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Serum Galectin-1 and Galectin-3 Levels in Benign and Malignant Nodular Thyroid Disease

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Background: Since the histological expression of galectins is increased in thyroid carcinoma, determination of their serum levels may provide useful preoperative information. The goal of this study was to determine if a difference in galectin serum levels could be detected between benign and malignant nodular thyroid diseases.
Design: Using validated ELISAs, the concentrations of several galectins were prospectively measured in serum samples from 30 healthy individuals and preoperatively in 90 patients with thyroid disease. Seventy-one patients had multiple thyroid nodules (MTN), 13 patients had a single thyroid nodule (STN), and 6 patients had Graves' disease. Nine of 71 patients with MTN had fine-needle aspiration biopsy (FNAB) of their nodules and in 7 patients a "benign" diagnosis was made, in 0 patient a "malignant" diagnosis was made, and in 2 patients a "suspicious" diagnosis was made. Six of 13 patients with STN had FNAB of their nodules and in 2 patients a "benign" diagnosis was made, in 3 patients a "malignant" diagnosis was made, and in 1 patient a "suspicious" diagnosis was made.
Results: Thyroid disease was associated with higher levels of galectins-1 and -3 compared to normal subjects. Using a threshold value of 3.2 ng/mL as a cut-off point, the measurement of serum galectin-3 separated micro- and macropapillary thyroid carcinoma (PAP_CA) from patients with nonmalignant thyroid disease with 74% specificity, 73% sensitivity, 57% positive predictive value, and 85% negative predictive value. Elevated serum galectin-3 concentrations (>3.2 ng/mL) detected 87% of macropapillary thyroid carcinomas and 67% of micropapillary thyroid carcinomas.
Conclusions: Serum levels of galectins-1 and -3 are relatively high in patients with thyroid malignancy but there is considerable overlap in serum galectin-3 concentrations between those with benign and malignant nodular thyroid disease and, to a lesser extent, between those with and without nodular thyroid disease.

Introduction

Among the endogenous lectins, special attention is currently paid to the galectins (Ca^{2+}-independent lectins with specificity to a nonsubstituted or substituted β-galactoside core) and that too for the following reasons: (i) galectins are potent regulators of adhesion and growth (1), (ii) they sense even subtle changes in cell surface glycosylation and glycan density (2–4), (iii) they are potentially able to interact with growth-relevant proteins such as protooncogenes H-ras or bcl-2 (5), (iv) they are widely expressed in tumor and stromal cells, and (v) cell regulators such as the tumor suppressor p53 can regulate galectin expression together with that of glycosyltransferases, which generate galectin ligands (5–7). Experimental studies are currently exploring the potential relevance of galectins to cancer biology. In this context, we examined whether galectins may eventually open the door to a simple and convenient assay procedure enabling one to preoperatively distinguish benign from malignant diseases of the thyroid.

Thyroid nodules are quite common in the general population and appear to be the most frequent disorder presented to endocrine surgeons. The estimated prevalence in the global population is from 4% to 7% (8). Further, postmortem

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examination and high-resolution ultrasound studies suggest that up to 50% of the general population may have thyroid nodule(s) (8–10). In absolute terms, the incidence of thyroid cancer runs up to about 18,000 new cases per year in the United States (11). Ideally, any thyroid nodule should be classified correctly in order to make an error-free decision regarding the need for surgical treatment. In this respect, the introduction of fine-needle aspiration biopsy (FNAB) 30 years ago provided the clinician with a valuable tool, resulting in a significant reduction in the number of needless surgical interventions (8,9). However, there are notable limitations to FNAB, such as its inherent inability to obtain a sample from all nodules in multiple thyroid nodules (MTN) patients and its lack of sensitivity in the evaluation of follicular neoplasms due to its failure to differentiate benign follicular adenomas from malignant lesions (12).

Previous immunohistochemical analysis has revealed an increase of galectin expression—especially in the case of galectins-1 and -3 during thyroid tumor progression from benign lesion to malignancy (13–16). In contrast, the pattern of galectin-7 expression differs markedly from that of galectin-1 since there is strong immunohistochemical staining in the multinodular goitre, the normofollicular adenomas, and most thyroid cancers (15). The absence of galectin-3 in adenomas and the prevalence of this marker in carcinomas, as initially seen in immunostaining and further confirmed by proteomic profiling (17,18), have highlighted the diagnostic value of galectins in the preoperative assessment of nodular thyroid lesions. Beside monitoring galectin presence in tissue, another reasonable option is to measure serum levels. In fact, a pilot study on 99 patients with 10 different types of tumor has disclosed an increase in serum level for galectin-3 in cancer patients as compared to healthy individuals (19). On the basis of these considerations, we endeavored to determine whether (i) there may be a change in serum levels for galectins in patients with thyroid disease, (ii) a difference could be detected between benign and malignant status of disease, and (iii) thyroid surgery would affect galectin serum levels.

Materials and Methods

Sera and patients

Blood samples were obtained from patients admitted to the Department of Surgery of the Hôpital Claude Hurriez (Lille, France). The patients and healthy control subjects were recruited for our study in full accordance with the ethical guidelines approved by the Claude Huriez (Lille) and the CHU Saint-Pierre (Brussels) Institutional Review Boards. Informed consent and detailed information on health status at admission and retrospectively were obtained from each patient. A total of 90 patients who enrolled in this study underwent thyroid surgery for several reasons: 71 patients for MTN, 13 patients for single thyroid nodule (STN), and 6 patients for Graves' disease (GD). FNABs were performed on a small number of MTN (9/71 cases) and STN (6/13 cases). Serum specimens were stored at -20°C until assay. Serum levels for galectins were measured before and 2 days after surgery. Samples were also obtained from 30 matched healthy blood donors with a mean age of 55 years (range, 22–75 years) and sex ratio of 1/6 (male/female). This group of healthy volunteers presented normal neck palpation and had no history of thyroid disease.

ELISA for galectin-3

Ninety-six–well microplates precoated with capture polyclonal antibody (goat) to human galectin-3 (BMS279; Bender MedSystems, Vienna, Austria) were rinsed three times with wash buffer (1% Tween-20 and 10% bovine serum albumin in phosphate-buffered saline). Samples (50 μL) were added in duplicate to each well, already containing 50 μL of sample diluent. Detection antibody (rabbit polyclonal anti-galectin-3) diluted in Reagent Diluent (as provided by the supplier) was then added to each well and plates were incubated for 2 hours at room temperature (RT) on an orbital microplate shaker set at 200 rpm. The wells were then rinsed three times with wash buffer before adding 100 μL of buffer containing horseradish peroxidase-conjugated anti-rabbit Ig antibody. After 1 hour of incubation at RT, wells were rinsed three times and then 100 μL of tetramethylbenzidine substrate solution was added to each well. After a 20-minute incubation period at RT, the enzymatic reaction was stopped by adding 100 μL of stop solution (1 M phosphoric acid). The absorbance of each sample was determined at 450 nm in a Labsystems Multiskan MS microplate reader (Thermo Electron, Zellik, Belgium). Concomitantly, a standard curve with galectin concentrations ranging from 0.156 to 10 ng/mL was run in parallel with each experimental series.

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ELISAs for galectins-1 and -7

Ninety-six–well microplates were coated with capture antibody at 2000 ng/mL (100 μL/well; galectin-1: AF1152; galectin-7: AF1339; R&D Systems, Minneapolis, MN). After incubation overnight at RT, the wells were rinsed three times with wash buffer (0.05% Tween-20 in phosphate-buffered saline). Samples of serum (100 μL) were added to the wells and then incubated at RT for 2 hours. At the end of this period, plates were inverted to remove all liquid from the wells, which were then thoroughly washed with wash buffer. Biotinylated detection antibody (100 ng/mL, 100 μL; galectin-1: BAF 1152; galectin-7: BAF 1339; R&D Systems) diluted in Reagent Diluent (5% Tween-20 and 2% goat serum in phosphate-buffered saline; R&D Systems) was added to each well and the microplates were incubated at RT for 2 hours. The wells were then rinsed three times, and 100 μL of a solution containing streptavidin-horseradish peroxidase (1:200) was added to the wells. After 20 minutes of incubation at RT and washing, o-phenylene diamine substrate solution was added to the wells. The absorbance of each sample was determined at 450 nm after 30 minutes. A standard curve ranging from 5 to 160 ng of galectin-1 and from 1.25 to 40 ng/mL of galectin-7 (R&D Systems) was generated for each ELISA.

Data analysis

Data obtained from independent groups were compared using the nonparametric Kruskall–Wallis (more than two groups) or Mann–Whitney *U* tests (two groups). In the case of more than two groups, *post hoc* tests (Dunn procedure) were used to compare pairs of groups (to avoid multiple comparison effects). The threshold value was defined as two standard deviations above the mean in the control group. Pre- and posttherapy serum levels were compared thanks to the nonparametric Wilcoxon matched pair test. The statistical analysis was performed using Statistica software (Statsoft, Tulsa, OK).

Results

Clinical data

Among the 90 patients who underwent surgery, 55 presented a documented histopathological diagnosis of a benign lesion and 35 of a differentiated thyroid carcinoma. Table 1 shows the clinical characteristics (age, sex) of the 90 patients with thyroid lesions. Table 2 shows the clinical data of the 35 patients with a final diagnosis of papillary carcinoma as well as their individual galectin-3 serum levels measured before surgery. Twenty-five patients had a papillary lesion of less than 1 cm. Among them, surgery was carried out for MTN in 15 cases (60%), STN in 7 cases (28%), and GD in 3 cases (12%). Ten patients had a macronodule that was between 11 and 50 mm in diameter. Among them, the majority (8/10) underwent surgery for MTN and only 2/10 for STN. In the MTN group (71 cases), FNABs were benign in seven patients (among these, four patients presented a papillary lesion at the final histopathology) and suspicious in two patients (with a benign lesion at the final histopathology). In the STN group (13 cases), FNABs were benign in two patients (with two benign lesions at the final histopathology), malignant in three patients (with three malignant lesions at the final histopathology), and suspicious in one patient (with a malignant lesion at the final histopathology).

Six females underwent total thyroidectomies for GD (mean age: 38 years, range from 30 to 46 years). Among these, three patients presented a micropapillary carcinoma and three patients a benign lesion at the final histopathology.

Serum levels of galectin-3

The level of galectin-3 in the sera of 30 healthy individuals varied between 1.4 and 3.1 ng/mL (median: 2.22 ng/mL), while the concentration of galectin-3 in the sera of patients with benign thyroid lesions (see Table 1) was in the range between 1.0 and 9.1 ng/mL (median: 2.76 ng/mL). In Micro_PAP, the galectin-3 serum levels were between 1.0 and 5.0 ng/mL (median: 3.21 ng/mL), whereas concentrations between 2.3 and 7.8 ng/mL (median, 3.4 ng/mL) were determined for patients with Macro_PAP. The median galectin-3 serum levels in benign thyroid lesions and in cancer patients (grouping Micro_PAP and Macro_PAP together) were significantly higher than in healthy individuals (Kruskall–Wallis: $p < 10^{-6}$) (Fig. 1A). Using *post hoc* comparisons between selected groups, this parameter difference was significant in both benign thyroid lesions ($p = 0.004$) and PAP_CA ($p = 0.00007$) when compared to the control (CT) group (Fig. 1A). The median galectin-3 concentration value determined in the sera from the 35 PAP_CA patients (grouping Micro_PAP and Macro_PAP) was also significantly higher (Mann–Whitney, $p = 0.03$) than the median concentration value determined in the sera from the 55 patients with benign thyroid lesions (Fig. 1A) (the 2.5th to 97.5th percentile values were chosen for this test).

With a threshold value of 3.2 ng/mL, the serum level enabled us to set PAP_CA patients apart from CT patients with 100% level of specificity, 74% level of sensitivity, 100% of positive predictive value (PPV), and 83% of negative predictive value (NPV) (Fig. 1B). Using the same threshold in an attempt to discriminate benign thyroid lesion from PAP_CA (grouping MICRO_PAP and MACRO_PAP) in a series of 71 multinodular goiters (MNG), the level of sensitivity was stable at 74% and the level of specificity decreased slightly to 73% (Tables 3 and 4) (Fig. 1B). The PPV and NPV of PAP_CA were, respectively, 57% and 85%. The same threshold made it possible to correctly discriminate MACRO_PAP from benign lesions (in a series of 56 MNG) with an 87.5% level of sensitivity, a 73% level of specificity, 35% of PPV, and 97% of NPV (Table 4). Table 4 shows similar data for MICRO_PAP versus benign histology in a series of 63 MNG. In contrast to the case with MTN patients, galectin-3 concentrations are inadequate in discriminating benign from malignant histology in the group of STN patients. Using the same threshold value to distinguish benign histology from PAP_CA in the STN group, the sensitivity decreased dramatically to 11% (Tables 3 and 4).

As illustrated in Figure 1C, the serum level of galectin-3 was also measured before and after thyroid surgery in 15 cases of PAP_CA. There was a significant (Wilcoxon matched pair test, $p = 0.001$) decrease in the posttreatment galectin-3 serum concentrations (2 days after surgery) as compared to the pretreatment level (Fig. 1C).

Serum levels of galectins-1 and -7

The level of galectin-1 in the sera of 20 healthy individuals varied between 0.6 and 13.4 ng/mL (median: 4.7 ng/mL). As seen previously in the case of galectin-3, the serum

Table 1. Patients with Benign and Malignant Histopathology

	Benign histopathology (55 cases)	*Micro_PAP histopathology (25 cases)*	*Macro-PAP histopathology (10 cases)*
Single thyroid nodule (13 cases)			
Female age[a]	38 ± 15 years (4 cases)	49 ± 10 years (3 cases)	38 ± 22 years (2 cases)
Male age[a]		44 ± 10 years (4 cases)	
Galectin-3 serum level[b]	2 (1.8–2.8)	3 (1–3.8)	2.55 (2.4–2.7)
Multiple thyroid nodules (71 cases)			
Female age[a]	39 ± 16 years (40 cases)	58 ± 15 years (12 cases)	48 ± 12 years (8 cases)
Male age[a]	56 ± 16 years (8 cases)	61 ± 9 years (3 cases)	
Galectin-3 serum level[b]	2.8 (0.9–9.1)	3.5 (1.8—5.0)	3.6 (2.4–7.8)
Graves' disease (6 cases)			
Female age[a]	33 ± 3 years (3 cases)	44 ± 3 years (3 cases)	
Male age[a]			
Galectin-3 serum level[b]	2.3 (1.5–2.4)	2.7 (1.7–3.3)	

[a]Correspond to the mean and standard deviation of age.
[b]Correspond to the median and extreme values in ng/mL.

concentration of galectin-1 (0–58.7 ng/mL, median: 17.9 ng/mL) increased in the sera of patients with benign thyroid lesions as compared to healthy individuals. Contrasting with galectin-3, the assessment of galectin-1 in sera of cancer patients revealed no further increase for this galectin. More specifically, galectin-1 serum levels varied between 0 and 50 ng/mL with median values of 12.6 ng/mL and 11.5 ng/mL in Micro_PAP and Macro_PAP, respectively. Overall, the median galectin-1 serum level in benign thyroid lesions was significantly higher than in CT samples (Kruskall–Wallis: $p = 0.003$) (Fig. 2A). With a threshold value of 7 ng/mL, these data reached a 95% level of specificity, 61% level of sensitivity, 97% level of PPV, and 48% level of NPV to distinguish between benign lesion and CT status. When measuring this parameter before and after thyroid surgery in 14 cases of thyroid CA patients, there was a significant (Wilcoxon matched pair test, $p = 0.01$) decrease in posttreatment galectin-1 serum concentrations (2 days after surgery) as compared to the pretreatment level (Fig. 2B). Using the same methodology for galectin-7, and on the basis of a corresponding standard curve, we found no detectable amount of this galectin in serum samples.

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Discussion

Recent studies report the expression of several galectins in thyroid malignancies [i.e., for galectin-1, (13,14); for galectin-2, (16); for galectin-3, (14,17,18); for galectin-7, (15)]. Our report presents the first study dedicated to the serum levels of galectins in patients with thyroid disease. Our data clearly show that the levels of circulating galectin-3 increase during thyroid

Table 2. Micro and Macro_PAP Clinical Data

n	Sex	Age	Reason of surgery	FNA	Surgical treatment	Histology: size of MICRO_PAP	Gal-3 serum levels (ng/mL)
Micro papillary carcinomas							
1	F	46	GD		TT	8 mm	3.3
2	M	45	STN	Suspicious	TT	6 mm	3.8
3	F	37	MTN		TT	5, 5 mm	3.3
4	M	30	STN	Malignant	TT	7, <1 mm (3 N+)	2.7
5	F	79	MTN		TT	3, 7 mm	4.0
6	F	38	STN	Benign	TT	1, 2 mm	3.0
7	F	56	STN		TT	4 mm	2.9
8	M	60	MTN		HT	2 mm	2.2
9	F	70	MTN		TT	<1, <1 mm	4.8
10	F	60	MTN	Benign	TT	3 mm	3.4
11	F	38	MTN	Benign	TT	1, 2 mm	1.8
12	F	44	MTN		TT	2.5, 2.5 mm	2.3
13	F	44	GD		TT	2 mm	1.7
14	F	71	MTN		TT	1, 3 mm	4.3
15	F	60	MTN		TT	1, 4 mm	2.7
16	F	59	MTN	Benign	TT	1, 1 mm (1 N+)	3.5
17	M	70	MTN		TT	3 mm	4.8
18	M	51	STN		HT	<1, <1 mm	3.0
19	F	49	MTN		TT	4 mm	2.7
20	F	77	MTN		TT	5, 9 mm (3 N+)	4.2
21	F	47	MTN		TT	1, 3 mm	4.0
22	F	41	GD		TT	2 mm	2.7
23	F	53	STN		HT	4 mm	1.0
24	M	52	MTN		TT	1.5 mm	5.0
25	M	51	STN		HT	2 mm	3.0
Macro papillary carcinomas							
1	F	47	MTN		TT	50 mm	3.7
2	F	31	MTN		TT	11 mm	7.8
3	F	52	MTN		TT	18 mm	3.5
4	F	55	MTN	Benign	TT	13 mm	3.3
5	F	33	MTN		TT	14 mm (3N+)	2.7
6	F	23	STN	Malignant	TT	11 mm	2.4
7	F	54	STN	Malignant	TT	30 mm (3N+)	2.7
8	F	49	MTN		TT	12 mm	5.1
9	F	67	MTN		TT	14 mm	5.4
10	F	49	MTN		TT	30 mm	3.5

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F, female; M, male; GD, Graves' disease; TT, total thyroidectomy; STN, single thyroid nodule; MTN, multiple thyroid nodules; N+, invaded lymph node; HT, hemithyroidectomy.

carcinoma progression, more specifically in the subgroups of patients suffering from MTN. In this pilot study, the galectin-3 serum concentration test detected 87% of MACRO_PAP and 67% of MICRO_PAP using our tentative cut-off point. This pilot study included 71 patients with MTN and 13 patients with STN, two types of disease requiring distinct approaches for medical and surgical management (8,9). The serum galectin-3 test is undoubtedly more sensitive in MTN than in STN, detecting 74% of PAP_CA in the MTN group and only 11% of PAP_CA in the STN group. Our preliminary estimates of sensitivity compare favorably with other widely used screening tests such as prostate-specific antigen for prostate cancer [sensitivity 60–80%, specificity 90%, (20)] and the Papanicolaou test for cervical cancer [sensitivity 30–87%, specificity 86–100%, (21)].

Iurisci *et al.* demonstrated the feasibility of developing an immunoligand assay for determining the circulating levels of galectin-3 in cancer patients as compared to healthy controls (19). These authors observed that galectin-3 serum levels in patients with breast, gastrointestinal, lung or ovarian cancers, melanomas, and non-Hodgkin's lymphomas are significantly elevated (19). Using commercial galectin-3 ELISA, we observed the same feature in a study on head and neck squamous cell carcinomas (HNSCCs) (22). Because galectin-3 is a substrate for matrix metalloproteinases-2 and -9, which thus might remove antigenic epitopes by truncation and suppress the immunoreactivity of the lectin to monoclonal antibodies, it is advisable to use polyclonal antibodies detecting both full-length and truncated proteins. This study showed that high galectin-1 and -3 serum levels were associated with a weak, but nevertheless significant, prognostic value in terms of a period of survival for HNSCC patients (22). The galectin-3 concentrations in sera from the patients with a metastatic disease were higher than in sera from the patients with localized tumors (22).

Recent studies have questioned the predictive value of galectin-3 in thyroid tumor histopathology (23–29). As discussed for immunocyto- and histochemical detection (18,30–34), a combination of markers may improve the accuracy in defining tumor status. The source of increased serum galectin-3 in PAP_CA patients remains unclear. According to present (in thyroid cancers) and previous [in HNSCC, (22)] results showing that removal of the tumor decreased serum galectin-1 and -3 concentrations, tumor tissue or peritumoral stromal cells (strongly influenced by the vicinity of the tumor) could be the source of galectins-1 and -3 in sera. Even though thyroid tumors prove to be the actual source of galectin secretion, it has been reported that galectin-3 serum level is also increased in other cancers (19), leading to the risk of confounding effects in case of multiple malignancies.

In conclusion, we report here the first results on galectin-1 and -3 serum levels in a group of patients with nodular thyroid

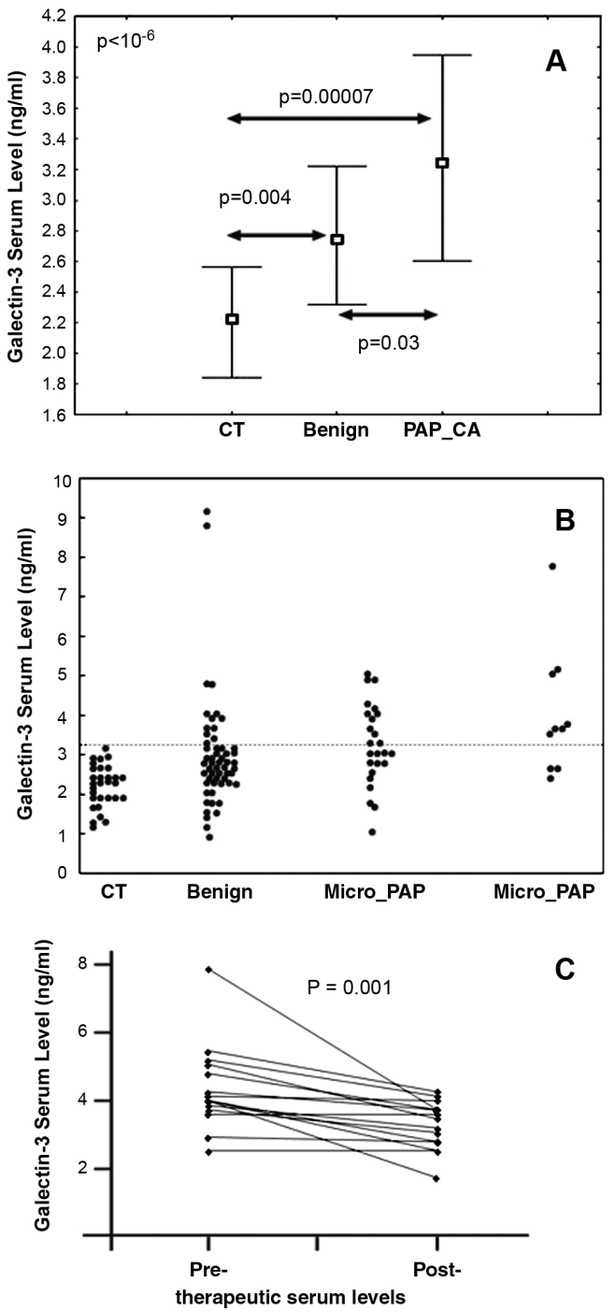

FIG. 1. (**A**) Quantitative determination of galectin-3 serum concentrations by ELISA in a series of 30 controls (CT), 55 benign thyroid lesions (Benign), and 35 papillary carcinomas (PAP_CA). Using *post hoc* comparisons between selected groups, we observed increased galectin-3 serum levels in benign thyroid lesions ($p = 0.004$) and in PAP_CA ($p = 0.00007$) comparing to the (CT) group. The median galectin-3 concentration value determined in the sera from the 35 PAP_CA patients (pooling Micro_PAP and Macro_PAP) was also significantly higher (Mann–Whitney, $p = 0.03$) than the median concentration value determined in the sera from the 55 benign thyroid lesions patients. Squares and bars correspond, respectively, to the median values and the 25% and 75% percentiles. (**B**) The galectin-3 serum level enabled us to set PAP_CA patients apart from CT and benign thyroid disease patients (see Table 4 for the sensitivity, specificity, positive predictive value, and negative predictive value using a threshold value of 3.2 ng/mL). (**C**) Moreover, in a series of 15 PAP_CA patients, we observed a significant decrease in the posttreatment galectin-3 serum concentrations as compared to the pretreatment galectin-3 concentrations.

Table 3. STN or MTN and Galectin-3 Serum Levels

	STN with benign histology	*STN with MICRO_PAP histology*	*STN with MACRO_PAP histology*
Galectin-3 serum level >3.2 ng/mL	0/4 cases	1/7 cases	0/2 cases
Galectin-3 serum level <3.2 ng/mL	4/4 cases	6/7 cases	2/2 cases
	MTN with benign histology	*MTN with MICRO_PAP histology*	*MTN with MACRO_PAP histology*
Galectin-3 serum level >3.2 ng/mL	13/48 cases	10/15 cases	7/8 cases
Galectin-3 serum level <3.2 ng/mL	35/48 cases	5/15 cases	1/8 cases

STN, single thyroid nodule; MTN, multiple thyroid nodules.

Table 4. Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value of the Clinical Series

	MTN/PAP_CA	*MTN/MICRO_PAP*	*MTN/MACRO_PAP*	*STN/PAP_CA*	*STN/MICRO_PAP*
Sensitivity[a]	74%	67%	87%	11%	14%
Specificity[b]	73%	73%	73%	100%	100%
Positive predictive value[c]	57%	43%	35%	100%	100%
Negative predictive value[d]	85%	87%	97%	33%	40%

[a]Sensitivity = true positives/(true positives + false negatives).
[b]Specificity = true negatives/(true negatives + false positives).
[c]Positive predictive value = true positives/(true positives + false positives).
[d]Negative predictive value = true negatives/(true negatives + false negatives).
MTN, multiple thyroid nodules; STN, single thyroid nodule.

disease and how these levels relate to the presence or absence of thyroid cancer. However, considering the degree of galectin-3 serum level overlap between benign and malignant thyroid nodules patient groups, we think that galectin-3 serum levels should be used in association with other potential serum thyroid markers [such as TSH receptor mRNA, (35)]. In order to further validate serum galectin-3 measurement in cases of MTN, the following steps are now to be taken: (i) we need to study a larger number of subjects with MTN in order to refine threshold value for serum galectin-3 as well as to determine more precisely the sensitivity and the specificity of the test, (ii) we need to evaluate other potential serum thyroid markers such as TSH receptor mRNA by RT-PCR (35) and refine our testing by combining both serum markers, and (iii) we also need to test follicular carcinomas, which are more aggressive and constitute a real challenge for pathologists. We are currently conducting a study aimed at reaching these objectives.

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