Original Article

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Effect and mechanism of Hashimoto thyroiditis on female infertility: A clinical trial, bioinformatics analysis, and experiments-based study

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SUMMARY Diagnosing Hashimoto thyroiditis (HT) relies on thyroglobulin antibody (TgAb) and thyroid peroxidase antibody (TPOAb) titers. The influence of these antibodies on female infertility remains a subject of debate. This study aims to explore the effect and mechanism of HT on female infertility. First, a single-center cross-sectional study was conducted to investigate whether TgAb and TPOAb are the key factors leading to female infertility. Second, bioinformatic analysis was performed to investigate the potential target molecules and pathways. Third, *in vivo* experiments were performed to explore the effects of elevated TgAb levels on embryo implantation in a mouse model of autoimmune thyroiditis (AIT). Four hundred and five infertile women and 155 healthy controls were enrolled in the cross-sectional study. Results indicated that the TPOAb titer was associated with female infertility, while the TgAb titer showed no significant association. The increased levels of TgAb and TPOAb are not significantly correlated with anti-Mullerian hormone. Bioinformatic analysis indicated that the common target molecules for HT and female infertility include interleukin (IL)-6, IL-10, matrix metalloproteinase 9, and tumor necrosis factor, suggesting potential regulation through multiple signaling pathways such as HIF-1, VEGF, MAPK, and Th17 cell differentiation. A certain dose of porcine thyroglobulin can successfully establish a mouse model of AIT. In this mouse model, embryo implantation and ovarian reserve remain unaffected by elevated TgAb levels. In conclusion, the serum TPOAb titer was associated with infertility due to female factors but the TgAb titer showed no significant association. A simple increase in serum TgAb titer does not affect embryo implantation and ovarian reserve in the AIT model.

Keywords female infertility, Hashimoto thyroiditis, thyroglobulin antibody, thyroid peroxidase antibody, embryo implantation

1. Introduction

Infertility is defined as the inability of a couple to achieve a clinical pregnancy after 12 months of regular, unprotected sexual contact (*1*). Over 186 million people are suffering from infertility (*2*), impacting 8 to 12% of couples of reproductive age globally (*3*). About 85% of infertile couples have a clear etiology. The most common reason is ovulation dysfunction, male infertility factors, and fallopian tubes, and the remaining 15% of infertile couples have unexplained infertility (*4*). Immune factors associated with infertility primarily encompass non-organ-specific antibodies, organ-specific antibodies, and natural killer cells. Although studies have shown that immune factors are involved in various links of reproduction, the mechanism has not been clarified (*5-11*). Insufficient evidence exists to identify the causal relationship between immune factors and infertility.

Hashimoto thyroiditis (HT), a chronic inflammation of the thyroid gland, is currently recognized as the most prevalent autoimmune disease, with a population prevalence of approximately 4.6% (*12,13*). The prevalence of HT in women is at least 8 times higher

than in men, and it is more prevalent among Whites and Asians compared to African-Americans. The diagnosis of HT relies on identification of thyroglobulin antibody (TgAb) and thyroid peroxidase antibody (TPOAb) (*12*). TgAb and TPOAb are collectively referred to as antithyroid antibodies (ATAb). Several clinical studies have suggested a relationship between TgAb, TPOAb, and fertility decline (*5-9*). However, the specific causal relationship between TgAb and TPOAb and fertility decline has not been clarified, and its mechanism still needs to be studied (*11*).

The prevalence of thyroid autoimmunity features is more frequent in infertile women than in healthy fertile women (*14,15*). However, asserting causation in the relationship between fertility and thyroid autoimmunity is a challenge, even when adequately controlling for other variables (*11*). Some researchers have pointed out that numerous studies have mainly explored the association between TPOAb and infertility. They advocate that research should also explore the effect of TgAb in fertility assessment (*10*). Most recently, some researches have simultaneously or independently studied the effects of TPOAb and TgAb on infertility, the pregnancy rate, outcomes of reproductive-assisted technology, and ovarian function, but the results have been inconsistent. A meta-analysis revealed an association between the presence of thyroid antibodies in euthyroid patients and unexplained subfertility (*16*). Research has also demonstrated that TPO-Ab is associated with reduced ovarian reserve and decreased embryo quality. High titers of TPO-Ab can reduce live birth rates. However, a recent prospective study has shown that while higher TPOAb titers should be considered one of the risk factors for miscarriage in infertile women, fertility is not affected by any thyroid-related factors (*17*). A population-based study and a case-control study have found no significant correlation between thyroid autoimmune antibodies and female infertility or cumulative pregnancy rate (*17,18*). TPOAb and/or TgAb's association with and causation of infertility are related to intervention with levothyroxine (L-T4) and other treatment regimens (*19,20*). This relationship also affects the rationality of screening infertile women for thyroid antibodies. At present, the causal relationship is still a subject of debate, and further research is of great value.

The current study sought to explore whether there is a correlation between infertility and HT and to elucidate the mechanisms underlying HT's impact on female infertility. This study consists of the following three parts. The first part explored whether TgAb and TPOAb are associated with female infertility based on clinical data. In the second part, the common target molecules for HT and female infertility were analyzed bioinformatically. The third part explored whether TgAb affects embryo implantation and ovarian reserve through animal experiments. This study combined clinical data, bioinformatics, and animal experiments to provide evidence for the treatment of HT and the improvement of female fertility.

2. Materials and Methods

2.1. A single-center cross-sectional study

2.1.1. Trial oversight

This study adopted a single-center cross-sectional design and was divided into two parts. The first part investigated the association between female infertility and peripheral blood TgAb and TPOAb positivity. The second part explored the correlation between peripheral blood TgAb or TPOAb titers and AMH levels.

The study included a group of infertile females and a control group. A total of 927 participants were screened. In the group of infertile females, 729 individuals were screened and 324 individuals were excluded, resulting in a remaining sample size of 405 individuals. Based on etiological factors, the group of infertile females was further divided into a group with ovulation disorders (47 individuals) and a group with pelvic factors (358 individuals, including 78 patients with endometriosis). Age-matched healthy adults made up the control group. In the control group, 198 people were screened, and 155 people were finally enrolled. The 405 individuals in the group of infertile females and the 155 individuals in the control group were used to study thyroid function and the association of peripheral blood TgAb and TPOAb with the disease (Figure 1).

Further screening of these patients was conducted to exclude confounding factors affecting the odds ratio (*21,22*). Of the 405 individuals in the group with infertility due to female factors, 300 were excluded, leaving 105 individuals. In the control group, 155 individuals were excluded, leaving 83 individuals. The association between peripheral blood TgAb titers, TPOAb titers, and AMH levels was examined based on the remaining 105 participants in the group of infertile females and the 83 participants in the control group. The study flow is shown in Figure 1. The Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University approved this clinical investigation (Kyy2018-85), which was conducted from January 1, 2018 to December 31, 2020.

2.1.2. Inclusion and exclusion criteria

The inclusion criteria were: 1) patients who were unable to achieve a clinical pregnancy after 12 months of regular sexual contact (*23,24*); 2) patients who have undergone hysterosalpingography, ultrasound, and a reproductive endocrine hormone examination suggesting female factors for infertility; 3) age between 20 and 40; 4) patients who have undergone a full set of thyroid laboratory examinations. Exclusion criteria were: 1)

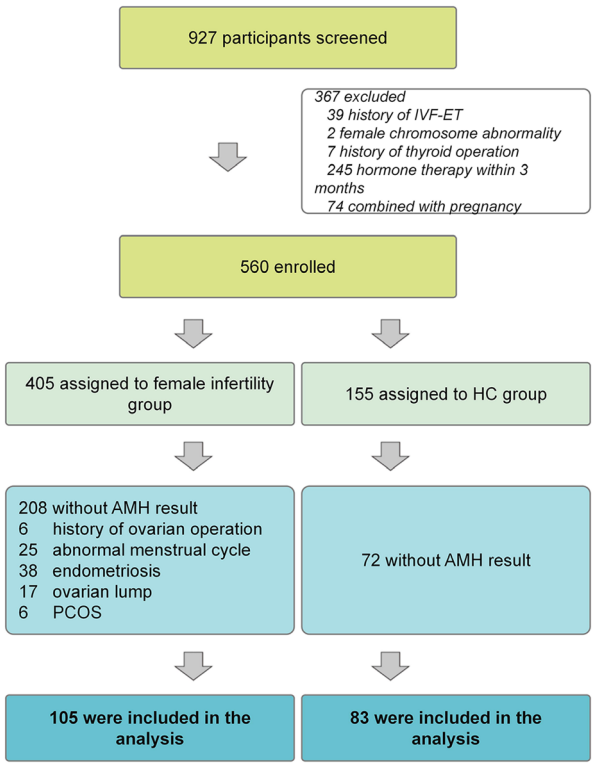

Figure 1. Flow chart showing the categorization of 560 participants based on diagnosis.

a history of significant respiratory, neurological, or cardiovascular diseases, or gastrointestinal, genitourinary system disorders; 2) severe infections, tumors, or immune deficiencies; 3) having previously undergone *in vitro* fertilization and embryo transfer; 4) female chromosomal abnormalities; 5) having previously undergone thyroid surgery; 6) the use of hormonal medications (such as L-T4, phenobarbital, and carbimazole) within three months of the determination of thyroxine hormones and antibodies; 7) patients whose thyroid hormone (TH), TgAb, and TPOAb results were only available during pregnancy; 8) having previously undergone an abortion within 6 months of measuring TH, TgAb, and TPOAb; 9) patients with factors affecting AMH, such as no measurement of AMH, having previously undergone ovarian surgery, an abnormal menstrual cycle, not having undergone a gynecological ultrasound examination, a gynecological ultrasound suggesting ovarian mass, endometriosis diagnosed *via* laparoscopy, and polycystic ovary syndrome (PCOS) diagnosed according to the 2003 Rotterdam diagnostic criteria. Participants in the control group had regular menstrual cycles, no history of adverse pregnancy, no abnormal gynecologic ultrasound examination, age 20-40 years, and had undergone a full set of laboratory thyroid examinations.

2.1.3. Medical evaluation

The basic information on each participant was collected in detail, including age, menstrual cycle, history of the present illness, pregnancy history, surgical history, past medical history, and family history. Venous blood samples were taken from the patients when seen, and the samples were centrifuged for five minutes at 3,000 r/min and 4°C. Before determination, serum samples were collected and kept at -20°C.

The serum AMH level was measured using sandwich immunoassay with a direct chemiluminescence technique. The serum thyrotropin (TSH) level was measured using the double antibody sandwich method; serum free thyroxine (FT4), TgAb, and TPOAb (Roche from Shanghai, China) were measured using the competition method. Transvaginal or transrectal ultrasonography was performed to determine ovarian and uterine morphology. Hysterosalpingography was performed 3-7 days after the patient experienced a clean menstrual period to observe the morphology of the uterus.

2.2. Bioinformatic analysis to explore the mechanism of female infertility caused by HT

2.2.1. Screening of disease-related target molecules

"Female infertility" and "Hashimoto thyroiditis" were used as keywords, which were input respectively into the DrugBank database (*https://go.drugbank.com/*), OMIM database (*https://omim.org/*), GeneCards database (*https://www.genecards.org/*), and Therapeutic Target Database (*http://db.idrblab.net/ttd/*). Information on target molecules related to diseases was downloaded from every database. After duplicates were removed, validated human target genes were screened by specifying "Homo sapiens" and "Reviewed" in the UniProt database (*https://www.uniprot.org/*). All relevant target molecules for female infertility and HT were identified. Cytoscape 3.8.2 was subsequently used to establish separate disease-target networks for female infertility and HT.

2.2.2. Construction of a protein-protein interaction network

A Venn diagram (*http://www.bioinformatics.com.cn/plot_basic_proportional_2_or_3_venn_diagram_028*) was drawn to obtain overlapping target molecules for female infertility-HT. The String 11.5 database *(https://cn.string-db.org/*) was used to construct a protein-protein interaction (PPI) network of overlapping target molecules. "Homo sapiens" was designated as the species, and a confidence level of 0.4 was the parameter.

2.2.3. Analysis of core target molecules

The network underwent topological analysis using CytoHubba (*25*), a plug-in of Cytoscape, to identify the top 20 core target molecules. The parameters for

CytoHubba were set as follows: Core target molecule = the top 20 nodes in order of highest clique centrality.

2.2.4. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis

The target molecules interacting in female infertility and HT were subjected to KEGG and GO enrichment analysis using the Metascape database (*https://metascape.org/gp/index.html*), with "Homo sapiens" and "$P < 0.01$" serving as the screening conditions. The acquired data included cellular components (CC), biological processes (BP), molecular function (MF), and signal pathways that are closely related to HT and female infertility. The results were plotted for visualization (*https://cloud.oebiotech.cn/spa#/bio/detail?number=5f6b0540-9c97-4d95-9528-bd0f3ed9d412*, *http://www.ehbio.com/ImageGP/index.php/Home/Index/GOenrichmentplot.html*).

2.3. Animal experiments

2.3.1. Reagents

Collagenase, Chicago Sky Blue, complete and incomplete Freund's adjuvants (CFA and IFA), and porcine thyroglobulin (pTg) were acquired from Sigma. A hematoxylin and eosin (HE) staining kit was obtained from Wuhan Servicebio Technology Co., Ltd. Tribromoethyl alcohol was purchased from Jiangsu Yihe Scientific Instrument Co., Ltd.

2.3.2. Animals

Four-week-old female NOD/LtJ mice were procured from Jiangsu JiCui Yaokang Biotechnology Co., Ltd. (Nanjing, Jiangsu). The mice were housed in a climate-controlled setting with a 12-hour light/dark cycle, where they had unlimited access to food and drink. A constant temperature of 25°C was kept. After one week of acclimation, the mice were used in experiments. All operations were carried out in strict compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (publication number 85-23, amended in 1985) and Fudan University's (Shanghai, China) relevant guidelines.

2.3.3. Establishment of a mouse model of AIT

Forty-two specific pathogen-free NOD/LtJ female mice were randomly divided into three groups - a normal group, a control group, and a Tg group - with each group consisting of 14 mice. Mice in the Tg group were immunized with pTg. The 0.375 g/L pTg solution was fully emulsified with an equivalent volume of CFA on ice, and 0.1 ml of emulsified agent was injected subcutaneously 1 cm proximal to the root of the tail of each mouse. Two weeks after the primary immunization, a booster immunization with pTg and IFA emulsifier was administered again to establish a mouse model of AIT (*26,27*). The control group served as the solvent control group, while the normal group did not undergo any treatment. Six weeks after the establishment of AIT, some mice were sacrificed. The thyroid, lymph nodes, spleen, and serum samples were collected. Serum TH levels, the TgAb titer, and the TPOAb titer were determined using electrochemiluminescence immunoassay (ECLIA). HE staining was used to count the follicles at different developmental stages. The remaining mice were mated. The pregnant mice were injected with Chicago blue dye during the window of implantation (WOI) period (4.5 days of gestation) to count the number of embryo implantation sites, and HE staining was used to observe and count the uterine glands during WOI.

2.3.4. Measurement of serum TgAb, TPOAb, FT3, and FT4

Following anesthesia, blood samples were extracted from the mice. After the blood was left stand for 4-6 hours, it was centrifuged for 10 minutes at 4000 g. The upper serum was then collected and stored at -20°C. ECLIA was used to determine the serum TgAb titer, TPOAb titer, FT3 level, and FT4 level.

2.3.5. HE staining of the thyroid, ovaries, and uterus

Ovarian, uterine, and thyroid tissues were dissected and promptly fixed in 4% paraformaldehyde solution for 24 hours. After dehydration and embedding in paraffin, the sections underwent HE staining. The main process was as follows: after baking in an oven at 60°C, the slices were deparaffinized with xylene and ethanol and then hydrated. Tissues were stained with hematoxylin for 3-5 minutes, rinsed with running water, and differentiated with a differentiation solution. They were then washed with running water, treated with blue liquid, and rinsed again with running water. Afterwards, the slices were stained with eosin for 5 min, dehydrated with absolute ethanol and xylene, and sealed with neutral gum. Ultimately, microscopy and image acquisition were performed. HE-stained sections of ovaries were observed under a microscope. The count of primordial, primary, secondary, antral, and preovulatory follicles was determined, followed by statistical analysis. The number of uterine glands was also quantified under a microscope based on HE-stained uterine sections.

2.3.6. Measurement of implantation sites

After the mice were anesthetized, 0.25 ml Chicago blue dye was injected into the inner canthus vein, and the palms and soles of the mice could be observed to turn blue. After waiting for 10 minutes, blood and the uterus were collected. The implantation sites were stained light

blue by the Chicago blue dye. The uterine glands were photographed, and the number of embryo implantation sites during the WOI period was counted.

2.4. Statistical analysis

Data were processed and analyzed utilizing SPSS 25.0. In clinical trials, for the two groups of quantitative data, an independent samples *t*-test was used if the data followed a normal distribution and displayed homogeneity of variances. A Mann-Whitney *U* test was utilized if not. If there were three or more groups of quantitative data, analysis of variance was used for intergroup comparisons. The Kruskal-Wallis test and the Conover test were used to perform intergroup comparisons in the event that the data did not satisfy the requirements of normality and homogeneity of variances. A chi-square test or Fisher's exact test was used for unordered categorical data. The associations between serum TgAb titers, TPOAb titers, gravidity, parity, TSH levels, FT4 levels, age, antral follicle count (MOV), and AMH were analyzed using the Spearman correlation coefficient.

For animal experiments, in the three groups of quantitative data that were normally distributed and had homogeneity of variance, analysis of variance and the Least Significant Difference test were used. The Kruskal-Wallis rank sum test was used for between-group comparisons if the conditions were not met. Image pro plus 6.0.0 was used to compute the ovarian area. Scale addition was performed using ImageJ 1.5.3. Graphs were created using GraphPad Prism 8.0.2. A two-tailed $P < 0.05$ indicated a significant difference.

3. Results

3.1. Comparison of general information, thyroid function, peripheral blood TgAb, and TPOAb between infertile female patients and the control group

In comparison to the control group, the female infertility cohort exhibited a marked increase in pregnancies ($P < 0.001$) and a markedly increased FT4 level ($P = 0.027$). The TPOAb titer in peripheral blood was elevated ($P = 0.031$), and positivity for TPOAb was markedly higher ($P = 0.019$) compared to that in the control group. Positivity for TgAb or/and TPOAb was also significantly higher compared to that in controls ($P = 0.013$). Conversely, there were no significant differences in age, TSH level, TgAb titer, positivity for TgAb, double positivity for TgAb and TPOAb, the rate of congenital hypothyroidism (CH), the rate of subclinical hypothyroidism (SCH), and the rate of normal thyroid function between the infertile female group and the control group ($P > 0.05$) (Table 1).

In accordance with the etiology of the disease, infertile female patients were stratified into those with ovulatory dysfunction and pelvic factors. Results indicated that the ovulation disorder group tended to be younger than the control group ($P < 0.001$) and exhibited a notably higher pregnancy rate ($P = 0.004$). The TSH level in the ovulation disorder group was significantly elevated compared to that in the control group ($P =$

Table 1. Distributions of age and serological thyroid function indicators between infertile female patients and the control group

Items	Female infertility			Control (n = 155)
	Ovulatory dysfunction (n = 47)	Pelvic factor (n = 358)	Total (n = 405)	
Age (year)	27.66 ± 0.51***	29.43 ± 0.21	29.23 ± 0.2	29.83 ± 0.32
Serum hormone levels				
TSH (μIU/mL)	3.92 ± 0.51**	2.72 ± 0.1	2.86 ± 0.1	2.51 ± 0.12
FT4 (pmol/L)	16.69 ± 0.32	17.35 ± 0.14*	17.27 ± 0.13*	16.82 ± 0.19
Serum antibody levels				
TgAb (IU/mL)	10.89 (10-4000)	11.92 (10-4000)	11.86 (10-4000)	11.68 (10-4000)
TPOAb (IU/mL)	12.34 (5-600)	14.13 (5-600)*	13.87 (5-600)*	12.78 (5-600)
TgAb-positive [n (%)]	12 (25.53)**	51 (14.25)	63 (15.56)	15 (9.68)
TPOAb-positive [n (%)]	9 (19.15)*	56 (15.64)*	65 (16.05)*	13 (8.39)
TgAb or/and TPOAb-positive [n (%)]	14 (29.79)**	73 (20.39)*	87 (21.48)*	19 (12.26)
TgAb and TPOAb-positive [n (%)]	7 (14.89)	34 (9.5)	41 (10.12)	9 (5.81)
Clinical diagnosis				
EU [n (%)]	37 (78.72)	298 (83.24)	335 (82.72)	133 (85.81)
CH [n (%)]	1 (2.13)	1 (0.28)	2 (0.49)	0 (0)
SCH [n (%)]	9 (19.15)	45 (12.57)	54 (13.33)	19 (12.26)

Data are means ± SD or Median (Min-Max) for continuous variables, or n (%) for categorical variables. AMH: anti-Müllerian hormone; MOV: mean ovarian volume; TSH: thyroid stimulating hormone; FT3: free triiodothyronine; FT4: free thyroxine; TgAb: thyroglobulin antibody; TPOAb: thyroid peroxidase antibody; CH: clinical hypothyroidism; SCH: subclinical hypothyroidism; EU: euthyroid. *$P < 0.05$ *vs.* controls, **$P < 0.01$ *vs.* controls, ***$P < 0.001$ *vs.* controls.

0.004). Moreover, there was a significant increase in the positivity for TgAb in peripheral blood ($P = 0.005$) and an elevation in the positivity for TPOAb ($P = 0.038$) in the ovulation disorder group compared to controls. Positivity for TgAb or/and TPOAb was also higher ($P = 0.004$). However, the FT4 level, TPOAb titer, TgAb titer, double positivity for TgAb and TPOAb, the rate of CH rate, the rate of SCH, and the rate of normal thyroid function did not differ significantly compared to those indices in the control group ($P > 0.05$).

In the pelvic factor group, the number of pregnancies was notably higher than that in the control group ($P < 0.001$). Significantly elevated FT4 levels ($P = 0.016$) and TPOAb titers ($P = 0.027$) were noted compared to levels and titers in controls. Positivity for TPOAb and positivity for TgAb or/and TPOAb were both considerably higher than those in the control group ($P = 0.027$). There were significant differences in age, the TSH level, TgAb titer, positivity for TgAb, double positivity for TgAb and TPOAb, the rate of CH, the rate of SCH, and the rate of normal thyroid function compared to those indices in the control group ($P > 0.05$).

3.2. Peripheral blood TgAb and TPOAb levels lead to differences in the proportion of patients in different stages of clinical hypothyroidism

In accordance with the development of CH caused by TgAb and TPOAb, CH can be divided into four stages: Normal thyroid function and double negativity for TgAb and TPOAb, normal thyroid function and positivity for TgAb or/and TPOAb, SCH and positivity for TgAb or/and TPOAb, and CH and positivity for TgAb or/and TPOAb. An analysis of the distribution of these stages among all patients showed that individuals with SCH and positivity for TgAb or/and TPOAb were substantially more prevalent in the group of infertile females compared to controls ($P = 0.038$). There were no substantial differences in the proportion of patients with normal thyroid function and double negativity for TgAb and TPOAb, normal thyroid function and positivity for TgAb and/or TPOAb, and CH and positivity for TgAb and/or TPOAb ($P > 0.05$) (Table 2).

3.3. No correlation between serum TgAb, TPOAb, and AMH

3.3.1. No correlation between the serum TgAb titer, TPOAb titer, and serum AMH level

Spearman correlation analysis was performed between AMH levels in the two groups and other quantitative data. Results revealed a substantial positive association with MOV ($r = 0.459$, $P < 0.001$) and a significant negative correlation with age in the group of infertile females ($r = -0.346$, $P < 0.001$). In the control group, the AMH level also exhibited a significant association with MOV ($r = 0.41$, $P < 0.001$). However, the AMH level and antibody titer were not significantly correlated in either group ($P > 0.05$) (Table 3).

3.3.2. No correlation between ATAb positivity and peripheral blood AMH

TgAb titers and TPOAb titers are used to determine whether TgAb and TPOAb are positive in clinical practice, so TgAb and TPOAb were converted into categorical variables to explore the association between TgAb, TPOAb, and ovarian reserve. The two groups were subdivided according to TgAb and/or TPOAb

Table 2. Proportion of participants in each group at different development stages of CH

Items	Female infertility ($n = 405$)	Control ($n = 155$)	P value
EU and double negative for TgAb and TPOAb [n (%)]	280 (69.14)	118 (76.13)	0.102
EU and positive for TgAb or/and TPOAb [n (%)]	55 (13.58)	15 (9.68)	0.211
SCH and positive for TgAb or/and TPOAb [n (%)]	21 (5.19)*	2 (1.29)	0.038
CH and positive for TgAb or/and TPOAb [n (%)]	2 (0.49)	0 (0)	1

Data are n (%). EU: euthyroid; TgAb: thyroglobulin antibody; TPOAb: thyroid peroxidase antibody; SCH: subclinical hypothyroidism; CH: clinical hypothyroidism. *$P < 0.05$ *vs.* controls.

Table 3. Correlation analysis between serum AMH and quantitative data

Correlation coefficient	Female infertility ($n = 105$)	P value	Control ($n = 83$)	P value
Age	-0.346***	< 0.001	-0.19	0.085
MOV	0.459**	< 0.001	0.41***	< 0.001
TSH	0.056	0.571	-0.11	0.321
FT4	0.105	0.286	0.018	0.870
TgAb	0.103	0.297	-0.114	0.305
TPOAb	-0.056	0.572	-0.096	0.389

Data are r. MOV: mean ovarian volume; TSH: thyroid stimulating hormone; FT4: free thyroxine; TgAb: thyroglobulin antibody; TPOAb: thyroid peroxidase antibody. **$P < 0.01$ *vs.* controls, ***$P < 0.001$. *vs.* controls.

positivity. Among them, 21 were positive for TgAb and/or TPOAb , and 84 were negative in the group of infertile females. In the control group, 14 patients were positive for TgAb or/and TPOAb, and 69 were negative. The age, MOV, TSH, FT4, and AMH levels of the positive and negative groups were compared. Results indicated that, in both the group of infertile females and control group, there were no notable differences in age, MOV, TSH, FT4, and AMH levels between the TgAb- or/and TPOAb-positive subgroup and the double-negative TgAb and TPOAb subgroup ($P > 0.05$) (Table 4).

3.4. Analysis of intersecting target molecules for infertility due to female factors and HT

3.4.1. Overlapping target molecules for female infertility-HT

Nine hundred ninety-five target molecules related to female infertility and 1,282 target molecules related to HT were screened, respectively, using GeneCards and other databases. There were 427 overlapping target molecules for female infertility-HT (Figure 2A).

3.4.2. PPI network analysis of core target molecules

A PPI network was established from 427 overlapping target molecules utilizing the String database and the software Cytoscape. The female infertility-HT network consists of 426 nodes and 14,839 edges. Each node represents a target of the interaction between female infertility and HT, the magnitude of which is proportional to the value for the target molecule. An edge indicates an interaction between the two target molecules, and its thickness is proportional to the strength of the interaction (Figure 2B). The PPI network was entered into the software Cytoscape and analyzed using a CytoHubba plug-in to obtain the highest 20 core target molecules according to the MCC (Figure 2C). Core target molecules for female infertility-HT include glyceraldehyde-3-phosphate dehydrogenase, vascular endothelial growth factor A, interleukin-6 (IL-

Table 4. Comparison of peripheral blood AMH after subgrouping according to ATAb positivity

Items	Female infertility			Control		
	ATAb positive ($n = 21$)	ATAb negative ($n = 84$)	P value	ATAb positive ($n = 14$)	ATAb negative ($n = 69$)	P value
Age (year)	31 ± 0.94	29.7 ± 0.48	0.293	31.57 ± 0.8	30.35 ± 0.5	0.131
MOV (cm^3)	6.98 ± 0.47	7.07 ± 0.27	0.882	6.96 ± 0.59	7.61 ± 0.27	0.323
TSH (μIU/mL)	3.54 ± 0.75	2.45 ± 0.16	0.767	2.57 ± 0.35	2.59 ± 0.22	0.481
FT4 (pmol/L)	16.56 ± 0.4	17.17 ± 0.23	0.231	17.9 ± 1.57	16.63 ± 0.19	0.913
AMH (ng/mL)	3.86 ± 0.55	3.05 ± 0.19	0.087	3.38 ± 0.52	3.45 ± 0.21	0.675

Data are the mean ± SD. MOV: mean ovarian volume; TSH: thyroid stimulating hormone; FT4: free thyroxine; ATAb: antithyroid antibody; TgAb: thyroglobulin antibody; TPOAb: thyroid peroxidase antibody; AMH: anti-Müllerian hormone.

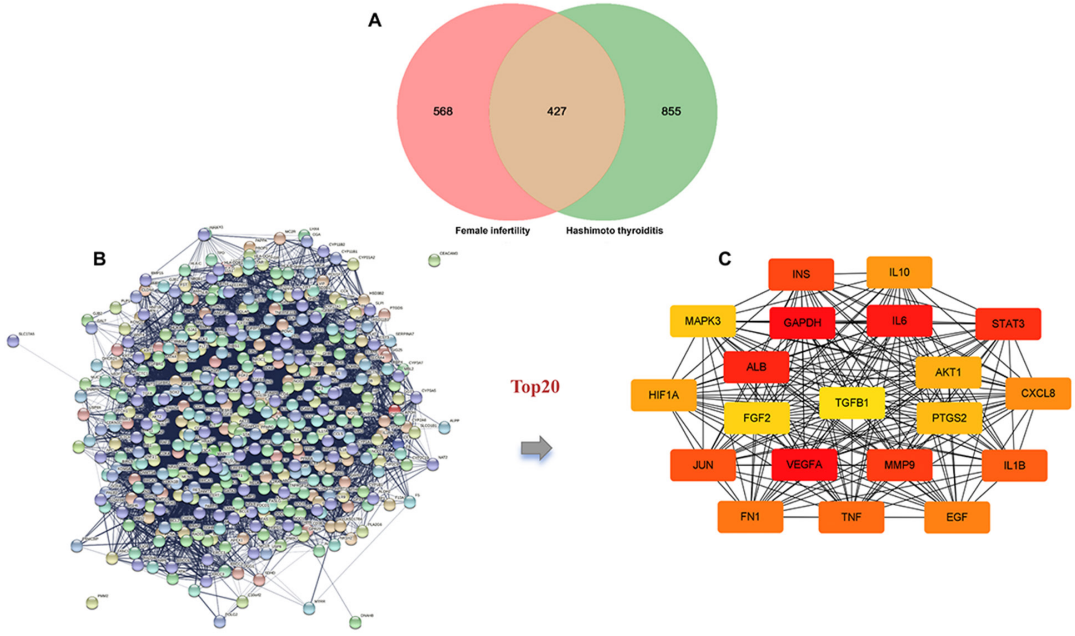

Figure 2. Analysis of target molecules for infertility due to female factors and HT. (A) Overlapping target molecules for female infertility-HT. **(B)** PPI network of overlapping target molecules for female infertility-HT. **(C)** PPI network of the top 20 target molecules. Rounded rectangles represent target molecules and those that are darker in color represent a greater degree of centrality. HT: Hashimoto thyroiditis, PPI: protein-protein interaction.

6), serum albumin, signal transducer and activator of transcription 3, matrix metalloproteinase 9 (MMP9), insulin, transcription factor AP-1, IL-1β, tumor necrosis factor (TNF), fibronectin 1, pro-epidermal growth factor (EGF), IL-8, IL-10, hypoxia-inducible factor 1-α (HIF 1-α), RAC-alpha serine/threonine-protein kinase (AKT1), prostaglandin G/H synthase, mitogen activated protein kinase 3 (MAPK3), fibroblast growth factor 2, and transforming growth factor-β1 (TGF-β1).

3.4.3. GO enrichment analysis and KEGG pathway enrichment analysis

GO enrichment and KEGG pathway enrichment analysis were performed for the top 20 target molecules to systematically explore their biological function. GO enrichment analysis revealed the function of genes by BP (green bars), CC (blue bars), and MF (red bars). A $P < 0.01$ was used, resulting in 671 entries. These included 634 BP entries, 15 CC entries, and 22 MF entries. The BP of female infertility-HT interaction, the positive regulation of cell migration, cell movement, and cell component movement were at the forefront. The endoplasmic reticulum lumen, cell membrane, outer cytoplasmic membrane, extracellular matrix, and membrane microdomain were at the forefront of CC. At the forefront of MF were cytokine activity, cytokine receptor binding, growth factor activity, chemokine receptor binding, and chemokine activity (Figure 3A).

KEGG signaling pathway enrichment analysis

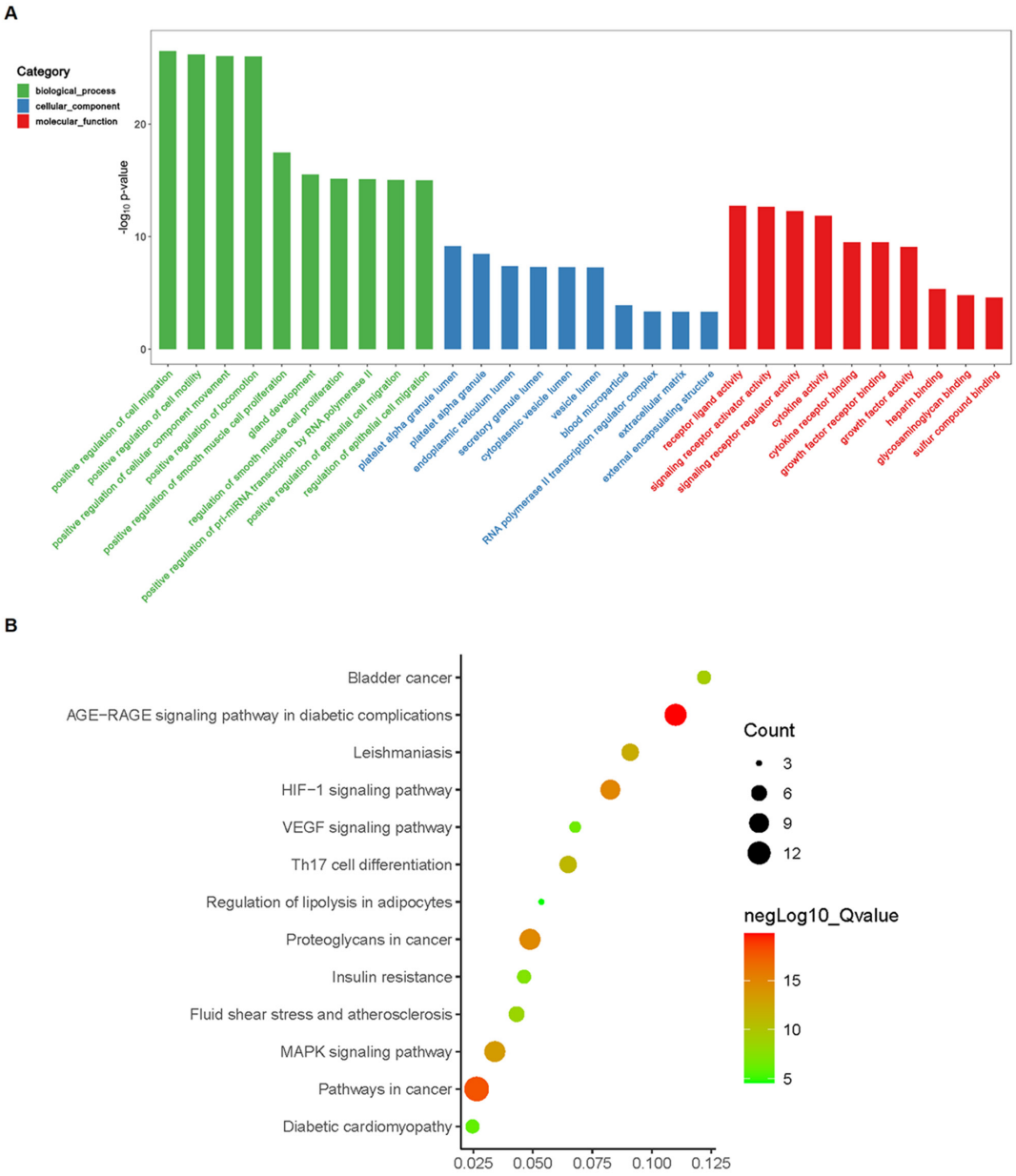

Figure 3. GO enrichment analysis and KEGG pathway enrichment analysis. **(A)** GO enrichment analysis. **(B)** KEGG pathway enrichment analysis. HT: Hashimoto's thyroiditis; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; AGE-RAGE: advanced glycosylated end product-glycosylated end product receptor; HIF-1: hypoxia-inducing factor 1; VEGF: vascular endothelial growth factor; TH17: type 17 helper T cells; MAPK: mitogen-activated protein kinase.

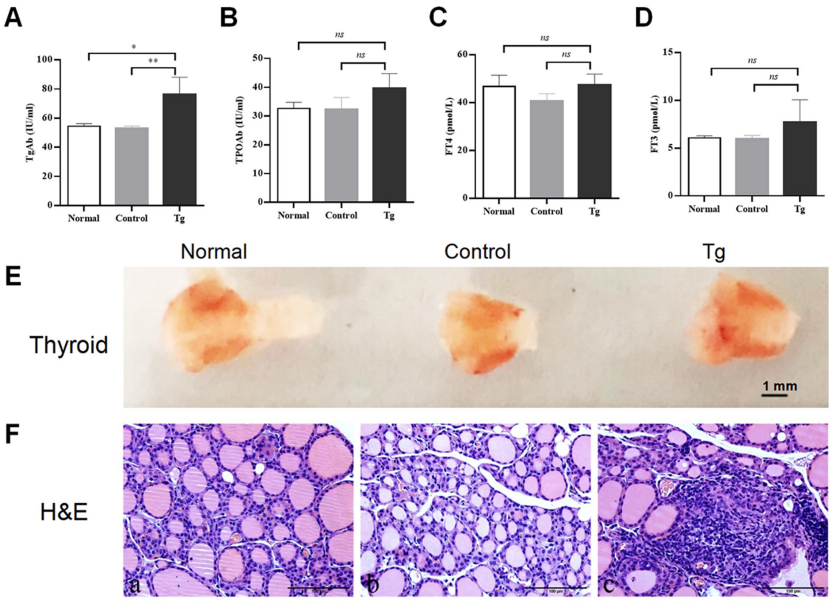

Figure 4. Serum thyroid hormone levels, antibody titers, and thyroid changes in each group. Comparison of serum **(A)** TgAb, **(B)** TPOAb, **(C)** FT4, and **(D)** FT3 in mice among the normal, control, and Tg groups. **(E)** Appearance of thyroid specimens in each group. Scale bars, 1mm. **(F)** Hematoxylin and eosin staining of thyroid tissue from rats. Magnification, ×400. Scale bars, 100 μm.

showed that the core target molecule for female infertility-HT was mainly enriched in 119 signaling pathways ($P < 0.01$) (Figure 3B). The pathways mainly included the advanced glycosylation end product (AGE) - receptor of AGE (RAGE) signaling pathway in diabetic complications, the cancer pathway, Chagas disease, HIF-1 pathway, and Kaposi sarcoma-associated herpes virus infection (Figure 3B).

3.5. Serum thyroid hormone levels and antibody titers in mice in each group

In this study, pTg was used to establish a mouse AIT model. On day 43, serum antibody titers and hormone levels in peripheral blood were determined. The serum TgAb titer in the Tg group was considerably higher than that in the normal group and control group ($P = 0.010$ in the normal group and $P = 0.008$ in the control group). The TPOAb titer tended to increase, while the serum FT4 and FT3 levels did not change substantially ($P > 0.05$), indicating that the model was successfully established (Figures 4A-4D).

3.6. Changes in the thyroid of mice in each group

The Tg group had larger thyroid volume, darker color, and tougher texture compared to both the normal and control groups. The HE-stained thyroid specimens from the normal, control, and Tg groups were observed under a light microscope. In the normal group and the control group, the thyroid follicle structure was intact, robust, and had a clear boundary. There was also no obvious destruction of the follicular epithelium. The cytoplasm was red, and the nucleus was blue. The follicular cavity had an abundance of glia-like substances and was pink. No lymphocytes were seen in the interfollicular space. The Tg group exhibited lymphocyte aggregation and partial destruction of follicular structures (Figures 4E and 4F).

3.7. Embryo implantation in mice in each group

Visual inspection of the uterus after Chicago staining revealed a V-shaped mouse uterus. Embryo implantation sites are stained dark blue and can be clearly counted in each uterus. No discernible difference in implantation sites was noted among the three groups ($P > 0.05$) (Figure 5G).

3.8. Changes in pathological sections of the mice uterus in each group

Pathological sections of the uterus showed that the mouse uterine cavity was located in the middle of the transverse section of the uterus and was surrounded by the luminal epithelium (Le), which was a single layer of columnar epithelium with the cytoplasm in red and nucleus in blue. Deep in the luminal epithelium is the lamina propria, which occupies most of the cross-sectional area of the mouse uterus and which contains stromal cells (S) and glands. The glands consist of glandular epithelium (Ge), which varies in size, with the cytoplasm in red and nucleus in blue. The Tg group's uterine pathological alterations did not differ markedly from those in the other two groups. The number of uterine glands did not differ significantly among the

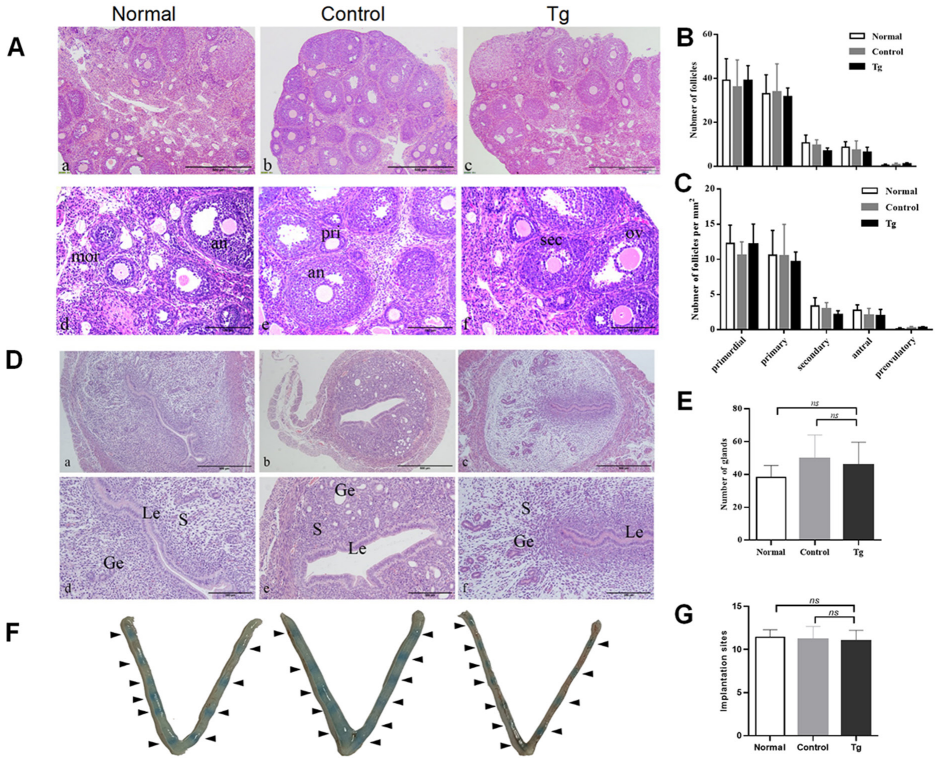

Figure 5. The ovaries, uterus, embryo implantation sites, and follicles of mice in each group. (A) Hematoxylin and eosin staining of mouse ovaries. a, b, c: Magnification, ×100. Scale bars, 500 μm. d, e, f: Magnification, ×200. Scale bars, 100 μm. mor: primordial follicle; pri: primary follicle; sec: secondary follicle; an: antral follicle; ov: preovulatory follicle. **(B)** Number of different types of follicles. **(C)** Number of different types of follicles per mm^2. **(D)** Hematoxylin and eosin staining of the uterus. a, b, c: Magnification, ×200. Scale bars, 500 μm. d, e, f: Magnification, ×200. Scale bars, 500 μm. Le: uterine luminal epithelium; S: uterine stromal cells; Ge: uterine glandular epithelium. **(E)** Comparison of counts in mouse uterine glands. ns: There was no significant difference. **(F)** Chicago Sky blue staining at the implantation site of mouse embryos in each group. **(G)** Comparison of the number of embryo implantation sites.

three groups ($P > 0.05$) (Figures 5D and 5E).

3.9. Ovarian pathological changes in mice in each group

The pathological sections of the mouse ovary suggested that the follicles were mainly distributed in the cortex and were of different sizes and shapes. Primordial follicles are formed by a single layer of spindle-shaped pregranulosa cells surrounding the primary oocyte (mor in Figure 5A). The primary follicle is larger in volume than the primordial follicle and consists of 1-2 layers of cuboid cells surrounding the oocyte (pri in Figure 5A). Secondary follicles further increased in size and had more cuboidal cell layers (sec in Figure 5A). The accumulation of follicular fluid between the granulosa cells increased, and fluid-filled spaces coalesced to form a follicular antrum, at which point the follicle is called antral (an in Figure 5A). Mature follicles are the final stage of follicular development, with a sharp increase in follicular fluid, enlargement of the follicular cavity, a significant increase in follicular volume, and the beginning of follicular projection toward the ovarian surface (ov in Figure 5A). The number of follicles at different developmental stages did not differ significantly among the normal, control, and Tg groups ($P > 0.05$). Similarly, the number of follicles per square millimeter at various developmental stages did not differ significantly among these groups ($P > 0.05$).

4. Discussion

The marked decrease in infertility is currently a substantial challenge for numerous countries (*28*). The etiology of female infertility is multifaceted, with ongoing exploration into immunological factors (*23,29*). However, the relationship between infertility due to female factors and HT remains inconclusive (*11,30*). In the current work, a cross-sectional study was designed to explore whether TgAb and TPOAb are associated with infertility due to female factors. Additionally, we conducted in-depth research based on bioinformatics and a murine AIT model to investigate the mechanism of TgAb and TPOAb on female fertility. Our findings verified the association between peripheral blood TPOAb and infertility due to female factors and suggested that peripheric TgAb may be not associated with infertility due to female factors or that TPOAb and TgAb may influence female reproductive capability synergistically. Elevated TgAb titers in the peripheral blood of mice were not significantly correlated with embryo implantation and ovarian reserve, which is consistent with our clinical results.

The thyroid gland, the body's largest endocrine organ, serves as a primary target of autoimmune assault, with HT regarded as the most prevalent autoimmune disorder (*10*). Our cross-sectional study found a positive correlation between serum TPOAb levels and infertility due to female factors, which accords with previous results (*11,14*). In a study by Poppe *et al*., among 197 patients with infertility due to female factors, positivity for TPOAb was considerably higher compared to that in the control group (18% *vs.* 8%, $P < 0.05$). Among these 197 patients with infertility due to female factors, 116 had ovulatory disorders, with no discernible difference in positivity for TPOAb compared to controls (16% *vs.* 8%, $P > 0.05$). In 21 patients with endometriosis, however, positivity for TPOAb was substantially higher than that in the control group (29% *vs.* 8%, $P < 0.05$) (*14*). The results of our investigation align closely with these findings, revealing a significantly higher positivity for TPOAb in patients with infertility due to female factors. Positivity for TPOAb was markedly higher in patients with ovulatory disorders, suggesting a potential correlation between TPOAb and infertility due to female factors.

The study by Poppe *et al*. did not include TgAb, whereas our study did, and we found a higher positivity for TgAb in the group with ovulatory disorders but no significant difference in any of the infertile female patients. Additionally, all of the patients with infertility due to female factors in our study demonstrated significantly higher parity compared to controls, which is probably because of the inclusion of mostly secondary infertility patients with a history of prior pregnancies. In summary, our results suggested a potential association between TPOAb and female infertility. However, it remains unclear whether TPOAb acts independently or in synergy with TgAb, whether TgAb and TPOAb are associated with clinical conditions manifesting as female infertility (*e.g.*, endometriosis, PCOS, primary ovarian insufficiency), or whether TgAb and TPOAb are directly related to female infertility. The diagnosis of infertility spans a year, adding to the complexity of cohort studies investigating the relationship between TgAb and TPOAb positivity and infertility. Consequently, further comprehensive clinical or foundational research needs to be conducted to provide conclusive evidence.

Several researchers have explored the relationship between TgAb, TPOAb, TSH, and AMH, but their conclusions have been inconsistent (*31-39*). The study by Poppe *et al*. and our research observed an increase in TSH levels in the group with ovulatory disorders (Poppe: 1.5 *vs.* 1.1, $P < 0.05$; ours: 3.92 *vs.* 2.51, $P < 0.01$). However, there is currently no evidence to suggest that TSH levels above the reference range (*i.e.*, SCH) impact ovarian response, oocyte/embryo quality, or pregnancy rates (*10*). AMH is mainly affected by biological characteristics, reproductive factors, environment, and lifestyle. In terms of reproductive factors, ovarian suppression, pregnancy status, and having previously undergone ovarian surgery have been linked to reduced AMH levels. Conditions like PCOS, granulosa cell tumors, and multiparity have been associated with elevated AMH levels. Smoking, chemotherapy, and low vitamin D levels have been linked to decreased AMH levels (*40,41*). Ovarian reserve is influenced by age and is negatively correlated with age (*40*). Few studies have focused on the impact of TgAb or TPOAb on AMH, and they did not exclude relevant factors. In our study, we excluded the factors that could interfere with AMH as much as possible to investigate the impact of TgAb and TPOAb on AMH. However, we found no correlation between AMH and TSH, FT4, TPOAb, or TgAb either in subjects with infertility due to female factors or in controls. Moreover, grouping based on TgAb and/or TPOAb positivity versus double negativity did not reveal any significant differences in AMH levels, indicating that TgAb and TPOAb do not affect ovarian reserve. A review by Zhu *et al*. posited that TPOAb may negatively affect oocyte fertilization, embryo, and placenta development (*42*). In contrast, other studies noted no impact of TgAb and TPOAb on assisted reproductive technology outcomes (*43-45*). Moreover, abnormal TH levels may lead to ovarian dysfunction throughout life, and especially during puberty and childbearing (*46*). Therefore, TgAb and TPOAb may affect ovarian reserve by influencing thyroid function. However, whether TgAb and TPOAb can directly influence ovarian reserve, oocyte development, fertilization, and other physiological processes remains unclear. Given our relatively small sample size and the fact that we did not exclude all potential factors influencing AMH (such as smoking and vitamin D levels), further well-designed clinical studies need to be conducted to establish whether there is an association among AMH, TgAb, and TPOAb.

Elevated peripheral blood TPOAb titers affecting infertility due to female factors may be related to inflammation. Our bioinformatic results indicated that the intersecting target molecules for HT and infertility due to female factors included IL-6, IL-10, TNF, and MMP-9, involving various signaling pathways such as HIF-1, VEGF, MAPK, and Th17 cell differentiation. The etiology of HT is a combination of genetic susceptibility and environmental factors, leading to the loss of immune tolerance (*47*). Lymphocyte infiltration, and especially T-cell infiltration, and follicular disruption are histological characteristics of HT (*47*). Cytokines and chemokines play crucial roles in the pathogenesis of HT, participating in the initiation and perpetuation of the autoimmune process (*47*). Infertility due to female factors can result from various factors, including age, anatomy, endocrine, and immunological factors. Pathological conditions affecting the uterine lining can also lead to implantation failure within the maternal uterus, thereby compromising fertility. Leukemia inhibitory factor, a cytokine belonging to the IL-6 family, can activate the Janus kinase-signal

transducer and activator of transcription pathway, which is essential for embryo implantation. The robust invasive capacity of trophoblast cells is attributed to the production of abundant MMPs, and particularly MMP2 and MMP9. The secretion of MMPs by trophoblast cells is regulated by factors such as IL-1β, TNF-α, IL-1α, macrophage colony-stimulating factor, TGF-β, EGF, and human chorionic gonadotropin, all of which are released by several types of cells at the fetal-maternal interface to promote trophoblast invasion. In addition, VEGF promotes embryo development, enhances endometrial receptivity, and facilitates interaction between developing embryos and the endometrium during implantation. Researchers have discovered that trophoblast cell migration and invasion are regulated by epidermal growth factor-like domain 7, which activates the NOTCH1, MAPK, and AKT signaling pathways in both trophoblast cell lines and primary cells. IL-10, an anti-inflammatory cytokine with important immune regulatory functions, plays a critical role in successful pregnancy. Starting from embryo implantation, fetal cells must survive under extremely adverse conditions within the maternal uterus, including severe hypoxia, lack of vascularization, and potential attacks from the maternal immune system.

The embryo implantation site serves as an indicator reflecting the uterine endometrial receptivity in mice. We constructed a murine model of AIT to investigate changes in implantation sites and ovarian reserve when peripheral blood TgAb titer increased. In this study, we used pTg instead of TPO for the AIT animal model due to various force majeure factors. During our exploratory phase of dosage modeling, we noticed that administering a dose of 25 μg led to a significant elevation in the peripheral blood FT4 levels in mice. When given a dose of 12.5 μg, murine serum antibody titers did not change markedly. Therefore, we opted for a dose of 18.75 μg in our study, resulting in a significant rise in serum TgAb titers and a noticeable rise in TPOAb titers. The results suggest that this model does not affect embryo implantation or ovarian reserve in mice, aligning with the results of our clinical cross-sectional study, in which patients with infertility due to female factors did not exhibit a notable increase in positivity for serum TgAb. This might be attributable to interspecies differences or the possibility that TPOAb plays a more dominant role during embryo implantation. TgAb is a diagnostic criterion for HT, but research has shown that women with isolated TgAb positivity exhibit significantly elevated blood TSH levels (*48*). However, TgAb cannot fix complement and is believed to have a less prominent role in thyroid damage, so it is considered less significant than TPOAb (*49*). Peripheral blood TPOAb and TgAb might play different roles or have a synergistic effect, influencing female fertility. TPOAb may act alone or synergistically with TgAb to affect female fertility.

Limitations of this study include its retrospective nature as a cross-sectional analysis, which makes it susceptible to selection bias (*21,22*). The study data rely on patient medical records, leading to the exclusion of some individuals due to the absence of relevant diagnostic indicators. Future research would benefit from prospective studies with larger sample sizes. Additionally, due to constraints in the modeling approach, mice in this study only exhibited an elevation in peripheral blood TgAb levels, hampering investigation of the influence of peripheral blood TPOAb on embryo implantation and ovarian reserve.

5. Conclusion

An elevated TPOAb titer in peripheral blood is associated with female infertility but has no significant correlation with AMH. The relative mechanisms may be related to the regulation of multiple signaling pathways such as HIF-1, VEGF, MAPK, and Th17 cell differentiation. The serum TgAb titer may be not associated with both female infertility and AMH. A certain dose of pTg can successfully establish a murine model of AIT, in which elevated peripheral blood TgAb levels did not affect embryo implantation and ovarian reserve.

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