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Thyroid Hormones and Morphological Features of Primary Breast Cancer

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Abstract. *Background/Aim: Many experimental studies have suggested the importance of thyroid hormones in breast cancer (BC) morphogenesis. The aim of this study was to evaluate the association of thyroid hormone levels in serum of patients with primary BC with morphological presentations of the disease in pathological specimens and prognosis. Patients and Methods: We measured the serum levels of thyroid-stimulating hormone (TSH), free triiodothyronine (FT3), and free thyroxine (FT4), along with serum thymidine kinase 1 activity and examined their relation to pathological features and prognosis of 158 patients with primary BC. Results: We found a significant positive association of serum FT3 level with the presence of carcinoma in situ component (CIS) (p=0.032) and its size (p=0.047), with the presence (p=0.022) and the number of multifocal/ multicentric tumors (MMTs) (p=0.002), as well as with increased proliferative activity in terms of serum thymidine kinase 1 (p=0.002). Moreover, we report that each 1.0 unit rise of FT3/FT4 ratio×10 was associated with an odds ratio of 1.77 (95% confidence interval=1.17-3.30, p=0.007), 1.97 (95% confidence interval=1.17-2.67, p=0.010) and 1.56 (95% confidence interval=1.02-2.37, p=0.039) for the detection of patients with CIS, MMTs and lymphovascular invasion, respectively, after adjusting for age. We did not find statistically significant associations of serum TSH level with breast cancer`s parameters. A Cox regression survival analysis identified serum FT3 level >5.95 pmol/l as a risk factor for BC recurrence (relative risk=2.65, p=0.017), a finding that retained significance in a multivariate model (relative risk=2.52, p=0.027). Conclusion: The FT3/FT4 ratio is a valuable parameter predicting the presence of CIS, MMTs and lymphovascular invasion in pathological specimens. An elevated serum FT3 level is associated with the presence of CIS, MMTs, increased proliferative activity and poor prognosis.*

Thyroid hormones (THs) are involved in the regulation of cell metabolism, maturation, and differentiation of the normal mammary gland and have been suggested to affect the growth and spread of breast cancer (BC) cells (1). Two main forms of TH are produced by the thyroid gland, the biologically active hormone T3 (3,3',5-triiodothyronine), and the prohormone T4 (3,5,3',5'-tetraiodothyronine), which is released 40-fold more than T3 (2). T3 binds to TH receptors with the highest affinity (3). Specific iodothyronine deiodinases (DIO) regulate the systemic concentrations of these hormones by converting T4 to T3 (4).

The involvement of T3 in the developmental morphogenesis of breast tissue has been established (5, 6). T3 contributes to the development of breast lobules by influencing ductal branching and alveolar budding (6). The basic developmental events underlying branching morphogenesis are closely related to pathways important to cancer progression, such as epithelial plasticity and epithelial–mesenchymal transition (EMT) (7).

Many findings from experimental studies have suggested that T3 might play an essential role in BC development and progression. In BC cell lines, T3 was shown to promote cell proliferation (8-12), and trigger remodeling of the cytoskeleton and focal adhesion formation (13). In *in vitro* models, T3 activated proangiogenic actions in endothelial cells by inducing the transcription of angiogenesis-related genes (14). In T-47D BC cells, T3 stimulated the EMT, which is fundamental in promoting cell adhesion, as well as

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migration and invasion processes (15, 16). All these data suggest the potential role of T3 in BC morphogenesis.

Despite abundant experimental data demonstrating a pleiotropic effect of T3 on BC cells, it appears that no clinical studies so far have identified the changes in morphological features in human BC specimens related to serum TH levels. In the present study, we assessed the effect of THs on some morphological features, proliferative activity and prognosis in human BC.

Patients and Methods

This prospective study, which was approved by the Institutional Ethical Review Board (380-23.04.04), included 158 women who underwent surgery for invasive breast carcinoma between 2004 and 2010, and were followed up at the Hadassah University Hospital in Jerusalem. Patients with known metastatic breast tumors and patients who had preoperative neoadjuvant chemo- or hormonal therapy, TH replacement therapy (for hypothyroidism) or treatment for thyroid hyperfunction were excluded, as were patients with only *in situ* BC. All participants provided signed informed consent.

Histological typing of the tumors was performed according to World Health Organization recommendations (17). Pathological staging was performed in line with the seventh edition of the Tumor, Nodes, Metastasis Classification of Malignant Tumors of the International Union Against Cancer (18). The grade of invasive tumors was assessed according to the Nottingham scoring system (19). Carcinoma *in situ* (CIS) was graded as low (grade 1), intermediate (grade 2) or high (grade 3). Regarding CIS as a component of invasive breast carcinoma (IBC), the samples were classified into pure IBC, IBC with small CIS component (≤1.0 cm), and IBC with large CIS component (>1.0 cm). Diagnosis of lymphovascular invasion (LVI) in the primary tumor was performed with hematoxylin and eosin staining and confirmed with CD31 and D2-40 immunostaining. Multifocal and multicentric tumors (MMTs) were considered as one category and defined as tumor with two or more foci of IBC in the same breast.

Serum samples were obtained from patients preoperatively, aliquoted and stored at –80˚C until analysis. Serum thymidine kinase 1 (TK1) activity was measured by a colorimetric enzyme-linked immunosorbent assay kit (DiviTum; Biovica International AB, Uppsala, Sweden), as described previously (20). TK1 levels were expressed in Divitum units/l (Du/l). The cut-off level for TK1 was 140.0 Du/l, based on the 95th percentile in a reference group of 149 healthy women (20). This cut-off was used for detection of patients with increased proliferative activity.

The testing of serum FT4, FT3 and TSH levels was performed using commercial IMMULITE kits, which are solid-phase, two-site chemiluminescent immunometric assays (Immulite; Siemens Healthcare Diagnostics, Llanberis, UK). The normal ranges for our laboratory were 0.35-5.50 mIU/l for TSH, 3.10-6.8 pmol/l for FT3 and 9.0-25.0 pmol/l for FT4.

Statistical analysis. FT4, FT3 and TSH concentrations were evaluated both as continuous and as categorical variables. The nonparametric Mann–Whitney and Kruskal–Wallis tests were used. Numeric variables are presented as medians with interquartile range (IQR). Associations between categorical variables were evaluated with Fisher's exact test. To detect an association between continuous TH levels and categorical pathological characteristics, bivariate logistic regression analysis was used with FT4, FT3, or (FT3/FT4 ratio)×10 as the dependent variables and pathological characteristics such as the presence of MMTs, CIS or LVI as the independent. The analysis was performed with adjustment for age as a continuous covariate.

Recurrence-free survival (RFS) was defined as the interval between the time of surgery and the detection of the first locoregional recurrence or distant metastasis or the last follow-up date. Patients who developed a second unrelated malignancy and those who died due to unrelated causes were censored. A Cox proportional hazards model was used to test the statistical independence and significance of predictors in RFS. Statistical calculations were performed using SPSS, version 17 for Windows (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered significant.

Results

This study included 158 women with a histological diagnosis of IBC who underwent surgery as the initial treatment. Age ranged between 24 to 88 years (median=56, IQR=46-65 years). One hundred and forty-two patients (89.9%) had invasive ductal carcinoma (IDC), and 16 patients (10.1%) had invasive lobular carcinoma (ILC). CIS component, MMTs and LVI were present in 125 (79.1%), 37 (23.4%) and 32 (20.3%) patients, respectively. Eighty-six patients (54.4%) had tumors ≤2.0 cm (T1), and 72 patients (45.6%) had tumors >2.0 cm (T2-T3). Histological tumor grade was I/II in 66 patients (41.8 %), and 79 patients had grade III tumors (50.0%). Ninety patients had node-positive disease (56.9%) and 68 (43.1%) node-negative. One hundred and twenty-six (79.8%) had estrogen receptor (ER)-positive disease, 115 cases progesterone receptor-positive (72.8%) and 32 cases were human epidermal growth factor receptor 2/neu-positive (20.3%).

Association of thyroid function tests with clinicopathological characteristics. A higher median basal FT3 level (Table I) was detected in younger (<50 years) compared to older (≥50 years) patients (5.24 *vs*. 4.64 pmol/l, $p=0.005$). There was no significant difference in median levels of FT3, FT4, FT3/FT4 ratio, TSH and TK1 between patients with IDC and ILC ($p=0.058$, $p=0.811$, $p=0.162$, $p=0.406$ and $p=0.059$, respectively).

The presence of a CIS component was associated with a higher level of FT3 (4.85 *vs*. 4.44 pmol/l, $p=0.032$), a lower level of FT4 (16.73 *vs*. 19.31 pmol/l, $p=0.010$), and a higher FT3/FT4 ratio (0.29 *vs*. 0.21, $p=0.001$). A similar pattern of changes in TH levels (Table I) was observed in patients with MMTs when compared to those with unicentric disease, namely higher FT3 (5.48 *vs*. 4.69 pmol/l, $p=0.022$), lower FT4 (15.44 *vs*. 18.02 pmol/l, $p=0.031$) and higher FT3/FT4 ratio (0.31 *vs*. 0.26, $p=0.015$), respectively.

The association of LVI with FT3 and FT4 levels was not significant (4.80 *vs*. 4.94 pmol/l, $p=0.203$ and 17.64 *vs*. 16.73 pmol/l, $p=0.381$, respectively). However, the presence of LVI was associated with a higher FT3/FT4 ratio (0.31 *vs*.

Table I. *Association of serum thyroid hormones and thymidine kinase 1 activity with patient characteristics (N=158). Data are median values (interquartile range).*

Characteristic	Subgroup	n	FT3 (pmol/l)	FT4 (pmol/l)	FT3/FT4 ratio	TSH (mIU/l)	TK1 activity (Du/l)
Whole cohort		158	4.82 (4.21-5.65)	17.25 (15.44-20.11)	0.28 (0.22-0.34)	1.57 (0.98-2.70)	35.7 (18.5-69.6)
Age	<50 Years	55	5.24 (4.58-5.76)	18.02 (14.93-20.59)	0.30 (0.23-0.36)	1.64 (0.98-2.71)	24.8 (15.6-71.7)
	≥50 Years	103	4.64 (4.06-5.41)	16.86 (15.44-20.08)	0.27 (0.21-0.33)	1.45 (0.98-2.70)	35.6 (19.1-66.5)
	p-Value		**0.005**	0.938	0.122	0.810	0.417
Histology	IDC	142	4.82 (4.22-5.65)	18.02 (15.44-20.15)	0.28 (0.22-0.34)	1.58 (0.98-2.75)	33.7 (18.4-66.6)
	ILC	16	4.35 (3.78-4.87)	16.73 (15.38-19.63)	0.24 (0.20-0.31)	1.33 (0.99-1.99)	72.1 (32.3-101.4)
	p-Value		0.058	0.811	0.162	0.406	0.059
Presence of CIS	No	33	4.44 (3.95-5.31)	19.31 (16.60-22.20)	0.21 (0.18-0.31)	1.37 (1.02-2.46)	22.1 (14.3-56.6)
	Yes	125	4.85 (4.31-5.73)	16.73 (15.32-19.31)	0.29 (0.24-0.35)	1.63 (0.97-2.83)	38.0 (20.5-76.6)
	p-Value		**0.032**	**0.010**	**0.001**	0.254	**0.049**
MMTs	No	121	4.69 (4.17-5.40)	18.02 (15.44-20.15)	0.26 (0.21-0.33)	1.51 (0.99-2.62)	34.3 (19.3-67.9)
	Yes	37	5.48 (4.28-6.08)	15.44 (14.16-19.95)	0.31 (0.25-0.39)	1.64 (0.93-3.33)	22.1 (12.6-62.4)
	p-Value		**0.022**	**0.031**	**0.015**	0.307	0.331
LVI	No	126	4.80 (4.14-5.58)	17.64 (15.44-20.59)	0.27 (0.21-0.33)	1.46 (0.95-2.73)	30.4 (15.9-61.1)
	Yes	32	4.94 (4.37-5.86)	16.73 (14.80-19.31)	0.31 (0.24-0.38)	1.66 (1.13-2.45)	55.1 (23.2-109.9)
	p-Value		0.203	0.381	**0.042**	0.549	**0.023**
Grade	1-2	66	4.80 (4.04-5.54)	17.25 (15.16-19.37)	0.27 (0.21-0.34)	1.36 (0.97-2.66)	25.2 (15.9-64.5)
	3	79	4.95 (4.36-5.73)	18.02 (15.44-20.59)	0.30 (0.23-0.34)	1.64 (0.95-2.87)	38.4 (19.2-84.6)
	p-Value		0.206	0.666	0.564	0.332	0.066
T size	<2 cm	86	4.82 (4.12-5.59)	18.02 (15.44-19.56)	0.27 (0.21-0.34)	1.64 (0.95-2.83)	31.5 (14.4-69.6)
	≥2 cm	72	4.82 (4.30-5.69)	16.73 (15.25-20.59)	0.29 (0.23-0.33)	1.51 (1.02-2.59)	35.6 (20.0-64.8)
	p-Value		0.648	0.511	0.524	0.853	0.663
Nodal status	Negative	90	4.87 (4.18-5.74)	16.93 (15.44-20.21)	0.28 (0.22-0.34)	1.45 (0.93-2.67)	30.6 (17.0-67.8)
	Positive	68	4.72 (4.23-5.56)	17.64 (15.44-20.05)	0.28 (0.22-0.33)	1.64 (1.08-2.80)	37.2 (18.9-67.3)
	p-Value		0.432	0.863	0.688	0.195	0.669
ER status	Negative	32	5.32 (4.36-5.90)	18.79 (15.44-20.59)	0.27 (0.21-0.36)	1.64 (0.78-2.39)	50.9 (24.5-142.0)
	Positive	126	4.72 (4.16-5.43)	17.64 (15.44-20.59)	0.28 (0.22-0.34)	1.59 (0.99-2.85)	27.4 (15.9-54.5)
	p-Value		0.098	0.403	0.801	0.264	**0.036**
PR status	Negative	43	5.02 (3.98-5.84)	18.66 (15.44-20.59)	0.26 (0.20-0.35)	1.40 (0.91-2.39)	37.9 (22.3-110.8)
	Positive	115	4.79 (4.24-5.55)	16.73 (15.44-19.56)	0.28 (0.23-0.34)	1.61 (1.01-2.78)	31.0 (16.5-64.6)
	p-Value		0.578	0.578	0.382	0.341	0.160
HER2/neu status	Negative	126	4.82 (4.21-5.58)	17.25 (15.44-20.11)	0.28 (0.22-0.34)	1.46 (0.96-2.65)	36.5 (18.5-65.7)
	Positive	32	4.88 (4.10-5.73)	17.38 (15.44-20.27)	0.29 (0.22-0.34)	1.71 (1.07-3.05)	22.9 (13.6-101.0)
	p-Value		0.969	0.867	0.885	0.151	0.435

CIS: Carcinoma *in situ* including ductal carcinoma *in situ* (n=114) and lobular carcinoma *in situ* (n=11); ER: estrogen receptor; progesterone receptor; FT3: triiodothyronine; FT4: free thyroxine; HER2: human epidermal growth factor receptor 2; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; TK1: thymidine kinase 1; MMTs: multifocal/multicentric tumors; LVI: lymphovascular invasion; TSH: thyroid-stimulating hormone. *p*-Values were derived from Mann–Whitney test. Statistically significant *p*-values are shown in bold.

0.27, $p=0.042$). There was no association of FT3, FT4 or FT3/FT4 ratio with tumor grade, tumor size, nodal or hormone receptor status. The TSH level was not associated with any of the BC parameters investigated (Table I).

Figure 1 depicts the distribution of THs by the number of invasive lesions (A, B and C), the size of the CIS component (D, E and F) and the grade of CIS (G, H and I). The FT3 level was positively associated with the number of invasive lesions (Figure 1A, $p=0.002$), as was the FT3/FT4 ratio (Figure 1C, $p=0.013$). The size of the CIS component was positively associated with the FT3 level (Figure 1D, $p=0.047$), negatively with the FT4 level (Figure 1E, $p=0.011$) and positively with the FT3/FT4 ratio (Figure 1F, $p=0.001$). There was no association between TH levels and grade of CIS (Figure 1G; FT3, $p=0.803$; Figure 1H; FT4, $p=0.756$; Figure 1I; FT3/FT4 ratio, $p=0.973$).

The relationship between TH levels considered as continuous variables and MMTs, CIS and LVI was assessed by logistic regression with adjustment for patient age (Table II). Each rise of 1.0 pmol/l of FT3 level was associated with an odds ratio (OR) of 1.46 ($p=0.044$) for the detection of MMTs. Opposite trends in the changes in the FT3 and FT4 levels resulted in significant changes of the ratio between the two hormones (Table II) in predicting the presence of MMTs (OR=1.77, $p=0.007$), CIS component (OR=1.97, $p=0.010$) and LVI (OR=1.56, $p=0.039$).

Serum TK1 activity was measured in all patients with primary BC (Table I). TK1 activity above the median was

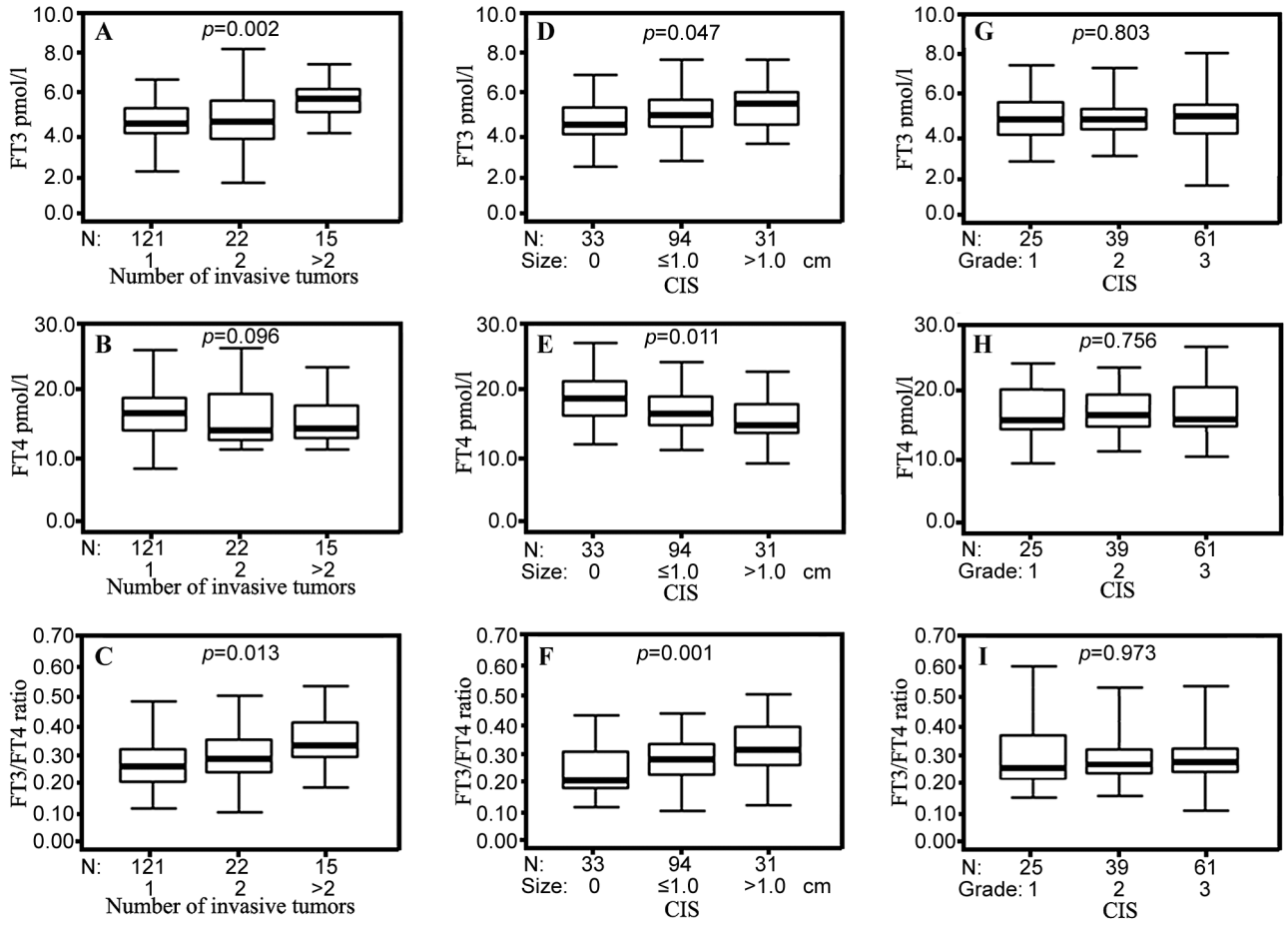

Figure 1. *The distribution of triiodothyronine (FT3), free thyroxine (FT4) and the FT3/FT4 ratio by the number of invasive tumors (A, B and C), the size of the carcinoma in situ (CIS) component (D, E and F) and the grade of CIS (G, H and I). Each box plot shows the median (line), quartiles (box ends), and extreme values (whiskers) within the category shown. p-Values were derived from Kruskal–Wallis test.*

associated with accompanying CIS (38.0 *vs*. 22.1 Du/l, p=0.049), presence of LVI (55.1 *vs*. 30.4 Du/l, p=0.023), and ER negativity (50.9 *vs*. 27.4 Du/l, p=0.036).

Previously, we had reported that the serum FT3 level was positively correlated with serum TK1 activity in patients with BC (21). Here, we extended this by finding that when FT3 was considered as a continuous variable, each increase of 1.0 pmol/l was associated with an OR of 1.69 (p=0.029) for detection of patients with increased serum TK1 activity, after adjusting for age (Table II). For dichotomization of FT3 levels, we used a cut-off of 5.95 pmol/l, which corresponded to the upper top sixth, which included 26 patients. In this group, the activity of TK1 was significantly higher compared to those with FT3 below the cut-off [median (IQR): 63.7 (42.6-144.4) *vs*. 30.9 (17.1-65.1) Du/l, p=0.002]. An FT3 level >5.95 pmol/l was significantly associated with MMTs (MMTs *vs*. unicentric, 29.7% *vs*. 12.4%, p=0.021). The rate of cases with elevated FT3 (>5.95 pmol/) was higher in patients that developed BC recurrence compared to those who did not (34.6% *vs*. 13.6%, p=0.019).

Analysis of RFS. Univariate analysis based on Cox regression (Table III) demonstrated that >5.95 pmol/l FT3, MMTs, LVI, T2-3 stage, and TK1 activity >140 Du/l were all predictors of disease recurrence. In order to select the most important variables the method of forward stepwise regression (likelihood ratio) was used. The model resulted in three variables: Elevated FT3 [relative risk (RR)=2.52, p=0.027], MMTs (RR=2.21, p=0.046) and T2-3 stage (RR=2.49, p=0.024).

Discussion

BC is a heterogeneous disease with varied morphological presentation. In this study, we focused on some morphological features of BC tissue specimens and evaluated

Table II. *The relationship between serum thyroid hormones as continuous variables and pathological characteristics, adjusted for age.*

Thyroid hormone	Characteristic for	Wald	OR	95% CI	*p*-Value
FT3 (for 1.0 pmol/l rise)	MMTs	4.050	1.46	1.01-2.11	**0.044**
FT3/FT4 ratio ×10 (for 1.0 rise)	MMTs	7.259	1.77	1.17-2.67	**0.007**
FT3 (for 1.0 pmol/l rise)	CIS	4.518	1.52	1.03-2.24	**0.034**
FT3/FT4 ratio ×10 (for 1.0 rise)	CIS	6.573	1.97	1.17-3.30	**0.010**
FT3 (for 1.0 pmol/l rise)	LVI	2.671	1.37	0.94-2.00	0.102
FT3/FT4 ratio ×10 (for 1.0 rise)	LVI	4.255	1.56	1.02-2.37	**0.039**
FT3 (for 1.0 pmol/l rise)	TK1 activity >140 Du/l	4.767	1.69	1.06-2.69	**0.029**
FT3/FT4 ratio ×10 (for 1.0 rise)	TK1 activity >140 Du/l	1.067	1.33	0.77-2.30	0.302

CI: Confidence interval; CIS: carcinoma *in situ*; FT3: triiodothyronine; FT4: free thyroxine; LVI: lymphovascular invasion; MMTs: multofocal/multicentric tumors; OR: odds ratio; TK1: thymidine kinase 1. Statistically significant *p*-values are shown in bold.

Table III. *Association of patient characteristics with recurrence-free survival in 158 patients with breast cancer.*

		Univariate analysis			Forward stepwise analysis (likelihood ratio)		
Characteristic		RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
Age	<50 *vs*. >50 Years	2.04	0.52-5.05	0.125			
Histology	IDC *vs*. ILC	1.11	0.40-3.70	0.860			
Presence of CIS	No *vs*. yes	1.16	0.44-3.07	0.761			
MMTs	No *vs*. yes	2.38	1.10-5.12	**0.027**	2.21	1.01-4.84	**0.046**
LVI	No *vs*. yes	2.19	1.01-4.88	**0.049**			
Grade	1-2 *vs*. 3	1.52	0.70-3.34	0.288			
T size	≤2.0 *vs*. >2.0 cm	2.19	1.01-4.79	**0.047**	2.49	1.13-5.85	**0.024**
Nodal status	Negative *vs*. positive	1.99	0.93-4.30	0.078			
ER status	Positive *vs*. negative	1.48	0.63-3.50	0.373			
PR status	Positive *vs*. negative	1.41	0.63-3.13	0.405			
HER2/neu status	Negative *vs*. positive	1.11	0.45-2.76	0.817			
TK1 activity	<140 *vs*. ≥140 Du/l	2.50	1.01-6.18	**0.048**			
FT3	<5.95 *vs*. ≥5.95 pmol/l	2.65	1.19-5.89	**0.017**	2.52	1.11-5.69	**0.027**

CIS: Carcinoma *in situ* including ductal carcinoma *in situ* and lobular carcinoma *in situ*; ER: estrogen receptor; progesterone receptor; FT3: triiodothyronine; HER2: human epidermal growth factor receptor 2; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; LVI: lymphovascular invasion; MMTs: multifocal/multicentric tumors; RR: relative risk; TK1: thymidine kinase 1. Statistically significant *p*-values are shown in bold.

whether they are related to circulating THs and to proliferative activity in terms of serum TK1.

Earlier we reported the significant positive correlation between serum FT3 levels and TK1 activity that suggested the involvement of FT3 in proliferative signaling in patients with BC (21). TK1 is an enzyme participating in DNA synthesis and considered a proliferative marker. Proliferating cells release TK1 into the circulation during mitotic exit, and this process is mediated by the ubiquitin system (22). The expression of the *TK1* gene is regulated by the transcription factor E2F (23, 24). Mitogenic signaling from T3 is bound to the transcription factor E2F (9) activating the expression of TK1 (23, 24). Here we show that an elevated level of FT3 predicted increased proliferative activity. Each 1.0 pmol/l rise of the serum FT3 level was associated with an OR of 1.69 (p=0.029) for the detection of increased serum TK1 activity, suggesting FT3 as a stimulator of proliferation in BC. These data are in line with previous data on different BC cell lines where T3 was able to stimulate cell proliferation (10-12, 25). In addition, we found that FT3 proliferative signaling appears to be more active in ER-negative BC that showed higher TK1 activity compared to ER-positive BC (p=0.036).

A significant number of patients with invasive BC have accompanying noninvasive CIS: Ductal CIS or lobular LCIS. CIS is an overgrowth of abnormal cells in the milk ducts and lobules of the breast. Lobular CIS, unlike ductal, is not considered a precursor of invasive disease but rather a

marker of increased risk of developing an invasive disease (26). A feature common among neoplastic epithelial cells in these lesions is that they have not acquired the ability to detach from the primary lesion and migrate throughout interstitial tissues. In our study, accompanying CIS was found in 79.1% patients with IDC and in 81.3% patients with ILC. Ductal CIS and lobular CIS were considered as one entity. We found that the presence of CIS as an accompanying component in invasive BC was significantly associated with increased median FT3 level. By applying logistic regression, it was shown that each 1.0 pmol/l rise of this hormone was associated with an OR of 1.52 (p=0.034) for the presence of a CIS component, after adjusting for age. Moreover, FT3 was positively associated with the size of CIS, with larger lesions observed with higher serum concentrations of FT3. These data clearly relate serum FT3 to accompanying CIS. In addition, we found an increased TK1 activity in serum of patients with accompanying CIS compared to those with pure IBC, suggesting that FT3 may be a factor stimulating the proliferation of epithelial cells filling the breast ducts and lobules in non-invasive lesions.

Regarding the prognostic significance of CIS, the data available in literature are contradictory. In fact, some studies showed that the presence of a CIS component was associated with better survival (27, 28), while opposite results have also been reported (29). We did not find a significant association of accompanying CIS with disease-free survival (p=0.761).

Another finding was the relationship between FT3 and MMTs. MMTs may result from either intramammary spread from a single primary tumor or multiple synchronous primary tumors arising as monoclonal or independent lesions (30). The frequency of MMTs diagnosed in resected specimens ranges from 9% to 75% (31). In our study, MMTs were detected in 24% of patients and their presence was significantly associated with an increased FT3 level. When FT3 concentrations were used as a continuous variable in a logistic regression model adjusting for age, the OR was 1.46 (p=0.044) for MMTs. This value is the relative increase of MMT risk associated with a rise of 1.0 pmol/l of FT3. In addition, FT3 was significantly positively associated with the number of invasive tumors, relating this hormone to invasive tumor multiplicity. It should be noted that the prevalence of a CIS component in patients with MMTs was three times higher than in patients with unicentric BC (27.3% *vs.* 9.1%, p=0.036), suggesting a relationship between these two features that might be modulated by the circulating FT3. Unlike others (32), we did not find an association between elevated T3 and larger tumors or nodal involvement.

We also considered the relation of THs to LVI, which refers to the presence of tumor cells within lymphatic spaces, blood vessels, or both, in the peritumoural area, and is considered the first of the critical steps in metastasis (33). The presence of LVI was not associated with significant changes in FT3 and FT4 levels. However, the ratio between these two hormones was significantly positively associated with the probability of LVI in pathological specimens.

Thus, MMTs, CIS and LVI all share an association with an increased FT3/FT4 ratio. The FT3/FT4 ratio is a TH index that represents the conversion of T4 to T3. Deiodinases 1, 2 and 3 (DIO1, DIO2 and DIO3) are involved in the regulation of balance between the two hormones (4). DIO1 and DIO2 are key enzymes that participate in the production of T3, while DIO3 promotes its inactivation. In the current study, the majority of patients had TH levels within the euthyroid range. In these patients, DIO2 is considered as the primary source of circulating T3 (34). The opposite trends in the changes of FT3 (increase) and FT4 (decrease) observed with presence of MMTs, CIS and LVI indicate that DIO2 is possibly the main contributor to FT3 at these conditions.

According to The Cancer Genome Atlas database analysis (35) of the three deiodinases, only *DIO2* showed significantly higher expression in BC samples (n=1,085) than in normal breast tissue samples (n=291), while the expression of DIO1 and DIO3 was similar. An increased concentration of circulating FT3 appears to contribute to intracellular biologically active T3 and enhance its effects.

By logistic regression analysis, adjusted for age, it was shown that each 1.0 unit rise of FT3/FT4 ratio×10 was associated with an OR of 1.77 (p=0.007), 1.97 (p=0.010) and 1.56 (p=0.039) for the detection of patients with BC with CIS, MMTs and LVI, respectively. According to these data, the relationship between FT3 or FT3/FT4 ratio and morphological features does not only seem to be threshold-dependent, since it was observed with a rise of serum concentration of FT3 in the wide euthyroid range.

Earlier, Weissenbacher *et al.* reported that compared to unifocal BC, MMTs had down-regulation of E-cadherin (36). This cell adhesion-related glycoprotein, encoded by the *CDH1* gene, has the main function of regulating cellular adhesion and migration, and acts as an invasion suppressor system (37). In experiments on T-47D BC cells, T3 induced the progressive decrease of E-cadherin expression and an increase in vimentin expression, indicating the involvement of T3 in EMT (13, 16). EMT plays a key role in tumor invasion by disrupting intercellular contacts and enhancing motility and migration of tumor cells to surrounding tissues (38, 39). It could be suggested that T3, through its suppressive effect on E-cadherin, might have an effect on cell migration and the emergence of multiple invasive lesions and LVI.

Our data on increased FT3 level and FT3/FT4 ratio suggest that enhanced production of FT3 may be a factor modulating tumor phenotype. In experiments on T-47D BC cells, T3 *via* integrin avb3, was shown to stimulate the cortactin/neural-Wiskott–Aldrich syndrome protein/actin-related protein 2/3 complex signaling pathway, induce actin cytoskeleton reorganization, trigger focal adhesion formation

and promote actin nucleation (16). According to these authors, T3 may exert an integrated regulation of tumor progression by modifying the extracellular matrix and the signaling pathways implicated in cancer cell proliferation, migration and invasion. All these effects of T3 may reflected in the changes of BC morphology, namely in appearance of CIS, MMTs and LVI.

Many studies have shown that MMTs are more aggressive and carry worse overall outcomes than unifocal disease (36, 40, 41). LVI is also strongly associated with a poor prognosis in early BC (42-44). In accordance with these data, we also found that patients with MMTs and LVI had a poorer prognosis. Another important characteristic of tumor aggressiveness is cell proliferation. In this study, we confirmed our previous observation (20) that elevated serum TK1 activity is associated with a high proliferative potential of tumor, and is associated with increased risk of BC recurrence.

The significant association of increased FT3 or its ratio with FT4 with morphological features of known prognostic significance and cell proliferation suggests that this hormone might have an impact on the behavior of BC with increasing propensity for disease progression. In fact, the survival analysis identified elevated FT3 as a risk factor for cancer recurrence. The patients with a serum FT3 level below the cut-off of 5.95 pmol/l had a better RFS compared with those with FT3 levels above this cut-off (RR=2.65, p=0.017). Forward stepwise linear regression model used for selection of the most important prognostic variables showed elevated FT3 to be an independent predictor on RFS (RR=2.52, p=0.027). These findings are in accordance with the data from the population-based cohort study reporting a significant positive association between higher pre-diagnostic T3 levels with BC risk (45), more aggressive forms of disease and higher mortality (32).

In summary, this study showed that the serum level of FT3 and its ratio to FT4 were associated with some characteristic morphological features of BC. The FT3/FT4 ratio, reflecting the production of FT3, was shown to be a predictor of CIS, MMTs and LVI in pathological specimens. Furthermore, an increased serum level of FT3 was associated with the presence of CIS component and its size, with the presence of MMTs and their number and with increased proliferative activity in terms of serum TK1 activity. The higher the serum level of FT3, the higher was the risk of detection of CIS and MMTs in pathological specimens. A level of FT3 >5.95 pmol/l (upper top sixth of the population) was associated with MMTs, increased proliferative activity and disease recurrence. The multivariate survival analysis identified elevated FT3 as independent predictor of poor prognosis in terms of RFS.

The current study raises questions regarding the care of women with disorders or treatments resulting in an increased level of FT3, and its impact on BC development and progression. Obviously, the harmful effects of a high FT3 level should be taken into account in the development of new strategies for the treatment of BC. It remains to be determined whether manipulation of TH levels might have an effect on prognosis of patients with BC. Since this is, to our knowledge, the first study demonstrating a significant relation of serum FT3 and FT3/FT4 ratio to morphological features of prognostic significance, these data need to be validated in a larger prospective study.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conception and design: AM, BN, TA, TP. Acquisition of data: TA, AM, BN, EC, TP, LK, OM. Analysis and interpretation of data: BN, AM, TA, TP, LK, AM. Histological examination of the breast: BM. Writing, review, and/or revision of the article: AM, BN, TA, TP.

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