INTRODUCTION

Autoimmune thyroid diseases (AITD) is the most common organ-specific autoimmune disease, mainly caused by genetic predisposition, environmental factors and dysfunction in the microbiome. The main types of AITD are Hashimoto's thyroiditis (HT) and Graves' disease (GD). HT, considered the most common endocrine disorder, is characterized by invasion of the thyroid gland by inflammatory lymphocyte cells. This infiltration induced the destruction and the replacement of follicular tissue leading to hypothyroidism. Anti-thyroperoxidase antibodies are considered the main serological marker for the diagnosis of HT. Anti-thyroglobulin antibodies are less sensitive and less specific than anti-thyroperoxidase antibodies. The incidence of GD is 20 to 50 cases per 100,000 subjects. During this...
syndrome, the thyroid gland is enlarged and overactive, the heart rate is accelerated, and there are eye abnormalities. Antibodies activating thyrotropin receptors induce overproduction of thyroid hormones. Additionally, B and T lymphocytes and antigen-presenting cells produce cytokines that induce inflammation and change the behavior of thyroid epithelial cells.  

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by the persistent antiphospholipid antibodies (aPL), along with occurrence of vascular thrombosis and pregnancy morbidity. Lupus anticoagulant, anti-cardiolipin antibodies (aCL) and anti-β2 glycoprotein I (aβ2GPI) are the most commonly detected aPL antibodies.  

In AITD, few studies have determined only the frequency of aCL (IgG and IgM). However, aCL-IgA and aβ2GPI have never been determined in patients with GD or HT. So, the objective of this study to try to explain why these antibodies are produced in AITD.

2 | MATERIALS AND METHODS

2.1 | Patients

In this retrospective study, we included 195 AITD patients. The inclusion criteria were patients older than 18 years and suffering from HT or GD. Patients with another autoimmune disease associated to AITD were excluded. Sera were consecutively collected between January 2017 and December 2018 from four hospitals in the center of Tunisia. Ninety age-matched healthy blood donors (HBD) served as a control group (Table 1).

All sera were stored at −80°C until the use. All sera were tested for aCL and aβ2GPI. We obtained the approval for this study from ethical committee of Farhat Hached hospital.

2.2 | Methods

2.2.1 | aCL assays

aCL (IgG, IgA and IgM) were determined by using an enzyme-linked immunosorbent assay (ELISA) (Orgentec Diagnostika®, Mainz, Germany) as described in our previous study. Antibodies present in positive samples bind to the antigen coated on the surface of the two reaction wells forming an antibody antigen complex. After incubation, a first washing step removes unbound and unspecific bound molecules. Subsequently added enzyme conjugate binds to the immobilized antibody-antigen complex. After incubation, a second washing step removes unbound enzyme conjugate. Addition of enzyme substrate solution results in hydrolyzation and color development during incubation. The intensity of the color correlates with the concentration of the antibody-antigen-complex and can be measured photometrically at 650 nm. Results were expressed with a cut-off of positivity of 7 U/ml for IgM and 10 U/ml for IgA and IgG.

2.2.2 | aβ2GPI assays

aβ2GPI (IgG, IgA and IgM) were evaluated by an ELISA (Orgentec Diagnostika®, Mainz, Germany) using highly purified human β2GPI as described in our previous study. β2GPI is bound to microwells. Specific antibodies in the patient sample bind to the antigen coated on the surface of the reaction wells. After incubation, a washing step removes unbound and unspecifically bound serum or plasma components. Subsequently added enzyme conjugate binds to the immobilized antibody-antigen-complexes. After incubation, a second washing step removes unbound enzyme conjugate. After addition of substrate solution, the bound enzyme conjugate hydrolyses the substrate forming a blue coloured product. Addition of an acid stops the reaction generating a yellow end-product. The intensity of the color can be measured photometrically at 450 nm. The results were expressed in arbitrary units and the cut-off for positivity was 8 U/ml.

2.2.3 | Thyroperoxidase antibodies and thyroglobulin antibodies assays

Anti-thyroid peroxidase antibodies (TPO-Ab) and anti-thyroglobulin antibodies (Tg-Ab) were assessed with ELISA kit (Euroimmun®, Lübeck, Germany) as we have described in our previous study. The ELISA is performed on microplate wells coated with recombinant TPO or highly purified native Tg isolated from human thyroid gland. The assay was performed according to the manufacturer’s recommendations. Results are expressed in international units (IU/ml). The

| TABLE 1 Characteristics of autoimmune thyroid disease (AITD) patients and the control group |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Autoimmune thyroid disease (n = 195)          | Hashimoto’s thyroiditis (n = 139)              | Graves’ disease (n = 56)                       | Control group (n = 90)                        | p Autoimmune thyroid disease/Control group |
| Sex-ratio (F/M)                               | Sex-ratio (F/M)                               | Sex-ratio (F/M)                                | Sex-ratio (F/M)                               | Sex-ratio (F/M)                               |
| Median age (IQR) years                        | Median age (IQR) years                        | Median age (IQR) years                         | Median age (IQR) years                        | Median age (IQR) years                        |
| Age range (years)                             | Age range (years)                             | Age range (years)                              | Age range (years)                             | Age range (years)                             |
| 3.8 (154/41)                                  | 4.3 (113/26)                                  | 2.7 (41/15)                                   | 1.6 (56/34)                                  | 0.002                                         |
| 45 (34–55)                                    | 45 (34–55)                                    | 46 (32–54)                                    | 38.5 (26–44)                                  | NS                                            |
| 13–83                                        | 13–83                                        | 14–78                                         | 20–64                                        | -                                             |

The bold values indicates statistically significance of p values.
cut-off limit recommended by Euroimmun is 50IU/ml for TPO-Ab and 100IU/ml for Tg-Ab.

2.2.4 | Thyroid-stimulating hormone receptor antibodies assay

Human autoantibodies against thyrotropin (TSH) receptor (TRAb) were assessed with an ELISA kit (Euroimmun®, Lübeck, Germany). The test kit contains microplate wells coated with TSH receptor. In the first reaction step, patient sera are incubated in the wells. If samples are positive, specific antibodies bind to the TSH receptors. Bound antibodies are able to inhibit the binding of biotin-labelled TSH, which is added in a second incubation step. To detect the bound TSH-Biotin, a third incubation is carried out using enzyme-labeled avidin (enzyme conjugate), catalyzing a color reaction. The intensity of the color formed is inversely proportional to the concentration of antibodies against TSH receptor. The results were expressed in international units (IU/L) and the cut-off for positivity was 2 IU/L.

2.2.5 | Statistical analysis

All statistical analyses were done using Epi Info 3.5. The comparison of frequencies of aPL was conducted using the Chi-square test or Fisher exact test. A p-value less than 0.05 was considered statistically significant.

3 | RESULTS

Table 1 shows patients and control group characteristics. Forty-one patients were men and 154 patients were women. The median age of AITD patients was 45 years (range 13–83 years).

| Autoantibodies | Autoimmune thyroid disease (n = 195) | Control group (n = 90) | p    |
|----------------|--------------------------------------|------------------------|------|
| aPL (aCL or aβ2GPI) n (%) | 65 (33.3) | 10 (11.1) | <10^-3 |
| aCL (IgG, IgA or IgM) n (%) | 25 (12.8)^a | 5 (5.6) | 0.06 |
| aCL-IgG n (%) | 10 (5.1) | 2 (2.2) | NS |
| aCL-IgA n (%) | 3 (1.5) | 2 (2.2) | NS |
| aCL-IgM n (%) | 16 (8.2) | 4 (4.4) | NS |
| aβ2GPI (IgG, IgA or IgM) n (%) | 57 (29.2)^a | 10 (11.1) | <10^-3 |
| aβ2GPI-IgG n (%) | 8 (4.1)^a | 3 (3.3) | NS |
| aβ2GPI-IgA n (%) | 45 (23.1)^b,c | 7 (7.8) | 0.002 |
| aβ2GPI-IgM n (%) | 15 (7.7)^c | 4 (4.4) | NS |
| Only aβ2GPI-IgA n (%) | 31 (15.9) | 3 (3.3) | 0.002 |

^aComparison between aCL (IgG, IgA or IgM) and aβ2GPI (IgG, IgA or IgM) in autoimmune thyroid disease (p < 10^-3).

^bComparison between aβ2GPI-IgA and aβ2GPI-IgG in autoimmune thyroid disease (p < 10^-3).

^cComparison between aβ2GPI-IgA and aβ2GPI-IgM in autoimmune thyroid disease (p < 10^-3).

The bold values indicates statistically significance of p values.

3.1 | Frequencies of antiphospholipid antibodies in autoimmune thyroid diseases

aCL and aβ2GPI frequencies in patients with AITD were summarized in Table 2. Compared to HBD, aPL, aβ2GPI (IgG, IgA or IgM) and aβ2GPI-IgA were significantly more frequent in patients with AITD (33.3% vs 11.1%, p < 10^-3, 29.2% vs 11.1%, p < 10^-3 and 23.1% vs 7.8%, p = 0.002, respectively).

In patients, the frequency of aβ2GPI (29.2%) was significantly higher than that of aCL (12.8%) (p < 10^-3). The IgA isotype of aβ2GPI (23.1%) was predominant compared to IgG (4.1%) and IgM (7.7%) (Table 2).

Among 65 AITD patients with aPL, 31 had only IgA isotype of aβ2GPI, while three healthy blood donors had isolated aβ2GPI-IgA (15.9% vs 3.3%, p = 0.002).

3.2 | Frequencies of antiphospholipid antibodies in Hashimoto’s thyroiditis

aPL and aβ2GPI were significantly more frequent in HT patients than in the control group (38.1% vs 11.1%, p < 10^-3 and 34.5% vs 11.1%, p < 10^-3). In patients with HT, aβ2GPI (34.5%) were significantly more frequent than aCL (13.6%) (p < 10^-3). The IgA isotype of aβ2GPI (27.3%) was predominant compared to IgG (5%) and IgM (8.6%). In HT patients, isolated aβ2GPI-IgA (19.4%) was significantly more frequent than in the control group (3.3%) (p < 10^-3) (Table 3).

3.3 | Frequencies of antiphospholipid antibodies in Hashimoto’s thyroiditis according to gender

In females, aPL, aβ2GPI and aβ2GPI-IgA were more frequent in HT patients than in the control group (36.3% vs 8.9%, p = 0.0017; 31.8% vs 11.1%, p = 0.002).
Frequency of aPL according to gender in Hashimoto's thyroiditis

| Autoantibodies | Hashimoto's thyroiditis (n = 139) | Control group (n = 90) | p       |
|----------------|----------------------------------|------------------------|---------|
| aPL (aCL or a2GPI) n (%) | 53 (38.1) | 10 (11.1) | <10^-3 |
| aCL (IgG, IgA or IgM) n (%) | 19 (13.6)^a | 5 (5.6) | 0.05   |
| aCL-IgG n (%) | 6 (4.3) | 2 (2.2) | NS     |
| aCL-IgA n (%) | 0 (0) | 2 (2.2) | NS     |
| aCL-IgM n (%) | 15 (10.7) | 4 (4.4) | NS     |
| a2GPI-IgG n (%) | 48 (34.5)^a | 10 (11.1) | <10^-3 |
| a2GPI-IgA n (%) | 7 (5)^b | 3 (3.3) | NS     |
| a2GPI-IgA n (%) | 38 (27.3)^b,c | 7 (7.8) | <10^-3 |
| a2GPI-IgM n (%) | 12 (8.6)^c | 4 (4.4) | NS     |
| Only a2GPI-IgA n (%) | 27 (19.4) | 3 (3.3) | <10^-3 |

^a in Hashimoto's thyroiditis aCL (IgG, IgA or IgM) vs. a2GPI (IgG, IgA or IgM) p < 10^-3.
^b in Hashimoto's thyroiditis a2GPI-IgA vs. a2GPI-IgG p < 10^-4.
^c in Hashimoto's thyroiditis a2GPI-IgA vs. a2GPI-IgM p < 10^-3.
The bold values indicates statistically significance of p values.

The frequency of aPL was higher in females than in males, the same result was found (Table 4).

3.4 | Comparison of frequencies of antiphospholipid antibodies between Hashimoto's thyroiditis and Graves' disease

Compared to the control group, the frequency of aPL was higher in GD patients but the difference was not significant (21.4% vs 11.1%, p = 0.09).

aPL, a2GPI and a2GPI-IgA were significantly more frequent in HT patients than in GD patients (38.1% vs 21.4%, p = 0.025; 34.5% vs 16.1%, p = 0.01 and 27.3% vs 12.5%, p = 0.02, respectively). Compared to HT, aCL-IgA was significantly more frequent in GD (5.3% vs 0%, p = 0.02).

The isolated presence of a2GPI-IgA was higher in HT than in GD (19.4% vs 7.1%, p = 0.03) (Table 5).

4 | DISCUSSION

The current study demonstrated a higher frequency of aPL in AITD patients compared to healthy population (33.3% vs 11.1%, p < 10^-3). Few studies have evaluated only aCL-IgG and aCL-IgM frequencies while, to our knowledge, aCL-IgA and a2GPI have never been determined in patients with AITD.6–12 We found that frequencies of a2GPI and a2GPI-IgA were significantly higher in AITD patients than in healthy subjects (29.2% vs 11.1%, p < 10^-3 and 23.1% vs 7.8%, p = 0.002, respectively). In agreement with our previous studies on several autoimmune diseases (celiac disease, primary biliary cholangitis, APS, systemic lupus erythematosus, rheumatoid arthritis),13,14,16–18 we have observed a predominance of the IgA isotype of a2GPI in AITD patients (Table 6). Moreover, our patients are of African origin and it has been reported that IgA was the predominant isotype of a2GPI in Afro-Americans.19 Interestingly, 15.9% of patients with AITD had only a2GPI-IgA. IgA a2GPI has gained relevance in recent years. Better sensitivity of IgA a2GPI compared to IgM a2GPI and IgM/IgG aCL has been established for the diagnosis of APS.20 Hu et al.21 have shown that a2GPI-IgA provides added value in the diagnosis of APS. Moreover, several current evidences demonstrated the role of a2GPI-IgA in APS from a clinical point of view.22

aCL frequency was not significantly higher in AITD than in HBD. The same result was found by Diez et al.,8 who demonstrated the absence of association between aCL and AITD. In our HT patients, the frequency of aCL was lower than that of Osundeko et al.11 (13.6% and 21%, respectively). This discrepancy between results could be
explained by the sample size of patients. Indeed, we had 139 patients compared to 19 in the study of Osundeko et al. In addition, in the present study, the frequency of aCL was higher in HT patients than in healthy subjects but reached a borderline significance (13.6% vs 5.5%, p = 0.05). Similarly, Petri et al. and Diez et al. demonstrated that aCL frequency was not significantly different between patients with HT and control group.

### 4.1 Why are the aβ2GPI synthesized in HT?

1. aβ2GPI is a ubiquitous protein, which is expressed, among other organs, in the thyroid gland. Indeed, aβ2GPI has been identified as a megalin ligand, expressed on the apical surface of thyroid epithelial cells, directly facing the lumen of the follicle. Dimeric aβ2GPI can interact with different members of the low-density lipoprotein receptor family, including megalin. Megalin plays a key role in organ development and function and it serves as a thyroidoglobulin receptor and can mediate thyroglobulin endocytosis. HT is characterized by an autoimmune attack mediated by helper T cells (Th1, Th2 and Th17). Th1 produces cytokines, which activate cytotoxic T cells inducing, therefore, thyrocyte apoptosis. IL-17 released by Th17 lymphocytes stimulates fibroblasts, epithelial cells and macrophages contributing to an inflammatory state. During inflammation, the activated aβ2GPI is transformed from circular to open hockey stick conformation and this change induces aβ2GPI synthesis.

2. In HT, Th2 cells stimulate B lymphocytes and, therefore, antibodies synthesis against thyroid autoantigens. The immune complexes formed induce complement activation, thus allowing thyrocyte apoptosis. On the other hand, aβ2GPI have several roles in both complement cascade and mechanism of coagulation. It is considered as a scavenger protein that removes apoptotic cells, dead cells and immune complexes from the circulation. aβ2GPI also mediates the clearance of inflammatory cellular debris by macrophages. So, the synthesis of aβ2GPI can occur via dysregulated clearance of apoptotic bodies associated with aβ2GPI.

3. An association between the infectious history of patients and the presence of autoimmune diseases has been described. Thus, molecular mimicry between microbes and certain autoantigens could be responsible for the triggering of an autoimmune response. It is generally recognized that environmental factors contributing to the development of HT include bacterial and viral (enterovirus) infections. Moreover, it has been reported a possible relationship between aβ2GPI and infections. Indeed, serum aβ2GPI-IgA levels may experience a transient increase during multiple infections. Hepatitis C is described to be associated with endocrine disorders. Interestingly, we have previously reported a higher frequency of aβ2GPI-IgA in chronic hepatitis C patients compared to healthy controls.

### Table 5 Frequency of aCL and aβ2GPI in patients with Hashimoto’s thyroiditis and in patients with Grave’s disease

| Autoantibodies          | Hashimoto’s thyroiditis (n = 139) | Grave’s disease (n = 56) | p     |
|-------------------------|-----------------------------------|-------------------------|-------|
| aPL (aCL or aβ2GPI) n (%) | 53 (38.1)                         | 12 (21.4)               | 0.025 |
| aCL (IgG, IgA or IgM) n (%) | 19 (13.6)                         | 6 (10.7)                | NS    |
| aCL-IgG n (%)           | 6 (4.3)                           | 4 (7.1)                 | NS    |
| aCL-IgA n (%)           | 0 (0)                             | 3 (5.3)                 | 0.02  |
| aCL-IgM n (%)           | 15 (10.7)                         | 1 (1.8)                 |       |
| aβ2GPI (IgG, IgA or IgM) n (%) | 48 (34.5)                        | 9 (16.1)                | 0.01  |
| aβ2GPI-IgG n (%)        | 7 (5)                             | 1 (1.8)                 | NS    |
| aβ2GPI-IgA n (%)        | 38 (27.3)                         | 7 (12.5)                | 0.02  |
| aβ2GPI-IgM n (%)        | 12 (8.6)                          | 3 (5.3)                 | NS    |
| Only aβ2GPI-IgA n (%)   | 27 (19.4)                         | 4 (7.1)                 | 0.03  |

The bold values indicates statistically significance of p values.

### Table 6 Predominance of aβ2GPI-IgA in our previous studies

| Authors              | Autoimmune diseases           | aβ2GPI-IgA (%) | aβ2GPI-IgA (%) | aβ2GPI-IgA (%) |
|----------------------|------------------------------|---------------|---------------|---------------|
| Mankaï et al.        | Celiac disease               | 1.6           | 14.3          | 1.6           |
| Mankaï et al.        | Primary biliary cholangitis  | 12.5          | 62.5          | 21.2          |
| Mankaï et al.        | Antiphospholipid syndrome    | 22            | 83.1          | 1.2           |
| Mankaï et al.        | Systemic lupus erythematosus | 19.8          | 50.9          | -             |
| Melayah et al.       | Rheumatoid arthritis         | 6.7           | 26.7          | 5.6           |

The bold values indicates statistically significance of p values.
described to be frequent in Tunisia 3.32%, so, could we imagine that the high frequency found in the present study is due to hepatitis C virus?

4. In addition to environmental triggers of HT, the microbiota has been described as involved in thyroid autoimmunity. Microbiome studies have clearly shown that gut microbiota composition can discriminate between healthy population and patients with HT and GD, correlating with disease stage, anti-thyroid antibodies titers and response to treatment. In addition, the microbiome may be the source of autoantigens that induce an autoreactive T cell response. Ruff et al. hypothesized that human autoimmunity can be triggered by the persistent presence of microbiota mimotopes via a cross-reactive mechanism. In order to test the cross-reactivity of commensal mimotopes, the authors used APS as a model of systemic autoimmunity with well-defined autoepitopes. Thus, it is possible that commensal bacteria could induce breakdowns of tolerance and induce anti-β2GPI synthesis in genetically predisposed individuals.

This study has some limitations. The control group, on one hand was not sex-matched with patient’s group and on the other hand, did not benefit from biological investigation looking for a possible AITD. The number of GD patients was smaller than that of HT.

In conclusion, this is the first study evaluating the frequency of anti-β2GPI in AITD. Anti-β2GPI and particularly anti-β2GPI-IgA were frequent in AITD patients. We provided elements explaining the synthesis of these antibodies especially in HT. Further and prospective studies are needed to investigate the involvement of these antibodies on the clinical manifestations of HT.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
Data available on request from the authors.

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