ab76935, used for WB and IF experiments, has been produced against the TPO peptide AA 672-780 and the first half of this region is similar to LPO (encoded by TPO gene exon 12), but the second part (encoded by TPO gene exon 13) is exclusive to TPO, since a correspondent sequence in LPO protein does not exist. The ab12500, used for IHC, recognizes a small portion of the TPO peptide sequence used to generate ab76935, containing only 4/13 (30.77%) identical AA and not in sequence.

Figure 3-20: TPO and LPO amino acidic sequence alignment (TPO AA 672-780)



LPO = lactoperoxidase; TPO = thyroid peroxidase. Numbers indicate the amino acids (indicated by letters). Amino acids identical between TPO and LPO are reported in red. AA 672-780 represent the primary sequence of TPO peptide used to generate ab76935. The square identifies the epitope recognized by ab12500 (TPO = 710-722; LPO = 685-697).

Despite the large similarity of the first epitope part, WB pre-adsorption experiments of ab76935 with LPO clearly demonstrated that this antibody does not bind LPO, since the signal was not reduced after its pre-adsorption with bovine LPO. Bovine LPO has been used instead of purified human LPO; this is possible since mammalian LPOs have a very high rate of identity, in particular bovine and human LPO have 85 % homology (281). For this reason bovine LPO is routinely used for similar cross-reaction studies in place of human LPO (290, 291). In particular Ruf et al. tested MoAb47, recognizing the same epitope as ab12500 used for IHC in my study, for cross-reactivity with bovine LPO, human MPO and horseradish peroxidase (HRP) in an enzyme-linked immunosorbent assay (ELISA), with negative results. On the contrary, 3/13 (23.1%) of tested mouse monoclonal TPOAb (raised against the whole TPO protein) cross-reacted with bovine LPO, more strongly compared with human MPO, therefore the authors concluded that some TPO epitopes are phylogenetically conserved through evolution and shared by different peroxidases (290).

I therefore conclude that, despite TPO and LPO proteins possibly share similar epitopes, TPO protein expression results obtained with monoclonal antibodies in my study are specific for TPO and any cross-reactivity with LPO can be excluded,

considering that: 1) Western Blot (WB) showed the expected 105-110 kDa band, relative to TPO, while the expected molecular weight of LPO is 80-90 kDa, 2) WB signal reduction was obtained after pre-adsorption of ab76935 with TPO but not with LPO, 3) The epitope recognized by ab12500 used for IHC has already been demonstrated to be absent in LPO (290).

In this study not only were monoclonal antibodies to TPO used, since IF experiments were performed using human sera positive for TPOAb and a possible cross-reactivity with LPO has not been tested. However, this has already been investigated by different authors (291) and will be further discussed in chapter 5, paragraph 5.2.1.

3.5.3 TPO isoforms

Standard PCR gave the best results with primer pair F13/R17 amplifying TPO mRNA from exon 13 to 17, probably because the PCR product is shorter compared with the other primer pairs (Table 3-5). In addition it excludes exon 8, which is large (519 bp) and rich in G and C nucleotides, therefore difficult to amplify. In particular PCR F13/R17 revealed a new TPO mRNA variant without exons 14 and 16, mainly expressed in breast and adipose tissues (Figure 3-7). Standard PCR performed with different primer pairs identified new TPO mRNA variants too, as summarized in Figure 3-10.

In order to identify these new isoforms in their entirety, in particular that identified with F13/R17 PCR, LongRange RT-PCR was used to amplify the full-length TPO mRNA sequence; to my knowledge this is the first study of such kind. A special technique had to be used, considering the extreme fragility and instability of long RNA sequences and consequent difficulty to obtain good quality long cDNA sequences. In fact, due to these existing limitations, the TPO mRNA isoforms known so far and reported in Table 3-1 have been identified amplifying TPO mRNA with standard PCR only from exon 9 to exon 17; therefore, nothing is known about their sequence from exon 2 to 8 (253). LongRange RT-PCR has improved efficiency in amplifying long and difficult tracts of RNA/cDNA by using enzymes and buffer solutions designed to work at elevated temperatures, resulting in improved denaturation of difficult templates. In particular the PCR enzyme mix is a blend of thermostable DNA polymerases with proofreading-assisted fidelity and a high extension rate and the RT buffer contains the RNase H quencher, much more effective in preventing RNA degradation compared with different RNAse inhibitors. Different amplicons were obtained with or without Q solution, usually bigger in the first case: this because Q solution modifies the melting behavior of DNA, therefore facilitates the amplification of cDNA regions rich in C and G nucleotides,

or in general difficult to amplify in normal conditions because of the presence of secondary structures.

LongRange RT-PCR revealed a great variety of newly described isoforms in all examined tissues, mainly preserving exons from 2 to 7, while exons from 8 to 16 were strongly subjected to deletion. Surprisingly, neither the previously described TPO mRNA isoforms (TPO2, TPO3, etc…), nor the newly TPO mRNA variant missing exons 14 and 16 were identified; only TPO5 (missing only exon 8) and one new variant identified with F9/R17 PCR (missing exons from 10 to 14), were identified (Figure 3-11). I hypothesized two possible explanations: 1) many other LongRange RT-PCR amplicons were present but not successfully identified because of intense overlapping of TPO sequences of similar size, 2) alternative START codons not located in exon 2 may exist in TPO mRNA sequence, therefore the above mentioned TPO isoforms would not be detected in a PCR reaction with a forward primer designed in exon 2, as the case of LongRange RT-PCR F2/R17. The last hypothesis is plausible since other in-frame methionine AA (encoded by START codon AUG) are present in TPO mRNA sequence, with one of them (located in exon 3) followed by AA sequences essential for protein insertion into the endoplasmic reticulum (251).

The results of this study suggest that the real number of different TPO mRNA isoforms, resulting from different combinations between exons, is large. If TPO mRNA isoforms corresponding to virtually all the possible combinations between exons from 2 to 17 really exist, their identification would be quite challenging, since they would correspond to transcripts of every length, many having the same size but different nucleotide sequence, therefore impossible to separate on an agarose gel and sequence. However, the highest variety of TPO mRNA isoforms was found in thyroid tissue (TT), while breast cancer (BC) and adipose tissue (AD) showed a much smaller number of transcripts and, more importantly, different between them. In particular, it is important to state that the two different BC analyzed by LongRange RT-PCR, BC 5 and 6, represented respectively ductal and mucinous invasive carcinomas (Table 3-3), therefore it is plausible that they have a different pattern of gene expression. Furthermore, the TPO mRNA variant identified by PCR F13/R17 was expressed only by breast and adipose tissue and expressed in thyroid tissue only as a faint band, while in adipose/breast tissues it was sharper and BC cell lines expressed it much more than TPO1 and the other known isoforms. We could hypothesize that TPO isoforms are not only tissue specific, but in case of BC also may be related to different histological and molecular classification. In order to clarify this aspect, further studies on a larger number of samples are needed.

Another key point is to establish whether these isoforms are translated in the correspondent protein. WB results identified the presence of several additional bands of lower molecular weight compared with entire 105-110 kDa TPO protein (Figure 3-15), disappearing after pre-adsorption of primary antibody with TPO fragments but no LPO, suggesting a specific binding to TPO (Figure 3-14). If these proteins really exist, further studies are needed to investigate their function and immunogenic properties. The region included from exon 5 and exon 12 encodes for the myeloperoxidase (MPO) like sequence which contains important catalytic residues, so we can hypothesize that TPO isoforms that lack exons in this region are not functional, as in fact has been already demonstrated for TPO 2 that lacks exon 10 (252) and TPO 5 that lacks exon 8 (253). However, 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. Furthermore, smaller TPO isoforms are likely to fold differently compared with full-length protein, therefore exposing different conformational epitopes; TPO epitopes will be discussed in chapter 5, paragraph 5.2.2. In an alternative hypothesis, smaller MW bands present on WB could represent degraded parts of full length TPO protein. WB also showed the presence of bigger bands of 150-170 kDa and above, already described in previous studies and found to be TPO homodimers (292). In order to solve the question of additional bands, further experiments including protein sequencing need to be performed.

Finally, in presence of a so intense TPO mRNA alternative splicing phenomenon, the possibility of template switching was taken into consideration. Template switching is a phenomenon that may happen during RT-PCR processes, leading to the amplification of an artificially deleted cDNA, which can be wrongly interpreted as an alternative transcript (293). Several mechanisms may be involved: 1) template switching (ability to switch from a template to another presenting a homologous region) of the reverse transcriptase enzyme during cDNA synthesis, 2) mRNA hydrolysis, 3) cDNA synthesis interruption caused by strong secondary RNA structures, 4) template switching of the DNA polymerase enzyme during cDNA copying (294). In this study specific experiments in order to exclude the above mentioned possibilities were not performed. However, the phenomenon of template switching is unlikely to explain the presence of TPO mRNA alternative and shorter transcripts since: 1) the cDNA sequencing revealed that TPO isoforms transcripts corresponded only to exonic sequences, with the intronic sequences as the only exclusively missing, 2) LongRange RT-PCR, working at higher

temperature, is less subject to RNA/cDNA secondary structures potentially leading to template switching phenomenon. Future experiments searching for the correspondent protein of TPO isoforms transcripts would also help to solve this doubt.

4 TPOAb IMPACT ON BREAST CANCER PROGNOSIS

4.1 INTRODUCTION

As previously discussed in chapter 1, paragraph 1.4.3, thyroid autoimmunity (TA) and in particular anti-thyroid peroxidase (TPO) autoantibodies (TPOAb), have been found to be more prevalent among patients with breast cancer (BC) compared with the general population: 36% versus 19% (*P*<.001)(179), 23.5% versus 8% (*P*=.002)(180) and 15% versus 9.8% [Odds Ratio= 3.0; 95% Confidence Interval (95%CI), 1.4-6.5](166). However, these findings have not been confirmed in another study that observed no differences in the incidence of thyroid autoimmune diseases between BC patients and controls (163).

In the literature the prevalence of TPOAb and TgAb among the general population has been estimated to be 13% (8.7% males, 17% females) and 11.5% (7.6% males, 15.2% females) respectively (63), with other authors describing an even higher prevalence of general autoantibodies to the thyroid (TAb: TPOAb & TgAb combined) of 26.4%, (64). All authors agree that TPOAb and TgAb prevalence in general population increases with age (63-67).

Regarding thyroid function, hypothyroidism has been associated with both an increased (166, 295, 296) or reduced (167, 168) risk of developing BC, whilst other authors did not report any correlation (159, 191, 297-299).

Of relevance, TA but more specifically TPOAb, have been found to be significantly associated with an improved prognosis for BC. These results were obtained in small-scale studies, with the majority agreeing but with also some not confirming, as discussed in the next paragraph 4.1.1. Considering that, as shown in chapter 3, TPO is expressed in BC tissue, albeit at a lower level compared with thyroid tissue, this could provide a possible mechanistic link: a thyroid/breast shared cytotoxic autoimmune response against a common antigen (likely TPO) might target tumour cells and improve BC outcome. The increased prevalence of circulating TPOAb could represent the epiphenomenon of such protective immune activity and blood TPOAb could therefore represent an indicator of good BC prognosis.

The aim of the present study was to clarify the impact of circulating TPOAb on BC prognosis in a large, well powered cohort of patients, following the "REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines" (300). The Taxotere as Adjuvant Chemotherapy Trial (TACT) recruited

4162 women diagnosed with early BC and an indication for adjuvant chemotherapy to evaluate whether sequential docetaxel (Taxotere) after traditional anthracycline therapy would improve patient outcome compared with standard anthracycline chemotherapy (301). Of relevance, stored plasma was available in a significant number of these patients, permitting retrospective analysis of the impact of TPOAb and thyroid hormone status on BC prognosis with 97.5 months medium follow-up available (302).

Currently there are no major validated blood prognostic markers in BC: as previously discussed in chapter 1, paragraph 1.3.5, cancer antigen 15.3 (Ca 15.3) and carcinoembryonic antigen (CEA) are characterized by low specificity and sensitivity, while circulating tumour DNA (ctDNA) and circulating tumour cells (CTCs) represent very promising blood tumoural markers, but are not yet validated in routine clinical practice.

Therefore, assessing TPOAb as a prognostic marker, measurable in blood, for BC in a large and mature cohort would provide an important clinical observation with possibilities to develop TPO-targeted therapies in the future. In this study the thyroid function parameter has also been taken into consideration, since thyroid hormones have been also described as potential factors influencing BC prognosis, even if with contrasting conclusions, as described in the next paragraph 4.1.1.2.

4.1.1 Impact of thyroid autoimmune diseases on breast cancer prognosis

4.1.1.1 Thyroid autoimmunity

A better BC outcome has been reported in TPOAb positive (TPOAb+) versus TPOAb negative (TPOAb-) patients in most (182-184, 260), but not all (185) studies.

In particular, Smyth et al. evaluated the 5 years disease-free survival (DFS) and overall survival (OS) of 142 women affected with BC. TPOAb absence resulted in a poor prognostic factor for both DFS [Relative Risk (RR) 1.84, p=0.047)] and OS (RR 3.46, p=0.016) and this effect was confirmed in the multivariate analysis with other BC risk factors (182). Fiore et al. analyzed the 5 years OS of a small but homogeneous group of 47 women affected with high malignancy ductal infiltrating BC and found a 5 years mortality of 6.7% in patients positive for TAb, mainly TPOAb, compared with 46.9% in negative patients and this protective effect was confirmed in the multivariate analysis with other BC risk factors (183). In contrast, Jiskra et al. prospectively followed 84 patients with BC for a median of 136 months and found no impact of TPOAb on BC prognosis in terms of both DFS and

OS, registering a BC recurrence in 26.7% and 25.9% of patients affected respectively with and without TA (185).

A protective role for TPOAb was also suggested by Farahati et al. in a study conducted among 314 patients newly diagnosed with BC. Authors found an absence of distant metastasis among BC patients with TPOAb positivity (0/56) compared with metastasis prevalence of 6.6% (17/258) among TPOAb negative patients (184). Kim et al. prospectively followed 564 patients affected with BC for a median time of 36 months and incidentally found that an increased thyroid uptake of ^{18}F-FDG on PET/CT images due to the presence of thyroid autoimmunity was related to a significantly improved DFS, especially for locally advanced and secondary cancer stage (260).

4.1.1.2 Thyroid function

The role of thyroid function on BC prognosis has been debated during the last century and conflicting results are present in the literature. As previously introduced in paragraphs 1.4.1 and 1.4.2, since 1896 thyroid extract was proposed as a treatment for BC, based on the suspicion that hypothyroidism could represent a poor prognostic factor for BC (153, 303). In contrast, subsequent studies proposed thyroid supplementation as a possible risk factor for BC (304), not confirmed by other studies (305, 306).

However, subsequent findings supported the possibility that increased levels of thyroid hormones could be a poor prognostic factor for BC. In particular, in 2005 an American retrospective study based on 2226 women, reported that hypothyroid BC patients are more likely to be diagnosed with a BC at an earlier stage, of smaller size and without pathologic lymph node involvement (167). In agreement with these findings, a recent Swedish population-based prospective cohort study based on 2185 women (followed for more than 20 years) showed that baseline triiodothyronine (T3) serum levels were associated with an increased risk of BC specific death and with the presence of more aggressive BC, in particular larger, ER and PR negative and with a higher prevalence of positive lymph node metastases (307, 308).

Well powered prospective studies evaluating the effects of thyroid function on BC prognosis in terms of recurrence and survival are lacking.

4.1.2 Impact of thyroid autoimmunity on other cancer prognosis

TAb impact on cancer prognosis has also been analyzed in other tumours. Franke et al. analyzed 329 patients with metastatic renal cell cancer and found that patients positive for circulating TAb, present both before or triggered by the treatment with IL-2 and IFN-α2, had a statistically significant better 5 years OS

compared with patients negative for TAb (270). In contrast, Fyssas et al. showed that 33 patients with pancreatic adenocarcinoma had an increased prevalence of TAb (25%), mainly TPOAb, compared with healthy controls, but their presence was associated with a survival reduction of 2.4 months: 9.4 and 11.8 months respectively in TAb positive and negative patients (271).

4.1.3 Major breast cancer known prognostic factors

In order to correctly analyze the effects of two potential prognostic factors for BC such as TPOAb and thyroid function and exclude possible biases, it is essential to evaluate in the same study the impact of already known prognostic factors for BC that will be described in the present paragraph. Furthermore, a brief overview of the currently available treatments for BC will be provided, in order to better comprehend this observational study where patients were exposed to BC treatments. The key-elements of BC molecular classification already described in chapter 1, paragraph 1.3.3.2, such as the expression of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2), represent known BC prognostic factors and are also crucial for BC treatment decision making.

4.1.3.1 Tumour size, lymph node metastasis and TNM staging

When distant metastases are present, irrespective of their size, number and location, the survival of patients affected with BC suddenly drops. After distant metastases, tumour size and lymph node metastatic involvement are the two most important prognostic factors for BC (309). These 3 elements represent the basis of the TNM staging classification for cancer, that has been adopted worldwide for many years and initially developed by Denoix of France, based on the description of the primary tumour (T), the regional lymph nodes involvement (N) and the presence or absence of distant metastases (M) (310).

There are many different subcategories of T, N and M listed in ascending order of numbers and letters, directly proportional to the increasing tumoural size and extension. For example, T category is described with a number from 0 (absence of evidence) to 4 (any tumour size with extension to chest wall or skin), with letters (a, b, c, d) providing additional information. The acronym *Tis* indicates the presence of a carcinoma in situ, characterized by malignant epithelial cells without evidence by light microscopy of invasion through the basement membrane. The acronym *T1mi* indicates a tumour with dimensions of 0.1 cm across or less. A similar system is used for N, but the maximum number and letter used are respectively 3 and c. Distant metastases are assessed by only 2 numbers, 0 and 1, indicating respectively absence or presence of distant

metastases, without distinction in their number and location. The letter X indicates that the parameter cannot be assessed and it can be applied to all 3 categories T, N and M. The subcategories of T, N and M are then grouped in various combinations to describe four different tumoural staging, as summarized in Table 4-1. TNM staging is a good prognostic indicator, with worsening of BC prognosis according with increasing TNM stage (311).

Table 4-1: BC TNM staging of breast cancer

BC Stage		T	N	M
0		*Tis*	N0	M0
I		T1*	N0	M0
II	IIA	T0 or T1*	N1	M0
		T2	N0	M0
	IIB	T2	N1	M0
		T3	N0	M0
III	IIIA	T0 or T1* or T2	N2	M0
		T3	N1	M0
		T3	N2	M0
	IIIB	T4	N0 or N1 or N2	M0
	IIIC	Any T	N3	M0
IV		Any T	Any N	M1

Adapted from (311). BC= breast cancer. N= regional lymph nodes involvement. M= distant metastases. T = primary tumour. *Tis*: carcinoma in situ. *T1 includes *T1mi*.

Based on this classification, generally BC is commonly subdivided into 3 different categories: 1) "Early BC", when potentially curable by surgery since it is not locally advanced and with absence of distant metastasis (stage 0, I, II and T3N1M0 case of stage IIIA) 2) "Locally advanced BC" (stage III except T3N1M0) 3) "Metastatic BC" (stage IV).

4.1.3.2 Breast cancer histological grading

Invasive BC are routinely graded based on the assessment of 3 biological parameters: 1) tubule and gland formation, 2) nuclear pleomorphism, 3) mitotic count. Each parameter is classified with an individual score from 1 to 3 and the final grading reflects the total score deriving from the 3 parameters taken together: 1 or low (score: 3-5), 2 or medium (score: 6-7), 3 or high (score: 8-9) (136).

The initial grading score system based on methods from Patey & Scarff (312) and Bloom & Richardson (313) was characterized by a high variability across different centres and became more objective after its modification by Elston & Ellis, known as the "Nottingham grading system" (314). Since then, histological

grading has been routinely adopted to assess BC prognosis and many studies demonstrated a significant association between histological grade and survival of BC patients (inverse correlation: high grades are associated with shorter survivals), assessing histological grade as the fourth main prognostic factor for BC after the presence of distant metastases, tumour size and metastatic lymph node involvement (315, 316).

4.1.4 Prognostic tools and decision-making for breast cancer treatment

BC treatment is highly dependent on BC classification and phenotype, as shown in this paragraph. The main types of treatment for BC are surgery, radiation therapy (RT), chemotherapy (CT), hormonal therapy and targeted therapy:

- Surgery. Breast conservation surgery, consisting in the removal of just the lump or area of cancer and also called wide local excision (WLE), is the trending approach in the treatment of localized BC. The alternative is mastectomy, where the whole breast is removed; usually it is required when the tumour is large or multifocal (i.e. multiple areas of cancer in different parts of the breast). Sometimes surgery can be preceded by neo-adjuvant therapy (either RT or CT) in order to shrink the tumour bulk (317). In the case of lymph node metastasis, the surgery of the axilla is recommended, however axillary dissection may be omitted in patients with one or two positive sentinel nodes following breast-conserving surgery when whole breast RT is planned (318).
- RT is a process where cancer cells are exposed to high level of radiation directly. This is extremely recommended as adjuvant treatment (i.e. following the surgery) especially after performing WLE, since it may kill cancerous cells not seen during surgery and therefore not removed (317).
- Systemic CT is usually recommended as adjuvant treatment in particular cases, especially those not-responding to endocrine treatments because of histological type (e.g. apocrine, medullary, adenoid cystic and metaplastic) or molecular phenotype (ER negative, high Ki-67 levels) (318). The main drugs used in CT for BC, usually in combinations, are: anthracyclines (epirubicin, doxorubicin, mitoxantrone), cyclophosphamide, fluorouracil, methotrexate, mitomycin, docetaxel (Taxotere) and gemcitabine (319). A discussion of different chemotherapy protocols for BC is beyond the aims of the present thesis.
- Endocrine treatment aims to block the oestrogen stimulation pathway in BC and it is indicated in all patients with detectable ER expression, defined as

≥1% of ER+ cells. The choice of medication is primarily determined by the patient's menopausal status (317). The first treatment choice for premenopausal patients is a selective oestrogen receptor modulator called "Tamoxifen", usually taken for 5 years, exerting anti-oestrogen effects on BC cells (therefore acting as an ER antagonist), but paradoxically having ER agonist effects on different cells and tissues, e.g. bone and blood lipids (320). In case of contraindications to the use of Tamoxifen, an alternative treatment is the use of a gonadotropin-releasing hormone (GnRH)-agonist (e.g. goserelin), determining a reduction in oestrogen release. GnRh-agonists may be prescribed alone or in association with aromatase inhibitors, that block the peripheral conversion of androgens into oestrogens (317). In postmenopausal women, both Tamoxifen and aromatase inhibitors are valid options, usually prescribed for 5-10 years (317).

- Targeted therapy. As described below, HER2 protein expression allows the use of highly effective targeted treatments. The most renowned is trastuzumab (Herceptin®), a monoclonal antibody interfering with HER2 receptor. It can be used also in combination with endocrine treatments, RT and CT however, due to its cardiotoxicity, it should not be routinely administered concomitantly with anthracyclines. However new alternative target therapy drugs have been developed, such as lapatinib, a dual tyrosine kinase inhibitor which interrupts both HER2 and epidermal growth factor (EGF) receptor pathways. Their clinical use is likely to increase and substitute trastuzumab in the future (317).

In conclusion BC molecular biomarkers, previously described in paragraph 1.3.3.2, have become more and more important in BC prognostic assessment and treatment decision-making. In particular:

- The positivity of oestrogen receptor (ER) and/or progesterone receptor (PR) (range: ≥1% - 100%) represents the indication to hormonal therapy and the likelihood of response to this treatment is directly correlated to the level of their expression; only entirely negative tumours (0%) are essentially unresponsive (321). Four possible phenotypes of ER/PR combined expression are possible and each of them is associated with significantly different rates of response to hormonal therapy, which would not be apparent measuring ER or PR only (322). The phenotype ER-positive/PR-positive is the most frequent (70%) and associated with the best rate of response (60%). ER-negative/PR-negative is the next common combination (25%) and these tumours are essentially unresponsive (0%). The remaining two discordant phenotypes are associated with intermediate response rates (136).

- Recent studies demonstrate that HER2-positive invasive BC respond favorably to therapies that specifically target the HER2 protein, e.g. trastuzumab (Herceptin®) or lapatinib. The best response to HER2-targeted therapy in any clinical setting is shown by positive HER2 tumours showing strong (3+) or moderately strong (2+) HER2 protein expression by IHC (136, 323).

Complex multivariate outcome prediction models combining different clinical, histological, biological and molecular prognostic factors for BC have been developed as prognostic tools to choose the best regimen of treatment and to predict the probability of response (324). The two most often used are The Nottingham Prognostic Index and Adjuvant! Online (http://www.adjuvantonline.com) (325, 326).

For example, in ER-positive/HER2-negative BC, the option of adding chemotherapy to hormonal therapy is evaluated according to the tumour size, nodal status, occurrence of peri-tumoural vascular invasion, grade and proliferative fraction (Ki67) (141).

4.1.5 Aims of the study

The aim of the present study was to clarify the impact of circulating TPOAb and thyroid function on BC prognosis in a large and well-powered cohort of patients affected with BC having blood samples available, previously enrolled in the TACT study (301). The median long-term follow-up was 97.5 months, resulted from a subsequent more mature analysis of the same trial population (302).

4.2 MATERIALS AND METHODS

4.2.1 Patients

The Taxotere as Adjuvant Chemotherapy Trial (TACT) was a multicentre, open-label, phase III, randomized controlled trial of women aged more than 18 years diagnosed with operable early BC (pT1-3a,pN0-1,M0), with an indication for adjuvant chemotherapy, including patients both lymph-node positive (node+) and high risk (eg, tumour grade 3, hormonal-receptor expression negative, or lympho-vascular invasion) lymph-node negative (node-). A total of 4162 women were enrolled between February 2001 and June 2003 across 103 UK centres and one Belgian centre (including specialist cancer hospitals, university teaching hospitals, and smaller community general hospitals). Additional inclusion criteria were normal hematological, hepatic and renal function. Exclusion criteria included locally advanced or distant disease, bilateral breast cancer, pregnancy, and previous invasive malignancy within 10 years (301).

All subjects underwent surgery (mastectomy or wide local excision) and were randomized in a 1:1 ratio to the experimental regimen FEC-D [n=2073; fluorouracil 600mg/m^2, epirubicin 60mg/m^2, cyclophosphamide 600mg/m^2 (FEC) at 3-weekly intervals for four cycles followed by docetaxel 100mg/m^2 at 3-weekly intervals for four cycles] or centre's choice of control chemotherapy, either FEC for eight cycles (n=1265) or epirubicin 100mg/m^2 at 3-weekly intervals for four cycles followed by CMF (cyclophosphamide 600mg/m^2, methotrexate 40mg/m^2, and fluorouracil 600mg/m^2 at 4-weekly intervals) for four cycles (E-CMF, n=824).

Adjuvant radiotherapy, starting within 4 weeks of chemotherapy, was mandatory after breast conserving surgery or used after mastectomy according to local guidelines.

Endocrine treatments (tamoxifen monotherapy, tamoxifen followed by aromatase inhibitor or aromatase inhibitor monotherapy) were administered to patients with positive oestrogen receptor (ER) expression (ER+). Patients positive for human epidermal growth factor receptor 2 (HER2) expression (HER2+) were allowed to enter clinical trials assessing trastuzumab.

Analyses at 62 months (301) and 97.5 months (302) of follow-up respectively showed no evidence of a difference and little clear benefit in terms of BC outcome in patients treated with sequential docetaxel after traditional anthracycline therapy (FEC-D), compared with the control chemotherapy regimens (FEC, E-CMF).

4.2.2 Laboratory measurements

In November 2002, following a protocol amendment, patients were asked to provide one 10ml EDTA blood sample to extract plasma for future translational research. Consenting patients who had been already recruited provided a blood sample at their next follow-up visit; new patients had blood taken at the time of randomization.

Samples were stored at -20°C for a range of 6.5-13 years at the Institute of Cancer Research - Clinical Trials & Statistics Unit (ICR-CTSU, London, Surrey). All available samples (one aliquot each) were transferred to the Institute of Molecular & Experimental Medicine (Cardiff) for TPOAb, thyroid stimulating hormone (TSH) and free-thyroxine (FT4) analysis. Stored plasma samples were thawed, aliquoted and analyzed in the same assays in October 2014 in the Department of Medical Biochemistry, School of Medicine, Cardiff University, using an ADVIA Centaur automated immunoassay analyzer (Bayer plc, Newbury, Bucks, UK) and Chemiluminescent Microparticle Immunoassay (CMIA) methods by the ARCHITECT®

System (ABBOTT Laboratories, Abbott Park, USA). TPOAb positivity cut-off was ≥6 kU/L; FT4, TSH normal ranges respectively 9.0–19.1 pmol/L and 0.30–4.40 mU/L.

TPOAb values were dichotomized as ≥6 kU/L (positive: TPOAb+) versus <6 kU/L (negative: TPOAb-); TPOAb+ were also categorized into tertiles (exploratory analysis). FT4 and TSH were combined in a thyroid function status variable: euthyroidism (FT4 and TSH within the normal ranges), hypothyroidism (FT4 <9.0 pmol/L and/or TSH >4.40 mU/L); hyperthyroidism (FT4 >19.1 pmol/L and/or TSH <0.3 mU/L).

4.2.3 Statistical analysis

When formulating the study hypothesis, it was anticipated that 20% of individuals with BC will be TPOAb+ in keeping with its prevalence in females in the general population (64, 327). Assuming the average 5-year OS from BC in UK is 82% (328, 329), the TACT trial, with more than 1900 available plasma samples and 5-year DFS and OS rates of 74.9% and 82.7% respectively (301), was a suitable candidate trial in which to test the study hypothesis. Power calculations indicated that 1158 and 1430 samples would be needed to provide respectively 80% and 90% of power to detect a 81% 5-year DFS in TPOAb+ versus 73% in TPOAb- subjects (HR 0.64; two-sided log rank test with a .05 probability of a type I error), consistent with a DFS rate in the whole cohort of 74.9%.

To assess whether the thyroid antibody project samples were representative of the TACT population, baseline characteristics, BC treatments and DFS-related characteristics for all patients with blood samples available for analysis were compared with TACT patients not included in our study and all patients recruited in the TACT trial (n=4162). Baseline characteristics were also presented by dichotomized TPOAb (TPOAb+, TPOAb-) and thyroid function status (hypothyroid, euthyroid, hyperthyroid). Correlations between biomarkers (TPOAb, TSH, FT4) were assessed using the Spearman rank method.

The primary outcome was to assess the prognostic significance of circulating TPOAb in relation to DFS as defined in the main TACT trial (301). Secondary outcomes were the prognostic significance of circulating TPOAb in relation to OS and time to recurrence (TTR) and blood TSH and FT4 levels in relation to DFS, OS and TTR.

For DFS, OS and TTR, Kaplan-Meier curves were plotted and biomarkers compared by use of the log-rank test. Hazard Ratio (HR) with 95% CI were obtained from Cox proportional hazards regression models stratified by each centre's choice of control chemotherapy regimen and ER status, with HR<1 indicating a better BC prognosis. The proportionality assumption of the Cox model

was tested with Schoenfeld's residuals and found to hold. All biomarkers were assessed initially in a univariate Cox model and subsequently included in a multivariable Cox model along with known prognostic factors for BC: age, HER2 status and trastuzumab use, nodal involvement, tumour size and tumour grade. Additional variables such as trial treatment (experimental versus control), type of surgery, radiotherapy use and menopausal status were included if, by stepwise selection ($p<0.05$), was shown to add value. Once the optimal model for the primary endpoint was established, TSH and T4 were considered for inclusion to investigate whether they added independent prognostic information in addition to TPOAb. Interaction tests were also used to explore differential effects within subgroups (TPOAb with trial treatment, age, HER2 status, nodal involvement, tumour size and tumour grade).

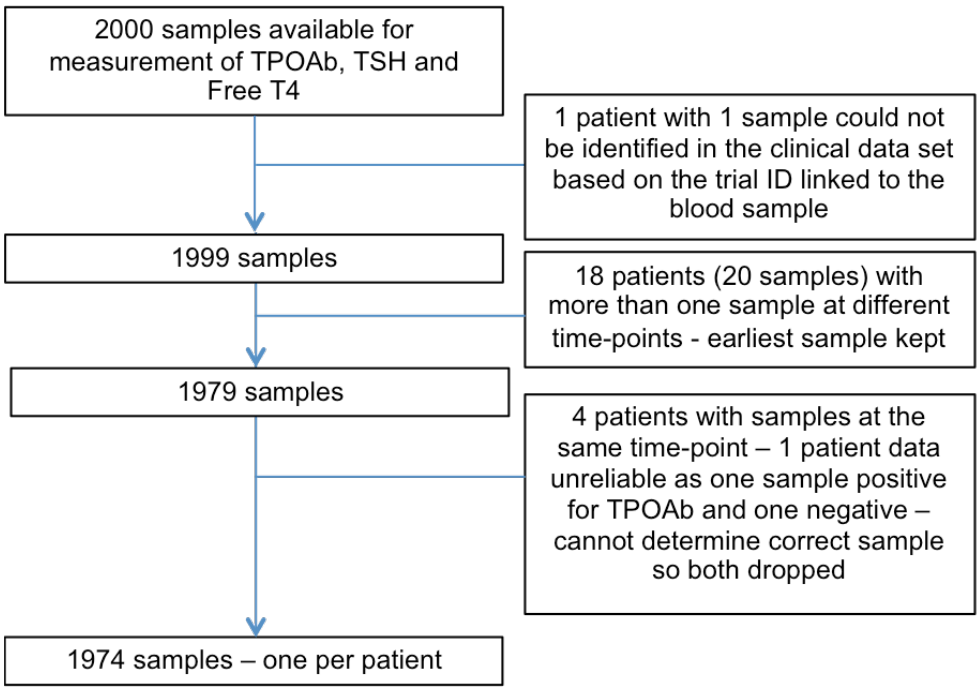
All patients with a biomarker value available were included in the analysis, regardless of whether they were found to be ineligible, a protocol violator, given the wrong treatment allocation, never treated, etc. in the main TACT trial (as per an intention to treat analysis). Comparisons were made by randomized treatment where appropriate. Sensitivity analyses including node+ patients who did not receive radiotherapy (similar to Fiore *et al*. population (183)) and patients with blood samples taken after surgery but before any adjuvant cancer therapy were conducted. All analyses were conducted using Stata version 13.1 (STATACORP, College Station, TX). This analysis was based on a database snapshot taken on 25 November 2011 when median follow-up was 97.5 months [Interquartile Range (IQR): 87.6-107.4, range 0-126.9](301, 302).

My part in this study was: i) to formulate and design the study, in collaboration with my supervisors (e.g. establishment of the principal and secondary outcomes); ii) to collect and aliquot the blood samples from ICR-CTSU and ensure they were measured in the Department of Medical Biochemistry, School of Medicine, Cardiff University, with results correctly reported on an Excel database; iii) to interpret the blood results (e.g. subdividing patients in different categories of thyroid function); iv) to direct the statistical analysis (e.g. suggesting to analyze the correlation between TPOAb, TSH and FT4 or suggesting specific sensitivity analyses); v) to analyze and interpret the results according to the current available literature. The calculations of the statistical analysis have been performed by Lucy Kilburn, statistician at the ICR-CTSU, since this Department holds the clinical data and preferred to directly deal with them.

4.3 RESULTS

All available plasma samples taken for the TACT trial (n=2000) were analyzed for thyroid biomarkers. After excluding multiple samples from the same patients, retaining the sample with the earliest time-point, a final number of 1974 (47.4%) samples (defined as the "analysis population"), one for each patient, was considered for the statistical analysis (Figure 4-1).

Figure 4-1: Flowchart regarding sample availability and data handling



Blood collection date was available in 1914/1974 (97%) patients and its median was 15.5 months (IQR= 7.0-24.0 months; range= 0.5-57.2 months] after surgery. Median follow-up in the analysis population was 96.7 months (IQR: 87.4-106.3, range 3.4-126.4). According to our power calculation, this number was adequate for this assessment, with a margin for error for the final proportion found to be TPOAb+ and differences in DFS in the subpopulation studied. In summary, patients included in the analysis population (TPOAb study: n=1974) had a mean age of 49.1 years [standard deviation (SD)= 8.4 years], 1567 (79.4%) were node+, 1079 (54.7%) had a mastectomy, 1745 (88.4%) had radiotherapy, 1396 (70.7%) were ER+ of which 1378 (98.7%) received hormonal therapy and 408 (20.7%) were HER2+, of which 48 (11.8%) were treated with trastuzumab (Table 4-2 and Table 4-3). Patients included in the analysis population were not comparable with not included TACT patients (not having blood available: n=2188) in terms of baseline characteristics (Table 4-2), BC treatments received and events contributing to DFS analysis (Table 4-3). In particular, patients within the

analysis population were more often treated with trastuzumab (11.8% vs 6.4%) and had less distant recurrence (20.5% vs 26.1%) and less deaths (20.1% vs 28.3%), both BC-related (18.7% vs 26%) or not (1.4% vs 2.4%), compared with not included patients.

Table 4-2: Baseline characteristics of all TACT trial participants, those in the TPOAb study and non-studied women

		TACT trial N=4162	TPOAb study N=1974	Not-studied N= 2188
Age (years), mean (SD);	*p<0.001*	48.6 (8.5)	49.1 (8.4)	48.2 (8.6)
Age group (years), n (%);	*p=0.02*			
<40		718 (17.3)	306 (15.5)	412 (18.8)
40-49		1567 (37.7)	726 (36.8)	841 (38.4)
50-59		1487 (35.7)	757 (38.4)	730 (33.4)
≥60		390 (9.4)	185 (9.4)	205 (9.4)
Nodal status, n (%);	*p=0.09*			
Node negative		835 (20.1)	407 (20.6)	428 (19.6)
1-3 positive nodes		1839 (44.2)	890 (45.1)	949 (43.4)
≥4 positive nodes		1488 (35.8)	677 (34.3)	811 (37.1)
Tumour grade, n (%);	*p=0.56*			
Grade 1		229 (5.5)	100 (5.1)	129 (5.9)
Grade 2		1536 (36.9)	758 (38.4)	778 (35.6)
Grade 3		2382 (57.2)	1111 (56.3)	1271 (58.1)
Unknown		15 (0.4)	5 (0.3)	10 (0.5)
Tumour size, n (%);	*p=0.003*			
≤2cm		1436 (34.5)	725 (36.7)	711 (32.5)
>2 and ≤5cm		2330 (56.0)	1077 (54.6)	1253 (57.3)
>5cm		392 (9.4)	171 (8.7)	221 (10.1)
Unknown		4 (0.1)	1 (0.1)	3 (0.1)
ER & HER2 status, n (%);	*p=0.01*			
ER+		2875 (69.1)	1396 (70.7)	1479 (67.6)
& HER2+		494 (11.9)	247 (12.5)	247 (11.3)
& HER2-		1963 (47.2)	973 (49.3)	990 (45.2)
& HER2 unknown		418 (10.0)	176 (8.9)	242 (11.1)
ER-		1287 (30.9)	578 (29.3)	709 (32.4)
& HER2+		355 (8.5)	161 (8.2)	194 (8.9)
& HER2-		761 (18.3)	350 (17.7)	411 (18.8)
& HER2 unknown		171 (4.1)	67 (3.4)	104 (4.8)
Molecular subgroup, n (%);	*p=0.01*			
ER+/HER2-*		2001 (48.1)	987 (50.0)	1014 (46.3)
HER2+		849 (20.4)	408 (20.7)	441 (20.2)
Triple negative		723 (17.4)	336 (17.0)	387 (17.7)

* includes ER-, PgR+, HER2-

ER = oestrogen receptor. HER2 = human epidermal growth factor receptor 2. PgR = progesterone receptor. SD = Standard deviation.

Table 4-3: BC treatments and DFS events of all TACT trial participants, those in the TPOAb study and non-studied women

	TACT trial N=4162	TPOAb study N=1974	Not-studied N= 2188
Type of surgery and radiotherapy, n(%); *p=0.08*			
Mastectomy	2265 (54.4)	1079 (54.7)	1186 (54.2)
with radiotherapy	1814 (43.6)	865 (43.8)	949 (43.4)
breast	413 (9.9)	159 (8.1)	254 (11.6)
chest wall	1402 (33.7)	709 (35.9)	693 (31.7)
supraclavicular fossa	980 (23.5)	480 (24.3)	500 (22.9)
axilla	188 (4.5)	85 (4.3)	103 (4.7)
Wide local excision	1897 (45.6)	895 (45.3)	1002 (45.8)
with radiotherapy	1841 (44.2)	880 (44.6)	961 (43.9)
breast	1777 (42.7)	856 (43.4)	921 (42.1)
chest wall	78 (1.9)	31 (1.6)	47 (2.1)
supraclavicular fossa	574 (13.8)	291 (14.7)	283 (12.9)
axilla	173 (4.2)	103 (5.2)	70 (3.2)
Endocrine treatment in ER+ patients, n (%); *p=0.01*			
Tamoxifen monotherapy	1790 (62.3)	863 (61.8)	927 (62.7)
Tamoxifen followed by AI	893 (31.1)	454 (32.5)	439 (29.7)
AI monotherapy	137 (4.8)	61 (4.4)	76 (5.1)
No endocrine treatment/unknown	55 (1.9)	18 (1.3)	37 (2.5)
Trastuzumab in HER2+ patients, n (%); *p=0.02*			
Yes	76 (9.0)	48 (11.8)	28 (6.4)
No/Not known	773 (91.0)	360 (88.2)	413 (93.7)
Chemotherapy, n (%); *p<0.001*			
Control (FEC)	1265 (30.4)	626 (31.7)	639 (29.2)
Control (E-CMF)	824 (19.8)	332 (16.8)	492 (22.5)
FEC-D	2073 (49.5)	1016 (51.5)	1057 (48.3)
Number of patients with event contributing to DFS analysis; *p<0.001*	**1329 (31.9)**	**551 (27.9)**	**778 (35.6)**
Local recurrence	183 (4.4)	76 (3.8)	107 (4.9)
Distant recurrence	977 (23.5)	405 (20.5)	572 (26.1)
New breast disease	91 (2.2)	43 (2.2)	44 (2.0)
Death from other cause (no recurrence)	78 (1.9)	27 (1.4)	51 (2.3)
Distant relapse ever reported; *p<0.001*	**1117 (26.8)**	**462 (23.4)**	**655 (29.9)**
New breast disease ever reported; *p=0.78*	**124 (3.0)**	**57 (2.9)**	**67 (3.1)**
All non-breast cancer second primary; *p=0.77*	**106 (2.5)**	**52 (2.6)**	**54 (2.5)**
All deaths; *p<0.001*	**1017 (24.4)**	**397 (20.1)**	**620 (28.3)**
Breast cancer	937 (22.5)	369 (18.7)	568 (26.0)
Death from other causes	80 (1.9)	28 (1.4)	52 (2.4)
Cancer (non-breast)	36 (0.9)	15 (0.8)	21 (1.0)
Treatment toxicity	5 (0.1)	0	5 (0.2)
Other	39 (0.9)	13 (0.7)	26 (1.2)

* includes ER-, PgR+, HER2-

AI = Aromatase Inhibitors. DFS = disease-free survival. ER = oestrogen receptor. E-CMF = epirubicin 100 mg/m^2 for 4 cycles followed by CMF (cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2 and fluorouracil 600 mg/m^2) for 4 cycles. FEC = fluorouracil 600 mg/m^2, epirubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 for 8 cycles. FEC-D = FEC for 4 cycles followed by docetaxel 100 mg/m^2 for 4 cycles. HER2 = human epidermal growth factor receptor 2.

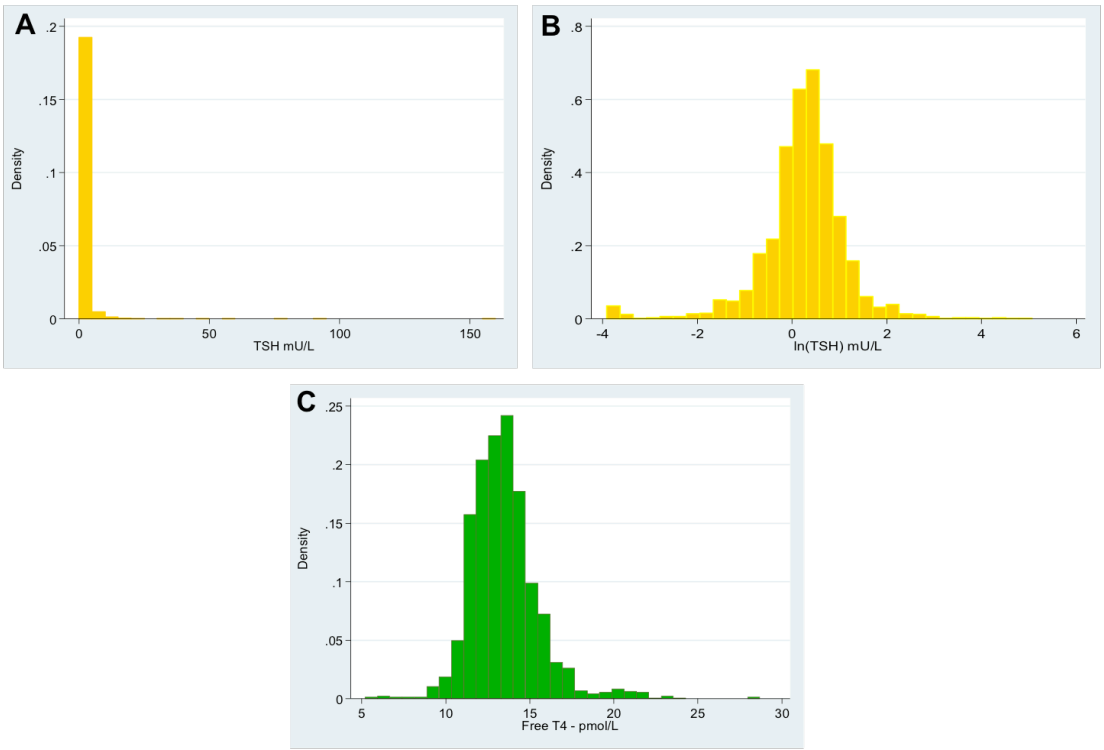
The total number of events available in the analysis population for DFS was 551 (27.9%), with overall 5-year estimate of 79.5% (95%CI 77.6-81.2). For OS, 397 (20.1%) events were available, with overall 5-year estimate of 87.4% (95%CI 85.9-88.8). For TTR, 498 (25.2%) events were available, with overall 5-year estimate of 81.1% (95%CI 79.3-82.8). TACT patients without blood samples available, therefore not included in the analysis population, had overall 5-year estimates of 71.4% (95%CI, 69.5-73.3), 79.0% (95%CI, 77.3-80.7) and 73.7% (95%CI, 71.7-75.5) for DFS, OS and TTR respectively.

4.3.1 Distribution of TPOAb and thyroid function

TPOAb positivity was detected in 406 (20.6%) patients, distributed in the following tertiles: 137 (6.9%) 6-40 kU/L (T1), 134 (6.7%) 41-238 kU/L (T2) and 135 (6.8%) 240-2000 kU/L (T3).

Mean FT4 value was 13.5 pmol/L ± 2.1 standard deviation (SD), range 5.2 – 28.7 pmol/L. TSH mean value was 2.05 mU/L ± 5.49 SD, range: 0.02 – 159.83 mU/L. FT4 values were normally distributed, while TSH values were right skewed, therefore the logarithm of TSH values, ln(TSH), was used in the statistical analyses involving TSH as a continuous variable (Figure 4-2). When FT4 and TSH were considered combined as thyroid function, 1760 (89.2%), 96 (4.9%) and 118 (6.0%) patients were found to be respectively euthyroid, hypothyroid and hyperthyroid.

Figure 4-2: Histograms of TSH, ln(TSH) and FT4 values



Free T4 = free thyroxine; TSH = thyroid-stimulating hormone; ln(TSH) = logarithm of TSH values.

Baseline characteristics (Table 4-4) and BC treatments (Table 4-5) by TPOAb status were largely comparable, apart from age, where TPOAb+ patients tended to be slightly older than TPOAb- patients, as expected (mean age ± SD = 50.2±7.7 years versus 48.8±8.5 years; p=0.005). Baseline characteristics (Table 4-4) and BC treatments (Table 4-5) were similar between the different thyroid function classifications.

Table 4-4: Baseline characteristics by TPOAb and thyroid function status

	TPOAb- N=1568	TPOAb+ N=406	Hypothyroid N=96	Euthyroid N=1760	Hyperthyroid N=118
Age (years): mean (SD)	48.8 (8.5)	50.2 (7.7)	50.5 (6.6)	48.9 (8.5)	50.7 (7.6)
Age group (years): n (%)					
<40	257 (16.4)	49 (12.1)	8 (8.3)	287 (16.3)	11 (9.3)
40-49	575 (36.7)	151 (37.2)	36 (37.5)	647 (36.8)	43 (36.4)
50-59	590 (37.6)	167 (41.1)	45 (46.9)	657 (37.3)	55 (46.6)
≥60	146 (9.3)	39 (9.6)	7 (7.3)	169 (9.6)	9 (7.6)
Nodal status: n (%)					
Node negative	314 (20.0)	93 (22.9)	18 (18.8)	367 (20.9)	22 (18.6)
1-3 positive nodes	719 (45.9)	171 (42.1)	33 (34.4)	808 (45.9)	49 (41.5)
≥4 positive nodes	535 (34.1)	142 (35.0)	45 (46.9)	585 (33.2)	47 (39.8)
Tumour grade: n (%)					
Grade 1	77 (4.9)	23 (5.7)	4 (4.2)	88 (5.0)	8 (6.8)
Grade 2	603 (38.5)	155 (38.2)	35 (36.5)	681 (38.7)	42 (35.6)
Grade 3	883 (56.3)	228 (56.2)	57 (59.4)	986 (56.0)	68 (57.6)
Unknown	5 (0.3)	0 (0.0)	0 (0.0)	5 (0.3)	0 (0.0)
Tumour size: n (%)					
≤2cm	578 (36.9)	147 (36.2)	25 (26.0)	659 (37.4)	41 (34.8)
>2 and ≤5cm	857 (54.7)	220 (54.2)	61 (63.5)	952 (54.1)	64 (54.2)
>5cm	132 (8.4)	39 (9.6)	10 (10.4)	148 (8.4)	13 (11.0)
Unknown	2 (0.1)	0 (0.0)	0 (0.0)	2 (0.1)	0 (0.0)
ER & HER2 status: n (%)					
ER+	1107 (70.6)	289 (71.2)	69 (71.9)	1248 (70.9)	79 (67.0)
& HER2+	198 (12.6)	49 (12.1)	13 (13.5)	220 (12.5)	14 (11.9)
& HER2-	772 (49.2)	201 (49.5)	46 (47.9)	873 (49.6)	54 (45.8)
& HER2 unknown	137 (8.7)	39 (9.6)	10 (10.4)	155 (8.8)	11 (9.3)
ER-	461 (29.4)	117 (28.8)	27 (28.1)	512 (29.1)	39 (33.1)
& HER2+	118 (7.5)	43 (10.6)	8 (8.3)	141 (8.0)	12 (10.2)
& HER2-	289 (18.4)	61 (15.0)	15 (15.6)	313 (17.8)	22 (18.6)
& HER2 unknown	54 (3.4)	13 (3.2)	4 (4.2)	58 (3.3)	5 (4.2)
Molecular subgroup: n (%)					
ER+/HER2-*	784 (50.0)	203 (50.0)	47 (49.0)	885 (50.3)	55 (46.6)
HER2+	316 (20.2)	92 (22.7)	21 (21.9)	361 (20.5)	26 (22.0)
Triple negative	277 (17.7)	59 (14.5)	14 (14.6)	301 (17.1)	21 (17.8)

* includes ER-, PgR+, HER2-

ER = oestrogen receptor. HER2 = human epidermal growth factor receptor 2. PgR = progesterone receptor. SD = Standard deviation.

Table 4-5: BC treatments by TPOAb and thyroid function status

	TPOAb- N=1568	TPOAb+ N=406	Hypothyroid N=96	Euthyroid N=1760	Hyperthyroid N=118
Type of surgery and radiotherapy use: n (%)					
Mastectomy	854 (54.5)	225 (55.4)	53 (55.2)	962 (54.7)	64 (54.2)
with radiotherapy ^	688 (80.6)	177 (78.7)	47 (88.7)	772 (80.2)	46 (71.9)
Wide local excision	714 (45.5)	181 (44.6)	43 (44.8)	798 (45.3)	54 (45.8)
with radiotherapy #	704 (98.6)	176 (97.2)	41 (95.3)	787 (98.6)	52 (96.3)
Endocrine treatment in ER+ patients: n (%)*					
Tamoxifen monotherapy	696 (62.9)	167 (57.8)	43 (62.3)	772 (61.9)	48 (60.8)
Tamoxifen followed by AI	354 (32.0)	100 (34.6)	20 (29.0)	409 (32.8)	25 (31.7)
AI monotherapy	46 (4.2)	15 (5.2)	6 (8.7)	53 (4.3)	2 (2.5)
No treatment/unknown	11 (1.0)	7 (2.4)	0 (0.0)	14 (1.1)	4 (5.1)
Trastuzumab in HER2+ patients: n (%)**					
Yes	40 (12.7)	8 (8.7)	1 (4.8)	44 (12.2)	3 (11.5)
No/Not known	276 (87.3)	84 (91.3)	20 (95.2)	317 (87.8)	23 (88.5)
Chemotherapy: n (%)					
Control (FEC)	498 (31.8)	128 (31.5)	27 (28.1)	568 (32.3)	31 (26.3)
Control (E-CMF)	271 (17.3)	61 (15.0)	16 (16.7)	301 (17.1)	15 (12.7)
FEC-D	799 (51.0)	217 (53.4)	53 (55.2)	891 (50.6)	52 (44.1)

^ denominators calculated using patients treated with mastectomy

denominators calculated using patients treated with wide local excision

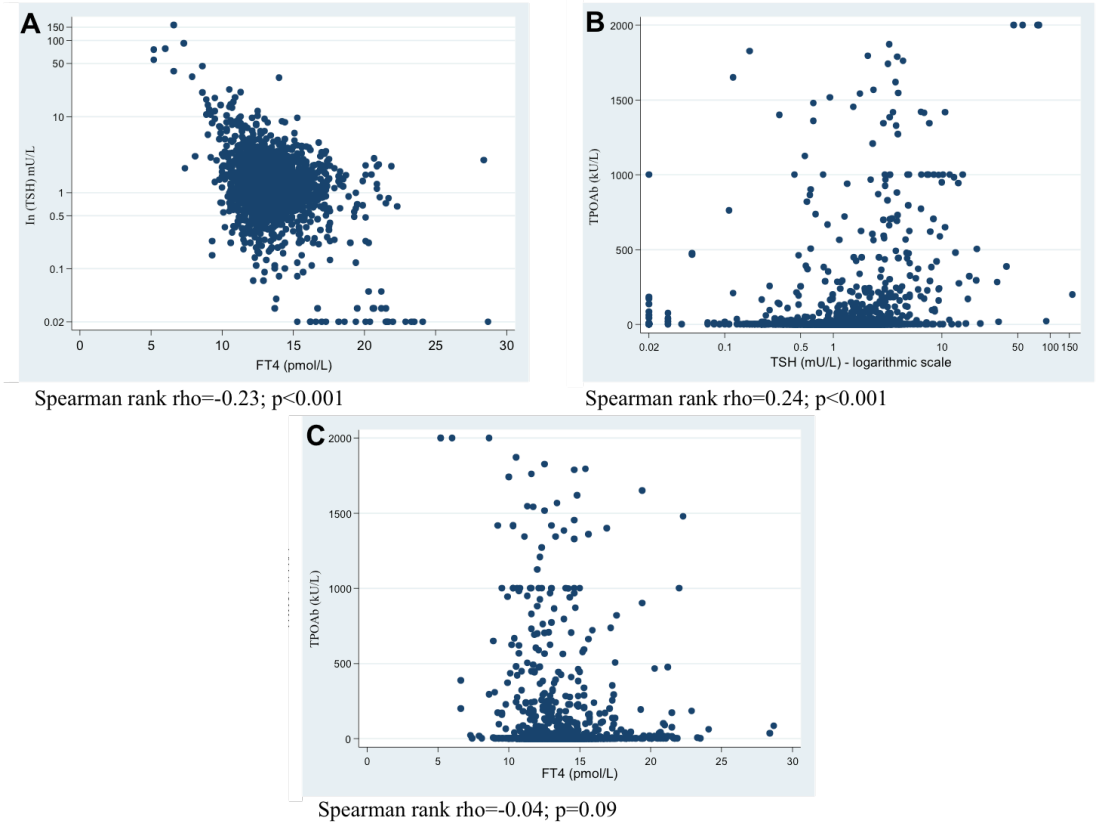
* denominators calculated using ER+ patients

** denominators calculated using HER2+ patients

ER = oestrogen receptor. E-CMF = epirubicin 100 mg/m^2 for 4 cycles followed by CMF (cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2 and fluorouracil 600 mg/m^2) for 4 cycles. FEC = fluorouracil 600 mg/m^2, epirubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2 for 8 cycles. FEC-D = FEC for 4 cycles followed by docetaxel 100 mg/m^2 for 4 cycles. HER2 = human epidermal growth factor receptor 2.

As expected, FT4 and TSH were inversely correlated (Spearman rank= -0.23, p<0.001; Figure 4-3 panel A) and TPOAb were positively associated with TSH (Spearman rank= 0.24, p<0.001; Figure 4-3 panel B). The inverse correlation between TPOAb and FT4 was weak (Spearman rank= -0.04, p=0.09; Figure 4-3 panel C). When considering FT4 and TSH combined together (thyroid function status), TPOAb positivity was more prevalent among hypothyroid and hyperthyroid patients compared with the euthyroid group, as expected [TPOAb+: 73 (76.0%) hypothyroid, 288 (16.4%) euthyroid, 45 (38.1%) hyperthyroid; p<0.001].

Figure 4-3: Correlation between thyroid markers



Panel A: inverse correlation between thyroid-stimulating hormone (TSH) in logarithmic scale (ln) and free-thyroxine (FT4). Panel B: positive correlation between autoantibodies to thyroid peroxidase (TPOAb) and TSH. Panel C: inverse correlation between TPOAb and FT4. p = p value

4.3.2 TPOAb and breast cancer prognosis

In both TPOAb+ and TPOAb- groups the majority of events contributing to DFS related to distant recurrence; similarly, the major cause of death was BC (Table 4-6).

Table 4-6: Events contributing to DFS and numbers of deaths by dichotomized TPOAb status

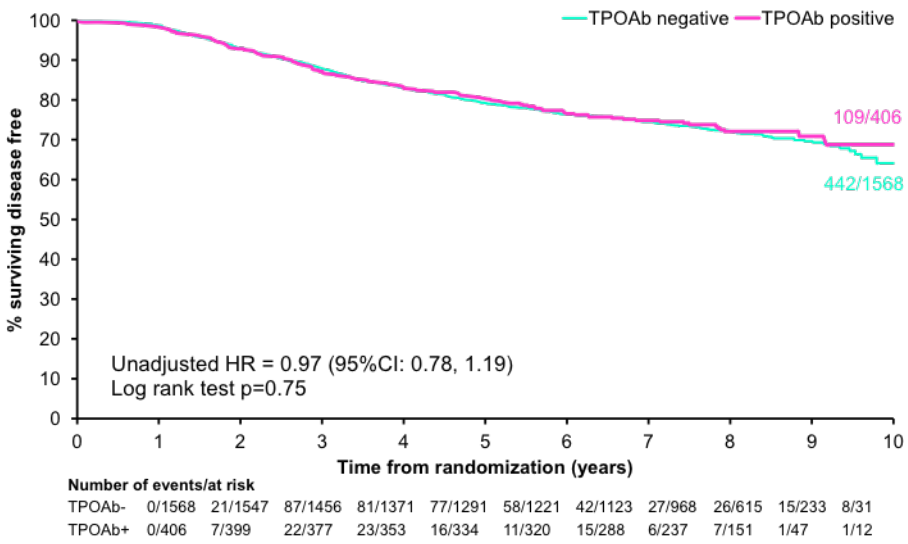
	TPOAb- N=1568 n (%)	TPOAb+ N=406 n (%)
Number of patients with event contributing to DFS analysis	**442 (28.2)**	**109 (26.8)**
Local recurrence	59 (3.8)	17 (4.2)
Distant recurrence	327 (20.9)	78 (19.2)
New breast disease	33 (2.1)	10 (2.5)
Death from other cause (no recurrence)	23 (1.5)	4 (1.0)
All deaths	**325 (20.7)**	**72 (17.7)**
Breast cancer	301 (19.2)	68 (16.7)
Death from other causes (without distant recurrence)	24 (1.5)	4 (1.0)
Cancer (non-breast)	14 (0.9)	1 (0.2)
Treatment toxicity	0 (0.0)	0 (0.0)
Other	9 (0.6)	3 (0.7)
Vascular (cardiac)	1 (0.1)	1 (0.2)
Vascular (cerebral)	1 (0.1)	0 (0.0)
Vascular (thromboembolic)	0 (0.0)	0 (0.0)
Respiratory	0 (0.0)	0 (0.0)
Accident, suicide, alcoholism	5 (0.3)	0 (0.0)
Infection (not treatment related)	0 (0.0)	1 (0.2)
GI bleed	0 (0.0)	0 (0.0)
Chronic liver disease	1 (0.1)	0 (0.0)
Unknown	2 (0.1)	1 (0.2)

DFS = disease free survival. TPOAb = autoantibodies to thyroid peroxidase.

4.3.2.1 Dichotomized TPOAb

There was no evidence of a difference in DFS between TPOAb+ and TPOAb- patients in both univariate (unadjusted-HR = 0.97, 95%CI, 0.78 - 1.19; p=0.75; Figure 4-4) and multivariable (adjusted-HR=1.00, 95%CI,0.81-1.23; p=1.00; Table 4-7) analyses.

Figure 4-4: Univariate analysis of DFS by dichotomized TPOAb



DFS = disease free survival. HR = Hazard Ratio. TPOAb = autoantibodies to thyroid peroxidase. 95%CI = 95% confidence interval. p= p value.

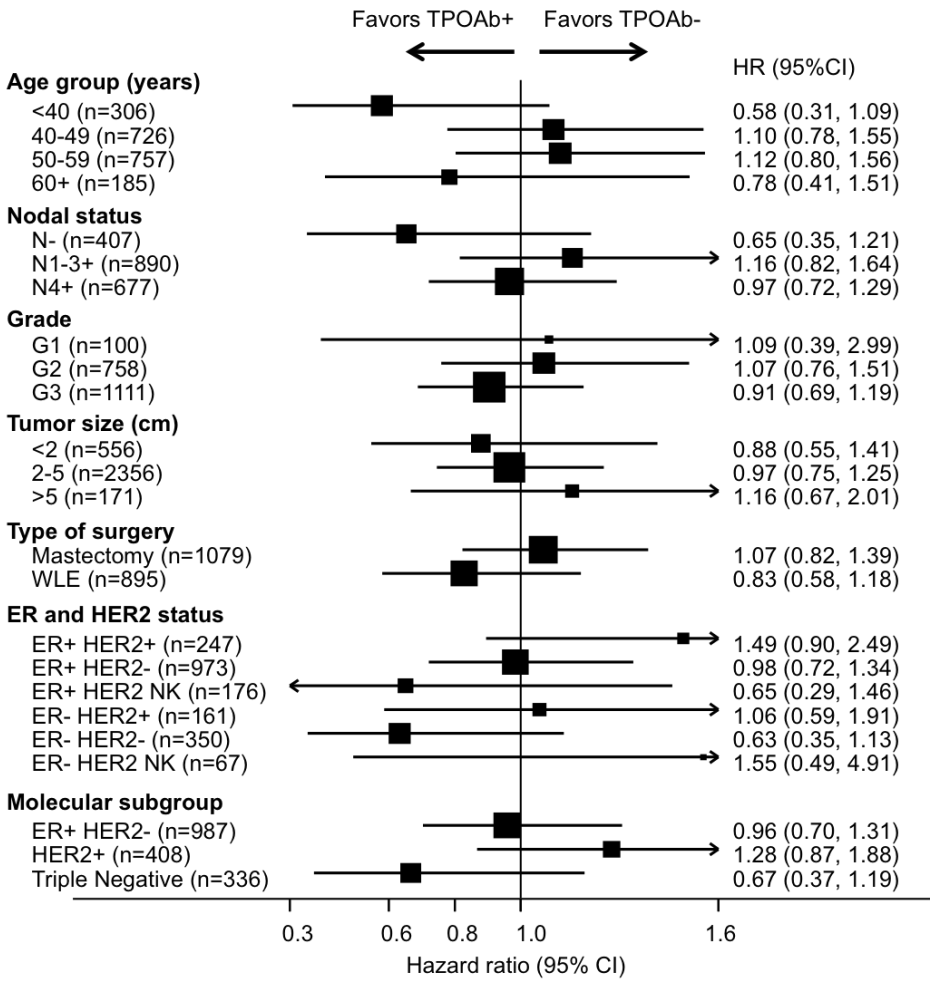
Table 4-7: Multivariable analysis of DFS by dichotomized TPOAb

		HR	95%CI	P-value
TPOAb status	negative	1.00	-	-
	positive	1.00	0.81, 1.23	1.00
Nodal status	positive	1.00	-	-
	negative	0.49	0.38, 0.64	<0.001
HER2 status and trastuzumab use	negative	1.00	-	-
	positive, trastuzumab	0.91	0.52, 1.59	0.74
	positive, no trastuzumab	1.23	1.00, 1.52	0.05
	unknown	0.93	0.71, 1.23	0.63
Age group	<40	1.00	-	-
	40-49	0.79	0.62, 1.00	0.05
	50-59	0.75	0.59, 0.95	0.02
	60+	0.94	0.69, 1.30	0.72
Tumour grade	G1	1.00	-	-
	G2	1.15	0.74, 1.78	0.55
	G3	1.39	0.89, 2.16	0.15
	unknown	0.75	0.10, 5.63	0.78
Tumour size (cm)	≤2	1.00	-	-
	>2 and ≤5	1.36	1.12, 1.66	0.002
	>5	1.89	1.41, 2.53	<0.001
Type of surgery	Mastectomy	1.00	-	-
	WLE	0.79	0.66, 0.95	.01

DFS = disease free survival. HER2 = human epidermal growth factor receptor 2. HR = Hazard Ratio. TPOAb = autoantibodies to thyroid peroxidase. WLE = wide local excision. 95%CI = 95% confidence interval.

Analyses in clinical subgroups according to age group, nodal status, tumour grade, tumour size, type of surgery, ER and HER2 status and molecular subgroup showed consistency with the overall effect, with no evidence of any significant interaction effects of TPOAb on DFS (Figure 4-5).

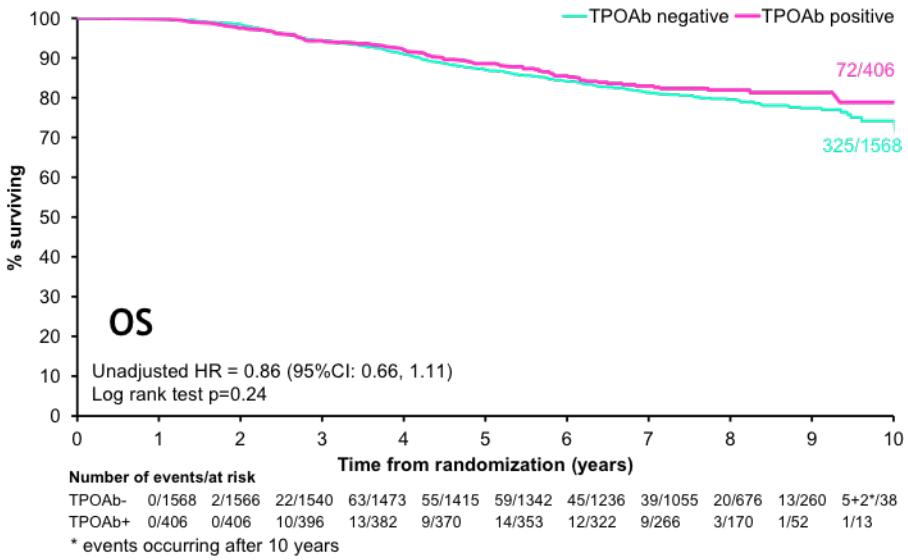
Figure 4-5: Subgroup analyses of DFS by dichotomized TPOAb

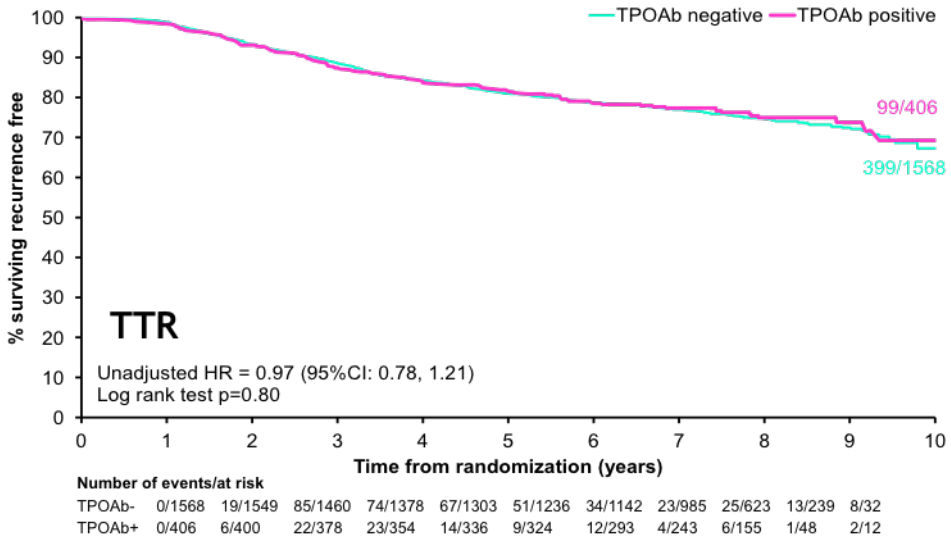


DFS = disease free survival. ER = oestrogen receptor. HER2 = human epidermal growth factor receptor 2. HR = Hazard Ratio. NK = not known. TPOAb = autoantibodies to thyroid peroxidase. WLE = wide local excision. 95%CI = 95% confidence interval.

Similarly to DFS, there was no evidence of a difference by TPOAb status on OS and TTR in both univariate (respectively unadjusted-HR = 0.86 and 0.97, 95%CI: 0.66,1.11 and 0.78-1.21, p=0.24 and p=0.80; Figure 4-6 panels A and B respectively) and multivariable (respectively adjusted-HR=0.89 and 1.02, 95%CI 0.69-1.15 and 0.82-1.27, p=0.36 and p=0.88; Table 4-8) analyses.

Figure 4-6: Univariate analysis of OS and TTR by dichotomized TPOAb





HR = Hazard Ratio. OS = overall survival. TPOAb = autoantibodies to thyroid peroxidase. TTR = time to recurrence. 95%CI = 95% confidence interval.

Table 4-8: Multivariable analysis of OS and TTR by dichotomized TPOAb

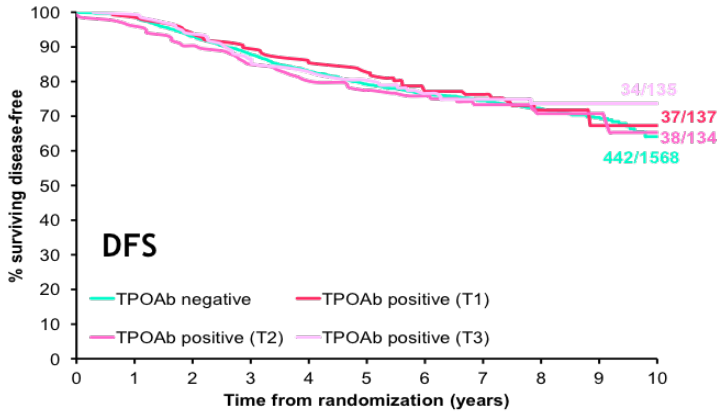
		OS			TTR		
		HR	95%CI	p-value	HR	95%CI	p-value
TPOAb	negative	1.00	-	-	1.00	-	-
	positive	0.88	0.68, 1.14	0.34	1.01	0.81, 1.26	0.91
Nodal status	positive	1.00	-	-	1.00	-	-
	negative	0.43	0.31, 0.60	<0.001	0.42	0.32, 0.57	<0.001
HER2 status and trastuzumab use	negative	1.00	-	-	1.00	-	-
	unknown	1.04	0.76, 1.42	0.81	0.99	0.75, 1.31	0.93
	positive, no trastuzumab	1.33	1.04, 1.69	0.02	1.25	1.00, 1.55	0.05
	positive, yes trastuzumab	0.89	0.45, 1.73	0.73	1.02	0.58, 1.78	0.95
Age group	<40	1.00	-	-	1.00	-	-
	40-49	0.84	0.62, 1.13	0.24	0.78	0.60, 1.01	0.06
	50-59	0.93	0.69, 1.24	0.62	0.74	0.57, 0.95	0.02
	60+	1.17	0.81, 1.70	0.40	0.95	0.68, 1.32	0.74
Grade	G1	1.00	-	-	1.00	-	-
	G2	1.35	0.76, 2.39	0.30	1.36	0.83, 2.25	0.22
	G3	1.69	0.96, 2.98	0.07	1.64	1.00, 2.71	0.05
	unknown	1.17	0.15, 9.06	0.88	0.98	0.13, 7.42	0.98
Tumour size (cm)	≤2	1.00	-	-	1.00	-	-
	>2 and ≤5	1.31	1.04, 1.66	0.02	1.31	1.06, 1.60	0.01
	>5	1.84	1.31, 2.57	<0.001	1.94	1.44, 2.62	<0.001
Type of surgery	Mastectomy	1.00	-	-	1.00	-	-
	WLE	0.71	0.57, 0.88	0.002	0.75	0.62, 0.91	0.003

HER2 = human epidermal growth factor receptor 2. HR = Hazard Ratio. OS = overall survival. TPOAb = autoantibodies to thyroid peroxidase. TTR = time to recurrence. WLE = wide local excision. 95%CI = 95% confidence interval.

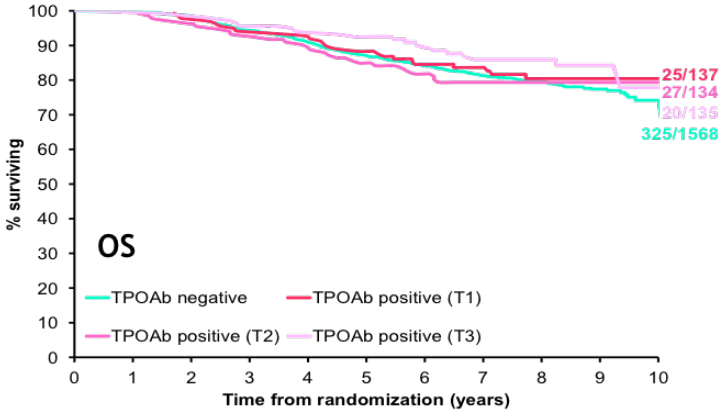
4.3.2.2 TPOAb tertiles

TPOAb+ cases when categorized into tertiles showed no evidence of a prognostic effect in both univariate (Figure 4-7) and multivariable (Table 4-9) analyses for DFS, OS or TTR.

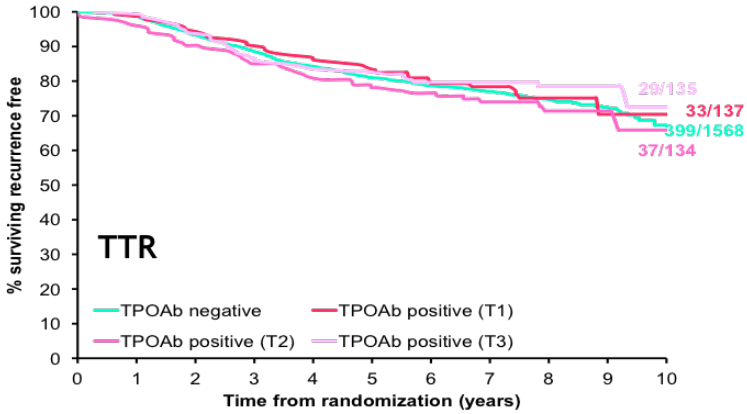
Figure 4-7: Univariate analysis of DFS, OS and TTR by TPOAb tertiles



	Unadjusted HR	95%CI	p-value
TPOAb negative	1.00	-	-
TPOAb positive (T1: 6-40kU/L)	0.97	0.69, 1.36	0.86
TPOAb positive (T2: 41-238kU/L)	1.06	0.76, 1.48	0.73
TPOAb positive (T3: 240-2000kU/L)	0.88	0.62, 1.25	0.47



	Unadjusted HR	95%CI	p-value
TPOAb negative	1.00	-	-
TPOAb positive (T1: 6-40kU/L)	0.90	0.60, 1.35	0.61
TPOAb positive (T2: 41-238kU/L)	1.03	0.70, 1.53	0.87
TPOAb positive (T3: 240-2000kU/L)	0.67	0.42, 1.05	0.08



	Unadjusted HR	95%CI	p-value
TPOAb negative	1.00	-	-
TPOAb positive (T1: 6-40kU/L)	0.96	0.68, 1.37	0.84
TPOAb positive (T2: 41-238kU/L)	1.15	0.82, 1.61	0.43
TPOAb positive (T3: 240-2000kU/L)	0.82	0.56, 1.20	0.31

DFS = disease free survival. HR = Hazard Ratio. OS = overall survival. TPOAb = autoantibodies to thyroid peroxidase. TTR = time to recurrence. T1, T2, T3 = first, second, third tertile. 95%CI = 95% confidence interval.

Table 4-9: Multivariable analysis of DFS, OS and TTR by TPOAb tertiles

		DFS			OS			TTR		
		HR	95%CI	p	HR	95%CI	p	HR	95%CI	p
TPOAb	neg	1.00	-	-	1.00	-	-	1.00	-	-
	pos T1	1.01	0.72, 1.41	0.97	0.94	0.62, 1.41	0.76	1.01	0.70, 1.43	0.98
	pos T2	1.07	0.77, 1.50	0.68	1.02	0.69, 1.52	0.91	1.16	0.83, 1.63	0.39
	pos T3	0.92	0.65, 1.31	0.65	0.70	0.44, 1.10	0.12	0.88	0.60, 1.28	0.50
Nodal status	pos	1.00	-	-	1.00	-	-	1.00	-	-
	neg	0.49	0.38, 0.64	<0.001	0.43	0.31, 0.60	<0.001	0.42	0.32, 0.57	<0.001
HER2 status and trastuz. use	neg	1.00	-	-	1.00	-	-	1.00	-	-
	unk.	0.93	0.71, 1.23	0.62	1.04	0.76, 1.42	0.81	0.99	0.74, 1.31	0.93
	pos	1.23	1.00, 1.52	0.05	1.33	1.05, 1.70	0.02	1.25	1.00, 1.55	0.05
	pos T*	0.91	0.52, 1.59	0.73	0.89	0.45, 1.74	0.73	1.01	0.58, 1.78	0.96
Age group	<40	1.00	-	-	1.00	-	-	1.00	-	-
	40-49	0.79	0.62, 1.00	0.05	0.84	0.62, 1.13	0.24	0.78	0.60, 1.01	0.06
	50-59	0.75	0.59, 0.95	0.02	0.93	0.69, 1.24	0.62	0.74	0.57, 0.95	0.02
	60+	0.94	0.68, 1.30	0.72	1.17	0.80, 1.70	0.42	0.94	0.67, 1.31	0.72
Grade	G1	1.00	-	-	1.00	-	-	1.00	-	-
	G2	1.14	0.73, 1.77	0.57	1.33	0.75, 2.36	0.33	1.35	0.82, 2.22	0.24
	G3	1.38	0.89, 2.15	0.16	1.66	0.94, 2.94	0.08	1.62	0.98, 2.67	0.06
	unk.	0.75	0.10, 5.59	0.78	1.15	0.15, 8.94	0.89	0.96	0.13, 7.32	0.97
Tumour size (cm)	≤2	1.00	-	-	1.00	-	-	1.00	-	-
	>2 -≤5	1.36	1.12, 1.66	0.002	1.32	1.04, 1.66	0.02	1.31	1.06, 1.60	0.01
	>5	1.89	1.41, 2.53	<0.001	1.83	1.31, 2.57	<0.001	1.94	1.43, 2.61	<0.001
Type of surgery	Mast.	1.00	-	-	1.00	-	-	1.00	-	-
	WLE	0.79	0.66, 0.95	0.01	0.71	0.57, 0.88	0.002	0.75	0.62, 0.91	0.003

pos T* = HER2 positive treated with trastuzumab

DFS = disease free survival. HER2 = human epidermal growth factor receptor 2. HR = Hazard Ratio. Mast. = mastectomy. neg = negative. OS = overall survival. pos = positive. TPOAb = autoantibodies to thyroid peroxidase. trastuz. = trastuzumab. TTR = time to recurrence. T1 = TPOAb 6-40kU/L. T2 = TPOAb 41-238kU/L. T3 = TPOAb 240-2000kU/L. unk. = unknown. WLE = wide local excision. 95%CI = 95% confidence interval.

4.3.2.3 DFS sensitivity analyses with dichotomized TPOAb

2 sensitivity analyses for DFS were performed, based on different populations:

1) 126 node+ patients not treated with radiotherapy, therefore similar to Fiore *et* al. cohort (183). The median time of blood collection after surgery was 12.4 months (IQR=4.9-21.6; range=0.7–47.2 months).
2) 123 patients whose blood had been taken after surgery but before any adjuvant BC therapy. The median time of sera collection after surgery was 1.1 months (IQR=0.9-1.4; range=0.5-5.9).

There was no evidence of a significant impact on DFS by TPOAb status in either of the sensitivity analysis populations, with unadjusted-HRs of 1.48 (95%CI,0.68-3.25; p= 0.32) and 0.83 (95%CI, 0.35-2.03; p= 0.69) respectively.

4.3.3 Thyroid function and breast cancer prognosis

4.3.3.1 Univariate analysis

When considering separately FT4 and TSH both as continuous or categorical variables in univariate analyses, no evidence of a significant effect for DSF, OS and TTR was found (Table 4-10).

Table 4-10: Univariate analysis of DSF, OS and TTR by FT4 and TSH variables

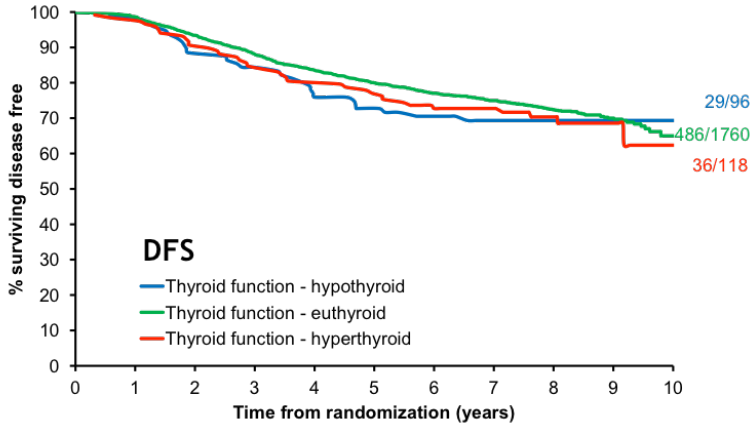
		DFS			OS			TTR		
		HR	**95%CI**	**p**	**HR**	**95%CI**	**p**	**HR**	**95%CI**	**p**
FT4	Continuous	1.00	0.96-1.04	0.91	1.01	0.96-1.06	0.68	1.01	0.97-1.05	0.70
	Hypo	1.61	0.67-3.88	0.29	2.23	0.92-5.40	0.07	1.80	0.75-4.36	0.19
	Eu	1.00	-	-	1.00	-	-	1.00	-	-
	Hyper	1.08	0.62-1.87	0.79	0.88	0.44-1.77	0.71	1.10	0.62-1.96	0.74
TSH	Continuous*	1.03	0.94-1.13	0.48	1.03	0.92-1.14	0.65	1.04	0.94-1.14	0.43
	Hypo	1.08	0.73-1.59	0.71	1.03	0.65-1.63	0.91	1.15	0.78-1.71	0.48
	Eu	1.00	-	-	1.00	-	-	1.00	-	-
	Hyper	1.19	0.82-1.72	0.36	1.27	0.83-1.93	0.27	1.24	0.84-1.81	0.28

* referred to ln(TSH) continuous values

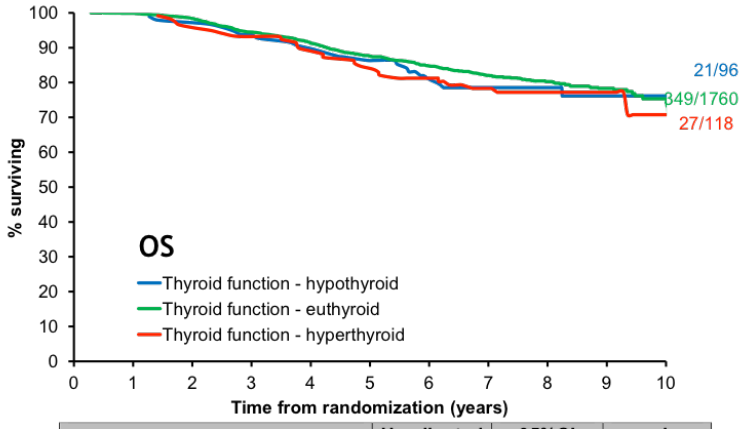
DFS = disease free survival; Eu = hypothyroidism; FT4 = free thyroxine; HR = Hazard Ratio; Hyper = hyperthyroidism; Hypo = hypothyroidism; ln = logarithmic scale; OS = overall survival; p = p value; TSH = thyroid stimulating hormone; TTR = time to recurrence; 95%CI = 95% confidence interval.

When considering together TSH and FT4 as unique thyroid function categorical variable in univariate analyses, absence of evidence of a significant effect was confirmed in DFS, OS and TTR (Figure 4-8).

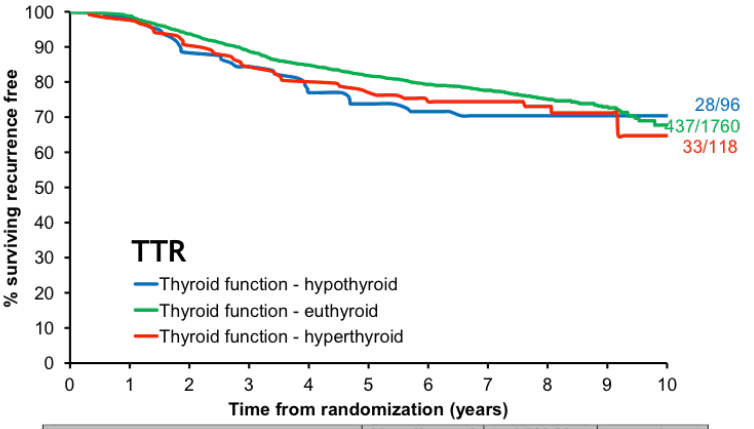
Figure 4-8: Univariate analysis of DFS, OS and TTR by thyroid function status (TSH/FT4 combined categories)



	Unadjusted HR	95%CI	p-value
Thyroid function - hypothyroid	1.15	0.79, 1.68	0.46
Thyroid function - euthyroid	1.00	-	-
Thyroid function - hyperthyroid	1.14	0.82, 1.61	0.44



	Unadjusted HR	95%CI	p-value
Thyroid function - hypothyroidism	1.13	0.73, 1.76	0.58
Thyroid function - euthyroid	1.00	-	-
Thyroid function - hyperthyroidism	1.17	0.79, 1.74	0.43



	Unadjusted HR	95%CI	p-value
Thyroid function - hypothyroidism	1.24	0.85, 1.82	0.27
Thyroid function - euthyroid	1.00	-	-
Thyroid function - hyperthyroidism	1.18	0.82, 1.67	0.37

DFS = disease free survival. HR = Hazard Ratio. OS = overall survival. TTR = time to recurrence. 95%CI = 95% confidence interval.

4.3.3.2 Multivariable analyses

The multivariable analyses confirmed the absence of evidence of a significant impact of thyroid function on DFS, OS and TTR, both when considering FT4 (Table 4-11) and TSH (Table 4-12) separately or combined into a unique thyroid function status categorical variable (Table 4-13).

Table 4-11: Multivariable analysis of DFS, OS and TTR by FT4 variable

		DFS			OS			TTR		
		HR	**95%CI**	**p**	**HR**	**95%CI**	**p**	**HR**	**95%CI**	**p**
FT4	cont.	1.00	0.96, 1.05	0.83	1.01	0.97, 1.06	0.55	1.01	0.97, 1.06	0.60
Nodal status	pos	1.00	-	-	1.00	-	-	1.00	-	-
	neg	0.49	0.38, 0.64	<0.001	0.43	0.31, 0.59	<0.001	0.42	0.32, 0.57	<0.001
HER2 status and trastuz. use	neg	1.00	-	-	1.00	-	-	1.00	-	-
	unk.	0.93	0.71, 1.23	0.63	1.04	0.76, 1.42	0.82	0.99	0.75, 1.31	0.94
	pos	1.23	1.00, 1.51	0.06	1.32	1.04, 1.68	0.02	1.24	1.00, 1.55	0.05
	pos T*	0.91	0.52, 1.59	0.73	0.89	0.45, 1.74	0.73	1.01	0.58, 1.78	0.96
Age group	<40	1.00	-	-	1.00	-	-	1.00	-	-
	40-49	0.79	0.62, 1.00	0.05	0.83	0.62, 1.12	0.22	0.78	0.61, 1.01	0.06
	50-59	0.75	0.59, 0.95	0.02	0.92	0.69, 1.23	0.56	0.74	0.57, 0.95	0.02
	60+	0.94	0.69, 1.30	0.72	1.16	0.80, 1.69	0.43	0.94	0.68, 1.32	0.74
Grade	G1	1.00	-	-	1.00	-	-	1.00	-	-
	G2	1.15	0.74, 1.78	0.55	1.36	0.77, 2.40	0.30	1.36	0.83, 2.25	0.22
	G3	1.39	0.89, 2.16	0.15	1.69	0.96, 2.99	0.07	1.64	1.00, 2.71	0.05
	unk.	0.75	0.10, 5.65	0.78	1.22	0.16, 9.43	0.85	0.98	0.13, 7.67	0.99
Tumour size (cm)	≤2	1.00	-	-	1.00	-	-	1.00	-	-
	>2 -≤5	1.36	1.12, 1.66	0.002	1.32	1.04, 1.66	0.02	1.31	1.06, 1.61	0.01
	>5	1.89	1.41, 2.53	<0.001	1.83	1.31, 2.57	<0.001	1.94	1.44, 2.62	<0.001
Type of surgery	Mast.	1.00	-	-	1.00	-	-	1.00	-	-
	WLE	0.79	0.66, 0.95	0.01	0.70	0.56, 0.87	0.002	0.75	0.62, 0.91	0.003

pos T* = HER2 positive treated with trastuzumab

cont. = continuous variable; DFS = disease free survival; FT4 = free-thyroxine; HER2 = human epidermal growth factor receptor 2; HR = Hazard Ratio; Mast. = mastectomy; neg = negative; OS = overall survival; pos = positive; unk. = unknown; TTR = time to recurrence; WLE = wide local excision; 95%CI = 95% confidence interval.

Table 4-12: Multivariable analysis of DFS, OS and TTR by TSH variable

		DFS			OS			TTR		
		HR	95%CI	p	HR	95%CI	p	HR	95%CI	p
TSH(ln)	cont.	1.03	0.94, 1.13	0.55	1.02	0.91, 1.13	0.75	1.04	0.94, 1.14	0.49
Nodal status	pos	1.00	-	-	1.00	-	-	1.00	-	-
	neg	0.48	0.37, 0.63	<0.001	0.42	0.30, 0.58	<0.001	0.43	0.32, 0.57	<0.001
HER2 status and trastuz. use	neg	1.00	-	-	1.00	-	-	1.00	-	-
	unk.	0.94	0.71, 1.23	0.64	1.04	0.76, 1.60	0.81	0.99	0.75, 1.31	0.94
	pos	1.24	1.00, 1.52	0.05	1.33	1.05, 1.69	0.02	1.25	1.00, 1.56	0.05
	pos T*	0.91	0.52, 1.60	0.76	0.90	0.46, 1.75	0.75	1.02	0.58, 1.79	0.94
Age group	<40	1.00	-	-	1.00	-	-	1.00	-	-
	40-49	0.78	0.62, 1.00	0.05	0.83	0.62, 1.12	0.22	0.78	0.61, 1.01	0.06
	50-59	0.74	0.58, 0.95	0.02	0.91	0.68, 1.22	0.53	0.73	0.57, 0.95	0.02
	60+	0.94	0.69, 1.30	0.72	1.16	0.80, 1.69	0.42	0.95	0.68, 1.32	0.74
Grade	G1	1.00	-	-	1.00	-	-	1.00	-	-
	G2	1.14	0.73, 1.78	0.55	1.36	0.77, 2.40	0.30	1.36	0.83, 2.24	0.22
	G3	1.39	0.89, 2.16	0.15	1.69	0.95, 2.98	0.07	1.64	0.99, 2.70	0.05
	unk.	0.75	0.10, 5.60	0.78	1.20	0.16, 9.30	0.86	0.96	0.13, 7.31	0.97
Tumour size (cm)	≤2	1.00	-	-	1.00	-	-	1.00	-	-
	>2 -≤5	1.36	1.11, 1.65	0.002	1.31	1.04, 1.65	0.02	1.30	1.06, 1.60	0.01
	>5	1.88	1.41, 2.52	<0.001	1.83	1.30, 2.56	<0.001	1.93	1.43, 2.61	<0.001
Type of surgery	Mast.	1.00	-	-	1.00	-	-	1.00	-	-
	WLE	0.80	0.66, 0.96	0.01	0.71	0.57, 0.88	0.002	0.75	0.62, 0.91	0.003

pos T* = HER2 positive treated with trastuzumab

cont. = continuous variable; DFS = disease free survival; HER2 = human epidermal growth factor receptor 2; HR = Hazard Ratio; ln = logarithmic scale; Mast. = mastectomy; neg = negative; OS = overall survival; pos = positive; TSH = thyroid stimulating hormone; TTR = time to recurrence; unk. = unknown; WLE = wide local excision; 95%CI = 95% confidence interval.

Table 4-13: Multivariable analysis of DFS, OS and TTR by thyroid function status (TSH/FT4 combined categories)

		DFS			OS			TTR		
		HR	95%CI	p	HR	95%CI	p	HR	95%CI	p
Thyroid function	eu	1.00	-	-	1.00	-	-	1.00	-	-
	hypo	1.14	0.79, 1.67	0.48	1.08	0.69, 1.68	0.73	1.24	0.84, 1.82	0.27
	hyper	1.14	0.81, 1.59	0.47	1.16	0.78, 1.72	0.46	1.17	0.82, 1.66	0.40
Nodal status	pos	1.00	-	-	1.00	-	-	1.00	-	-
	neg	0.49	0.38, 0.64	<0.001	0.43	0.31, 0.59	<0.001	0.42	0.32, 0.57	<0.001
HER2 status and trastuz. use	neg	1.00	-	-	1.00	-	-	1.00	-	-
	unk.	0.93	0.71, 1.22	0.61	1.03	0.76, 1.41	0.84	0.98	0.74, 1.30	0.91
	pos	1.23	1.00, 1.51	0.06	1.32	1.04, 1.68	0.02	1.24	1.00, 1.55	0.05
	pos T*	0.91	0.52, 1.59	0.74	0.89	0.46, 1.74	0.73	1.02	0.58, 1.79	0.95
Age group	<40	1.00	-	-	1.00	-	-	1.00	-	-
	40-49	0.78	0.61, 0.99	0.05	0.82	0.61, 1.11	0.21	0.77	0.60, 1.00	0.05
	50-59	0.74	0.58, 0.94	0.02	0.91	0.68, 1.22	0.53	0.72	0.56, 0.94	0.01
	60+	0.94	0.68, 1.29	0.70	1.16	0.80, 1.63	0.43	0.94	0.67, 1.31	0.71
Grade	G1	1.00	-	-	1.00	-	-	1.00	-	-
	G2	1.15	0.74, 1.79	0.54	1.36	0.77, 2.41	0.29	1.37	0.83, 2.25	0.22
	G3	1.39	0.89, 2.16	0.15	1.69	0.96, 2.99	0.07	1.64	0.99, 2.70	0.05
	unk.	0.77	0.10, 5.73	0.80	1.22	0.16, 9.47	0.85	1.00	0.13, 7.59	1.00
Tumour size (cm)	≤2	1.00	-	-	1.00	-	-	1.00	-	-
	>2 -≤5	1.36	1.12, 1.65	0.002	1.31	1.04, 1.66	0.02	1.30	1.06, 1.60	0.01
	>5	1.88	1.41, 2.52	<0.001	1.82	1.30, 2.56	<0.001	1.93	1.43, 2.60	<0.001
Type of surgery	Mast.	1.00	-	-	1.00	-	-	1.00	-	-
	WLE	0.79	0.66, 0.95	0.01	0.70	0.56, 0.87	0.002	0.75	0.62, 0.91	0.003

pos T* = HER2 positive treated with trastuzumab

DFS = disease free survival; HER2 = human epidermal growth factor receptor 2; HR = Hazard Ratio; Mast. = mastectomy; neg = negative; OS = overall survival; pos = positive; TSH = thyroid stimulating hormone; TTR = time to recurrence; unk. = unknown; WLE = wide local excision; 95%CI = 95% confidence interval.

4.3.4 Impact of TPOAb and thyroid function on breast cancer prognosis

As expected, considering together dichotomized TPOAb and thyroid function status in the multivariable model for DFS, no evidence of a significant impact was found (Table 4-14). Similar analyses for OS and TTR were not performed because they were secondary outcomes. Furthermore, if 2 variables (i.e. TPOAb and thyroid function) give negative results when analyzed separately, they will give negative results also when considered together.

Table 4-14: Multivariable analysis of DFS by TPOAb (dichotomized) and thyroid function status (TSH/FT4 combined categories)

		HR	95%CI	P-value
TPOAb status	negative	1.00	-	-
	positive	0.96	0.77, 1.21	0.76
Thyroid function	Euthyroid	1.00	-	-
	Hypothyroid	1.17	0.78, 1.74	0.44
	Hyperthyroid	1.14	0.81, 1.61	0.44
Nodal status	positive	1.00	-	-
	negative	0.49	0.38, 0.64	<0.001
HER2 status and trastuzumab use	negative	1.00	-	-
	unknown	0.93	0.71, 1.22	0.61
	positive, no trastuzumab	1.23	1.00, 1.51	0.05
	positive, yes trastuzumab	0.91	0.52, 1.59	0.74
Age group	<40	1.00	-	-
	40-49	0.78	0.61, 1.00	0.05
	50-59	0.74	0.58, 0.95	0.02
	60+	0.94	0.68, 1.29	0.70
Tumour grade	G1	1.00	-	-
	G2	1.15	0.74, 1.79	0.54
	G3	1.39	0.89, 2.16	0.15
	unknown	0.76	0.10, 5.70	0.79
Tumour size (cm)	≤2	1.00	-	-
	>2 and ≤5	1.36	1.12, 1.65	0.002
	>5	1.88	1.41, 2.52	<0.001
Type of surgery	Mastectomy	1.00	-	-
	WLE	0.79	0.66, 0.95	0.01

HER2 = human epidermal growth factor receptor 2; HR = Hazard Ratio; WLE = wide local excision; 95%CI = 95% confidence interval.

4.4 CONCLUSIONS

An association between thyroid autoimmunity (TA) and breast cancer (BC) has been debated for years and the presence of circulating anti-thyroid peroxidase (TPO) autoantibodies (TPOAb) has been suggested as a favorable BC prognostic factor (182-184, 260). Consequently, a common immune response between thyroid and BC has been proposed, with TPO the most likely shared antigen involved, as discussed in Chapter 3.

Since around 20% of women are positive for TPOAb (TPOAb+) and currently there are few useful blood prognostic markers for BC validated for clinical practice, it is important to verify TPOAb positive prognostic role for BC in patient cohorts appropriately powered for population size and length of follow-up.

The present patient cohort is the largest to address this topic to date (n=1974), with one of the longest follow-ups (96.7 months). This population study

is generally representative of patients affected with moderate-high risk early BC receiving adjuvant treatments (both systemic and local). In this patient cohort we found that neither the presence nor the titre of serum TPOAb measured with standard assays seemed to have a substantial impact on long-term recurrence or mortality; similar findings were observed for thyroid status.

4.4.1 Comparison with results from previous studies

There may be several reasons why our results do not confirm previous findings of a TPOAb+ protective role in BC prognosis. These include the fact that previous studies used smaller patient cohorts with shorter follow-ups (182, 183, 185), considered BC at different stages (185) or provided no information about BC stage (182) and may thus be susceptible to bias and random findings. As previously stated, the TACT trial is not representative of the whole BC population but of moderate-high risk early BC very similar to that analyzed by Fiore *et al.*, who recruited women with non-metastatic aggressive BC all treated with chemotherapy (183).

Thanks to the discovery and use of effective treatments for BC, the survival of patients affected with BC has increased very much during last decades. In particular, the whole TACT trial population had 5-year estimates of 82.7% and 74.9% for respectively overall survival (OS) and disease-free survival (DFS), very similar to the average 5-year OS for BC in UK (82%) calculated in an analogue time period (patients diagnosed in the period 2001-2006 and followed until 2007) (328, 329).

When re-analyzing the data and preparing the manuscript for submission to a peer-reviewed journal, I realised that the analysis population described in the present study (TACT patients having blood available) was not representative of the TACT population as a whole. In fact the analysed population had a better breast cancer prognosis compared with not included TACT patients (without blood available); mainly because blood collection was started nearly 21 months after the beginning of the study. For this reason the blood was usually taken after randomization and consequently early-relapsing patients were less likely to provide blood. Furthermore, a higher proportion of patients included in this study were treated with trastuzumab: 11.8% versus 6.4% of patients not included in the study. The likely explanation is that clinical trials assessing trastuzumab were initiated around the same time of blood collection, therefore patients providing blood were more likely to receive this additional treatment. This could also have contributed to a further increase of the survival among patients with available blood. The increased use of trastuzumab in the analysis population group is

unlikely to have biased TPOAb and thyroid function results on BC prognosis since: 1) trastuzumab was given only to a few patients, 2) the "use of trastuzumab" variable was considered combined to HER2 expression in all the performed multivariable analyses and did not significantly change the results for DFS, OS and TTR by TPOAb and/or thyroid function status.

The longer survival of the BC population analyzed in the present study could obscure a minor TPOAb prognostic effect, hypothetically detectable only among patients not suitable for standard treatments (e.g. elderly or with medical contraindications) and targeted therapies (e.g. triple negative BC). Although possible it remains unlikely, since our exploratory analysis conducted among different BC subtypes confirmed our negative results.

4.4.2 Impact of other examined factors on breast cancer prognosis

The multivariable analysis confirmed nodal status and tumour size as the two most important BC prognostic factors (309, 330). Similarly, the better BC prognosis characterizing the intermediate age group (50-59 years) confirms results of a recent wide cohort study conducted on 4453 women (331).

The role of tumour size and lymph node metastasis is well-known and for this reason they are included as major factors of TNM staging, as described in paragraph 4.1.3.1. On the contrary, the impact of age on BC prognosis is still debated and contradictory results have been obtained in the literature, likely because of the wide heterogeneity of BC and its subtypes, different adjuvant treatments and lack of adjustment for other prognostic factors. Many authors confirmed a worse BC prognosis among young (332-334) and elderly women (332, 335-337), not supported by others (338-340). A plausible explanation for a worsening of prognosis among young women is that they tend to be affected with a higher grade BC, consequently more aggressive, and BC is also more prone to be ER negative and therefore less susceptible to treatment with adjuvant endocrine therapy (334, 341, 342). On the contrary, BC in elderly women is often ER positive (343), therefore responds well to endocrine therapy (344, 345). However, although elderly women usually have less aggressive cancers which are susceptible to a wide range of treatments, the most likely reason for having worsened survival rates is potentially not receiving treatment according to the guidelines because of age or comorbidities (331).

HER2 status and tumour grade were not significant outcome factors in the great majority of cases and all the multivariable analyses conducted, respectively. The first likely explanation is that the present study has not been powered in order to assess the role of these prognostic factors.

As expected, HER2 positive patients not treated with trastuzumab clearly tended to have a worse prognosis compared with HER2 negative patients and this difference reached statistical significance in several multivariable analyses conducted.

Tumour grade, although it missed reaching the statistical significance (likely because BC tissues were graded in 104 different centres, without universal agreement among tumour grading systems), showed a constant and convincing trend of concordance between increasing tumour grade and progressive worsening of prognosis. Therefore, all the considered known risk factors for BC showed clear or partial influence on BC prognosis, proving that the cohort studied was appropriate and the model reasonably sensitive. On the contrary, TPOAb and thyroid function were constantly not significant in all analyses, with TPOAb tertiles showing no trend at all.

4.4.3 Principal limitations of the study

The principal limitations of the present study are principally a consequence of its retrospective nature: 1) lack of clinical history for thyroid diseases or medications; 2) plasma samples having been collected in most cases during or after adjuvant BC therapy.

In this study cohort all patients were treated with chemotherapy and 1745/1974 (88.4%) patients were treated with adjuvant radiotherapy for BC. Some authors suggested that in some cases the homolateral thyroid lobe can be included in the radiation field used for irradiation of internal mammary, supra- and infra-clavicular nodes (346), with a partial thyroid irradiation more likely to happen using fields including supra- and infra-clavicular node chains (347). Both studies suggested an increased risk of hypothyroidism after radiotherapy for BC but their sample size was small and no previous thyroid history was available (346, 347). Other authors observed an increased rate of hypothyroidism in patients treated with chemotherapy for BC; in particular, the thyroid function was compared at baseline and during/after the treatment, i.e. before the 6^{th} cycle of chemotherapy (348) or up to 6 months after completing chemotherapy (349). However, Smith et al. evaluated 38'255 patients with BC and found a significantly higher incidence of subsequent hypothyroidism in a 5-year follow-up compared with age-matched controls, but unrelated to radiotherapy or chemotherapy treatments (350). The main differences between studies reporting an effect of BC therapy (348, 349) and Smith's cohort (350) were the length of follow-up and the method used to evaluate thyroid function; in Smith's study the thyroid diagnosis was extrapolated from diagnostic codes taken from registers, without direct

assessment of thyroid function. A possible explanation for these discrepant results could be a temporary thyroid function reduction caused by chemotherapy, which would explain why De Groot and Kumar, evaluating thyroid function maximum 6 months after the end of chemotherapy treatment, observed an increased incidence of hypothyroidism (348, 349), not confirmed in a 5-year follow-up as in Smith's study (350). Furthermore, chemotherapy and radiotherapy could act synergistically on thyroid tissue. In fact thyroid damaging is known to be caused by treatments for head/neck tumours and blood cells tumours, i.e Hodgkin disease. Hancock et al. evaluated 1787 patients affected by Hodgkin disease and found that chemotherapy associated to radiotherapy was a major risk factor for hypothyroidism, because chemotherapy sensitize the thyroid gland to the effects of concomitant radiation therapy (351).

Regarding the therapy with tamoxifen, in this study cohort 1378 ER positive patients were submitted to hormonal therapy and in particular 1317 to tamoxifen. Some authors reported that tamoxifen may exert a modulation of thyroid function, mainly determining an anti-thyroid effect (352-354).

To my knowledge no studies have been carried out exploring possible thyroidal effects (autoimmune and non) of the treatment with trastuzumab.

Finally, the stress related to the surgical procedure itself has been suggested to have a possible immunomodulating effect that could therefore affect autoantibodies levels (355).

In conclusion, a possible influence of various BC treatments on thyroid function and autoimmunity has been suggested, with not all authors agreeing and contrasting results being produced. Well designed and powered studies are needed to definitively address this topic in the future. Furthermore, the impact of those treatments on thyroid autoantibody levels has been rarely investigated so far. For these reasons serum/plasma samples for the study of thyroid autoimmunity should be ideally collected not only before adjuvant treatments but also before breast surgery; unfortunately this was not possible in this study cohort.

However, no clear large-scale effects of adjuvant treatments for BC on thyroid function and immunity have been described to date. In addition, the sensitivity analysis conducted in a subgroup of 123 patients with plasma collected before BC adjuvant therapy also showed no evidence of TPOAb prognostic ability, even if the wide 95%CI suggests a lack of statistical power (95%CI, 0.35-2.03; $p = 0.69$). For these reasons, the results of the present study are unlikely being significantly biased by BC treatments.

4.4.4 Conclusions about thyroid autoimmunity and breast cancer prognosis

Our study indicates that circulating TPOAb measured by standard assays seem not to have any impact on the prognosis of moderate-high risk early BC. However, we cannot exclude a role of different thyroid autoimmunity (TA) parameters on breast cancer (BC) prognosis, considering that a positive prognostic effect on BC has also been suggested for the presence of goitre (182) or incidental TA related ^{18}F-FDG PET/CT uptake (260). Since in chapter 3 differences in the alternative splicing of TPO in the breast as compared to the thyroid have been suggested, this may result in different epitopes being targeted. This topic will be further discussed in the next chapter (5).

The overall TPOAb prevalence in our cohort was 20.57%, very similar to our *a priori* predicted value. This value reflects TPOAb prevalence among women in the general population, reported as varying from 17% (63) to 26.4% (64), with all authors agreeing that TAb prevalence increases with age (64-67) and after iodization programs (327), explaining why TPOAb+ women were older than TPOAb-. It remains possible that TPOAb+ rates are higher in the BC population (generated, for example, by antigen cross-reactivity) even if they do not predict outcome, as this study was not designed to compare TPOAb prevalence among BC patients and the general population.

In conclusion, the present study is to my knowledge the largest currently available investigating the impact of circulating TPOAb and thyroid function on BC prognosis. In contrast to previous results from small-scale studies, blood TPOAb measured with standard assays and thyroid function did not substantially influence the long-term recurrence and mortality of moderate-high risk early BC in the modern era. Considering the limitations of the present observational study due to its retrospective nature, before drawing definitive conclusions it would seem appropriate to perform a similar study in a prospective patients cohort, with available clinical data about thyroid disorders and treatments and collecting the blood samples before any cancer treatment. However, major confounding factors in this study due to the effects of BC treatments on circulating thyroid markers seems unlikely, even if not 100% excluded. Future studies might also explore non-conventional or breast-specific immune responses to TPO to determine whether other aspects of thyroid autoimmunity may be relevant to breast cancer outcome, as will be further discussed in the next chapter (5: general discussion).

5 GENERAL DISCUSSION

5.1 Summary of main findings

As summarized in chapter 1, paragraph 1.4, an association between breast cancer (BC) and benign thyroid disorders (BTD) has been debated for decades, supported by many authors (154-158), with others not agreeing (159-163). In particular, BC was suggested to be associated with thyroid autoimmunity (TA) (157); similarly to BTD this association has not been confirmed in different studies (161). More specifically, thyroid peroxidase (TPO) is one of the major antigens involved in TA and autoantibodies to TPO (TPOAb) have been found to be more common among patients affected with BC compared with healthy controls (166, 179-181). Furthermore, several small-scale studies observed that patients affected with BC and positive for blood TPOAb had a better prognosis compared with TPOAb negative individuals, suggesting a possible protective role of TPOAb (182-184), even if not confirmed in another study (185). BC and thyroid cells share similar properties (199), e.g. they both express the sodium iodide symporter (NIS) (200) and have a peroxidase activity, TPO in thyroid and lactoperoxidase (LPO) in breast (257). In the present study a shared immune activity between thyroid and BC tissues has been hypothesized, leading to increased tumour destruction and therefore to an improved prognosis.

The first aim of the present study was to identify the hypothetical shared antigen between BC and thyroid cells. Considering that NIS is expressed in both thyroid and BC cells, in chapter 2 NIS has been hypothesized as common antigen and the presence of circulating autoantibodies to NIS (NISAb) has been investigated, expecting a higher prevalence among patients affected with TA and BC. NISAb were found to be rare and apparently not related to the presence of TA and/or BC therefore, although NIS is present in both thyroid and BC cells, it does not seem to represent an important antigen, either for TA, or as shared antigen between thyroid and BC cells.

Considering the higher prevalence of TPOAb among patients affected with BC (166, 179-181), in chapter 3 TPO has been investigated as possible alternative shared antigen between thyroid and BC cells. The results indicated that TPO, both mRNA and protein, is expressed (at a much lower level compared to thyroid) in BC and breast peri-tumoural tissues, as well as different tissues, e.g. adipose tissue. Therefore, TPO is the most likely candidate as shared antigen between thyroid and BC tissue, since even trace amounts could suffice to trigger lymphocyte activity. Other unknown shared antigens, e.g. expressed by thyroid colloid, could

also be involved, even if unlikely, giving the huge number of studies trying to find novel thyroid autoantigens, with negative results. The TPO gene is highly subjected to alternative splicing and transcripts for novel TPO isoforms never described before have been discovered with this study. Interestingly, some TPO mRNA isoforms could be tissue specific, some in particular breast specific, therefore if confirmed they could represent possible bio-markers for BC. However, despite TPO mRNA and/or protein were expressed in all the BC tissues examined in chapter 3, only 1/8 (12.5%) patients was found positive for serum TPOAb (Table 3-3). Conversely, the prevalence of TPOAb among BC patients should be higher, in accordance to data from literature (summarized in paragraph 4.1), further confirmed by 20.6% TPOAb prevalence among BC patients observed in chapter 4. There are several possible explanations for this discrepancy; first, 8 patients are too few in order to validate an epidemiological data (TPOAb prevalence among BC patients). Second, thyroid and breast TPO could express different antigenic properties, even if preliminary studies seem to exclude this hypothesis (356). Third, BC could mainly express TPO isoforms with epitopes different from complete TPO protein and therefore generating TPOAb not identifiable by standard TPOAb assays.

In chapter 4 the prognostic role of TPOAb has been investigated in a large cohort of patients affected with BC and extensively followed for 96.7 months; the prognostic role of thyroid function has been investigated as well. Surprisingly, the present observational study clearly indicated that neither TPOAb (measured with one standard assay) nor thyroid function seemed to significantly affect the prognosis of BC, therefore these results did not confirm previous findings suggesting a protective role of TPOAb for BC (182, 183). However, considering the retrospective nature of the present study, a confirmation of its results in a prospective patients cohort would be needed before definitively excluding from breast cancer prognosis a role for TPOAb (measured by standard assays) and thyroid function.

5.2 How do my results compare with those of others?

5.2.1 Cross-reactivity of circulating TPOAb with different peroxidases

An alternative mechanism to explain the increased prevalence of TPOAb among patients with BC could be the presence of cross-reactivity of TPOAb with different peroxidases, in particular LPO, the most abundant peroxidase expressed in breast tissue (257), as previously discussed in chapter 3, paragraphs 3.1.1.2 and 3.5.2.

The study of Banga *et al.* (291) revealed that 40% and 45% of human sera positive for TPOAb cross-reacted with native bovine LPO, when detected respectively by enzyme-linked immunosorbent assay (ELISA) and western blot (WB). In reducing conditions, the percentage of TPOAb cross-reactivity with LPO decreased to 25% for ELISA and increased to 60% for WB. Pre-adsorption experiments of human sera with human TPO and/or LPO in order to test the signal specificity gave poor and satisfactory results in ELISA and WB assays respectively, as summarized in Table 5-1. In particular, pre-adsorption with native TPO caused the signal to disappear in TPO WB and not in LPO WB as expected; on the contrary, pre-adsorption with native LPO was not able to reduce the signal in LPO WB.

Table 5-1: Adsorption experiments with TPO and LPO in ELISA and WB

	ELISA (Native)		WB (Reduced)	
Human serum	**TPO**	**LPO**	**TPO**	**LPO**
Normal	+	+	+	+
PA with liver microsomes	+	+	+	+
PA with native TPO	/	/	/	+
PA with reduced TPO	/	/	/	+
PA with native+reduced TPO	/	/	/	/
PA with native LPO	+	+	+	+
PA with reduced LPO	+	+	+	/
PA with native+reduced LPO	+	+	+	/

Summary of pre-adsorption (PA) experiments of human serum with thyroid peroxidase (TPO) and lactoperoxidase (LPO) in enzyme-linked immunosorbent assay (ELISA) and western blot (WB) performed by Banga et al. (291). + = positive signal. / = negative signal.

TPOAb cross-reactivity with native myeloperoxidase (MPO) was less common, 0% of sera tested with ELISA and 15% with WB, however it increased to 70% and 50% respectively when using reduced MPO (291).

Nishikawa *et al.* further investigated TPOAb cross-reactivity using four monoclonal human TPO-Fab (TPOAb's variable regions only) directed towards the immunodominant region of TPO, defined as the region recognized by the majority of sera from patients positive for TPOAb. None of the four TPO-Fab cross-reacted with MPO or LPO, therefore the authors concluded that circulating TPOAb cross-reacting with other peroxidases are likely to bind TPO outside the immunodominant region (357).

In conclusion, different patients affected with TA are positive for different kinds of circulating TPOAb, some of them cross-reacting with the other peroxidases. TPOAb cross-reactivity with LPO could represent an additional mechanism to explain why TPOAb are more prevalent among patients with BC. However, LPO is abundantly expressed in breast tissue, both normal and tumoural,

where it is present as a secreted protein and therefore normally accessible to the immune system. As a consequence, the mechanism of antigen unmasking triggered by the tumoural process is unlikely to be involved. Different mechanisms are needed in order to explain the rise in TPOAb mainly in patients affected with BC compared with the general population. An alternative hypothesis could be the expression of a more antigenic form of LPO, e.g. with a different glycosylation level, in BC tissue compared with normal breast, or a general over-activation of the immune system secondary to tumoural processes, leading to an increase of auto-reactivity. Considering that TPO is expressed in BC and breast peri-tumoural tissue and different TPO isoforms exist, as described in chapter 3, similar mechanisms leading to different TPO epitopes being exposed in BC patients could be involved.

5.2.2 TPO isoforms and epitopes

Lai et al. have already described TPO gene expression in orbital tissue (261). The present study (chapter 3) confirmed TPO presence in orbital fat but also indicated that TPO is expressed in many different tissues: other depots of adipose tissue (abdominal, subcutaneous, from knee), BC and breast peri-tumoural tissue, and other cancers. So far, TPO's role and function in these different tissues have not been investigated. Furthermore, it is unclear which TPO isoforms are more expressed and if some of them could be tissue specific, as suggested by the present study. In chapter 3, a TPO mRNA isoform missing both exons 14 and 16 was found to be more prevalent in breast and adipose tissue and in BC cell lines; similarly, LongRange RT-PCR showed the presence of different TPO mRNA isoforms among the different tissues studied (one thyroid tissue, one adipose tissue and two BC tissues). Further experiments using a higher number of tissues are needed in order to draw substantial conclusions about tissue specificity of TPO mRNA isoforms.

Furthermore, it would be important to establish whether mRNA is translated into the corresponding protein; this would be essential to establish if TPO isoforms have functional or antigenic activity. In order to be recognized by the immune system, TPO has to express certain epitopes and different isoforms may fold differently and therefore express alternative epitopes compared with full-length TPO. In fact epitopes may be of two kinds: linear and conformational. Linear epitopes, mainly recognized by T lymphocytes, consist of short linear peptides (8-20 amino acids) processed from protein antigens and subsequently bound to MHC class II (triggering CD4+ cells) or class I (triggering CD8+ cells) (246), as previously introduced in chapter 1, paragraph 1.2.2.1. Many authors identified TPO epitopes

recognized by T lymphocytes in the MPO-like sequence (246, 358-361). Furthermore, two T-lymphocyte epitopes have been found also in the C-terminal part of TPO protein, in particular in the transmembrane region (359, 360) and in the intracellular region (359). On the contrary conformational epitopes consist of juxtaposed amino acids occurring at intervals in the sequence (or "remote in the sequence") but brought closely together by the three-dimensional folding of the molecule (362). TPO epitopes recognized by human serum autoantibodies and B lymphocytes are for the great part conformational (242, 246, 255, 363, 364), therefore strongly related to tertiary and quaternary protein structures. Only a few TPO epitopes recognized by B-lymphocytes are linear: C21 and C2 (365, 366), as previously reported in chapter 3, paragraph 3.1.1.

Therefore, TPO isoforms lacking one or more exons probably fold into a different conformation when compared with the full-length TPO and hence may lose their antigenic property for B cells and autoantibodies, or expose alternative conformational epitopes. Considering that standard laboratory assays to detect TPOAb are based on the full-length TPO protein, they are likely to fail in detecting TPOAb directed towards epitopes present in TPO isoforms lacking one or more exons. This could be an explanation of the negative results obtained in chapter 4: TPOAb detected by standard assays (and therefore recognizing the full-length TPO) seem not to have any impact on BC prognosis. However, different results could be obtained measuring blood circulating TPOAb directed towards alternative epitopes present on hypothetical smaller TPO isoforms, whose gene lack one or more exons.

Similarly, the absence of TPOAb influencing BC prognosis does not exclude the presence of a hypothetical T lymphocytes common response between thyroid and BC cells.

Finally, metastatic BC patients at diagnosis were systematically excluded in our large-cohort prognostic study (chapter 4). Therefore, our findings of an absence of any substantial effect of TPOAb on the prognosis of non-metastatic BC cannot exclude the presence of a TPOAb protective effect on patients affected with metastatic BC, as described by other authors (184).

5.3 Future directions

First, it is important to state what I would do differently if I had to start my PhD again. During this time I learned the importance of a proper study design in order to deliver good quality science and research projects. Therefore, I would initially spend more time studying the literature related to my research project, delaying the start of laboratory experiments. In fact having a better knowledge of

laboratory techniques and work already performed by different research groups would definitively save time in experimental optimizations and the identification of specific targets and goals. For example, if I had known before that phenol red exerts an oestrogenic stimulatory activity on MCF-7 cells, in chapter 3 I would have used a different cell culture medium for BC cells experiments and I may have obtained more defined conclusions about hormonal regulation of TPO gene expression. Similarly, I should have used charcoal stripped foetal calf serum, in order to avoid the presence of any hormones that could interfere with my *in vitro* assays.

Second, the results of the present thesis open multiple possibilities of future studies, as detailed below.

5.3.1 Functional and immunological characterization of breast cancer-specific TPO isoforms

First, in order to identify potential tissue specific TPO isoforms, a higher number of *ex vivo* human tissues should be tested with LongRange RT-PCR investigating the whole length of TPO mRNA. The following tissues (fresh frozen) should be included: thyroid tissue as positive control, BC, normal breast (from reductive mammoplasty), adipose tissue from different depots, other cancers (from kidney, pancreas, and also different sites) and relative normal tissues obtained from benign diseases affecting the same organs. Regarding BC, not only different histological types should be investigated but, even more importantly, different molecular types. In fact, as previously explained in paragraph 1.3.3.2, the clinical behavior of BC is more determined by molecular parameters, e.g. the expression of human epidermal growth factor receptor type 2 (HER2) and oestrogen receptor (ER) or progesterone receptor (PR). Similarly, it could be possible that TPO gene and different isoforms expression in BC is regulated and dependent on similar molecular factors. Different forward (F) and reverse (R) TPO primers in addition to F in exon 2 and R in exon 17 could also be used, in order to investigate for the presence of alternative START and STOP codons in TPO gene and therefore to identify TPO isoforms eventually missing exon 2 and/or 17.
Moreover, the mRNA sequence of all TPO isoforms could be better investigated with more recent high throughput techniques for the analysis of transcriptomics: RNA-Seq (367, 368).

Secondly, the corresponding protein should be investigated for the most promising potentially tissue-specific TPO isoforms identified. To this purpose the development of TPO-isoform specific antibodies would be recommended. For example the peptide of the TPO isoform apparently more abundant in breast and

adipose tissues, missing amino acids encoded by exons 14 and 16 (paragraph 3.4.2.2), could be generated by chemical synthesis (e.g. creating a peptide composed of sequential amino acids encoded by exons 13, 15 and 17). This peptide could then be used to generate a TPO antibody directed towards this specific TPO isoform, whose presence in human *ex vivo* tissues would be screened using WB in denaturing (loss of hydrogen and hydrophobic bonds) and reducing (loss of disulfide bonds) conditions. This would be essential in order to ensure that the peptide does not fold in tertiary structures potentially hiding the linear epitope recognized by the newly synthesized antibody. Using this technique, it could be possible to test several *ex vivo* human tissues for the expression of specific TPO isoforms that could be then verified by protein sequencing.
Considering that WB requires homogenized tissues and therefore it is impossible to distinguish between different tissue compartments (e.g. breast contains both breast cells and adipocytes), additional techniques such as immunohistochemistry (IHC) should be used, in order to exactly localize TPO expression within the tissue. Different antibodies may be needed for this technique, since with IHC TPO protein would be present preserving its tertiary and quaternary structure and therefore potentially hiding linear epitopes, while exposing conformational epitopes.

Similarly, different antibodies and different techniques from WB should be applied in order to investigate for the immunogenic properties of tissue-specific TPO isoforms, since they may also involve conformational epitopes. In fact TPO denaturation and reduction conditions typical of WB cause a considerable modification of the antigenic structure of TPO, with loss of many conformational epitopes; in the study of Ruf *et al*. 13 monoclonal TPO antibodies recognized native TPO, but only 5/13 (38.5%) recognized also denatured and/or reduced TPO (290). Specific monoclonal TPO antibody directed towards conformational epitopes of TPO isoforms could be produced by genetic immunization, as previously described by Costagliola *et al*. (369). Briefly, a plasmid DNA harboring one TPO isoform cDNA would be inoculated in the anterior tibialis muscle of mice; as a consequence, some mouse cells would internalize the plasmid DNA and express the TPO isoform and corresponding protein, leading to immunization and production of TPO isoform-specific antibodies, subsequently identified and purified. Considering that TPO is a transmembrane protein, TPO expression by mouse cells is essential in order to maintain the original conformation that would likely be lost if immunizing mice directly with the recombinant TPO isoforms produced in bacteria, since cell membrane-free. Antibodies produced with this technique could then be used in IHC to localize TPO isoforms expression within the examined tissues.

In order to test the functional and immunogenic properties of these TPO isoforms, *in vitro* assays could be used. CHO cells should be transfected with expression vectors like pcDNA3 containing the gene of a specific TPO isoform, so that the correspondent protein would be expressed by CHO cells, as performed in this study with NIS (chapter 2). The presence of circulating human TPOAb specific for these isoforms in the blood of individuals with TA and/or BC, compared with healthy controls, could be tested by flow cytometry, as performed in chapter 2 when searching for autoantibodies to NIS. Considering the presence of aspecific human immunoglobulins binding to antigens expressed on CHO cells surface (chapter 2, paragraph 2.4.2), it is likely to obtain the same limitation when investigating for TPOAb. In order to reduce this aspecific binding, it would be advisable to perform the pre-adsorption of human sera with a higher number of CHO cells compared to what was done in the present thesis (each 2µl of serum wee adsorbed approximately with $9x10^4$ cells: see paragraph 2.2.2.4). In a careful analysis of related literature, other authors suggested and performed a 10 times higher ratio of pre-adsorption of human sera for flow cytometry experiments in order to reduce the aspecific signal: each 2µl of serum were adsorbed approximately with $6x10^5$ cells (230). This is an additional example of the importance of carefully checking the related literature during the study design and before performing the laboratory experiments. If I had to start again my PhD, I would perform differently the pre-adsorption step in flow cytometry experiments of chapter 2, according to these indications.

Regarding the functional activity of TPO isoforms, it is likely to be preserved if they contain the amino acidic sequence 239-494 AA (encoded by exons 7-8-9), corresponding to the catalytic site (247), as previously explained in chapter 3, paragraph 3.1.1. In order to test it, specific enzymatic assays based on guaiacol oxidation should be performed, as previously described (370).

If the presence of tissue-specific TPO isoforms should be confirmed, they could be used as diagnostic biomarkers and validated in longer prospective studies, as later proposed.

5.3.2 Prospective study

The results of chapter 4 indicated that TPOAb measured by standard assays and thyroid function do not seem to affect the prognosis of BC. However, this study has 2 main limitations: the lack of clinical history of thyroid diseases and the blood collection performed during or after adjuvant treatments for BC at different time-points, that could have affected TPOAb, FT4 and TSH values, even if major confounding is unlikely. For this reason, I would like to start a new large-

scale prospective study enrolling patients newly diagnosed with BC, in order to assess baseline parameters in a homogeneous and correct manner.

- Aims:
  - To correlate the expression of TPO isoforms in BC tissue with circulating isoform-specific TPOAb.
  - To investigate a hypothetical prognostic role of: 1) TPO isoforms expressed by tumoural tissue 2) TPOAb measured by standard assays collected before any treatment 3) isoform-specific TPOAb if present 4) thyroid function 5) thyroid volume.
  - To prospectively evaluate the impact of BC treatments on thyroid function and autoimmunity. This would represent one of the first principal studies in this field.
- Inclusion criteria: considering that the high BC survival as a consequence of highly effective BC treatments could hide a minor positive prognostic role of TPOAb, I would include in the study patients affected with BC not suitable for usual treatments, e.g. chemotherapy because elderly or having medical contraindications, or target therapy because triple negative BC.
- Materials:
  - Accurate clinical history including thyroid diseases, thyroid medications and use of drugs potentially affecting thyroid function (e.g. amiodarone), familiarity for BC and thyroid diseases.
  - Blood for serum extraction collected before any treatment, including breast surgery, in order to avoid any treatment-related possible bias. Blood would be subsequently collected every year until the end of follow-up (initially 5 years, pre-planning a more mature analysis): this would allow to document eventual effects of treatments on thyroid function and autoimmunity.
  - Fresh tissues collected during breast surgery, immediately snap frozen in liquid nitrogen and stored at -80 °C.
- Methods:
  - mRNA expression of TPO isoforms: RNA extraction from tissues, LongRange RT-PCR, gene sequencing, RNA-seq analysis
  - Protein expression of TPO isoforms: protein extraction from tissues and analysis with WB using isoform-specific monoclonal TPOAb and protein sequencing for confirmation
  - Serum would be tested for autoantibodies recognizing specific TPO isoforms by flow cytometry using CHO cells transfected with different TPO isoforms.

- The same statistical analyses performed in chapter 4 would be applied in order to assess the prognostic impact of: 1) TPO isoforms (mRNA and/or protein) expressed in tumoural tissue; 2) circulating TPOAb (both measured by standard assays detecting full-length TPO and specific for TPO isoforms, when present); 3) thyroid function (FT4 and TSH); 4) additional parameters of TA with a suggested role on BC prognosis, such as the thyroid volume (182), measured for each patient by ultrasound examination.

5.3.3 In vitro studies investigating the regulation of TPO expression in breast cancer cell lines

Finally, *in vitro* assays investigating the regulation of TPO expression in BC cell lines (MCF-7, T-47D and MDAMB-231) should be repeated with the following modifications: 1) using phenol red-free culture medium and charcoal stripped foetal calf serum in order to eliminate uncontrolled hormonal interferences 2) trying different concentrations of estradiol and Insulin-like growth factor type II (IGF-II). Furthermore, considering that TPO is expressed also in adipose tissue and that adipocytes within mammary gland carcinomas seem to be dynamic cells that may contribute to human BC progression (274), accurate studies of adipokines should be performed in order to add the most important ones to the cell culture medium.

5.4 Summary conclusions

In the present thesis, a shared immune response between thyroid and breast cancer (BC) tissues has been hypothesized, in order to explain the association between thyroid autoimmunity (TA) and BC, summarized in chapter 1. Considering that the sodium iodide symporter (NIS) is expressed by both thyroid and BC tissues, the presence of circulating autoantibodies to NIS has been investigated among patients affected with TA and/or BC, with negative results (Chapter 2). Therefore, considering that thyroid peroxidase (TPO) is one of the major antigens involved in TA and that autoantibodies to TPO (TPOAb) are more prevalent among patients affected with BC compared with healthy controls, the presence of TPO expression has been investigated in BC tissue, obtaining positive results, even if expressed at a much lower level compared with thyroid tissue. TPO expression has been found also in different examined tissues, e.g. adipose tissues or other cancers and, more importantly, the results of the present study suggest the possibility that different TPO isoforms obtained by alternative splicing of TPO gene may be tissue-specific and therefore potentially used as biomarkers for BC (Chapter 3). Despite the presence of circulating TPOAb being previously suggested

as a positive prognostic factor for BC in small-scale studies, the results of the present retrospective study obtained in a large patients cohort with an extensive follow-up showed no apparent evidence for a significant prognostic role for BC of TPOAb measured by standard assays, as well as of thyroid function (Chapter 4). Future studies should further investigate TPO isoforms expression in BC tissue and their potential as biomarkers, as well as searching for different TPO isoforms-specific TPOAb, not detectable by standard assays. A prospective study should validate the findings of the present retrospective study and also investigate whether these alternative TPO isoforms-specific TPOAb, differently from TPOAb recognizing full-length TPO, could have a prognostic role for BC.

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