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Assessment of Oxidative Stress in Hashimoto's Thyroiditis Patients: Effects of Levothyroxine Sodium Treatment

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Abstract

Background: Hashimoto's thyroiditis (HT) is a prevalent autoimmune disease characterized by chronic inflammation of the thyroid gland, leading to hypothyroidism. Oxidative stress has been implicated in the pathogenesis of HT, influencing disease progression and therapeutic outcomes. Understanding the dynamics of thiol/disulfide balance, a marker of oxidative stress, in HT patients receiving levothyroxine treatment is crucial for elucidating its role in disease management. Our study has the largest cohort on this topic.

Methods: This study enrolled 357 euthyroid HT patients, divided into groups based on levothyroxine treatment status. Thiol/disulfide homeostasis was assessed using the Erel method. Clinical parameters, including thyroid function tests and antibody levels, were measured. Statistical analyses were performed to compare oxidative stress markers between groups.

Results: Patients on levothyroxine therapy showed lower native and total thiol levels than untreated patients, indicating potential antioxidant depletion. Higher disulfide/native thiol and disulfide/total thiol ratios in the treated group suggest elevated oxidative stress. There were no correlations between thyroid antibodies (TPO-Ab, TG-Ab) and thiol/disulfide levels.

Conclusion: This study highlights alterations in thiol/disulfide balance among euthyroid HT patients, with implications for oxidative stress management in clinical practice. Levothyroxine treatment appears to be associated with oxidative stress markers, suggesting associations that warrant further investigation.

Keywords: Autoimmunity, Oxidatvie Stress, Hashimoto Disease

INTRODUCTION

Hashimoto's thyroiditis (HT), the most prevalent organ-specific autoimmune disease, is characterized by chronic thyroid gland inflammation driven by autoimmune mechanisms. It stands as the leading cause of hypothyroidism in iodine-sufficient areas, impacting approximately 10% of the population, predominantly women, with prevalence rising with age (1). The etiology of HT is believed to stem from a blend of genetic predisposition and environmental influences. Clinically, HT manifests as gradual thyroid failure. This is due to the infiltration of lymphocytes and the autoimmune-induced degradation of thyroid gland tissue, which involves programmed cell death of thyroid epithelial cells (2,3). In the United States National Health and Nutrition Examination Survey (NHANES III), hypothyroidism was found in 4.6% and hyperthyroidism in 1.3% (1). In another study, more than 90% of HT patients have high serum concentrations of autoantibodies to thyroglobulin (TG-Ab) and thyroid peroxidase (TPO-Ab) (4,5). The

failure of immunological self-tolerance initiates a series of cascading events. This includes the dysfunction of T regulatory cells, the activation and proliferation of T-helper cells, and the differentiation of auto-reactive B cells, leading to anti-thyroid autoantibodies. These processes culminate in tissue inflammation and subsequent damage (6). The release of pro-inflammatory cytokines by infiltrating T and B cells exacerbates tissue damage and inflammation, fostering a cycle of worsening pathology (7). In this context, an imbalance arises between the endogenous production of reactive oxygen species (ROS) and the antioxidant defenses, emerging as a significant factor in the onset and advancement of the autoimmune process and associated glandular dysfunction. When thyroid dysfunction occurs, it exacerbates oxidative stress, as changes in thyroid hormone levels can impact the production of ROS and the synthesis of antioxidants, potentially affecting both processes (8). Several studies have investigated the correlation between oxidative stress and HT to gauge the oxidant-antioxidant balance in the body. However, findings have occasionally been conflicting (9-12).

The oxidant radicals cause oxidation of the thiol groups of sulfur-containing amino acids (cysteine) of proteins and form disulfide (-S-S-) bonds (13). These disulfide bonds are the earliest sign of protein oxidation and are reversible (14). Reducing reversible disulfide bonds to thiol groups maintains dynamic thiol/disulfide homeostasis (15). Unlike previous methods, the method developed by Erel et al. allows individual measurement of both thiol and disulfide levels (16). This method is also superior to former methods in terms of fast and accurate results, the possibility of remeasurement, and being both a manual and colorimetric method (17). Abnormal thiol/disulfide homeostasis is implicated in several diseases characterized by chronic inflammation (18-20). In this study, we aimed to investigate dynamic thiol/disulfide homeostasis in euthyroid patients with Hashimoto's thyroiditis, both with and without levothyroxine medication, using the Erel method.

To our knowledge, this represents the largest cohort specifically examining thiol/disulfide homeostasis in euthyroid HT patients using the Erel method.

METHODS

Study participants

A total of 357 subjects aged over 18 years, consecutively referred to our outpatient clinics over six months, were enrolled in the study. HT was diagnosed using the currently accepted laboratory and ultrasonographic criteria (serum anti-thyroid antibodies positivity and/or heterogeneous echo-structure with diffuse or patchy hypoechogenicity at ultrasonography). All patients were euthyroid. 176 (49.3%) of the patients were euthyroid on levothyroxine treatment. We assessed dynamic thiol/disulfide homeostasis in euthyroid patients with Hashimoto's thyroiditis, both with and without levothyroxine medication, using the Erel method.

Exclusion criteria;

1. Unwilling to participate
2. Active malignancy
3. Pregnancy
4. Inflammatory disease
5. Infection disease
6. Trauma or acute injury
7. Smoking, alcohol consumption
8. Taking any form of antioxidant agents or vitamin supplements

Patients who met the study criteria and agreed to participate were given details about the study and signed a free informed consent form.

Study Design

The study is a single-centered, cross-sectional clinical trial conducted at the Internal Medicine Department outpatient office of Ataturk Research and Training Hospital in Turkey. The study protocol received approval from the local ethical committee (17 May 2017, Yildirim Beyazit University Faculty of Medicine Ethical Committee, number 26379996/110, decision no 109) following the principles of the Helsinki Declaration.

Demographic data and patients' clinical history were reviewed. We examined thyroid stimulating hormone (TSH), free thyroxine (fT4), free triiodothyronine (fT3), TPO-Ab, and TG-Ab. Serum TSH, fT3, and fT4 levels were measured using chemiluminescence methods (Immulite 2000, Diagnostic Products Corp., Los Angeles, California and UniCel DXI 800, Beckman Coulter, Brea, California). Normal ranges for TSH, fT3, and fT4 were 0.27–4.2 uIU/mL, 1.57–4.71 pg/mL, and 0.61–1.2 ng/dL, respectively. Venous blood samples from the patients and healthy controls were collected after 12 hours of fasting and put in EDTA tubes.

Thiol/disulfide Homeostasis

We conducted thiol/disulfide homeostasis tests following the method described by Erel and Neselioglu (16). First, disulfide bonds were reduced to form free functional thiol groups. Any unused sodium borohydride reducer was neutralized and removed using formaldehyde. Next, we determined native and total thiol levels by reacting with 5,5'-dithiobis-2-nitrobenzoic acid. The dynamic disulfide amount was calculated by determining half of the difference between the total and native thiol. After the native thiol, total thiol, and disulfide amounts were determined, the ratios of disulfide/total thiol, native thiol/total thiol, and disulfide/native thiol were calculated. Plasma disulfide levels were 17.29 ± 5.32 μmol/L, native thiol levels were 397 ± 62 μmol/L, and disulfide/native thiol percent ratios were 4.32 ± 1.49 in healthy subjects (16).

STATISTICAL ANALYSIS

The data were entered into an Excel spreadsheet (Microsoft, Redmond, Washington) for analysis. Statistical analyses were performed using the SPSS. To assess the normal distribution of the variables, we used both analytic (Shapiro-Wilk test) and visual methods, utilizing histograms and probability plots. Independent t-tests were used to compare continuous variables between groups. Chi-square tests were used for categorical variables. A significance level of 5% (type-I error) was employed to determine statistical significance. Descriptive analyses were presented using means and standard deviations for normally distributed variables and medians with minimum-maximum values for non-normally distributed variables. Statistical significance was established when $p<0.05$. Pearson correlation tests were used to explore the relationships among the variables and determine their significance. For this study, we conducted a power analysis to determine the required sample size for detecting significant differences between group means. We set the alpha at 0.05 and aimed for a power of 95%, which led us to calculate a necessary sample size of 342 participants to detect actual differences between the groups effectively.

RESULTS

The study included 370 patients. Thirteen patients were excluded from the study due to non-acceptance of informed consent. A total of 357 patients, 313 women and 44 men, were included in the study (**Figure 1**). The study was terminated because a sufficient number was reached.

Diabetes Mellitus (DM) was present in 26 (7.3%) patients and hypertension in 39 (10.9%). The patients had no additional diseases other than DM and hypertension. All patients were euthyroid. One hundred seventy-six (49.3%) patients were using levothyroxine sodium therapy. The average dose of levothyroxine sodium was 76.46±13.97 mcg/d. Regarding thyroid function tests, the mean TSH level was 2.25±1.11 mIU/mL, ranging from 0.50 to 4.20 mIU/mL. The mean fT4 level was 1.26±0,21 ng/dL. The mean fT3 level was 2.95±0,43 ng/dL. The mean TPO-Ab titer was 96±148 IU/mL. The mean TG-Ab titer was 148±351 IU/mL (**Table 1**). When all patients were evaluated, the mean disulfide level was 18.08±10.20 µmol/L, ranging from 0.80 to 45.45 µmol/L. The mean native thiol level was 445.41±46.06 µmol/L, ranging from 256.60 to 594.40 µmol/L. The mean total thiol level was 481.61±50.52 µmol/L, ranging from 331.60 to 623.40 µmol/L. The mean disulfide/native thiol (%) was 4.07±2.33. The mean disulfide/total thiol (%) was 3.84±2.34. The mean native/total thiol (%) was 92.57±3.96 (**Table 1**). The mean disulfide and native thiol levels were higher than healthy subjects (16).

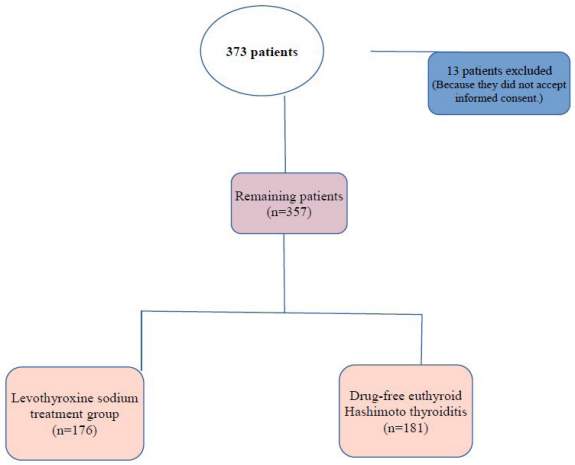

Figure 1. Study design, basic flowchart

Then, the patients were divided into two groups: those who received levothyroxine sodium treatment and those who did not. The mean age of the group that received treatment and did not was 44.85±14.03 and 31.17±12.86, respectively ($p<0.001$). While 157 (89.2%) of the patients in the group that received treatment were women, 156 (86.2%) were women in the group that did not ($p=0.38$). The number of patients diagnosed with DM was 15 (8.5%) in the group that received treatment and 11 (6.1%) in the group that did not ($p=0.37$). The number of patients diagnosed with hypertension was 29 (16.5%) in the group that received treatment and 10 (5.5%) in the group that did not ($p<0.001$). The mean

Table 1. Characteristics of the study participants

Patient number, n	357
Female gender, n (%)	313 (87.7)
Age, years	40.96 ± 13.95 (18-79)
Diabetes Mellitus present, n (%)	26 (7.3)
Hypertension present, n (%)	39 (10.9)
Usage of levothyroxine sodium yes, n (%)	176 (49.3)
The average dose of levothyroxine sodium	76.46 ± 13.97
TSH mIU/mL	2.25 ± 1.11 (0.5-4.2)
fT3 ng/dL	2.95 ± 0.43 (2.0-5.0)
fT4 ng/dL	1.26 ± 0.21 (0.8-2.0)
TPO-Ab IU/mL	96 ± 148 (5-600)
TG-Ab IU/mL	148 ± 351 (9-4000)
Mean disulfide level µmol/L	18.08 ± 10.20
Mean native thiol level µmol/L	445.41 ± 46.06
Mean total thiol level µmol/L	481.61 ± 50.52
Mean disulfide/native thiol (%)	4.07 ± 2.33
Mean disulfide/total thiol (%)	3.84 ± 2.34
Mean native/total thiol (%)	92.57 ± 3.96

TSH, thyroid-stimulating hormone; **fT3**, free triiodothyronine; **fT4**, free thyroxine; **TPO-Ab**, thyroid peroxidase antibody; **TG-Ab**, thyroglobulin antibody; **µmol/L**, micromoles per liter; **ng/dL**, nanograms per deciliter; **mIU/mL**, milli-international units per milliliter; **IU/mL**, international units per milliliter; %, percentage.
Statistical significance was established when $p<0.05$.

Table 2. Demographic characteristics and laboratory findings among groups

	Levothyroxine sodium treatment group	Drug-free euthyroid Hashimoto thyroiditis	p value*
Patient number, n (%)	176 (49.3)	181 (50.7)	
Female gender, n (%)	157 (89.2%)	156 (86.2%)	0.38
Age, years	44.85 ± 14.03	31.17 ± 12.86	<0.001
Diabetes Mellitus present, n (%)	15 (8.5%)	11 (6.1%)	0.37
Hypertension present, n (%)	29 (16.5%)	10 (5.5%)	0.00
TSH mIU/mL	2.28 ± 1.17	2.16 ± 1.06	0.29
TPO-Ab IU/mL	125 ± 170	67 ± 116	<0.001
TG-Ab IU/mL	180 ± 374	117 ± 324	0.09
Mean disulfide level µmol/L	17.88 ± 9.73	18.27 ± 10.68	0.72
Mean native thiol level µmol/L	437.07 ± 48.82	453.52 ± 41.77	<0.001
Mean total thiol level µmol/L	472.63 ± 52.03	490.36 ± 47.5	<0.001
Mean disulfide/native thiol (%)	4.10 ± 2.26	4.05 ± 2.41	0.83
Mean disulfide/total thiol (%)	3.97 ± 2.60	3.72 ± 2.06	0.31
Mean native/total thiol (%)	92.48 ± 3.79	92.66 ± 4.13	0.67

TSH level of the group that received treatment and did not was 2.28±1.17 [SD] and 2.16±1.06 mIU/mL, respectively (p=0.29). The mean TPO-Ab titer of the group that received treatment and did not was 125±170 and 67±116 IU/mL, respectively (p<0.001). The mean TG-Ab titer of the group that received treatment and did not was 180±374 and 117±324 IU/mL, respectively (p=0.09). The native and total thiol levels were lower in those who received the levothyroxine sodium treatment group (p<0.001, p<0.001). Although not statistically significant, the disülfide levels and native/total thiol ratio were also lower in the treatment group (p=0.72, p=0.67). The disulfide/native thiol and disulfide/total thiol ratios were higher in the treatment group (p=0.83, p=0.31) (Table 2).

When patients with diabetes were excluded, as this might affect the results, 331 patients were identified. While 161 (48.6%) of these patients received levothyroxine treatment, 170 (51.4%) were not. The mean age of those who received treatment was 43.73±13.95, while the mean age of those who did not receive treatment was 36.44±12.67. The native and total thiol levels were lower in those who received the levothyroxine sodium treatment group (p<0.001, p<0.001). Although not statistically significant, the disülfide levels, disulfide/total thiol, and native/total thiol ratios were also lower in the treatment group (p=0.68, p=0.27, p=0.62). The disulfide/native thiol ratio was higher in the treatment group (p=0.78) (Table 3).

No correlation was detected between TSH levels and native thiol, total thiol, and disulfide levels (p=0.06, p=0.051, p=0.54). No correlation was detected between TPO-Ab titer and native thiol, total thiol, and disulfide levels (p=0.29, p=0.37, p=0.56). No correlation was detected between TG-Ab titer and native thiol, total thiol, and disulfide levels (p=0.65, p=0.71, p=0.41).

DISCUSSION

HT represents a multifaceted autoimmune condition characterized by persistent inflammation of the thyroid gland, ultimately culminating in hypothyroidism. Our investigation sought to elucidate the dynamic thiol/

Table 3. Demographic characteristics and laboratory findings among groups without diabetes

	Levothyroxine sodium treatment group	Drug-free euthyroid Hashimoto thyroiditis	p value*
Patient number, n (%)	161 (48.6)	170 (51.4)	
Female gender, n (%)	144 (89.4%)	149 (87.6%)	0.61
Age, years	43.73 ± 13.95	36.44 ± 12.67	<0.001
TSH mIU/mL	2.30 ± 1.17	2.17 ± 1.06	0.29
TPO-Ab IU/mL	129 ± 172	65 ± 110	<0.001
TG-Ab IU/mL	184 ± 386	123 ± 334	0.12
Mean disulfide level µmol/L	17.66 ± 9.47	18.11 ± 10.65	0.68
Mean native thiol level µmol/L	435.76 ± 48.63	454.52 ± 42.11	<0.001
Mean total thiol level µmol/L	470.83 ± 50.54	491.06±47.81	<0.001
Mean disulfide/native thiol (%)	4.07 ± 2.25	4.00 ± 2.39	0.78
Mean disulfide/total thiol (%)	3.96 ± 2.66	3.68 ± 2.05	0.27
Mean native/total thiol (%)	92.52 ± 3.77	92.74 ± 4.12	0.62

disulfide homeostasis among euthyroid HT patients, with and without medication, utilizing the Erel method. Our findings offer valuable insights into the underlying mechanisms of oxidative stress in HT pathogenesis and management. Our study uncovered significant alterations in thiol/disulfide homeostasis parameters among euthyroid HT patients compared to healthy controls, as defined by established cut-off values (16). Recent studies have elucidated the intricate relationship between thyroid dysfunction and oxidative stress. Studies have highlighted the augmentation of free radical generation and oxidant production in hyperthyroidism (21-24), juxtaposed with the attenuation of antioxidant defense mechanisms in hypothyroidism (23-25), thus exacerbating oxidative stress. Ruggeri RM's study, encompassing a sizable cohort (n=134) of euthyroid HT patients without thyroxine therapy, corroborated these findings by revealing a pro-oxidative shift in the oxidative/antioxidative balance (12). They later published another study examining advanced glycation end products (AGEs) and their receptors(sRAGEs), demonstrating oxidative stress in patients with HT. They showed that sRAGEs were decreased and AGEs increased, suggesting a dysregulation of AGE/sRAGEs-related oxidative homeostasis in euthyroid HT patients (26). Thyroid hormones can target, influence, or alter the metabolism of numerous cells in the body by accelerating cellular reactions and enhancing oxidative metabolism. Excessive generation of free radicals and inadequate antioxidant defense systems lead to oxidative stress. Unchecked, these free radicals eventually harm cell membranes' essential cellular components such as DNA, proteins, and lipids. Each cell possesses mechanisms to mitigate the effects of free radicals through DNA repair enzymes and antioxidants. Poor control of pro-oxidants and oxidative stress can contribute to various chronic and degenerative diseases, aging, and pathological conditions. Specifically, heightened levels of disulfide and native thiol, indicative of oxidative stress imbalance, were observed in our study cohort. Consistent with prior research implicating oxidative stress in autoimmune thyroid disorders, our findings underscore the pertinence of oxidative stress markers in elucidating HT pathophysiology. Notably, the methodology employed in our study, involving the meticulous removal of sodium borohydride using formaldehyde, offers a nuanced approach to delineating thiol/disulfide homeostasis (16). This methodological refinement allows for the distinct measurement of these elemental constituents, potentially enhancing our understanding of the intricate interplay between oxidative stress and HT progression.

Besides inflammatory processes, hormonal imbalances can also contribute negatively to oxidative stress. Therefore, we selected euthyroid patients for our study. It represents the largest cohort in the literature that evaluates a similar patient group. We divided the patients into two groups based on levothyroxine usage (n=176, n=181)and assessed their oxidative stress status (**Table 2**). There was no statistically significant difference between the two groups in terms of age and prevalence of hypertension. While 89.2% of the group using levothyroxine were female, compared to 86.2% in the non-levothyroxine group. TPO-Ab levels did not show a statistically significant difference between the groups, whereas TG-Ab levels were higher in the levothyroxine group. This suggests that patients with higher antibody levels may require more levothyroxine. Native and total thiol levels were statistically significantly higher in the group not using medication, indicating potentially lower antioxidant levels in the medication-requiring group. Although not statistically significant, the higher mean disulfide/native thiol and mean disulfide/total thiol ratios in the levothyroxine group suggest higher oxidative stress in this group. Since a statistically significant difference in the prevalence of diabetes between the groups was observed, patients with DM were excluded, and the two groups (n=161, n=170) were re-evaluated (**Table 3**). Similarly, there was no statistically significant difference between the two groups in terms of age and prevalence of hypertension. TPO-Ab levels did not show a statistically significant difference between the groups, whereas TG-Ab levels were higher in the levothyroxine group. The oxidative stress markers showed similar results in both groups, even after excluding patients with diabetes. DM did not significantly alter the oxidative stress outcomes. Csiha et al. investigated the association between serum AGE, sRAGE, and thyroid function in HT patients receiving levothyroxine substitution and healthy controls. In their study, AGE levels were lower in the patient group than controls, while sRAGE levels were higher. However, not all patients in their study were euthyroid; some had overt or subclinical hypo/hyperthyroidism (27). Another study from Ates I. Et al. investigated the effects of levothyroxine replacement on oxidative stress in HT. Thirty-six patients recently diagnosed with HT-related hypothyroidism and 36 healthy controls were included in the study. Levothyroxine replacement was started for patients with hypothyroidism and had been followed up for 6 months. The study showed that levothyroxine replacement decreased oxidant status and increased antioxidant status following six 6 months of levothyroxine replacement in hypothyroidism developed by the HT (28). The outcomes may differ because the study designs, patient numbers, and methodologies varied among studies evaluating the effects of levothyroxine treatment on oxidative stress. Further studies would be beneficial in evaluating the effect of levothyroxine supplementation on subclinical inflammation in euthyroid HT patients.

No correlation was found between TSH, TPO-Ab, and TG-Ab levels and native thiol, total thiol, and disulfide levels in our study. Ates I. et al. investigated the effects

of oxidative stress on the pathogenesis and progression of HT, and their study revealed significant positive correlations between both TPO-Ab and TG-Ab levels with total oxidant status and negative correlations with total antioxidant status (29). Nanda et al. found a positive correlation of the oxidant molecules malondialdehyde and protein carbonyl with TPO-Ab (30). Highlighting discrepancies in existing literature, our study advocates for further research to clarify the inflammation in euthyroid HT patients.

Strengths of our study: (I). Our study includes a substantial number of participants, enhancing the statistical power and generalizability of findings within euthyroid HT patients. (II). The use of the Erel method for assessing thiol/disulfide homeostasis is detailed and scientifically sound, offering precise measurements and allowing for reproducibility. (III). By focusing on euthyroid HT patients both with and without levothyroxine medication, the study addresses a clinically relevant question about the impact of treatment on oxidative stress markers.

Limitations of the Study

This study has several limitations that should be acknowledged. The cross-sectional design precludes establishment of causal relationships between levothyroxine treatment and oxidative stress markers. The significant age difference between treatment groups represents a potential confounding factor that may influence our findings. Additionally, the clinical significance of the observed laboratory differences in thiol/disulfide parameters requires validation through longitudinal studies with clinical endpoints. Despite these limitations, our findings provide valuable insights into oxidative stress patterns in euthyroid HT patients.

CONCLUSION

Our study delves into the intricate dynamics of thiol/disulfide balance in euthyroid HT patients, offering novel insights into the role of oxidative stress in autoimmune thyroid disorders. By leveraging the robustness of the Erel method, we meticulously evaluated thiol/disulfide homeostasis, uncovering significant alterations influenced by levothyroxine therapy. These findings underscore the relevance of oxidative stress as a pivotal factor in HT pathogenesis and treatment response. Moving forward, further research exploring the interplay between thyroid function, oxidative stress markers, and clinical outcomes will be indispensable in refining therapeutic strategies and advancing personalized medicine for HT patients.

DECLARATIONS

Authors' contributions: All authors contributed to the study conception and design. Material preparation, and analysis were performed by B.C.H. Data collection was performed by B.C.H, and GD. The first draft of the manuscript was written by B.C.H. and all authors commented on previous versions of the manuscript. The study was supervised by O.H, S.N, and O.E. All authors read and approved the final manuscript.

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Conflict of interest: The authors declare that they have no conflict of interest. In addition, the authors have no financial relationship with the companies that manufactured the materials used in this study.

Ethics approval: All procedures performed in studies involving human participants were by the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol received approval from the local ethical committee.

Consent to participate and written consent for publication Informed consent was obtained from all participants.

Availability of data and material Data is available upon reasonable request from the corresponding author.

AI: Artificial intelligence tools were used to assist in language editing and improving the clarity of the manuscript. However, all scientific content, data analysis, and interpretations were carried out by the author, who bear full responsibility for the content and conclusions of this work.

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