

Anti-TPO Antibodies in Euthyroid Neck Swelling Patients with Cytologically Proven Autoimmune Thyroiditis: A Cross-sectional study for Early Identification of Thyroid Failure.

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Abstract

Background: Autoimmune thyroiditis, including Hashimoto's and lymphocytic thyroiditis, may be cytologically evident on fine-needle aspiration cytology (FNAC) before biochemical thyroid dysfunction develops. Anti-thyroid peroxidase (anti-TPO) antibodies may identify euthyroid patients with underlying thyroid autoimmunity who require follow-up.

Objectives: To determine the frequency of euthyroidism among cytologically proven autoimmune thyroiditis cases and characterize their anti-TPO antibody profile.

Methods: In this hospital-based, cross-sectional study of 100 satisfactorily aspirated thyroid FNAC cases conducted over 18 months (February 2019–August 2020), autoimmune thyroiditis (Hashimoto's and lymphocytic thyroiditis) was diagnosed in 18 cases; granulomatous thyroiditis (n=3), being of non-autoimmune (post-viral) aetiology, was analysed separately as a comparator. appropriate statistical tests were applied wherever necessary.

Results: Of the 18 autoimmune thyroiditis cases, one-third (6, 33.3%) were euthyroid at presentation, while 6 (33.3%) were hyperthyroid and 6 (33.3%) hypothyroid. A detectable anti-TPO titre (>9 IU/L) was present in 19 of 21 thyroiditis cases (90.5%); across the cohort, 22 of 41 patients with a detectable titre (53.7%) were euthyroid, indicating antibody elevation often precedes biochemical dysfunction. Mean anti-TPO titre was markedly higher in Hashimoto's (813.36 ± 487.51 IU/L) and lymphocytic thyroiditis (252.81 ± 228.30 IU/L) than granulomatous thyroiditis (31.87 ± 55.21 IU/L; one-way ANOVA, $F=7.21$, $p=0.005$). Detectable anti-TPO was significantly more frequent in thyroiditis than non-thyroiditis lesions (90.5% vs 27.8%; $\chi^2=24.37$, $p<0.001$; odds ratio 24.6).

Conclusion: A substantial proportion of patients with cytologically proven autoimmune thyroiditis remain biochemically euthyroid at diagnosis; anti-TPO positivity is more frequent among euthyroid than dysfunctional patients, identifying a group at increased future risk of hypothyroidism.

Recommendation: Anti-TPO estimation is recommended as a routine adjunct to FNAC in cytologically diagnosed autoimmune thyroiditis, irrespective of thyroid-function status, with periodic follow-up of antibody-positive euthyroid patients to enable early detection of progression to hypothyroidism.

Keywords: Autoimmune thyroiditis; anti-thyroid peroxidase antibody; euthyroid; Hashimoto's thyroiditis; lymphocytic thyroiditis; subclinical hypothyroidism

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Introduction

Autoimmune thyroiditis – encompassing Hashimoto's thyroiditis and its milder variant, lymphocytic thyroiditis – is a progressive, immune-mediated destructive process of the thyroid gland that frequently evolves over years, passing through a prolonged subclinical or euthyroid phase before culminating in overt hypothyroidism.¹ Fine needle aspiration cytology (FNAC) is capable of detecting the characteristic lymphocytic infiltration, Hurthle cell change

and lymphoid tangles of autoimmune thyroiditis at a morphological level that may considerably predate any measurable derangement of thyroid function, offering a potential window for early identification of at-risk patients.^{2,3}

Serum anti-thyroid peroxidase (anti-TPO) antibody is the serological hallmark of autoimmune thyroiditis and has long been recognized, from large community-based cohort studies, as a powerful predictor of future thyroid failure in

individuals who are biochemically euthyroid at the time of testing.¹ However, most published correlations of anti-TPO antibody with thyroid disease have been framed around biochemically overt hypothyroid or hyperthyroid populations, with comparatively less emphasis on the specific subgroup of cytologically confirmed autoimmune thyroiditis patients who remain euthyroid at the time of diagnosis – arguably the group in whom early biochemical risk-stratification would be of the greatest clinical value.

The present study was therefore undertaken to determine, within a cytology-based thyroid FNAC cohort, what proportion of cytologically proven autoimmune thyroiditis presents in a euthyroid state, and to characterize the anti-TPO antibody profile of this subgroup, with the aim of clarifying the potential role of anti-TPO estimation as an early marker for the identification of patients at risk of progressing to overt thyroid failure.

Materials and Methods

Study design and setting

This was a hospital-based, cross-sectional observational study conducted in the Department of Pathology, Index Medical College, Hospital and Research Centre, Indore, Madhya Pradesh, India. Index Medical College, Hospital and Research Centre is a multispecialty tertiary-care teaching hospital affiliated to Malwanchal University that functions as a major referral centre for central India. It offers comprehensive inpatient and outpatient clinical services across major medical and surgical specialties, including general medicine, general surgery, otorhinolaryngology (ENT), obstetrics and gynaecology, paediatrics and orthopaedics, supported by diagnostic and laboratory services in pathology, radiodiagnosis and biochemistry; the Cytology section of the Department of Pathology receives referrals for fine needle aspiration from across these clinical departments. The study period was 18 months, from February 2019 to August 2020. During this period, 108 patients were received in the Cytology department for thyroid FNAC. Blood samples for anti-TPO antibody estimation were collected on the day of FNAC. Serum TSH, T3 and T4 levels were estimated using a morning fasting sample collected before 10 am in the Biochemistry department. Of the 108 study subjects, 100 patients with satisfactory smears (Based on The Bethesda system for reporting thyroid cytopathology, 2017)² along with serum anti-TPO levels and thyroid function tests were included in the study. The study was structured and reported in accordance with relevant elements of the STROBE recommendations for observational studies.⁴

Study population

During the study period, 108 consecutive patients presenting with clinically palpable thyroid swellings and referred for

FNAC from the Medicine, Surgery and ENT outpatient and inpatient departments were enrolled using a consecutive (non-probability) sampling technique: every eligible patient presenting sequentially during the study period was invited to participate, without further selection, until the study period closed. Inclusion criteria were a clinically palpable thyroid swelling in a patient of either sex and any age willing to give written informed consent. Patients with a non-diagnostic (unsatisfactory) FNAC specimen on cytological assessment were excluded from the final analysis; 8 of 108 specimens (7.4%) were excluded on this basis, leaving 100 patients with satisfactory smears, together with paired serum anti-TPO and thyroid-function results, for final analysis.

Sample size calculation

The minimum sample size was estimated using the standard formula for estimating a population proportion: $n = Z^2 p(1-p)/d^2$, where $Z=1.96$ (for 95% confidence), $p=0.20$ (an assumed prevalence of anti-TPO antibody positivity among patients undergoing thyroid FNAC, based on prior literature), and $d=0.08$ (absolute precision). This yielded a minimum required sample size of approximately 96 evaluable patients ($n = (1.96)^2 \times 0.20 \times 0.80 / (0.08)^2 \approx 96$). Anticipating an approximately 8–10% rate of non-diagnostic aspirates based on departmental experience, 108 patients were enrolled to ensure at least 96–100 evaluable cases; 100 patients ultimately had satisfactory smears and complete biochemical data, meeting the pre-specified target.

Minimisation of Bias

Several measures were adopted to minimize potential sources of bias. Selection bias was minimized by consecutively enrolling every eligible patient presenting during the study period, without discretionary inclusion or exclusion based on demographic or clinical characteristics. Diagnostic (information) bias was minimized by having cytological smears reported by senior pathologists applying uniform, pre-specified morphological diagnostic criteria,⁵ anti-TPO and thyroid-function assays were, in turn, performed by laboratory personnel on a single standardized analyser platform, to minimize measurement bias and inter-assay variability. Observer bias in the classification of thyroid function status was minimized by applying pre-defined, standard laboratory reference ranges uniformly to all participants.

Fine-needle aspiration cytology

FNAC of thyroid swellings was performed following standard aspiration and non-aspiration (capillary/FNC) techniques. Smears were prepared and stained using routine cytological stains – Giemsa, Papanicolaou and haematoxylin and eosin stains. The cytological diagnosis

was based on the morphological features and diagnostic criteria described by Jayaram G and Orell SR.⁵ For analytical purposes, thyroid lesions were classified into three broad groups: (1) benign non-neoplastic lesions other than thyroiditis – colloid goitre, nodular goitre, follicular hyperplasia/hyperplastic nodule and cystic lesions; (2) benign non-neoplastic thyroiditis – Hashimoto's thyroiditis, lymphocytic thyroiditis and granulomatous thyroiditis; and (3) neoplastic lesions – papillary carcinoma, follicular neoplasm/carcinoma and other suspicious neoplastic lesions. Where surgical specimens were available, histopathological findings were correlated with the cytological diagnosis, particularly for neoplastic lesions; histopathological confirmation was not routinely available for all benign/inflammatory lesions, as surgery was not clinically indicated in every patient.

Thyroid-function assessment

Thyroid-function tests were performed as part of the evaluation. Serum T3 and T4 were measured by radioimmunoassay, while TSH was measured using an immunoradiometric assay. Patients were classified as euthyroid, hyperthyroid or hypothyroid according to the thyroid-function results.

Anti-TPO antibody estimation

Serum anti-TPO antibodies were estimated using a solid-phase enzyme immunoassay kit (manufactured by XEMA Co. Ltd) on an Erba ELAN 30s analyser. The assay manufacturer's reference limits were: males, 30 IU/L; females <50 years, 30 IU/L; females >50 years, 50 IU/L. For descriptive analysis, this study also examined antibody concentrations above 9 IU/L. Because this threshold differs from the assay's stated reference limits, it is reported here as an exploratory threshold for describing the distribution of low-level antibody elevation, rather than as the formal definition of clinical anti-TPO positivity.

Statistical analysis

Data were analysed using suitable statistical software. Anti-TPO titres (mean $\pm$ SD) were compared across groups using one-way ANOVA. Categorical variables, including the distribution of thyroid function status and the frequency of detectable anti-TPO antibody, were compared using the chi-

square test; Fisher's exact test was used in place of the chi-square test wherever more than 20% of expected cell counts were below 5, as is the case for the granulomatous thyroiditis subgroup (n=3). A two-tailed p-value <0.05 was considered statistically significant.

Ethical Consideration

The study was approved by the Institutional Ethics Committee of Index Medical College, Hospital and Research Centre, Indore (MP).

Results

Autoimmune thyroiditis (Hashimoto's and lymphocytic thyroiditis combined) was diagnosed cytologically in 18 of the 100 satisfactorily aspirated cases (18.0%), with a further 3 cases (3.0%) diagnosed as granulomatous thyroiditis and analysed separately as a non-autoimmune comparator.

Thyroid function status in autoimmune thyroiditis

Among the 18 autoimmune thyroiditis cases, thyroid function status was evenly distributed across the three functional categories: 6 patients (33.3%) were euthyroid, 6 (33.3%) were hyperthyroid, and 6 (33.3%) were hypothyroid at the time of diagnosis. Within the individual subtypes, hypothyroidism was more frequent in Hashimoto's thyroiditis (45.4%), whereas hyperthyroidism predominated in lymphocytic thyroiditis (57.1%); nonetheless, in both subtypes a substantial minority of patients (28.6–36.4%) remained euthyroid despite unequivocal cytological evidence of autoimmune thyroiditis. In contrast, all 3 granulomatous thyroiditis cases were hyperthyroid, consistent with the transient thyrotoxic phase characteristic of this self-limiting, non-autoimmune condition. The distribution of thyroid function status did not differ significantly between Hashimoto's, lymphocytic and granulomatous thyroiditis on chi-square testing ($\chi^2=7.73$, df=4, p=0.102); however, more than 20% of expected cell counts in this 3 $\times$ 3 comparison were below 5 owing to the small granulomatous subgroup (n=3), so this result should be interpreted with caution and is presented for descriptive completeness rather than as a definitive test of association (Table 1).

Table 1

Thyroid Function Status by Cytological Subtype of Thyroiditis

Cytological Diagnosis	Euthyroid, n (%)	Hyperthyroid, n (%)	Hypothyroid, n (%)	Total
Hashimoto's thyroiditis	4 (36.4)	2 (18.2)	5 (45.4)	11
Lymphocytic thyroiditis	2 (28.6)	4 (57.1)	1 (14.3)	7

Autoimmune thyroiditis (total)	6 (33.3)	6 (33.3)	6 (33.3)	18
Granulomatous thyroiditis	0 (0.0)	3 (100.0)	0 (0.0)	3

Page | 4 *Note.* Granulomatous thyroiditis is a self-limiting, non-autoimmune (post-viral) entity and is presented as a comparator rather than as part of the autoimmune thyroiditis total. χ^2 (Hashimoto's vs. lymphocytic vs. granulomatous) = 7.73, df = 4, p = .102; interpreted with caution as >20% of expected cell counts were below 5. A separate goodness-of-fit comparison showed that the euthyroid proportion within the autoimmune thyroiditis group (33.3%) was significantly lower than would be expected from the background thyroid-function distribution of the overall cohort (66% euthyroid, 19% hyperthyroid, 15% hypothyroid; $\chi^2=8.89$, df=2, p=0.012), confirming that a cytological diagnosis of autoimmune thyroiditis is statistically associated with a departure from the cohort's predominantly euthyroid background pattern, even though a third of such patients remain euthyroid in absolute terms.

Anti-TPO antibody titre: autoimmune versus non-autoimmune thyroiditis

The mean anti-TPO antibody titre was markedly elevated in both subtypes of autoimmune thyroiditis – 813.36 ± 487.51 IU/L in Hashimoto's thyroiditis and 252.81 ± 228.30 IU/L in lymphocytic thyroiditis (pooled mean across both subtypes, 595.37 ± 487.10 IU/L) – compared with a mean of only 31.87 ± 55.21 IU/L in granulomatous thyroiditis, an approximately 19-fold difference. This difference across the three thyroiditis subtypes was statistically significant on one-way ANOVA ($F(2,18)=7.21$, p=0.005), confirming that anti-TPO elevation is specific to autoimmune, rather than post-viral inflammatory, thyroiditis (Table 2).

Table 2

Serum Anti-TPO Antibody Titre by Cytological Subtype of Thyroiditis

Cytological Diagnosis	n	M (SD), IU/L
Hashimoto's thyroiditis	11	813.36 (487.51)
Lymphocytic thyroiditis	7	252.81 (228.30)
Autoimmune thyroiditis (pooled)	18	595.37 (487.10)
Granulomatous thyroiditis (non-autoimmune)	3	31.87 (55.21)

Note. M = mean; SD = standard deviation; IU/L = international units per litre. Pooled estimate for autoimmune thyroiditis derived statistically from the Hashimoto's and lymphocytic subgroup summary statistics. One-way ANOVA (Hashimoto's vs. lymphocytic vs. granulomatous): $F(2, 18) = 7.21$, p = .005.

Anti-TPO detectability in relation to thyroid function across the cohort

Across all thyroiditis subtypes combined, 19 of 21 cases (90.5%) had a detectable anti-TPO titre (>9 IU/L), compared with only 22 of the remaining 79 non-thyroiditis cases (27.8%). This difference was highly statistically

significant (χ^2 with Yates' correction=24.37, p<0.001; Fisher's exact p<0.001; odds ratio 24.6, 95% CI 5.3–114.6), confirming a strong and specific association between a cytological diagnosis of thyroiditis and anti-TPO detectability, independent of thyroid function status. Considering the cohort as a whole, 41 patients had a detectable anti-TPO titre, of whom the majority – 22 (53.7%) – were biochemically euthyroid, while 10 (24.4%) were hyperthyroid and 9 (22.0%) were hypothyroid. This confirms that antibody detectability was more often found in the absence of, rather than in association with, overt thyroid dysfunction (Table 3).

Table 3

Anti-TPO Antibody Detectability in Relation to Thyroid Function Status

Parameter	n/N	%
Thyroiditis cases with detectable anti-TPO (>9 IU/L)	19/21	90.5
Detectable anti-TPO patients (cohort) who were euthyroid	22/41	53.7
Detectable anti-TPO patients (cohort) who were hyperthyroid	10/41	24.4
Detectable anti-TPO patients (cohort) who were hypothyroid	9/41	22.0
Autoimmune thyroiditis cases euthyroid at diagnosis	6/18	33.3

Note. Anti-TPO = anti-thyroid peroxidase antibody. Thyroiditis vs. non-thyroiditis detectability: χ^2 (Yates' corrected) = 24.37, $p < .001$; Fisher's exact $p < .001$; odds ratio = 24.6, 95% CI [5.3, 114.6].

Discussion

This study demonstrates three principal findings, each supported by internal statistical evidence and consistent with external literature. First, a full third of patients with cytologically unequivocal autoimmune thyroiditis were biochemically euthyroid at diagnosis, and the euthyroid proportion within this group was itself significantly lower than the cohort's background euthyroid rate ($\chi^2=8.89$, $p=0.012$) – confirming that autoimmune thyroiditis measurably shifts thyroid function away from the euthyroid state even while a substantial minority remain compensated. Second, anti-TPO antibody titres were markedly and specifically elevated in autoimmune (Hashimoto's and lymphocytic) as opposed to non-autoimmune (granulomatous) thyroiditis (one-way ANOVA, $F(2,18)=7.21$, $p=0.005$), a finding that mirrors the assay-validation data of Engler et al and the population-based specificity data of Hoogendoorn et al.^{6,7} Third, detectable anti-TPO antibody was present in over 90% of thyroiditis cases regardless of thyroid function, and was dramatically more frequent in thyroiditis than in all other cytological categories combined (90.5% vs 27.8%; odds ratio 24.6, 95% CI 5.3–114.6) – a magnitude of association that provides direct internal evidence, beyond the external literature cited below, for the specificity and clinical utility of anti-TPO testing in this setting.

This temporal sequence – antibody and cytomorphological positivity preceding biochemical failure – has been most convincingly demonstrated by long-term community-based cohort data. In the Whickham Survey, a landmark twenty-year population follow-up study, Vanderpump et al showed that thyroid antibody-positive, biochemically euthyroid

individuals at baseline had a substantially and significantly higher annual incidence of progression to overt hypothyroidism than antibody-negative individuals, establishing anti-TPO positivity as one of the strongest independent predictors of future thyroid failure in an apparently well population.¹ The findings of the present study extend this principle from an asymptomatic population-screening context into a symptomatic, cytologically confirmed clinical cohort: even among patients who have already declared themselves through a palpable thyroid swelling and an unambiguous cytological diagnosis of autoimmune thyroiditis, a substantial proportion remain biochemically compensated, and anti-TPO estimation is what identifies this euthyroid-but-at-risk subgroup.

Rho et al similarly demonstrated, in a histopathologically confirmed surgical cohort, that antithyroid peroxidase antibody positivity correlates strongly and specifically with an autoimmune thyroiditis phenotype.⁸ Bromińska et al's observation that anti-TPO titres correlate with thyrotropin levels even among euthyroid individuals lends further biochemical support to the concept of a graded, antibody-driven continuum from euthyroid autoimmunity to overt hypothyroidism, rather than a binary distinction between 'disease' and 'no disease'.⁹ From a cytopathological standpoint, the ability of FNAC to detect autoimmune thyroiditis in its euthyroid, subclinical phase is itself clinically valuable and has been highlighted by several Indian cytology-serology correlation studies. Kumar et al and Singh et al both demonstrated that careful cytomorphological evaluation – attentive to lymphocytic infiltration, Hurthle cell change and lymphoid tangles – can identify autoimmune thyroiditis even in patients without overt biochemical abnormality, provided the cytopathologist remains alert to potentially confounding patterns of abundant colloid or follicular hyperplasia.^{10,11} Jayaram et al's classical correlation of the functional,

immunological and cytomorphological features of thyroiditis similarly underscored the value of combining cytology with serology, rather than relying on either modality in isolation, for a complete assessment of disease stage.³ Bhatia et al further demonstrated that cytological grading of lymphocytic thyroiditis can be meaningfully correlated with clinical, biochemical and radionuclide parameters, supporting the broader principle that cytological and serological data together provide a more complete picture of disease activity and stage than either alone.¹²

Generalizability

These findings derive from a single-centre, hospital-based cohort of symptomatic patients referred for FNAC at a tertiary care teaching hospital in central India, and several factors should be weighed before extrapolating them more broadly. First, the study population was, by definition, a clinically selected group with a palpable thyroid swelling severe enough to prompt referral; the findings may not directly generalize to asymptomatic, community-dwelling individuals of the kind studied in population-based surveys such as Wickham, where antibody prevalence and its predictive value may differ.¹ Second, the region's dietary iodine status, and the specific antibody assay platform, kit and threshold used in this study (including the exploratory 9 IU/L threshold, which falls below the assay's formal reference limit), may not be directly comparable to those of other centres, laboratories or countries, and multicentre replication using harmonized assay platforms would strengthen confidence in the observed thresholds. Third, the sample size, while adequate to characterize the overall pattern in this cohort, is modest for individual thyroiditis subtypes – particularly granulomatous thyroiditis (n=3) – limiting the precision and generalizability of subgroup-level estimates. With these caveats, the core, internally well-supported finding that a substantial proportion of cytologically proven autoimmune thyroiditis is biochemically silent at diagnosis, and that anti-TPO antibody helps identify this subgroup, is biologically plausible and consistent with international literature, and would be expected to generalize qualitatively, if not necessarily in its precise numerical estimates, to comparable tertiary-care thyroid FNAC populations elsewhere.

Recommendations

On the basis of these findings, the authors recommend that: (1) serum anti-TPO antibody estimation be performed routinely alongside FNAC in all patients with a cytological diagnosis of thyroiditis, irrespective of their thyroid-function status at presentation, given that a third of autoimmune thyroiditis cases in this cohort were biochemically euthyroid; (2) patients found to be anti-TPO antibody-positive but currently euthyroid be enrolled in a

programme of periodic thyroid-function follow-up (for example, annually), consistent with the risk of progression to hypothyroidism documented in long-term community cohorts; (3) cytopathologists remain alert to subtle features of autoimmune thyroiditis even when colloid or follicular hyperplasia predominates in the smear, given the recognized risk of cytological under-recognition in this setting; and (4) future research take the form of prospective, multicentric studies with longitudinal biochemical follow-up of antibody-positive euthyroid patients, to directly quantify the rate and time course of progression to hypothyroidism in a cytologically defined population, and to validate the exploratory 9 IU/L antibody threshold used descriptively in this study against hard clinical endpoints.

Limitations

This being a cross-sectional study, longitudinal follow-up data to directly confirm progression from the euthyroid to the hypothyroid state in antibody-positive patients were not available within the study period; this represents the most important direction for future prospective research arising from these findings. The pooled anti-TPO estimate for combined Hashimoto's and lymphocytic thyroiditis was derived statistically from the two subgroup means and standard deviations rather than from raw patient-level data, and should be interpreted as an approximation. The sample size, while adequate to characterize the overall pattern, remains modest for fine-grained subgroup analysis, particularly for granulomatous thyroiditis (n=3). The 9 IU/L threshold used for descriptive antibody detectability is exploratory and falls below the assay's formal reference limit; findings framed around this threshold should be interpreted as hypothesis-generating rather than as a formal clinical positivity criterion.

Conclusion

A third of patients with cytologically proven autoimmune thyroiditis in this cohort were biochemically euthyroid at diagnosis, and a detectable anti-TPO titre was, overall, more frequently observed among euthyroid than among dysfunctional patients. Anti-TPO titres were markedly and specifically elevated in autoimmune (Hashimoto's and lymphocytic) as opposed to non-autoimmune (granulomatous) thyroiditis. These findings support the use of anti-TPO antibody estimation, alongside FNAC, as a valuable early marker for identifying euthyroid patients with autoimmune thyroiditis who are at increased future risk of thyroid failure, and argue for a policy of periodic biochemical follow-up in this specific, cytologically defined but currently compensated patient group.

List of Abbreviations

ANOVA, analysis of variance; anti-TPO, anti-thyroid peroxidase (antibody); CI, confidence interval; df, degrees

of freedom; ENT, ear, nose and throat (otorhinolaryngology); FNAC, fine needle aspiration cytology; FNC, fine needle capillary (non-aspiration) sampling technique; IU/L, international units per litre; M, mean; OR, odds ratio; SD, standard deviation; STROBE, Strengthening the Reporting of Observational studies in Epidemiology; T3, tri-iodothyronine; T4, thyroxine; TSH, thyroid-stimulating hormone.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

The study was approved by the Institutional Ethics Committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Data Availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

MC: conceptualization, methodology, patient recruitment and data collection, cytological evaluation, formal analysis, writing – original draft. SN: conceptualization, methodology, supervision, writing – review and editing. SKN: methodology, supervision, writing – review and editing. NG: formal analysis (statistical), methodology review, writing – review and editing.

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