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Decreased circulating levels of angiopoietin – 1 (Ang-1) are associated with the presence of multinodular goiter or differentiated thyroid cancer

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ABSTRACT

Background: The aim of this study was to investigate whether the presence of benign or malignant nodular thyroid disease affects levels of circulating angiogenesis cytokines.
Methods: In this study we investigated levels of angiopoietin – 1 and -2 (Ang-1 and Ang-2 respectively), vascular endothelial growth factor –A (VEGF-A), galectin-3 (Gal-3), urokinase plasminogen activator receptor (uPAR) and plasminogen activation inhibitor – 1 (PAI-1) in 40 patients with differentiated thyroid cancer (DTC), 45 with thyroid papillary microcarcinoma (mPTC), 53 patients with multinodular goiter (MNG) and 58 controls. Six months after surgery 28 patients resubmitted blood samples. The diagnostic value of Ang-1 levels was evaluated with receiver operating characteristic (ROC) curves. Results: Statistically significant lower levels of Ang-1 were observed in DTC and MNG patients compared to controls ($p<.05$). No significant differences were observed in the levels of the other factors. The area under ROC curves for Ang-1 discerning DTC, mPTC and MNG from control were 0.68, 0.66 and 0.71 respectively. A significant increase in Ang-1 levels ($p<.05$) was documented in the subset of patients that underwent thyroidectomy. Thyroidectomy did not influence levels of the other factors.
Conclusions: Our results suggest an association between low levels of Ang-1 and the presence of underlying benign or malignant nodular thyroid disease.

Introduction

Tumor-induced angiogenesis is vital for tumor growth and metastatic potential and this observation has formed the basis for the development of novel therapeutic agents that target this specific mechanism [1]. Differentiated thyroid cancer (DTC) is the most common form of endocrine gland malignancy and to a certain degree is also dependent on angiogenesis [2].

Several growth factors participate in neovascularization by specifically targeting endothelial cells (ECs) and other cells in the tumor microenvironment. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are an important subset of growth factors that seem to have a considerable effect on angiogenesis. Both factors act on the Tie-2 receptor with differing effects. Ang-1 is a potent agonist, and through its effects on ECs, promotes vascular integrity [3,4]. Ang-2 has a more subtle role acting as a weak agonist in the absence of inflammation and as an antagonist in its presence [5]. In DTC Ang-1 mRNA production is significantly lower as compared to healthy adjacent tissue and some reports indicate an overexpression of Ang-2 in cancerous tissue [6,7].

Vascular endothelial growth factor-A (VEGF-A) is a member of the broader VEGF family and is the most significant angiogenic factor, promoting EC proliferation and differentiation. Levels of VEGF-A seem to be over-expressed in thyroid cancer tissue, while higher levels of circulating VEGF-A have been observed in patients with metastatic disease compared to patients with no distant organ involvement [8]. Results have not been consistent; however, most reports suggest an active role for VEGF-A in thyroid cancer and possibly in benign disorders such as multinodular goiter (MNG).

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Proteases and inhibitors of the fibrinolytic system such as urinary-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) respectively have also been implicated as modulators of tumor induced angiogenesis in several tumors including DTC [9,10].

In summary, angiogenesis is comprised of a complex web of intertwining processes where all the key cytokines have multiple roles. These cytokines are produced and function in an autocrine and paracrine fashion and they exert their action in low concentrations [11]. Detection in serum is known to be challenging due to this fact. Therefore significant differences in serum levels between groups could indicate significant differences in cytokine production and or release. However, differences in cytokine production or release do not necessarily translate into differences in local activity since cytokines are pluripotent and redundant in action [11].

Our objective in this study was to investigate levels of circulating angiogenic cytokines in patients with benign and malignant thyroid disease. Differential expression of these factors could provide insights into the angiogenic mechanisms involved in DTC and serve as possible markers of malignancy.

Materials and methods

Ethical approval

The study protocol was reviewed and approved by the Scientific Committee of our institution. All participants were informed about the objectives and methods of this study. Written consent was obtained from every participating individual.

Subjects

This study included 138 euthyroid patients undergoing thyroid procedures for multinodular goiter (MNG) and thyroid cancer. Euthyroid status was defined as having normal levels of TSH, total T4, free T4, total T3 and free T3. The diagnosis of MNG was established prior to surgery through ultrasonogrophic (US) findings suggestive of gland enlargement with nodularity and subsequently identified as multinodular hyperplasia by biopsy. All patients underwent total thyroidectomy or total thyroidectomy with lymph node dissection depending on the preoperative diagnosis. All patients were evaluated by US prior to surgery and fine needle aspiration (FNA) was performed on suspicious nodules. Based on current World Health Organization (WHO) definition, patients with a tumor equal or smaller than 10 mm in diameter were classified as having microcarcinoma (mPTC) [12]. This decision, to include mPTC as a separate group was made due to the high prevalence of mPTC in patients undergoing surgery for MNG [13]. Therefore, after biopsy was performed, patients were divided into three groups: cancer (DTC), mPTC and MNG. In summary, 40 patients (Table 1) were found to have DTC (34 PTC and 6 FTC), 45 patients were diagnosed with mPTC and 53 were found to have MNG. Patients who on final pathology met criteria for non-invasive follicular neoplasm with papillary-like nuclear features (NIFTP) were not included in data analysis. Patients with conditions known to influence neovascularization such as cardiovascular disease, chronic obstructive pulmonary disease, uncontrolled diabetes mellitus and arterial hypertension, autoimmune disorders, surgical intervention or major trauma in the past 6 months, pregnancy and prior history of malignancy were not included in this study. Patients undergoing thyroidectomy for DTC with concurrent chronic autoimmune thyroiditis (Hashimoto's thyroiditis) were not excluded from the study. Patients with laboratory indicators of active inflammation such as leukocytosis, thrombocytosis and/or elevated levels of C-reactive protein were excluded from the study. Fifty-eight volunteers were recruited for the control group using the same exclusion criteria. Data regarding age, gender, body mass index (BMI) and smoking habits were collected.

Table 1
Age and gender data as documented between groups.

Group	Number of individuals	Gender F/M	Age (yrs) ± SD
DTC	40	33/7	49.17 ± 13.18
mPTC	45	37/8	49.17 ± 13.52
MNG	53	39/14	49.11 ± 13.34
Control	58	38/20	48.84 ± 15.06

Sample collection and measurement

All samples were collected between 8 am and 10 am after an overnight fast. Peripheral venous blood samples were collected into appropriate vacutainers, for the separation of serum and plasma, respectively. The specimens were allowed to clot for 30 min at room temperature, were centrifuged, and then aliquoted and subsequently stored at -80 °C according to standard protocols. Levels of Ang-1, Ang-2, Galectin-3 (Gal-3), VEGF-A, uPAR and PAI-1 were quantitatively determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D SYSTEMS, Minneapolis, MN, U.S.A.). Patients that had undergone FNA had blood specimens drawn 2 weeks after the procedure to avoid any possible interference in the measurement of serum levels. Six months after surgery, a random subset of patients (n = 28) were asked to provide new blood samples, with the aim of evaluating the influence of thyroidectomy on serum levels. Ten patients had MNG, 7 patients DTC and 11 patients mPTC. These patients were previously screened by measuring thyroglobulin, thyroglobulin antibodies and/or by neck US to exclude tumor or disease recurrence.

Statistical analysis

A one-way multivariate analysis of variance (MANOVA) was applied to test the hypothesis that there would be one or more mean differences between patients with DTC, microcarcinoma or benign disorders as compared to the control group in terms of their measured levels of Ang-1, Ang-2, Gal-3, VEGF-A, uPAR and PAI-1. Following this, a series of post-hoc analyses were performed to examine individual mean differences comparisons between the groups. Tuckey's range test was used to control for possible Type I errors due to multiple comparisons. For better visualization of our results, a primary component analysis (PCA) was performed using the MANOVA results (Fig 1).

ANOVA was used to compare age and BMI amongst groups. Chi-square test was employed to compare smoking habits between groups. Analysis between preoperatively and postoperatively measured parameters was performed using the Wilcoxon Sign-Ranked test. Values were

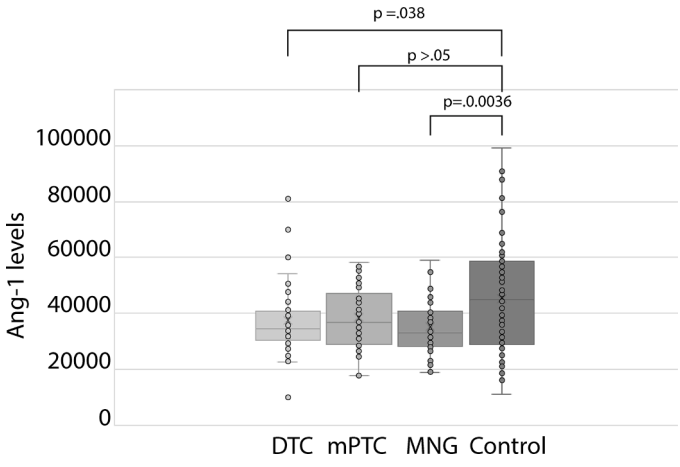

Fig. 1. Analysis of circulating levels of Ang-1 in controls and patients with DTC, mPTC and MNG. Significantly lower levels of serum Ang-1 were observed in DTC and MNG groups as compared to controls. No significant differences were observed between patient groups. All values are presented in pg/ml.

considered statistically significant at $p<.05$. Receiver-operating characteristic curves (ROC curves) were employed to estimate cut-off values of Ang-1 levels to diagnose DTC, mPTC and MNG respectively. In addition, the area under the curve (AOC) with 95% confidence intervals (CI), sensitivity and specificity for the cut-off values of Ang-1 were calculated. The optimal cut-off values were calculated using Youden's J statistic.

All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

Results

Patient characteristics

The age and gender make up of all groups is summarized in Table 1. Three patients in the DTC group had a prior history of autoimmune thyroiditis. No statistically significant effects were obtained regarding BMI, age, and smoking habits. In terms of BMI, no statistically significant ANOVA effect was obtained ($F(3192)=2.468$, $p>.05$, $\eta 2 = 0.036$). While the initial ANOVA test indicated that there might be a minor statistically significant difference in terms of age in our samples, the follow-up post-hoc tests did not indicate any, with the largest difference existing between the cancer and mPTC groups but with a p value greater than 0.1. No statistically significant Chi-Square effect was obtained regarding smoking ($p>.05$).

At the time of patient recruitment, the overwhelming majority of cancer patients (87%) had stage I cancer which is consistent with other published data (Table 2) [14]. Twelve patients in the mPTC group (22.2%) had histological changes compatible with MNG which is consistent with previously published data (15.6%) [13]. In contrast, 3 patients (7.5%) with DTC had concurrent histological findings consistent with multinodular hyperplasia.

Table 2
Pathology data. Staging was based on the 8th edition of the American Joint Committee on Cancer Tumor-Node-Metastasis (AJCC-TNM) staging system. Data of mPTC patients are included in this table.

Tumor Classification	Number of cases
Papillary	76 (89.4%)
Follicular	7 (8.2%)
Hürthle cell	2 (2.4%)
PTC variant	DTC/mPTC
Classic	18/19
Follicular	7/15
Sclerosing	2/8
Hürthle cell	2/3
Columnar	1/0
Warthin-like	1/0
Tumor size	
T1a	45 (52.7%)
T1b	26 (30.8%)
T2	11 (12.9%)
T3	2 (2.4%)
T4a	1 (1.2%)
T4b	–
Nodal status	
N1	12 (14.1%)
N2	4 (4.8%)
Tumor Stage	
I	74 (87%)
II	4 (4.7%)
III	4 (4.7%)
IVa	3 (3.6%)
IVb	–

Even though, all patients had TSH levels within normal range (0.4–4 mU/L), a significant difference was observed between DTC patients and MNG (1.85 ± 1.42 mU/L vs 1.15 ± 0.98 mU/L respectively). Twenty-five MNG patients (47%) were receiving thyroid suppression therapy prior to surgery, and this could have been a contributing factor to the observed difference. Thyroid suppression therapy did not lead to abnormal levels of T4, free T4, T3 and free T3.

Circulating levels

PCA/eigenvector analysis identified a projection axis in the six dimensional space of (Ang-1, Ang-2, Gal-3, VEGF, uPAR, PAI-1) parallel to the (6.00456, −0.85353, −1.39657, −3.12383, −2.27682, −0.91346) vector, which maximizes the statistical differences between the patient groups with Wilks = 0.79861, $F(24,695.44) = 1.92944$, $p <= 0.005$. Positive projection vectors were associated with the control group and negative projection vectors with the patient groups.

Levels of circulating angiogenesis related factors are summarized in Table 3. Statistically significant lower levels of Ang-1 were observed in patients with DTC compared to the control group (37,790 ± 11,270 pg/ml vs 47,720 ± 20,630 pg/ml, $p=.038$). No significant differences were observed between Stage III/IV and Stage I/II patients (38,310 ± 9730 pg/ml vs 37,711 ± 11,850 pg/ml). A pronounced effect was documented between patients with MNG and healthy controls (37,430 ± 11,370 pg/ml vs 47,720 ± 20,630 pg/ml, $p=.0036$) (Fig. 1).

Initially, mPTC patients had significantly lower levels of Ang-1 compared to control (37,190 ± 11,376 vs 47,720 ± 20,630 pg/ml, $p=.012$) but after correction for family-wise errors the p value was adjusted to $p=.053$, thus not achieving significance. As previously stated, 22.2% of the mPTC group also had MNG and therefore a separate comparison was performed, without these patients. No significant differences were observed between exclusively mPTC patients and controls (39,158 ± 11,850 vs 47,720 ± 20,630 pg/ml, $p=.780$). Comparison of patients with MNG and mPTC and mPTC documented lower levels in the former group but this was not significant.

Levels of Ang-2, VEGF, Gal-3 and uPAR did not exhibit any significant differences between groups.

In the subset of patients that underwent thyroidectomy, a significant increase in the levels of Ang-1 was noted as compared to their pre-operative status (41,690 ± 2570 pg/ml vs 53,680 ± 3250 pg/ml, $p<.05$) (Fig. 2). No significant differences were noted for Ang-2, VEGF, Gal-3, uPAR and PAI-1.

ROC curve analysis was selectively performed for Ang-1 levels which demonstrated significant differences in 2 patient subgroups (DTC and MNG) as compared to controls (Fig. 3). Analysis was also performed for the mPTC group where Ang-1 levels were lower than controls even though not significantly after correction. Data regarding ROC curve analysis is summarized in Table 4. AUC was greatest for the MNG group (0,710, CI: 0.613–0.795) and sensitivity and specificity were 86.54 and 59.62% at a cut-off value of 46,200 pg/ml. Similar results were observed in the DTC group with an AUC of 0.680 (CI: 0.575–0.773) and best threshold value of 43,780 pg/ml at which level sensitivity and specificity were 80.49% and 61.54% respectively.

Discussion

Angiopoietins

Excessive local expression of Ang-1 and Ang-2 has been documented in malignant thyroid tissue compared to normal tissue and higher levels of Ang-1 are associated with more aggressive tumor characteristics such as size, capsular invasion and lymph node involvement [15]. A more modest increase in these factors has been observed in benign disease compared to healthy tissue. MNG is a non-clonal aberrant growth of thyroid tissue where angiogenesis mechanisms contribute to disease development and therefore differences in expression compared to

Table 3
Comparison of circulating levels of Ang-1, Ang-2, VEGF-A, Gal-3, uPAR and PAI-1 between groups of patients and healthy controls. All values are presented in pg/ml.

Factor	Controls	DTC	p	mPTC	p	MNG	p
Ang-1	47,716 ± 20,633	4.65 ± 3.66	.038	37,190 ± 11,376	.053	37,430 ± 11,370	.0036
Ang-2	2336.29 ± 793.13	2422.58 ± 895.13	.804	2412.62 ± 883.66	.679	2412.10 ± 894.92	.940
VEGF-A	343.59 ± 293.73	366.02 ± 312.35	.943	345.35 ± 232.50	1.013	370.99 ± 300.14	.967
Gal-3	9440 ± 3250	10,690 ± 3612	.932	9860 ± 3550	.959	9950 ± 2830	.930
uPAR	859.35 ± 499.10	1996.74 ± 470.67	.734	1987.02 ± 473.90	.950	1991.68 ± 467.88	.624
PAI-1	4.65 ± 3.66	5.50 ± 5.00	1.029	5.54 ± 5.03	.779	5.50 ± 5.08	1.032

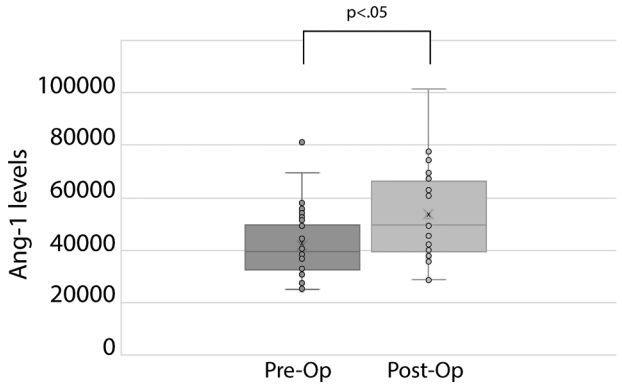

Fig. 2. Levels of serum Ang-1 in patients pre-operatively and 6 months after thyroid surgery. A significant increase was observed after surgery. All values are presented in pg/ml.

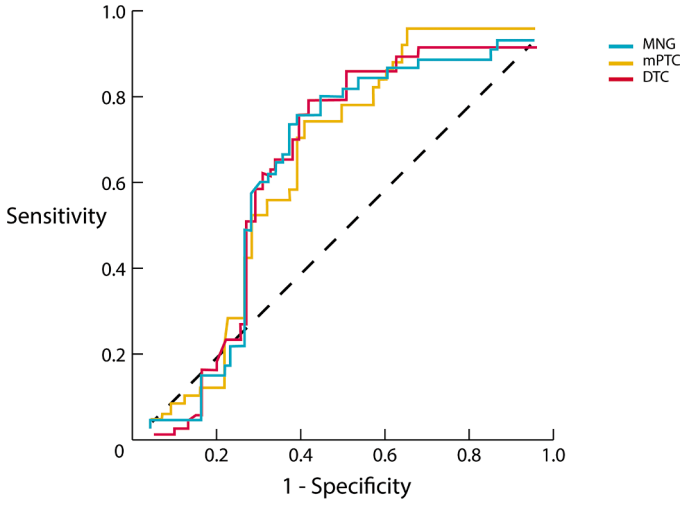

Fig. 3. ROC curve analysis for Ang-1 in DTC, mPTC and MNG patients.

Table 4
Results of ROC curves analysis of Ang-1 levels in patients with DTC, mPTC and MNG. Cut-off values are presented in pg/ml.

Diagnosis	AUC	95% CI	Sensitivity	Specificity	Cut-off value	Youden J index
DTC	0.68	0.575 - 0.773	80.49	61.54	43,780	0.462
mPTC	0.657	0.554 – 0.750	76.09	59.62	46,220	0.357
MNG	0.710	0.613 – 0.795	86.54	59.62	46,200	0.420

healthy tissue are expected [16]. In addition, increased tissue levels of Ang-2, as measured by semi-quantitative RT-PCR, are found in DTC.[7] These studies suggest an active role of these factors in the development and progress of DTC and to a lesser degree benign thyroid disease.

Our study documented a significant decrease in circulating Ang-1 levels in patients with MNG and malignant thyroid disease (DTC). Niedzwiecki et al. examined circulating levels of Ang-1 in a variety of thyroid cancer types and found significantly lower levels in DTC patients compared to healthy controls [17]. Makki et al. also demonstrated significantly lower levels in PTC patients compared to reference values of serum Ang-1 [18]. This previously published data is in accordance with the results of our current investigation. Lower serum levels of Ang-1 seem to be associated with the presence of DTC. This observation contrasts with the increases in Ang-1 expression on a local level. This in part could be attributed to the characteristics of our DTC cohort. The vast majority of DTC patients had stage I or stage II disease (92.5%) at presentation. More advanced disease stage could be associated with different Ang-1 levels. On preliminary analysis, Ang-1 levels were also lower in the mPTC group as compared to controls. After α value adjustment for multiple comparisons, this difference was not significant. The observed trend may be explained by the fact that 22% of the mPTC patients had concurrent multinodular hyperplasia. A reasonable approach for future investigations would be to exclude patients with concurrent MNG.

Data comparing DTC and benign thyroid disease is conflicting. In a recent study by Sun et al., significantly lower levels of Ang-1 were observed in DTC patients as compared to patients with MNG [19]. Details regarding disease stage were not reported. On the contrary, Makki et al. compared levels in malignant disease (PTC and mPTCs) and benign disease and did not observe significant differences [18]. As in our cohort, a considerable majority of cases were stage I & II disease.

Evaluation of Ang-1 as a potential biomarker for malignant thyroid disease and MNG was performed. Data from the ROC curve analysis indicated sensitivity ranging from 76.09–86.54% at specific cut-off levels (Table 4). However, specificity was far from ideal in all groups (59.62–61.54%). Even though these results are not insignificant they are inadequate for a potential screening biomarker. It should be reminded that strict exclusion criteria were employed during patient and control recruitment to limit the effect of confounding factors. Without these exclusion criteria, specificity could be even lower. To our knowledge no other study has evaluated Ang-1 levels as a potential biomarker in thyroid malignancy and MNG.

Thyroidectomy had a profound effect on Ang-1 levels. Six months after surgery a significant increase in Ang-1 levels was recorded approximating levels seen in healthy controls. This finding suggests that removal of underlying thyroid pathology affects Ang-1 serum levels.

Data regarding serum Ang-2 are less conclusive. Niedzwiecki et al. did not observe overall differences but in subgroup analysis levels of Ang-2 were significantly lower in PTC patients [17]. In a recent study with similar exclusion criteria as our own study design, levels of Ang-2 were elevated in PTC and benign thyroid disease compared to healthy controls [20]. Postoperative levels also exhibited a marked decline in both groups. Our investigation did not reveal significant differences overall or in subgroup analysis. Furthermore, no differences were noted in the postoperative setting. When comparing our findings to Ria et al., there is a considerable difference in Ang-2 levels in healthy controls. Our control levels are similar to levels reported in other series [21,22]. In addition, differences noted postoperatively could be attributed to

differences in sampling timing. In the aforementioned study, samples were obtained 2 weeks after surgery. In this limited time interval, the observed differences in Ang-2 levels could be the result of recent surgical trauma and not removal of the underlying pathology.

VEGF-A

Arguably VEGF-A is the most thoroughly studied angiogenesis related factor. Over-expression of VEGF-A in malignant tissue has been associated with tumor size and tumor invasiveness [23]. Increased local expression has also been observed in MNG [24]. The significance of serum levels of VEGF-A is less clear. Elevated levels have been observed in patients with metastatic disease compared to patients in remission [8]. In this same report patients with MNG were found to have significantly lower levels compared to healthy recruits. Yu et al. compared levels between PTC patients and patients with MNG. Increased levels were observed in the PTC group compared to the MNG group [25]. In a follow-up study the same investigators documented increased VEGF-A levels in patients with recurrent PTC (locoregional and/or distant metastasis) compared to benign cases [26]. On further analysis patients with distant metastasis had higher levels than patients with locoregional recurrence, suggesting that VEGF-A levels correlate with tumor stage. It should be noted that 40% of the PTC cohort had stage III or IV disease. Ria et al. reported significantly greater levels of VEGF-A in PTC and MNG patients as compared to healthy subjects [20]. Our results did not demonstrate differences between the studied groups. Our series had a limited number ($n = 7$, 8.2%) of patients with stage III and IV disease. Elevated circulating VEGF-A seems to be associated with advanced disease and therefore a larger sample size would be necessary to detect any possible differences.

Galectin-3

Gal-3 is pluripotent factor that has a salient role in cellular matrix interactions and by consequence in angiogenesis [27]. Immunohistochemical studies have consistently documented Gal-3 expression in DTC and non-expression in healthy thyroid tissue 28. Gal-3 has been used to facilitate recognition of malignancy in FNA samples [29]. An early report by Saussez et al., suggested that elevated serum galectin-3 levels were an indicator of the presence of malignancy in MNG [30]. However, no reference is made regarding the inflammation status of participants. In our investigation no significant differences were observed between groups. Gal-3 is actively involved in the modulation of the inflammatory response [31]. In our investigation, all participants were screened for indicators of active inflammation and history of autoimmune disease. Therefore, the differences observed could be attributed to differences in the vetting process. Our findings are in accordance with a previous study by Inohara et al. that did not document any significant differences between subjects with thyroid nodules and healthy individuals [32]. Larger studies are required to investigate Gal-3 levels in thyroid disease.

uPAR and PAI-1

A close connection has been established between VEGF-A and the plasminogen activation system. VEGF-A stimulates the expression of uPA and uPAR which leads to a flurry of proteolytic activity thus facilitating the initial steps of angiogenesis [33]. Built in feedback mechanisms activate PAI-1 which inhibits further plasmin activity. Increased expression of uPAR and PAI-1 has been reported in thyroid cancer cell lines as compared to healthy cells [34]. Similar findings have been observed in tumor specimens and increased mRNA levels have been associated with more advanced disease stages [10]. In our series, no significant differences were observed in circulating serum levels. Similarly, to VEGF-A, this could be partly attributed to the fact that the vast majority of patients had early-stage disease and therefore differences may not be detectable. In addition, uPAR and PAI-1 function in a paracrine fashion and therefore local increases in expression may not be evident in serum levels.

Study limitations

A major limitation of our study was the small number of patients with advanced thyroid cancer. In our cohort none of the patients had distant metastasis and only 7 patients had Stage III or IVa disease. Therefore, our results are only reflective of early-stage DTC.

Furthermore, the relatively small number of participants in each group may not be sufficient to detect possible subtle differences in serum levels of the investigated factors.

In addition, several exclusion criteria were implemented to eliminate possible confounding factors. However, it is impossible to discern if any unknown confounding factor had any effect on our results. All the studied factors participate in several physiological processes some of which certainly have not been fully investigated yet.

Finally, the observed differences are only suggestive of differences in Ang-1 production and cannot be used to make inferences about differences in local tissue activity.

Conclusion

Very few studies have examined the association of Ang-1 levels and thyroid disease (benign or malignant). The results of the current study suggest that decreased serum levels of Ang-1 are associated with the presence of underlying benign or malignant thyroid nodular disease, a finding which validates observations from two previous smaller studies which did not implement strict exclusion criteria. A novel finding from this study is that surgical removal of the affected thyroid gland results in normalization of Ang-1 levels, whereas it does not have an impact on levels of the other cytokines. These results suggest that Ang-1 levels could have a role in the development of benign nodular and malignant disease and further larger scale studies will be needed to validate this observed association. Interestingly no significant differences were observed between MNG and malignant disease. Furthermore, no significant differences were noted in serum levels of the other angiogenesis related factors amongst groups. Previous investigations have documented both differences and a lack of differences in the levels of these factors suggesting a weak association if any.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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