Autoantibody responses in autoimmune ovarian insufficiency and in Addison's disease are IgG1 dominated and suggest a predominant, but not exclusive, Th1 type of response

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Abstract

Objective: Steroid-producing cell autoantibodies (SCAs) directed against 21-hydroxylase autoantibodies (21OHAbs), 17α-hydroxylase autoantibodies (17OHAb), and cholesterol side-chain cleavage enzyme (side-chain cleavage autoantibodies, P450sccAb) characterize autoimmune primary ovarian insufficiency (SCA-POI). The aim of the study was to analyze IgG subclass specificity of autoantibodies related to adrenal and ovarian autoimmunity.

Design: We studied 29 women with SCA-POI, 30 women with autoimmune Addison's disease (AAD) without POI, and 14 patients with autoimmune polyendocrine syndrome type 1 (APS1). 21OHAb isotypes were also analyzed in 14 subjects with preclinical AAD. Samples from 30 healthy women served as control group to determine the upper level of normality in the isotype assays.

Methods: Immunoradiometric assays with IgG subclass-specific secondary antibodies.

Results: In 21OHAb-positive sera, IgG1 isotype was detected in 90% SCA-POI and non-POI AAD sera and 67% APS1 patients. IgG1 isotype was found in 69% 17OHAb-positive SCA-POI and 100% 17OHAb-positive APS1 sera, and in 60% P450sccAb-positive SCA-POI and 80% P450sccAb-positive APS1 sera. For 21OHAb, IgG4 isotype was detected in 17% SCA-POI, 7% non-POI AAD, and 8% APS1 sera. None of the 17OHAb-positive sera was positive for IgG4. In P450sccAb-positive sera, 15% POI and 20% APS1 sera were positive for IgG4. Two 21OHAb-positive SCA-POI (7%), one 21OHAb-positive AAD (3%), three P450sccAb-positive SCA-POI (15%), and two P450sccAb-positive APS1 (20%) sera were positive for IgG4, in the absence of IgG1. All preclinical AAD sera resulted as positive for IgG1-21OHAb, but not for IgG4-21OHAb.

Conclusions: The autoantibody responses in POI and AAD are IgG1 dominated, which suggests a predominant Th1 response. Selective IgG4 isotype specificity identified a small subset of patients with Th2-oriented response.

Introduction

Approximately 4–5% of women with primary ovarian insufficiency (POI), as defined by hypergonadotropic hypogonadism with hypoestrogenism and infertility before the age of 40 years, are positive for autoantibodies directed against steroidogenic cytochrome P450 enzymes, such as 21-hydroxylase autoantibodies (21OHAbs), 17α-hydroxylase autoantibodies (17OHAbs), and cholesterol side-chain cleavage enzyme (side-chain cleavage autoantibodies, P450sccAb) (steroid cell autoantibodies, SCAs) (1, 2). Positivity for these autoantibodies identifies POI due to autoimmunity to steroid-producing cells (SCA-POI) (1–8).

SCA-POI is almost invariably associated with biochemical signs of adrenal autoimmunity and with clinical or preclinical autoimmune Addison’s disease (AAD) (2, 4). In the absence of 21OHAb, the major immune marker of AAD, no SCAs are typically detected in women with POI (2), and no signs of autoimmune oophoritis are present at histology (3, 4). Accordingly, 21OHAbs are markers at the highest diagnostic
sensitivity for SCA-POI (4), even higher than that of either ovarian autoantibodies detected by indirect immunofluorescence or other autoantibodies to steroid-producing cells, such as 17OHAb and P450sccAb. No diagnosis of autoimmune POI can currently be formulated in the absence of 21OHAb, and concomitance with other autoimmune diseases is not a sufficient criterion to diagnose SCA-POI (2–4).

AAD results from the autoimmune destruction of the adrenal cortex, and is characterized by the appearance of circulating 21OHAb (9). Genetic predisposition for AAD is modulated by polymorphisms of genes influencing the function of the immune system, such as HLA class II genes, CTLA4, CIITA, PTPN22, and others (10). Though 17OHAb and P450scCAb can be detected in a fraction of patients with AAD, these markers are more frequently present in sera from patients with autoimmune polyendocrine syndrome type 1 (APS1) or in women with adrenal insufficiency and autoimmune oophoritis (1, 2, 11, 12).

Pathophysiology of ovarian insufficiency due to SCA-POI is characterized by the selective mononuclear cell infiltration of large, antral follicles (3, 4) and the selective autoimmune destruction of theca cells with concomitant preservation of granulosa cells (13, 14). In most cases, women with SCA-POI have a preserved pool of functioning follicles as demonstrated by normal serum concentrations of anti-Müllerian hormone (15).

Very little knowledge is currently available on the molecular mechanisms responsible for autoimmune-mediated ovarian or adrenal insufficiency. The autoimmune destruction of ovarian theca cells and adrenal cortex is thought to be a T-cell-mediated process (3, 9), but the molecular targets of autoreactive T-cells are not yet known. The characterization of the autoantibody isotype may provide indirect information on the immune pathways responsible for the ovarian and adrenal damage. This approach has extensively been used in other autoimmune diseases, such as type 1 diabetes mellitus (T1DM), in which the predominant IgG1 isotype of the related autoantibodies has been interpreted as a sign of Th1 type of immune response (16–18). An early study of adrenal cortex autoantibodies found these to be IgG1, IgG2, and IgG4, by using indirect immunofluorescence (19). A more recent study of a small number of AAD patients, in which western blotting technique was used, revealed the predominance of the IgG1 isotype among 21OHAb and P450scCAb (20). Also, P450scCAb associated with APS1 have been found to be of the IgG1 isotype in another study (21). Nonetheless, no information in this regard is currently available in women with SCA-POI or in subjects with preclinical AAD.

In the present study, we analyzed the IgG subclasses of 21OHAb, 17OHAb, and P450scCAb in women with SCA-POI, in women with AAD without clinical or biochemical signs of POI (non-POI AAD patients) and in APS1 patients, by using a novel immunoradiometric procedure. We also performed autoantibody isotyping in a group of subjects with preclinical AAD, identified by 21OHAb positivity in a large screening of patients with organ-specific autoimmune diseases.

Materials and methods

Patients and control subjects

The study design included 59 women with AAD, of whom 29 with SCA-POI and 30 without ovarian insufficiency, and 14 patients with APS1, all were positive for 21OHAb and/or 17OHAb/P450scCAb. Sera from 14 subjects were positive for 21OHAb, and subjects with preclinical AAD were also included in the study. The clinical characteristics of the patients studied are shown in Table 1.

Women with SCA-POI (all with 46, XX karyotype) were identified by the onset of clinical and biochemical signs of POI before the age of 40 years and concomitant positivity for autoantibodies to steroidogenic enzymes; they represented the entire population of women with SCA-POI identified by the Italian Addison network (IAN) from April 1998 to September 2009. Menarche occurred between 10 and 13 years, and blood sampling for the present study was taken 0–19 years

Table 1 Characteristics of the patients studied. Age at diagnosis of primary ovarian insufficiency in SCA-POI patients and of adrenal insufficiency in AAD and APS1 patients is indicated as medians (range). Disease duration after diagnosis of POI in SCA-POI patients and of AAD in AAD and APS1 patients is indicated as medians (range).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Female/male ratio</th>
<th>Age at diagnosis (years)</th>
<th>Disease duration at blood sampling (years)</th>
<th>21OHAb</th>
<th>17OHAb</th>
<th>P450scCAb</th>
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<tr>
<td>SCA-POI</td>
<td>29</td>
<td>29/0</td>
<td>33 (21–38)</td>
<td>6 (0–19)</td>
<td>29/29</td>
<td>13/29</td>
<td>20/29</td>
</tr>
<tr>
<td>Non-POI AAD</td>
<td>30</td>
<td>30/0</td>
<td>36 (17–66)</td>
<td>4 (0–20)</td>
<td>30/30</td>
<td>2/30</td>
<td>2/30</td>
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<tr>
<td>APS1</td>
<td>14</td>
<td>13/1</td>
<td>42 (21–61)</td>
<td>9 (5–17)</td>
<td>12/14</td>
<td>8/14</td>
<td>10/14</td>
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<td>13/1</td>
<td></td>
<td></td>
<td>NA</td>
<td>1/14</td>
<td>1/14</td>
</tr>
</tbody>
</table>

NA, not applicable; AAD, autoimmune Addison's disease; APS1, autoimmune polyendocrine syndrome type 1; POI, primary ovarian insufficiency; SCA-POI, autoimmune primary ovarian insufficiency due to steroidogenic cell autoimmunity; 21OHAb, 21-hydroxylase autoantibodies; 17OHAb, 17α-hydroxylase autoantibodies; P450scCAb, side-chain cleavage autoantibodies.

*Including three patients with SCA-POI.

*Age at diagnosis of preclinical AAD subjects was age at blood sampling for 21OHAb assay.

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(median 7 years) after diagnosis of POI. All the women with SCA-POI had clinical AAD, and were treated with substitutive doses of cortisone acetate and fludrocortisone. Out of the 29 patients, 19 (66%) were affected by other autoimmune diseases, including thyroid autoimmune diseases, T1DM, vitiligo, chronic atrophic gastritis, and rheumatoid arthritis.

The 30 AAD women with neither clinical nor biochemical signs of POI were consecutively recruited by the IAN between April 2008 and September 2009. AAD patients formed a control group for the SCA-POI group. APS2 was diagnosed in 18/30 (60%) patients.

The 14 APS1 patients (seven males and seven females, three of whom suffering from SCA-POI) enrolled in the study represented the entire population of APS1 subjects recruited by the IAN from April 1998 to September 2009.

Subjects with preclinical AAD were identified by screening over 1300 patients with organ-specific autoimmune diseases such as thyroid autoimmune diseases, T1DM, celiac disease, and vitiligo. Serum samples from 14 subjects were positive for 21OHAb, with normal baseline levels of cortisol and showing no clinical signs of adrenal insufficiency, consecutively recruited at the University of Perugia between April 1999 and June 2009, were assayed to determine the related IgG subclasses. Out of these 14 21OHAb-positive individuals, increased plasma renin activity was detected in 10 (71%), a subnormal response to the synthetic ACTH test in six (43%) and an increased basal ACTH plasma concentration in two (14%). Five out of the 14 (36%) subjects with preclinical AAD developed clinical signs of primary adrenal insufficiency during a 3- to 9.5-year follow-up period. In three subjects, consistently positive for 21OHAb, a follow-up serum sample was available after 8.5–10.5 years of disease-free time.

Serum samples from 30 healthy women (age 19–59 years, median 33 years) served as control group to determine the upper level of normality in each autoantibody subclass assay. The study was approved by the local ethics committee of Umbria region. All patients gave their written informed consent to be enrolled in the study.

**SCA assays**

21OHAb, 17OHAb, and P450sccAb were measured using radiobinding assays with recombinant human 21-hydroxylase, 17α-hydroxylase, and cholesterol side-chain cleavage enzyme radiolabeled with 35S, as described previously (22, 23). Briefly, in each assay, in vitro translated recombinant human autoantigen was immunoprecipitated with human serum at a 1:25 dilution, and immunocomplexes were separated using protein A-Sepharose. The immunoprecipitated radioactivity was analyzed in a liquid scintillation counter, and results were expressed as a relative index using a positive standard and two negative standard sera. The upper level of normality of each index had been previously established by testing sera from over 200 healthy control subjects (22, 23), and was 0.06 for 21OHAb, 0.08 for 17OHAb, and 0.06 for P450sccAb respectively.

The full-length cDNAs for human 17α-hydroxylase (24) and for human cholesterol side-chain cleavage enzyme (25) were donated by Dr Walter L Miller, Department of Pediatrics and Metabolic Research Unit, University of California, San Francisco, CA, USA.

**Isotype-specific RIAs for SCAs**

Isotype-specific RIAs for 21OHAb, 17OHAb, and P450sccAb were performed using modifications of a method described by Bonifacio et al. for islet autoantibodies (16). Briefly, Sepharose 4B-streptavidin beads (Zymed, San Francisco, CA, USA) were washed twice with ice-cold PBS and incubated overnight at 4°C on a rotating platform with 4 μg of biotinylated mouse anti-human IgG1 (BD Biosciences Pharmingen San Diego, CA, USA) cat. 555869), IgG2 (BD Biosciences Pharmigen cat. 555874), IgG3 (Zymed, cat. 05364), or IgG4 (BD Biosciences Pharmingen cat. 555879), alternatively. Biotinylated mouse anti-rat IgM (BD Biosciences Pharmingen cat. 550330) was used to correct for nonspecific binding. Beads were washed twice in PBS and once in assay buffer (50 mM Tris, 150 mM NaCl, and 1% Tween, pH 7.4) to remove the unbound biotinylated antibody and resuspended in assay buffer. In preliminary experiments, the biotinylated antibodies had been titrated to find out the optimal coating concentration.

In total, 0.5 μl of each serum sample was incubated with 25 000 c.p.m. of [35S]-methionine-labeled in vitro transcribed/translated autoantigen in 25-μl assay buffer overnight on a rotating platform at 4°C before the addition of antibody-coated beads suspension, incubation for 1 h at room temperature, washing, and counting. Results of duplicate samples were expressed as S.D. SDS: ((IgG subclass counts Δ c.p.m. − mean Δ c.p.m. of control subjects)/S.D. Δ c.p.m. of control subjects), where Δ c.p.m., IgG subclass c.p.m. − anti rat IgM c.p.m. Serum samples from 30 healthy women were used as a control group to define the upper level of normality. Samples with SDS > 3 were considered as positive.

**Statistical analysis**

Differences in the frequency of positivity among IgG subclasses of each autoantibody were tested by either the χ² test with Yates’ correction or Fisher’s exact test. Differences in autoantibody levels were tested with the nonparametric Mann–Whitney U test. A P value <0.05 was considered statistically significant. All calculations were carried out with SPSS release 17.0, SPSS Inc., Chicago, IL, USA, 2008.

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Venn diagrams were generated by Venn diagram plotter software release 1.3.250.34910, 2008, developed by Pacific Northwest National Laboratory, Richland, WA, USA.

Results

Prevalence of SCAs

Frequencies of SCAs in the patient groups are reported in Table 1. More specifically, 21OHAbs were detected in the sera from all patients included in the SCA-POI, non-POI AAD, and preclinical AAD groups and in 12/14 (86%) APS1 patients. 17OHAbs were detected in 13/29 (45%) SCA-POI sera and in 8/14 (57%) APS1 sera. Finally, P450sccAbs were present in 20/29 (69%) SCA-POI sera and in 10/14 (71%) APS1 sera.

By definition, the presence of 21OHAb in the sera from subjects with autoimmune diseases other than overt AAD identified the patients enrolled in the preclinical AAD group.

Among women with non-POI AAD, 17OHAb and P450sccAb were separately detected in two cases; in the same way, these autoantibodies were separately positive in one female subject with preclinical AAD. Because of such a limited number of positive samples, analysis of IgG subclasses of 17OHAb and P450sccAb was not performed in these cases.

No correlation between 17OHAb or SCCAb levels and age at disease onset or age at the time of blood sampling was detected either in SCA-POI or AAD.

IgG subclasses of SCAs in women with SCA-POI

21OHAb-IgG subclasses  IgG1-21OHAb was detected in 26/29 (90%) sera of patients with SCA-POI (SDS range: 3.78–121.52; Fig. 1; P<0.001 versus all other 21OHAb-IgG subclasses). IgG2-21OHAb was detected in 4/29 (14%) sera (SDS range: 4.46–12.64), and IgG4-21OHAb was detected in 5/29 (17%) sera (SDS range: 3.33–4.83). No SCA-POI serum was found positive for IgG3-21OHAb (Fig. 1). As displayed in Fig. 2, in 19 SCA-POI sera, 21OHAbs were exclusively of the IgG1 isotype. All the four cases positive for IgG2-21OHAb were also positive for IgG1-21OHAb and negative for IgG4-21OHAb. On the other hand, IgG1-21OHAb was detected in three of the five sera positive for IgG4-21OHAb, while in the remaining two cases 21OHAbs were exclusively of the IgG4 isotype (Fig. 2).

In one serum of this group (3%), the SDS of all IgG subclass assays was below the cut-off value, possibly because of low autoantibody titer.

17OHAb-IgG subclasses  Among the 13 sera of the patients with SCA-POI positive for 17OHAb, nine (69%) were positive in the IgG1 isotype assay (SDS range: 3.56–40.56; P<0.001 versus all other 17OHAb-IgG subclasses). In this group, one IgG1-17OHAb-positive serum was also positive for IgG2-17OHAb (SDS: 3.06), while no SCA-POI serum was found positive for either IgG3-17OHAb or IgG4-17OHAb (Fig. 1).

In four 17OHAb-positive sera (31%), the SDS of all IgG subclass assays did not reach the cut-off value.

P450sccAb-IgG subclasses  IgG1 isotype autoantibodies were detected in 12/20 (60%) P450sccAb-positive SCA-POI sera (SDS range: 3.04–20.39; Fig. 1; P=0.025 versus IgG2 and IgG4 isotypes; P<0.001 versus IgG3 isotype). Three sera (15%) tested positive for IgG2-P450sccAb (SDS range: 3.58–6.88). Similarly, IgG4-P450sccAb were detected in three sera (15%; SDS range: 5.11–32.32; Fig. 1). No SCA-POI serum was positive for IgG3-P450sccAb.

In the P450sccAb-positive sera of the women with SCA-POI, no serum positive for either IgG2-P450sccAb or IgG4-P450sccAb was also positive for IgG1-P450sccAb. On the other hand, one serum was

Figure 1  SDS of isotype-specific RIAs for 21OHAb, 17OHAb, and P450sccAb in patients with SCA-POI, APS1, or AAD without POI. Bars indicate the upper level of normality in each assay.
simultaneously positive for IgG2-P450sccAb and IgG4-P450sccAb (Fig. 2).

In three sera positive for P450sccAb (15%), the SDS resulting from the assays of the four IgG subclasses were below the cut-off value.

No correlation between subclass-specific autoantibody levels and age at disease onset or age at the time of blood sampling was detected in SCA-POI, for any tested autoantibody specificity.

IgG subclasses of 21OHAb in patients with non-POI AAD

IgG1-21OHAb was detected in 27/30 (90%) sera of non-POI AAD patients (SDS range: 3.25–135.52; Fig. 1; P < 0.001 versus all other 21OHAb IgG subclasses). Two sera (7%) were positive for IgG2-21OHAb (SDS range: 3.15–11.27), and two sera (7%) were positive for IgG4-21OHAb (SDS range: 8.65–9.87; Fig. 1). No serum was found positive for the IgG3 isotype.

In this group, 21OHAbs were exclusively of the IgG1 isotype in 26 sera (87%). One serum was simultaneously positive for IgG1-21OHAb, IgG2-21OHAb, and IgG4-21OHAb (3%). On the other hand, one serum resulted positive for both IgG2-21OHAb and IgG4-21OHAb: in this serum, IgG1-21OHAb was not detected (Fig. 2).

In two sera positive for 21OHAb (7%), no IgG subclass SDS resulted over the cut-off value.

No correlation between subclass-specific 21OHAb levels and age at disease onset or age at the time of blood sampling was detected in non-POI AAD.

IgG subclasses of SCAs in APS1 patients

21OHAb-IgG subclasses IgG1-21OHAb were detected in nine of the 12 (75%) positive sera from APS1 patients (SDS range: 3.53–23.3; P = 0.02 versus IgG2 and IgG4 isotypes, P = 0.003 versus IgG3 isotype). In one of the nine IgG1 21OHAb-positive sera, both IgG2 (SDS: 5.03) and IgG4 (SDS: 4.96) isotypes were also detected. No APS1 serum was found positive for IgG3-21OHAb (Fig. 1). The sera from all the three women with POI were exclusively positive for the IgG1 isotype.

In three 21OHAb-positive sera (25%), no related IgG subclass reached the cut-off value.

No correlation between subclass-specific 21OHAb levels and age at disease onset or age at the time of blood sampling was detected in non-POI AAD.

17OHAb-IgG subclasses All the eight sera positive for 17OHAb from patients with APS1 tested positive in the IgG1 isotype assay (SDS range: 3.41–27.26; P < 0.002 versus IgG3 and IgG4 isotypes); two of these sera (33%) were also positive at the IgG2 isotype assay (SDS range: 5.86–13.43; P = NS). No APS1 serum was found positive for either IgG3-17OHAb or IgG4-17OHAb (Fig. 1). As well as 21OHAb, the sera from all the three APS1 women with POI were exclusively positive for the IgG1 isotype.

P450sccAb-IgG subclasses IgG1 isotype was detected in eight of 10 (80%) P450sccAb-positive sera from APS1 patients (SDS range: 3.06–25.30; Fig. 1; P < 0.005 versus all other P450sccAb-IgG subclasses). Two sera (20%) were exclusively positive for IgG4-P450sccAb (SDS: 5.86–6.39; Fig. 2). No serum in this group resulted positive for either IgG2-P450sccAb or IgG3-P450sccAb. Two of the three sera from women with APS1 and SCA-POI were P450sccAb positive: in one case, the specificity belonged to the IgG1 isotype, while in the other case, it was of the IgG4 isotype.

IgG subclasses of 21OHAb in subjects with preclinical AAD

All the 14 sera from subjects with preclinical AAD tested positive in the IgG1 isotype assay (SDS range: 3.1–79.4; P < 0.001 versus all other 21OHAb-IgG subclasses). IgG2-21OHAb were detected in two sera (14%; SDS range: 4.35–16.9), while no serum was found positive for the IgG3 isotype. All three follow-up sera collected after 8.5–10.5 years of disease-free time resulted exclusively positive for IgG1-21OHAb (SDS range: 3.2–83.4), one of them having lost its concomitant positivity for the IgG2 isotype (Table 2).

IgG1-21OHAb levels were significantly higher among the five subjects who later developed clinical AAD than among the nine subjects who did not progress toward overt clinical disease during the follow-up period (P = 0.014).
Table 2 21-Hydroxylase autoantibody (21OHAb) subclasses in subjects with preclinical AAD.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age at blood sampling (years)</th>
<th>Duration of follow-up (years)</th>
<th>SDS IgG1</th>
<th>SDS IgG2</th>
<th>SDS IgG3</th>
<th>SDS IgG4</th>
<th>Progression to clinical AAD</th>
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<tr>
<td>1</td>
<td>F</td>
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AAD, autoimmune Addison’s disease; +, SDS > 3.0.

*Follow-up serum. Positive values are shown in bold character.

Discussion

In this study, we describe the development of novel radiobinding assays for the analysis of isotype-specific autoantibodies to steroidogenic enzymes. By means of these assays, we demonstrated the predominant expression of the IgG1 isotype in sera from SCA-POI, non-POI AAD, and APS1 patients, which suggests a Th1 type of immune response. In a small number of patients, a Th2 profile was suggested by the presence of IgG4 isotype autoantibodies in the absence of those of the IgG1 isotype. 21OHAbs detected in the sera of subjects with preclinical AAD were almost exclusively of the IgG1 isotype. The finding of IgG1-21OHAb several years before the appearance of clinical signs of disease demonstrates that production of these isotype-specific autoantibodies is an early event in the natural history of autoimmunity to steroid-producing cells. Their presence does not necessarily imply an evolution of the autoimmune process toward clinically overt disease, as IgG1-21OHAb was likewise found in the sera of subjects who did not develop clinical AAD after more than 10 years of follow-up. In our preclinical AAD group, an exclusive Th1 type of IgG subclass autoantibodies was observed both at baseline and in follow-up samples, in the sera from five individuals who later developed clinical signs of adrenal insufficiency (destructive adrenalitis) and in nine individuals who did not progress to overt AAD during the follow-up (nondestructive adrenalitis). These results show that selection of IgG1-21OHAb accompanies the autoimmune process irrespective of the type of inflammatory reaction (destructive versus nondestructive adrenalitis).

Radiobinding assays enable a more accurate and quantitative analysis of autoantibodies as compared with other methods, such as immunoblotting or immunofluorescence. In this sense, our isotype-specific autoantibody assays, developed by modification of a similar assay for detection of islet autoantibodies (16), may represent an improvement on methods, such as immunofluorescence or western blotting, which have been employed in previous studies (19–21). The use of a radiobinding approach enables future prospect of its standardization and application in clinical research and practice. It has, however, to be underlined that in the present study, no clear positivity for any of the IgG subclasses could be demonstrated in some sera positive for 21OHAb or 17OHAb or P450sccAb, which shows that isotype-specific assays may be associated with a somehow lower diagnostic sensitivity than commonly used autoantibody assays.

In agreement with previous reports on autoantibodies in AAD/APS1 (19–21, 26) and other endocrine autoimmune diseases (16–18, 27, 28), we confirm the predominant expression of the IgG1 subclass for autoantibodies to steroidogenic enzymes. In addition, we provide what is, to the best of our knowledge, the first line of evidence that, in women with SCA-POI, the distribution of the isotype-specific autoantibodies follows a similar pattern.

Both AAD and SCA-POI are considered as the result of an autoimmune, T-cell-mediated destructive inflammation with imbalance of the Th1/Th2 immune responses (3, 9). Although the Th1 paradigm has been challenged for human organ-specific autoimmune diseases by some studies (29), and direct measurement of Th1 and Th2 cytokines in the serum has not shown any significant difference between patients with autoimmune endocrinopathies and healthy subjects (30), the prevalent selection of steroidogenic enzyme autoantibodies of the IgG1 subclass is in line with the hypothesis of an antigen-driven, T-cell-dependent type of antibody response, and implies a Th1 profile (31, 32).
On the contrary, an autoantibody selection of the IgG4 subclass suggests a Th2 profile (33).

Several lines of evidence support the idea that SCAs have no direct pathogenetic role. First, 21OHAbs are detected in ~0.5–1.0% of healthy subjects who do not necessarily progress toward overt adrenal insufficiency (7, 9). Secondly, the transplacental crossing of adrenal autoantibodies in a mother with AAD does not determine any sign of preclinical or clinical adrenal insufficiency in the newborn (34). Finally, no biochemical signal of reduced 21-hydroxylase activity can be demonstrated in vivo during the natural history of the disease (35, 36).

Although autoantibodies to adrenal and ovarian targets do not possibly have a direct pathogenic action, they may influence T-cell responses by enhancing the uptake of antigen–antibody complexes by antigen-presenting cells (37, 38) and by promoting mixed Th1/Th2 cell cytokine responses (39).

At variance with a previous report (20), we could not document any significant positivity for IgG3-P450scgAb. By analyzing a small number of AAD patients, Boe et al. (20) found that autoantibodies of the IgG3 isotype may accompany those of the IgG1 in around one-third of P450scgAb-positive cases. Our negative findings, which are in agreement with the results of the 21OHAb isotyping of the previous study (20), may be related to the different types of assay utilized; in this sense, radiobinding assay may be associated with higher specificity and less subjective interpretation of the results. The use of a statistical approach to calculate a mathematical cut-off strongly limits the misinterpretation of raw data as compared with the operator’s evaluation of positive/negative signals in western blotting or immunofluorescence. Conversely, we could identify a small number of patients positive for IgG4 autoantibodies, this phenomenon having not been documented in the above study (20). This finding is especially important, as selective synthesis of IgG4 antibodies is controlled by a Th2 type of immune response. In this regard, similar findings were reported in studies on T1DM patients (16–18): accordingly, some patients with steroid cell autoimmunity have indirect immunological features of a different pattern of autoimmune response (Th2-oriented), independent from clinical manifestations of the disease (as both SCA-POI and non-POI AAD patients shared such reactivity) or genetic background (both monogenic APS1 and multifactorial APS2). At present, we cannot resolve the dilemma whether this subgroup of patients represents individuals with a distinct mechanism of immune response, or whether such phenomenon indicates a different stage in the natural history of the autoimmune process. In our study, no significant phenotypic or clinical characteristics discriminated the few subjects whose sera were both positive for IgG4 autoantibodies and negative for the IgG1 isotype from the large majority of patients being positive for the IgG1 isotype. Interestingly, all preclinical AAD individuals resulted positive for IgG1-21OHAb, and we can speculate that the production of specific IgG4 autoantibodies may be a late event, eventually intervening years after the manifestation of overt disease. However, further large, prospective studies of serial samples collected during the natural history of AAD and SCA-POI are needed to document the possibility of a switch from a Th1- to a Th2-type of response.

As already mentioned, other authors have similarly observed that autoantibodies to islet autoantigens, such as glutamic acid decarboxylase (GAD65Ab) and tyrosine phosphatase IA-2 (IA-2Ab) (16–18, 26), and to thyroid peroxidase (TPOAb) (27, 28), are predominantly of the IgG1 isotype, with minor contribution of the other IgG subclasses. More specifically, a Japanese study reported a different expression of IgG subclass TPOAb in patients with chronic thyroiditis (whose sera were predominantly positive for IgG1-TPOAb), as compared with healthy subjects (whose sera were exclusively associated with the IgG4 isotype) (27). On the contrary, Th2-linked IgG4-autoantibody responses do not seem to exercise protection from T1DM (16–18, 26). In our preclinical AAD group, an exclusive Th1 type of IgG subclass autoantibodies was observed both at baseline and in follow-up samples, in the sera from five individuals who later developed clinical signs of adrenal insufficiency (destructive adrenalitis) and in nine individuals who did not progress to overt AAD during the follow-up (nondestructive adrenalitis). These results show that selection of IgG1-21OHAb accompanies the autoimmune process irrespective of the type of inflammatory reaction (destructive versus nondestructive adrenalitis).

In conclusion, we have demonstrated the predominant Th1 type of autoimmunity to steriodogenic enzymes in SCA-POI, non-POI AAD, and APS1 patients by the largely prevalent identification of IgG1 isotype autoantibodies; on the other hand, we identified a restricted number of patients who showed a distinct IgG isotype specificity, suggesting an apparently Th2-oriented type of immune response. Future studies will be aimed at testing the further clinical applicability of our isotype-specific radiobinding assays, and to provide more information on the molecular mechanisms of autoantibody production in destructive endocrine autoimmune diseases.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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