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High Frequency of Thyroid Disorders in Patients Presenting With Neutropenia to an Outpatient Hematology Clinic STROBE-Compliant Article

Eleni Magdalini A. Kyritsi, MD, PhD, Xanthi Yiakoumis, MD, PhD, Gerasimos A. Pangalis, MD, PhD, Charalampos Pontikoglou, MD, PhD, Katerina Pyrovolaki, MD, PhD, Christina Kalpadakis, MD, PhD, Irini Mavroudi, PhD, Helen Koutala, PhD, Semeli Mastrodemou, PhD, Theodoros P. Vassilakopoulos, MD, PhD, George Vaiopoulos, MD, PhD, Evanthia Diamanti-Kandarakis, MD, PhD, Helen A. Papadaki, MD, PhD, and Maria K. Angelopoulou, MD, PhD

Abstract: Granulopoiesis abnormalities have been described in association with thyroid disorders (TD). However, data regarding systematic evaluation of adult neutropenia and concurrent or prior TD are scarce.

To investigate the frequency of TD among patients presenting with neutropenia, and the immunophenotypic and immunologic profile of neutropenic patients with concomitant thyroidopathy.

Two hundred eighteen consecutive neutropenic patients were prospectively evaluated in our outpatient Hematology Clinic, with a detailed laboratory screen, including thyroid function tests, antineutrophil antibodies, blood lymphocytes immunophenotyping, and detection of T-cell clonality by PCR.

Among 218 patients with neutropenia, 95 (43.6%) had TD, 65 chronic immunologic neutropenia, 20 clonal proliferation of T-large granular lymphocytes (T-LGL), 5 autoimmune disorders, and 33 other diagnoses. TD-patients had an increased frequency of recurrent infections compared with other patients ($P = 0.045$). The following correlations were found: negative correlation between FT3 and absolute neutrophil count (ANC) ($r^2 = -0.274$, $P = 0.007$), negative correlation between TPO-Abs/TG-Abs and C4 ($r^2 = -0.16$, $P = 0.045$; $r^2 = -0.266$, $P = 0.001$), and CD4$^+$ counts were inversely correlated to T4 and positively to TSH ($r^2 = -0.274$, $P = 0.024$; $r^2 = 0.16$, $P = 0.045$). In addition, TD-patients had significantly higher percentages of CD4$^+$ lymphocytes ($P = 0.003$). Among TD-patients, 23.4% had Hashimoto thyroiditis (HT), 4.1%, Graves disease (GD), 8.2% nontoxic multinodular goiter (NTMG), 5% subclinical hypothyroidism, and 2.8% had undergone total thyroidectomy associated with nodules (TTM). Thirteen TD-patients displayed T-LGL. Patients with autoimmune thyroidopathy had an increased frequency of concomitant autoimmune manifestations ($P = 0.03$). Significant differences between the different thyroidopathies included: HT-patients had higher percentages of B-lymphocytes, while the opposite was evident for the TTM-subgroup ($P = 0.009$, 0.02); GD-patients showed an increase of the proportion of NK cells and a decrease in the percentage of TCRγδ$+$ lymphocytes ($P = 0.001$, 0.045); and NTMG-patients had significantly higher ANC ($P = 0.004$) compared to other thyroidopathies. Antineutrophil antibodies were found in 37.2% of TD-patients tested. Anti-TPO titers were significantly higher in patients with positive antineutrophil antibodies ($P = 0.04$).

The frequency of TD among neutropenic patients may be higher than previously reported. The existence of antineutrophil antibodies, as well as the different distribution of lymphocyte subsets among patients with different TD, suggests both humoral and cellular mechanisms in the pathophysiology of thyroid disease-associated neutropenia.

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Abbreviations: Abs = antibodies, ANC = absolute neutrophil count, AN-SHP = antibody-negative subclinical hypothyroidism, Anti-TG = antithyroglobulin antibodies, Anti-PMN Abs = antineutrophil antibodies, Anti-TPO = anti-thyroid peroxidase antibodies, ATD = autoimmune thyroid disease, CBC = complete blood counts, FT3 = free triiodothyronine, FT4 = free thyroxine, GAT = granulocyte agglutination test, GD = Graves disease, GIFT = granulocyte immunofluorescence test, HT = Hashimoto thyroiditis, IQR = interquartile range, LIFT = lymphocyte immunofluerence test, NK = natural killer, NTMG = nontoxic multinodular goiter, T3 = total triiodothyronine, T4 = total thyroxine, TCR = T-cell receptor, TD = thyroid disorders, T-LGL(s) = T-cell large granular lymphocyte(s), TRAbs = anti-thyrotropin receptor antibodies, TSH = thyroid-stimulating hormone, TTM = total thyroidectomy associated with nodules.

INTRODUCTION

Hematologic disorders, especially single lineage abnormalities, have been described in association with thyroid disorders (TD).[1,2] Coexisting folic acid or B12 deficiency may result in a reduction of neutrophils, especially in patients

with autoimmune TD. Additionally, hyperthyroidism-associated neutropenia may be caused by either decreased neutrophil circulation time[3] or reduced marrow granulocytic reserve.[4] Furthermore, immune-mediated acute neutropenia or even agranulocytosis is a well-known serious side effect of antithyroid drugs.[5] However, reports on systematic evaluation of adults with neutropenia and concurrent or prior thyroid disease are limited. Moreover, data on the distribution of peripheral lymphocyte subsets in patients with thyroid disease with or without neutropenia are scarce.[6,7]

The present prospective study, expanding through a 4-year period aims to investigate the frequency of TD among patients presenting with neutropenia in the outpatient setting, describing in parallel their clinical and laboratory characteristics. Furthermore, we wanted to get insight into the pathophysiology of thyroid-associated neutropenia by evaluating possible differences in the distribution of peripheral lymphocyte subsets, the presence of antineutrophil autoantibodies and other immunologic parameters.

PATIENTS AND METHODS

In the present prospective observational and registrational study, we analyzed the clinical and laboratory findings of 218 consecutive patients, who presented with neutropenia as the dominant hematologic abnormality, to the Outpatient Hematology Clinic of our Department between 2010 and 2013. The study has been approved by the Ethics Committee of Athens Laikon General Hospital. Informed consent was not considered necessary, since all the tests performed are included in our routine work-up for the investigation of neutropenia. All patients enrolled in this study had an absolute neutrophil count (ANC) below 2×10^9/L, documented in at least 3 consecutive occasions within the last 3 months before study entry and were referred to our Outpatient Clinic by their family doctor. As the sole selection criteria was their referral from their primary care physician, cases with pancytopenia, systemic symptoms, or other prominent abnormalities would have been referred as inpatients. Patients' evaluation included detailed medical history, physical examination, complete blood counts, blood smear morphology, folate, vitamin B12 and iron status, serum liver enzymes, serum proteins, serology for HBV, HCV, HIV, EBV and toxoplasmosis, antinuclear (ANA) and anti-DNA antibodies (Abs) by immunofluorescence, rheumatoid factor (RF), complement factors C3 and C4 by nephelometry, and abdominal and thyroid ultrasonography. Peripheral blood lymphocyte subsets were analyzed by 3-color flow cytometry using Abs against CD2, CD3, CD4, CD8, CD19, CD20, CD56, CD57, CD16, T-cell receptor (TCR)αβ, and TCRγδ conjugated with the appropriate fluorochromes. Briefly blood samples were incubated with the Abs for 20 minutes in the dark, washed twice in PBS/FCS. Subsequently, red blood cells were lysed and samples were analyzed immediately in a FACSCalibur (BD Biosciences, Becton, Dickinson and Company, San Jose, CA, USA) cytometer. The appropriate isotypic controls were used.

Antineutrophil antibodies (anti-PMN Abs) were detected by granulocyte immunofluorescence test (GIFT), granulocyte agglutination test (GAT), and lymphocyte immunofluerence test (LIFT) methodology, using polyclonal rabbit F(ab′)2 anti-human IgG-FITC (DakoCytomation).[8,9] For both GIFT and GAT, neutrophils from a panel of 3 healthy donors with known phenotype were isolated from fresh whole blood using density gradient separation on the day of testing. In the GAT, patients' serum was incubated with the control neutrophils for 4 to 6 hours at 30°C on a microtiter plate, and cell agglutination was subsequently evaluated by microscopy. In the GIFT, patients' serum was incubated with the control neutrophils, which in this case had been previously treated with 1% paraformaldehyde to prevent nonspecific binding of Abs to granulocyte Fc-receptors and to stabilize the cell membranes. Following incubation for 30 minutes at 37°C, the flourochrome-conjugated secondary Ab was added to detect binding of antineutrophil Abs to granulocytes. The evaluation was performed by fluorescence microscopy. In order to distinguish anti-HLA class I from anti-PMN Abs, we used the LIFT methodology. This test was analogous to GIFT, but the evaluation was done by flow cytometry.

Since this was a prospective study, missing data were negligible in all parameters except of anti-PMN Abs. Anti-PMN Abs were intended to be measured only in patients with thyroidopathy. However, due to the technically challenging procedure of anti-PMN Abs detection in serum, only 43/95 samples were finally considered suitable for reliable analysis. Apart from anti-PMN Abs, the remaining data were available in more than 90% of the patients.

Serum free thyroxine (FT4), total thyroxine (T4), free triiodothyronine (FT3), total triiodothyronine (T3), and thyrotropin (thyroid-stimulating hormone [TSH]) were measured by radioimmunoassay (FT3-CTK RIA, FT4-CTK, SPART, T3-CTK, RIA, T4-CTK, RIA, TSH-CTK-3, IRMA, DiaSorin S.p.A., Italy). The normal range of thyroid hormones was as follows: TSH 0.4 to 4 mU/L, FT3 2.3 to 5.3 pg/mL, FT4 10 to 25 pmol/L, T3 1.23 to 3.08 nmol/L, and T4 58 to 154 nmol/L. The serum titers of antithyrotropin receptor Abs (TRAbs) were determined by a radioreceptor assay (TRAK, RIA, BRAHMS, Diachel S.A., Germany). The normal value of TRAbs was <1.5 IU/L. Antithyroglobulin Abs (anti-TG) and antithyroid peroxidase Abs (anti-TPO) were measured by ELISA. Normal values for anti-TG and anti-TPO were <55 and <30 IU/mL, respectively.

The diagnosis of thyroidopathy was based on clinical examination, laboratory tests, and ultrasonography of the thyroid gland. Patients with thyroid disease were classified in 5 diagnostic categories: Hashimoto thyroiditis (HT), Graves disease (GD), nontoxic multinodular goiter (NTMG), total thyroidectomy associated with nodules (TTM), and antibody-negative subclinical hypothyroidism (AN-SHP). The presence of serum antithyroid Abs, a sonographic heterogeneity of the thyroid gland with a micronodular pattern, with or without hypervascularity and/or goiter were indicative of HT. All patients of this subgroup were euthyroid with or without levothyroxine, for a median of approximately 6 years prior to presentation (median 74 months, range 2 months–18 years). Patients with GD were also euthyroid after cessation of the treatment with antithyroid drugs for at least 5 years. Long-term remission was achieved and further radioactive iodine administration was not needed. They all provided medical records and pathology reports according to which the diagnosis was established by the presence of diffuse goiter, thyrotoxicosis, a uniform pattern of uptake on scan with Tc-99m, positive serum antithyroid Abs, and/or TRAbs. Diagnosis of NTMG was established on the basis of several palpable thyroid nodules, confirmed by ultrasound, and fine needle aspiration in the presence of indicative sonographic features. All NTMG subjects were euthyroid, while serum thyroid Abs were negative. Patients who underwent TTM were receiving replacement therapy with levothyroxine. The thyroid specimens were classified as benign in all TTM patients

and showed no histopathological features of chronic lympho-cytic thyroiditis. Finally a TSH level above the upper normal limit (>4 mU/L) was considered as subclinical hypothyroidism. Patients in this subgroup had no hypothyroid symptoms, except of tiredness observed in a minority of them. Moreover, they had no laboratory or sonographic signs of autoimmune thyroiditis, that is, they were all antibody negative (AN-SHP). However, even if these criteria were fulfilled, an underlying thyroiditis cannot be definitely excluded. Maximum TSH levels of AN-SHP patients in our study (n = 11) were 6.27 mU/L, and one might argue, that TSH concentrations below 7 mU/L in the elderly could be normal and considered as a physiological adaptation to aging. According to recent studies, the use of age-specific reference ranges for TSH, rather than a common cut-off of 4.0 mU/L has only minor effects on thyroid status, except in the very old (≥85 years old).[10] Thus, a TSH level >4 mU/L was considered as subclinical hypothyroidism in the present study. Since only 3 patients with AN-SHP were older than 70 years, we consider the TSH cut-off of 4.0 mU/L as appropriate for the purposes of this study.

The diagnosis of T-cell large granular lymphocyte (T-LGL) expansion was based on the following criteria: relative increase of the percentage of T-cells with a T-LGL phenotype (CD3$^+$/CD8$^+$/CD57$^+$ with or without CD16$^+$), persisting for at least 6 months, and clonal rearrangement of the TCR gamma (γ) or beta (β) gene. Patients who fulfilled the above-named criteria and had in addition an absolute T-LGL count >2 × 10^9/L were diagnosed as having T-LGL leukemia.[11] TCR beta and gamma chain gene rearrangements were studied by polymerase chain reaction (PCR) using the commercially available kits "IdentiClone TCRB Gene Clonality Assay" and "IdentiClone TCRG Gene Clonality Assay" (Invivoscribe Technologies). Fragment analysis was carried out on a Beckman Coulter CEQ8000. Platform and results were interpreted according to the BIOMED-2 study.[12]

The diagnosis of myelodysplastic syndrome was established based on standard criteria.[13] The diagnosis of persistent polyclonal B-cell lymphocytosis was established by blood smear morphology, the identification of polyclonal B cell lymphocytosis by flow cytometry and nonclonal rearrangement of the immunoglobulin heavy chain genes.[14]

The diagnosis of chronic idiopathic neutropenia was established when other neutropenia states were excluded, in accordance with previous strict diagnostic criteria.[9]

Drug-related neutropenia was diagnosed in patients under treatment with drugs associated with neutropenia, in whom ANC reached normal values when the drug was withdrawn. Other diagnoses, such as viral infections, connective tissue disorders, and nutritional deficiencies were defined by the relevant clinical and laboratory criteria.

Patients who had T-LGL expansion or a connective tissue disorder, but fulfilled the appropriate criteria for any of the aforementioned categories of thyroidopathy, were classified as suffering from the respective thyroidopathy.

At 6 months intervals patients were followed by clinical examination, complete blood counts and blood smear, and further immunologic tests, if needed.

STATISTICAL ANALYSIS

Comparisons of continuous variables between categories was performed using nonparametric tests (Kruskal-Wallis and/or Mann-Whitney). Correlations between continuous variables were assessed using the Spearman correlation coefficient. Associations of categorical variables were carried out using the chi square (χ^2) and Fisher exact test. The results regarding quantitative variables were expressed as the median values and inter-quartile range (IQR). All statistical analyses were performed with the statistical package SPSS, version 17.00 (SPSS Inc, Chicago, IL). A P-value <0.05 was considered as statistically significant.

RESULTS

Among 218 patients presenting with neutropenia to our Outpatient Clinic, 65 had chronic idiopathic neutropenia, 12 connective tissue disorders, 75 thyroid disease with no other coexisting immunologic or hematologic disorders, 33 T-LGL expansion, and 33 other diagnoses. 13/33 patients with T-LGL expansion and 7/12 patients with connective tissue diseases suffered from thyroidopathy, as well. Hence, overall we detected 95 neutropenic patients with TD (Table 1).

The distribution of the 95 patients with TD in the 5 diagnostic subgroups was as follows: 51 (23.4%) presented HT, 9 (4.1%) GD, 6 (2.8%) TTM, 18 (8.2%) NTMG, and 11 (5%) AN-SHP. The clinical and laboratory characteristics of all

TABLE 1. Diagnostic Categories of Neutropenia in 218 Patients Enrolled in the Study

	Number of Patients, %
Thyroid disease	95 (43.6)
Hashimoto thyroiditis	51
Graves disease	9
Total thyroidectomy	6
Nontoxic multinodular goiter	18
Antibody-negative subclinical hypothyroidism	11
Chronic idiopathic neutropenia	65 (29.8)
T-large granular lymphocytosis (TLGL)	20* (9.2)
Connective tissue diseases	5† (2.3)
Systemic lupus erythematosus	3
Sjögren syndrome	1
Polymyalgia rheumatic	1
Infectious diseases	5 (2.3)
Hepatitis B	3
Hepatitis C	1
Ebstein barr	1
Drug related	11 (5.0)
B12 Deficiency	6 (2.8)
Iron deficiency	6 (2.5)
Other hematologic disorders	5 (2.3)
Myelodysplastic syndrome	2
MGUS‡	1
NHL§	1
Polyclonal B Lymphocytosis	1

* Overall 33 patients suffered from T-LGL, of whom 13 had thyroidopathy: 6 Hashimoto thyroiditis (2 had additionally systemic lupus erythematosus), 1 Graves disease, 1 hypothyroidism, 2 nontoxic multinodular goiter, and 3 had undergone total thyroidectomy.
† Overall 12 patients suffered from connective tissue diseases, of whom 7 had thyroidopathy: 1 systemic lupus erythematosus and Graves disease, 1 Sarcoidosis and Hashimoto thyroiditis, 3 rheumatoid arthritis, of whom 2 had Hashimoto thyroiditis and 1 had nontoxic multinodular goiter, 2 Sjögren syndrome of whom 1 had hypothyroidism and 1 Hashimoto thyroiditis.
‡ MGUS = monoclonal gammopathy of undetermined significance.
§ NHL = non-Hodgkin lymphoma: marginal zone lymphoma.

TABLE 2. Clinical and Laboratory Characteristics of All 218 Neutropenic Patients and 95 Neutropenic Patients With Thyroidopathy

	Neutropenia Without Thyroidopathy	Neutropenia and Thyroidopathy
Number of patients	123	95
Median age in years (range)	54 (15−81)	55 (20−80)
Gender (female/male)	41/82	83/12
Median Hb, g/dL (range)	13.2 (9.7−15.8)	12.9 (9.5−15.3)
Median Ht, % (range)	40.0 (29.7−47.5)	39.5 (31.3−45.3)
Median WBC, ×109/L (range)	3.60 (2.0−6.00)	3.77 (2.73−5.59)
Median ALC, ×109/L (range)	1.53 (0.69−3.66)	1.61 (1.00−3.00)
Median ANC, ×109/L (range)	1.63 (0.16−2.0)	1.70 (0.16−2.00)
Median PLT, ×109/L (range)	197 (123−471)	202 (85−315)
Median C3, mg/dL (range)	99.7 (62.9−135)	102.5 (61−162)
Median C4, mg/dL (range)	20.6 (7.9−41.2)	20.8 (0−42)

ALC = absolute lymphocyte count, ANC = absolute neutrophil count, C3 = complement component 3, C4 = complement component 4, Hb = he-hemoglobin, Ht = hematocrit, PLT = platelets, WBC = white blood cells.

neutropenic patients and patients with TD-associated neutropenia are shown in Table 2. The median follow-up time was 28 months (range: 6–80 months).

No major differences were detected in the ANC between patients with or without thyroidopathy. However, patients with TD had an increased frequency of recurrent infections (ie, ≥3 episodes/year) (genital, fungal, oral, and respiratory) compared to patients with no thyroidopathy ($P = 0.045$). Furthermore patients with autoimmune thyroid disease (ATD) had an increased frequency of concomitant autoimmune cutaneous or systemic manifestations (rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, autoimmune gastritis, and vitiligo) ($P = 0.03$). The prevalence of specific autoantibodies in patients with ATD did not differ compared with other neutropenic subjects, except for antithyroid Abs.

Associations Between Thyroid Function Parameters and Markers of Immunity

In the entire group of subjects (n = 218) the following significant associations were found: serum levels of FT3 inversely correlated to ANC ($r^2 = -0.274$, $P = 0.007$) (Figure 1A), absolute CD4+ lymphocyte counts correlated positively to TSH levels ($r^2 = 0.16$, $P = 0.045$) (Figure 1B) and negatively to T4 levels ($r^2 = -0.274$, $P = 0.024$) (Figure 1C), and serum TPO-Abs/TG-Abs titers inversely correlated to C4 levels ($r^2 = -0.16$, $P = 0.045$ and $r^2 = -0.266$, $P = 0.001$, respectively).

Immunophenotypic Findings in Patients With Neutropenia and Thyroidopathy

Next we investigated possible differences in the distributions of lymphocyte subsets in patients with thyroidopathy (Table 3). The percentage of CD4+ T-lymphocytes was significantly higher in patients with thyroid disease compared with the remaining diagnostic categories ($P = 0.003$), although absolute CD4+ counts did not differ.

Among patients with TD there was a statistically significant difference in the distribution of B-lymphocytes: in patients with HT (who were euthyroid), the percentage of B-lymphocytes was significantly higher ($P = 0.009$), compared to other subgroups of thyroidopathy. On the contrary, the TTM-subgroup had significantly lower percentages of B-lymphocytes ($P = 0.020$). Of note, all patients of the TTM-subgroup were

receiving high dose-replacement therapy with levothyroxine and presented with iatrogenic hyperthyroidism. GD patients displayed a significant increase of the percentage of natural killer (NK) cells ($P = 0.001$) and a decrease in the percentage of TCRγδ+ lymphocytes ($P = 0.045$). GD patients had been on antithyroid drug treatment for a period of 6 to 18 months. At the time of the study, none was thyrotoxic and TRAbs were found negative in all but one patient. Patients with NTMG, who were euthyroid, had significantly higher ANC ($P = 0.004$). Finally, patients with AN-SHP presented no significant differences regarding ANC or lymphocyte subsets.

T-LGL Expansion

Among the 218 patients, 33 displayed clonal proliferation of T-LGLs by PCR for the β and γ TCR rearrangement. Of note, none of the 33 patients fulfilled the strict criteria of T-LGL leukemia. Concomitant thyroid abnormalities were noted in 13/33 patients (39.4%): HT, the most common abnormality, occurred in 6 patients, GD in 1, NTMG in 2, and AN-SHP in 1 patient, while 3 patients had undergone TTM associated with nodules.
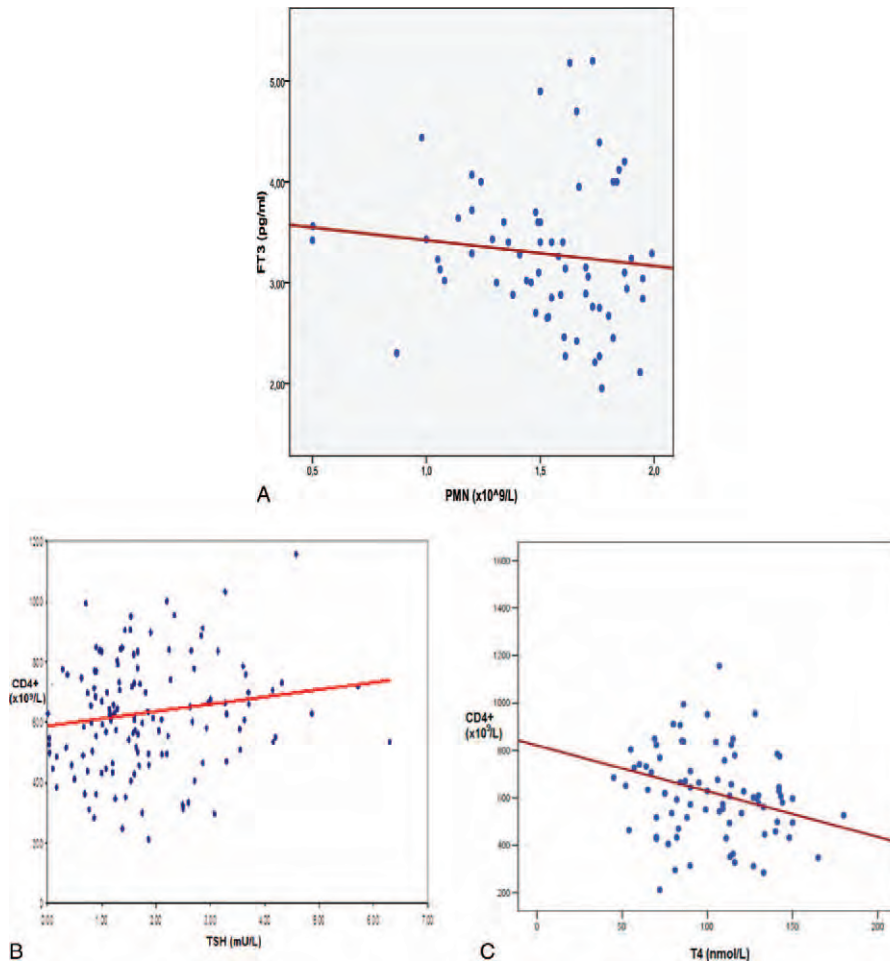
Anti-PMN Abs

Investigation for Anti-PMN Abs was carried out in 43 out of 95 patients with TD: 26 with HT, 5 with GD, 6 with TTM, 2 with NTMG, and 4 with AN-SHP. Overall anti-PMN Abs were detected in 16 patients (37.2%): 14 patients had autoimmune thyroidopathy (13 HT, 1 GD) and 2 NTMG. Anti-PMN Abs were positive by GIFT methodology in all patients, while in 4, anti-PMN Abs could be detected by GAT as well. LIFT was negative in all cases.

Patients with ATD had an increased incidence of anti-PMN Abs. This difference was of borderline significance, possibly due to small patient number ($P = 0.07$).

Furthermore anti-TPO titers were significantly higher in patients with positive anti-PMN Abs (median anti-TPO levels: 100 IU/mL, IQR: 425), compared to those without (median anti-TPO levels: 1.2 IU/mL, IQR: 54.75, $P = 0.04$), (Figure 2).

DISCUSSION

The association between thyroidopathy and neutropenia was first described more than 100 years ago and subsequently

FIGURE 1. (A) Negative correlation between FT3 levels and absolute neutrophil counts ($r^2 = -0.274$, $P = 0.007$). (B) Positive correlation between TSH levels and absolute CD4$^+$ counts ($\times 10^9$/L), ($r^2 = -0.16$, $P = 0.045$). (C) Negative correlation between T4 levels and absolute CD4$^+$ counts ($\times 10^9$/L), ($r^2 = -0.274$, $P = 0.024$). FT3 = free triiodothyronine, T4 = thyroxine (total), TSH = thyroid-stimulating hormone, PMN = polymorphonuclear/neutrophil cells (absolute counts), CD4$^+$ = subsets of T lymphocytes (absolute counts).

confirmed by other investigators.[15] However, there has been little mention of this association in the recent literature, except when due to thionamide therapy.[5] The present study revealed that thyroidopathy represents the most common disorder among apparently healthy patients with mild-to-moderate neutropenia, comprising 43.6% of this cohort of individuals. This frequency is significantly higher than the 1 reported in the recent study by Lima et al,[16] who identified TDs in 8.2% among 97 neutropenic individuals, of whom 7 patients had hypothyroidism and 1 GD. The main strength of our study is the fact that all patients were consecutive ones, presenting with neutropenia in the outpatient setting, and that they were prospectively studied with a detailed and homogenous laboratory screen. It is of interest, that the incidence of autoimmune TDs in neutropenic patients is considerably higher compared with the one reported in healthy individuals. Specifically, we identified HT in 23.4% of all neutropenic patients enrolled in this study, whereas the frequency of HT in several large series of healthy subjects ranges between 10.7% and 13.4%.[17,18] At this point, it has to be mentioned that the referral pattern may differ between studies, such as the one by Lima et al and ours. Certainly, this is not a population-based study and referral bias is likely to be present,

since patients were referred to a Tertiary Center. However, in our country, family physicians, biopathologists, internists, and other specialists can directly refer patients to tertiary Hematology Clinics without prior detailed work-up. Thus, this series of consecutive patients with incidental, persisting neutropenia can be considered as representative of asymptomatic neutropenia in the outpatient setting. Patients with systemic symptoms, the ones with pancytopenia or other prominent abnormalities are most likely investigated as inpatients and were not referred to the Outpatient Clinic. This might explain the low incidence of myelodysplastic syndromes or systemic diseases, such as systemic lupus erythematosus.

No major differences were detected in the ANC between the different diagnostic categories of neutropenia, both in our analysis and the study of Lima et al.[16] However, we found that patients with TD had an increased frequency of recurrent infections, although not life-threatening. To the best of our knowledge, this is the first study demonstrating that thyroidopathy may be involved in the development of infections in patients with neutropenia. In this regard, a potential basis for this observation could be that the production of proinflammatory cytokines, circulating immune complexes and cell-bound anti-PMN Abs

TABLE 3. Clinical Characteristics, Endocrine, Hematologic, and Immunophenotypic Findings in Neutropenic Patients With Thyroid Disorders

Parameters (Range)	HT	GD	TTM	NTMG	AN-SHP
Patients (n, F/M)	51 (46/5)	9 (7/2)	6 (4/2)	18 (17/1)	11 (9/2)
Median age (year, range)	56 (20−78)	51 (37−73)	51.5 (39−67)	55 (40−80)	55 (26−83)
FT3, pg/mL	3.29 (2.11−4.70)	3.43 (3.26−4.0)	3.40 (2.42−4.90)	2.92 (2.32−3.70)	3.19 (2.02−4.39)
FT4, pmol/L	15.2 (10.1−24.8)	15.2 (11.0−20.5)	17.1 (11.5−28.4)	15.3 (11.0−21.0)	14.0 (9.0−18.0)
T3, nmol/L	1.58 (0.80−2.51)	1.76 (1.09−2.28)	1.55 (0.88−4.90)	1.80 (1.25−2.22)	1.0 (0.61−1.75)
T4, nmol/L	111 (55−165)	85 (73−134)	141 (70−180)	110 (86−129)	78 (45−130)
TSH, mU/L	1.57 (0.42−4.0)	1.61 (0.41−3.90)	0.04 (0.02−0.35)	1.27 (0.43−3.14)	4.70 (4.16−6.27)
Anti-TPO, IU/mL	133.5 (0.1−2700)	95.0 (1.0−1000)	1.0 (0.0−4.0)	1.0 (0.0−28.7)	2.5 (0.3−25.9)
Anti-TG, IU/mL	66.7 (0.0−2700)	39.5 (8.9−100.0)	5.0 (0.0−10.0)	6.2 (0.0−17.0)	10.0 (1.7−32.7)
Ht, %	39.5 (27.6−46.5)	38.7 (31.3−44.3)	38.4 (31.5−45.5)	39.2 (34.7−43.1)	39.1 (33.8−43.3)
Hb, g/dL	13.0 (9.2−15.7)	12.8 (9.7−14.7)	12.6 (9.5−14.9)	12.8 (11.0−14.3)	13.0 (11.5−14.4)
WBC $\times 10^9$/L	3.74 (2.6−5.59)	3.58 (2.76−4.53)	3.70 (3.21−4.51)	3.94 (2.92−6.10)	3.42 (1.62−4.57)
ANC $\times 10^9$/L	1.66 (0.16−2.19)	1.58 (1.0−1.97)	1.42*(1.15−1.66)	1.9*(1.09−2.50)	1.53 (0.50−2.19)
ALC $\times 10^9$/L	1.64 (0.77−3.0)	1.58 (1.05−2.26)	1.79 (1.33−2.24)	1.57 (0.68−3.15)	1.48 (0.92−2.35)
PLT $\times 10^9$/L	198 (85−302)	196 (84−295)	206 (144−308)	212 (153−315)	176 (81−231)
CD2 %	81.0 (65.0−92.0)	82.0 (71.0−91.0)	82.0 (74.0−89.0)	80.7 (74.0−87.0)	78.1 (27.0−88.0)
CD3 %	71.0 (56.0−88.0)	66.4 (58.0−78.0)	71.2 (61.0−83.0)	71.2 (60.0−83.0)	69.8 (24.0−82.0)
CD4 %	44.8 (26.0−62.0)	46.6 (33.0−59.0)	39.5 (31.0−49.0)	45.5 (28.0−54.0)	41.5 (13.0−56.0)
CD8 %	32.6 (10.0−59.0)	28.3 (17.0−37.0)	39.3 (29.0−53.0)	35.1 (23.0−49.0)	32.3 (13.0−41.0)
TCRαβ+ %	66.4 (53.0−77.0)	63.3 (53.0−73.0)	66.3 (50.0−79.0)	63.8 (55.0−74.0)	69.3 (64.0−78.0)
TCRγδ+ %	4.6 (1.0−19.0)	2.40† (1.0−4.0)	5.7 (2.0−14.0)	4.6 (1.0−9.0)	4.7 (3.0−6.0)
CD19 %	12.3‡(5.0−25.0)	10.50 (4.0−17.0)	7.3§(2.0−13.0)	11.5 (6.0−22.0)	10.1 (6.0−18.0)
CD20 %	12.4‖(4.0−25.0)	10.50 (4.0−16.0)	7.3¶ (2.0−14.0)	10.9 (7.0−21.0)	10.2 (5.0−19.0)
HLA-DR %	30.6 (11.0−87.0)	33.2 (22.0−47.0)	31.5 (18.0−46.0)	27.7 (17.0−42.0)	26.8 (16.0−45.0)
CD38 %	57.5 (8.0−79.0)	62.5 (37.0−77.0)	54.0 (20.0−70.0)	60.2 (44.0−73.0)	57.3 (52.0−76.0)
CD8$^+$CD57$^+$%	19.2 (7.0−50.0)	17.8 (8.0−32.0)	28.2 (16.0−50.0)	19.8 (7.0−48.0)	14.0 (7.0−26.0)
CD4$^+$ $\times 10^9$/L	626 (212−1462)	725 (295−1310)	550 (499−650)	673 (299−1293)	633 (446−731)
CD8$^+$CD57$^+$ $\times 10^9$/L	278 (74−855)	272 104-572	426 (195−855)	295 (101−841)	187 (160−283)
CD8$^+$ $\times 10^9$/L	399 (83−1170)	367 (126−661)	496 (354−906)	502 (308−1099)	383 (120−937)
CD19$^+$ $\times 10^9$/L	158 (39−414)	153 (67−209)	93 (33−175)	167 (64−711)	107 (65−411)
CD20$^+$ $\times 10^9$/L	158 (39−414)	153 (84−209)	97 (33−189)	149 (75−679)	121 (65−411)
↑NK (% of patients)	15.4	50.0#	20.0	20.0	6.7

↑NK = increased percentage of NK (natural killer) cells, defined as >20% of total lymphocyte counts, ALC = absolute lymphocyte count, ANC = absolute neutrophil count, AN-SHP = antibody-negative subclinical hypothyroidism, Anti-TG = antithyroglobulin antibodies, Anti-TPO = anti-thyroid peroxidase antibodies, CD19/CD20 = subsets of B lymphocytes, CD2/CD3/CD4/CD8/TCRαβ+/TCRγδ+/CD8$^+$CD57$^+$ = subsets of T lymphocytes (CD2: also expressed on NK cells), F = female, FT3 = free triiodothyronine, FT4 = free thyroxine, GD = Graves disease, Hb = hemoglobin, HLA-DR/CD38 = markers of cell activation expressed on T and B cells, HT = Hashimoto thyroiditis, Ht = hematocrit, M = male, n = number, NTMG = nontoxic multinodular goiter, PLT = platelets, T3 = triiodothyronine (total), T4 = thyroxine (total), TSH = thyroid-stimulating hormone, TTM = total thyroidectomy, WBC = white blood cells.

* $P = 0.004$.
† $P = 0.045$.
‡ $P = 0.05$.
§ $P = 0.019$.
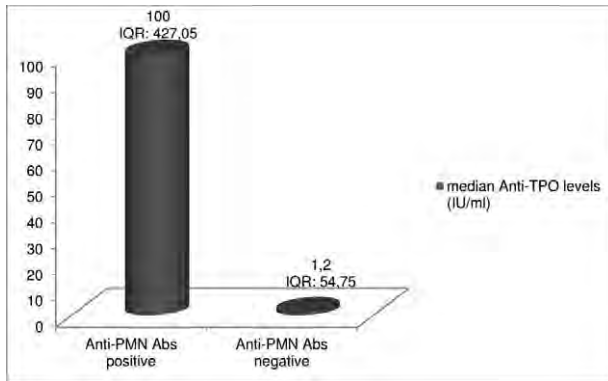‖ $P = 0.009$.
¶ $P = 0.02$.
# $P = 0.001$.

may disrupt the normal function, phagocytosis, chemotaxis, adherence, and metabolic activity of neutrophils.[19,20]

Three possible major mechanisms of thyroid-associated neutropenia should be discussed in relation to our findings: direct thyroid hormone-induced toxicity, humoral, and cellular mechanisms.

Excess of thyroid hormones could indeed have a direct toxic effect on neutrophils, since serum levels of FT3 inversely correlated to the ANC in the entire group of patients. In addition, patients of the TTM subgroup, who had iatrogenic hyperthyroidism, presented the lowest ANC, whereas patients with NTMG, who were euthyroid and had no detectable antithyroid Abs, displayed the highest ANC. In this regard, Shaw and Mehta[21] suggest that thyroid hormones may have a direct effect in early stages of hematopoiesis disturbing the maturation and differentiation of pluripotent stem cells. Furthermore, experimental data have demonstrated that exposure to both higher and lower than normal concentrations of thyroid hormone induced apoptosis in human CD34$^+$ enriched progenitor cells.[22] Pancytopenia associated with a reversible myelodysplastic syndrome has also been reported in patients with hyperthyroidism due to a direct toxic mechanism.[23−25]

ANC= Absolute Neutrophil Count

FIGURE 2. Anti-TPO Abs titers were significantly higher in patients, who had positive Anti-PMN Abs, compared with patients with negative Anti-PMN Abs tests ($P = 0.04$). Anti-PMN Abs = antineutrophil (antipolymorphonuclear) antibodies, Anti-TPO = antithyroid peroxidase antibodies, IQR = interquartile range.

We suggest that humoral mechanisms are more likely to be involved in the pathophysiology of thyroid disease-associated neutropenia at least in patients with ATD based on 2 major findings of our study: the statistically significant inverse correlation between TPO-Abs/TG-Abs titers and levels of C4 complement factor, and the finding of significantly higher anti-TPO titers in patients with anti-PMN Abs compared with the ones without.

The first finding may indirectly reflect the possible role of immune complexes,[26] leading to complement consumption. Elevated levels of circulating immune complexes have been reported in 63% of patients with HT.[27] Further data indicate that anti-TG Abs in patients with ATD, facilitate the formation of complement-activating thyroglobulin/anti-TG complexes.[28]

Our second finding, that is, the significantly higher anti-TPO titers in patients with anti-PMN Abs compared with the ones without, is another interesting finding pointing to the same direction. The association between neutropenia of autoimmune origin and autoimmune thyroidopathy has been mentioned in sparse case reports, but never confirmed in a systematic study. This association may be an indirect proof of the autoimmune nature of neutropenia at least in a proportion of patients with ATD. It has been suggested that, since these Abs behave in a manner similar to TRAbs, it is possible that these 2 types of Abs are directed against the same or similar antigens expressed on more than one tissue. The presence of anti-PMN Abs in serum of patients with GD has been recognized by Weitzman in 1985.[29] However, in this study anti-PMN Abs were found in 50 to 55% of patients with GD, while only 2 subjects were neutropenic. The investigators assumed that some of the Abs may not alter the survival of neutrophils against which they are directed, or increased bone marrow production might compensate a shortened cell lifespan. In our study, anti-PMN Abs were detected in 37.2% of patients with TD, the great majority of whom presented with ATD. It is noteworthy that the non-expression of the target antigen by the neutrophils used in the assays, the very low titer of the circulating autoantibodies, and/or a very high affinity, causing the Abs to be essentially bound to their targets may not allow the detection of anti-PMN Abs in some patients.[30] In concordance with previous observations, we also found an increased frequency of concomitant autoimmune cutaneous or systemic manifestations in patients with ATD.[31] These results are suggestive of disturbances of immune regulation, that may predispose to several immune-mediated hematologic and systemic disorders.

Regarding possible cellular mechanisms involved in thyroid disease-associated neutropenia, several investigators have reported changes in peripheral lymphocyte subsets in ATD, but results are conflicting.[32,33] Furthermore, there are no data regarding immunophenotypic findings in neutropenic patients with concomitant thyroidopathy, apart from rare case reports.[6,7] In the present analysis, a monoclonal proliferation of T-LGLs could be detected in 13 patients with thyroidopathy (13.7%), equally distributed between patients with or without ATD. T cell clonality was evidenced by molecular methods and a relative increase in T-LGLs (CD3$^+$/CD8$^+$/CD57$^+$), but the criteria for T-LGL leukemia were not fullfilled.[11] The pathogenetic role of T-LGL clones in the induction of neutropenia is well established in many disease states, such as T-LGL leukemia, aplastic anemia, and myelodysplastic syndromes, mainly through increased apoptosis. On the other hand, clonal proliferation of T-LGL has been noted in patients with altered B-cell function and autoimmunity and may be associated with autoimmune endocrine abnormalities.[34,35]

Another interesting finding from the present immunophenotypic analysis was the relative increase of CD4$^+$ T-lymphocytes in patients with thyroid disease compared to the remaining neutropenic patients. A similar finding has been reported in GD.[36]

The evaluation of lymphocyte subsets revealed different patterns in the different thyroid disease subgroups. In patients with HT an increase in B-cells was evident, compared to the other thyroidopathies. This finding, taking together the higher levels of antithyroid Abs among anti-PMN Abs-positive patients may indicate the important role of humoral mechanisms in the pathophysiology of thyroid disease-associated neutropenia. A different immunophenotypic profile was encountered in neutropenic GD patients: they displayed an increase of the proportion of NK cells, as well as a decrease in the percentage of TCRγδ+ lymphocytes compared to the remaining thyroidopathies. Unfortunately, there are no reported data regarding number or activity of NK cells in neutropenic subjects with GD, whereas numerous studies have mentioned a significant decrease of TCRγδ+ cells in patients with GD. In addition, some authors underline that this finding does not relate to thyroid hormone concentrations and does not normalize after methimazole or radical therapy of GD.[37] It is interesting that other investigators have found a higher proportion of TCRγδ+ cells among the thyroid gland-infiltrating lymphocytes in GD,[38] as well as in affected organs in other autoimmune diseases.[39] The TTM-subgroup showed decreased B-lymphocytes: of note, all TTM patients were receiving high dose-replacement therapy with levothyroxine and presented with subclinical iatrogenic hyperthyroidism. In concordance to our observation, a negative correlation between levels of thyroxine and both CD4$^+$ and CD19$^+$ lymphocytes has been reported suggesting that hyperthyroidism per se would exert a suppressor effect on both.[33] Overall, we assume, that the hematologic disturbances in the TTM subgroup may reflect the effect of thyroid hormone excess on neutrophils. On the contrary, patients with HT and GD were euthyroid subjects, with underlying autoimmune thyroidopathy (though of different pathophysiologic origin) and their immunophenotypic and immunologic characteristics point to humoral and cellular mechanisms, that are likely to be involved in the pathophysiology of thyroid disease-associated neutropenia.

Several limitations of this study should be acknowledged. Firstly, the possible referral bias has been already addressed at the beginning of the discussion. Secondly, the number of

patients with GD (n = 9) and TTM (n = 6) are small, so that firm conclusions cannot be drawn. However, this reflects the rarity of these two conditions.

In conclusion, the frequency of thyroid disease among patients with mild to moderate neutropenia is higher than previously reported. A comprehensive evaluation of thyroid function tests as well as antithyroid Abs in patients with neutropenia is mandatory, even in the absence of related symptoms. This may help to identify this indolent etiology of neutropenia in a substantial proportion of cases, avoiding patients' stress and cumbersome and expensive laboratory tests. Furthermore, our data may indirectly reflect the possible role of immune complexes in thyroid disease-associated neutropenia. The increased prevalence of anti-PMN Abs, their correlation with anti-TPO Abs, as well as the different distribution of lymphocyte subsets among patients with thyroid diseases point to humoral and cellular mechanisms in the pathophysiology of thyroid disease-associated neutropenia. Further investigation is required to elucidate the underlying pathophysiological mechanisms that associate neutropenia with TD.

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