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ORIGINAL ARTICLE

α-enolase: a novel autoantigen in patients with premature ovarian failure

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Summary

Objective Although controversial, the presence of circulating antiovarian antibodies (AOA) may be considered a marker of autoimmune premature ovarian failure (POF). The purpose of the present work was to evaluate the presence of AOA in POF patients, and to identify a possible autoantigen in order to develop a reliable diagnostic tool that might help to determine the real prevalence of autoimmune POF.

Design Non-randomised study. Blood sampling for determination of circulating AOA.

Patients One hundred and ten patients with POF and 60 normally menstruating women with no record of autoimmune diseases (controls).

Measurements Presence of circulating AOA was assessed by Western-blot, using cytosolic fraction from human ovarian homogenate as antigen.

Results Twenty-one of 110 women with POF presented circulating antibodies directed toward an antigen of approximately 50 kD. Sixty control subjects proved negative. After purification and analysis by mass spectrometry, the antigen was identified as α-enolase.

Conclusion Determination of the presence of circulating antiα-enolase antibodies might be instrumental in identifying those patients who may present a putative defect in immunoregulation and therefore a possible autoimmune aetiolgy for POF.

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Introduction

Premature ovarian failure (POF), a syndrome clinically defined by failure of the ovary before the age of 40, affects almost 1% of the Western female population.[1] It is characterized by primary or secondary amenorrhea, hypoestrogenism and elevated gonadotropin serum levels.[2] FSH level (> 40 mUI/ml) is the hallmark for diagnosis.[3] Though the phenotypic expression of POF is similar to that of age-appropriate natural menopause, in most cases the underlying pathophysiological mechanisms are diverse and unclear; any of the following: chromosomal, enzymatic, iatrogenic, autoimmune or infectious aberration, may be the cause of the disease.[4]

It was the association of POF with different autoimmune disorders, together with the findings of circulating antibodies to normal ovarian tissue in sera from patients with POF, that originally suggested an autoimmune mechanism in the pathophysiology of the disease.[5–9] Additional evidence was brought forward through the histological documentation of a lymphocytic infiltrate in the ovaries of some patients with this disorder.[6]

A small percentage of women develop POF in association with steroidogenic autoimmunity, leading to autoimmune oophoritis.[4,10] These patients may have antibodies that recognize several types of steroid-producing cells of the adrenal cortex, testis, placenta and ovary, the so-called 'steroid cell antibodies' (SCA).[11–14] To date, adrenal cortex autoantibodies may be the only validated marker of autoimmune oophoritis.[15]

On the other hand, several investigators have found other circulating antibodies directed toward human ovarian tissue in the sera from patients with ovarian infertility.[5,16–18] Reported antigenic ovarian structures include oocyte,[19,20] corpus luteum,[12,21,22] theca cells,[12,16,21,23] granulosa cells[20,21] and zona pellucida[24] among others. Nevertheless, although these antibodies may be common in POF, their pathophysiological significance remains obscure.[25] Further investigation into these ovarian targets may lead not only to a better understanding of the pathogenic mechanisms that may result in ovarian injury, but also to the development of more accurate diagnostic tools in order to determine the real prevalence of autoimmune aetiology in ovarian disease.

In the present study, we evaluated the presence of antiovarian antibodies (AOA) in a group of 110 patients with POF and in 60 normally menstruating women. By Western-blot analysis, we identified the presence of antibodies directed against an ovarian antigen, of approximately 50 kD, in 19·1% of patients. After purification and analysis by mass spectrometry, the antigen was identified as α-enolase.

Materials and methods

Sera

A study was carried out in 110 patients with POF, aged 15–37 years, all of them with normal 46, XX karyotype. Subjects presenting POF-related conditions such as ovarian surgery, previous chemo- or

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radiotherapy, or metabolic disorders (i.e. galactosaemia), were excluded from the present work.

Sera were obtained from diagnosed patients who were derived from several hospitals to our laboratory for determination of circulating antiovarian antibodies (AOA). Patients had been characterized as POF due to amenorrhea for over a year starting before the age of 40, with elevated serum FSH levels (> 40 mIU/ml) in two consecutive determinations. Serum LH concentrations were above 20 mIU/ml (normal follicular phase levels: 2–10 mIU/ml). Plasma 17 β-oestradiol was under 15 pg/ml (normal follicular phase levels: 20–120 pg/ml). Sera from 60 normally menstruating women within the same age range (average age 28 years) with proven fertility and no evidence of autoimmune diseases, were used as controls. Neither patients nor control subjects were receiving steroids at the time of blood sampling.

The Protocol was approved by the Instituto de Biología y Medicina Experimental Institutional Review Board. Informed consent was obtained from all patients and controls.

Preparation of tissue extracts

Normal human ovarian and muscle tissues were obtained from therapeutic surgery of young women (radical hysterectomy because of cervical cancer) or postmortem, respectively, and rapidly frozen. Frozen tissues were pulverized under liquid nitrogen and homogenized in buffer 50 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, pH 7·4 in the presence of 0·5 mM phenylmethylsulphonylfluoride (PMSF), 0·025 mM N-CBZ-L-phenylalanine chloromethyl ketone (ZPCK), 0·025 mM N′-p-tosyl-lysine chloromethyl ketone (TLCK) and 0·025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), as proteases inhibitors.

Homogenates were centrifuged at 1000 ***g*** for 10 min and the resulting supernatants were centrifuged at 7000 ***g*** for 10 min. Pellets (mitochondrial fractions) were retained, and supernatants were centrifuged at 105 000 ***g*** for 60 min. The resulting new pellets and supernatants were saved and called 'microsomal fraction' and 'cytosol', respectively.

Western-blot for determination of antiovarian antibodies

Cytosol fractions were depleted of endogenous IgG by means of a protein-A sepharose column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). For both microsomal and cytosol fractions, antigen (50 μg/well) was boiled for 3 min in sample buffer (60 mM Tris-HCl, pH 6·8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0·7 M 2β-mercaptoethanol and 0·01% bromophenol blue), and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were then transferred onto a nitrocellulose membrane at 100 V for 90 min. After blotting, the membrane was blocked overnight with 3% skim milk in phosphate-buffered saline (PBS) containing 0·05% Tween-20 (PBS-T-M). The nitrocellulose membrane was cut into strips, and each one was incubated with 2 ml of different sera, previously diluted 1 : 100 in PBS, 0·05% Tween-20 (PBS-T), for 2 h. The strips were washed for 20 min with four changes of PBS-T, and incubated with antihuman IgG peroxidase conjugate in PBS-T-M for 60 min, then washed again for 20 min in PBS-T. Enzyme activity was determined with 0·05% 3,3′diaminobenzidine (DAB) and 0·003% H_2O_2 in 100 mM Tris pH 7·4. Sera from six normal women were run each time.

Purification and identification of the 50-kD autoantigen

Five hundred μl of ovarian cytosol fraction – containing 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 mM sodium orthovanadate, 5 mM NaF, 20 mM sodium molybdate, 5 μg/ml spermin, 5 μg/ml spermidin and 10 mM β-glycerophosphate – were applied to a CM Affi-Gel Blue Gel (Bio-Rad, Hercules, CA, USA) column (1 cm^3 volume). After two washes with one volume each of running buffer (10 mM K_2HPO_4; 0·15 M NaCl, 0·02% NaN_3, pH 7·25), proteins were eluted with increasing molarity of NaCl in phosphate buffer as follows: 2 volumes of 0·3 M NaCl; 2 volumes of 0·6 M NaCl; 2 volumes of 1 M NaCl and 3 volumes of 1·4 M NaCl. Twelve fractions of approximately 1 ml each were collected, including the void volume, and protein concentration was determined in each one as described by Lowry *et al.*[26]. Column fractions were analysed by Western-blot. Thirty μl of each column fraction were boiled for 3 min in sample buffer, and proteins were separated on SDS-PAGE (10% polyacrylamide). In addition, 4·5 μl of cytosol fraction from human ovary homogenates were run on a separate line as control. Western-blot was performed as described above, using an AOA positive serum as primary antibody. The fraction where the ovarian antigen was detected was concentrated and enriched centrifuging in Ultrafilters Centricon-30 (Amicon, Inc., Beverly, MA, USA) at 5000 ***g*** during 35 min Proteins of this concentrated product were run on a 10% polyacrylamide-SDS gel of 15 cm long. The immunoreactive band was excised from Coomassie Brilliant Blue G-250-stained polyacrylamide gel with a sterile scalpel, and was analysed by nano-LC-ESI-MS/MS (electrospray ionization mass spectrometry coupled with a nanoflow liquid chromatography system) at Proteome Factory AG (Berlin, Germany).

Western-blot using recombinant human α-enolase

Recombinant human α-enolase was produced as described,[27] and stored in Tris HCl pH 8, 10% glycerol at –70 °C. Protein (0·5 μg/line) was boiled 3 min in sample buffer, and subjected to SDS-PAGE (10% polyacrylamide). After electroblotting onto nitrocellulose as explained above, membrane was blocked overnight in PBS-T-M. Nitrocellulose membrane was cut into strips, and each one was incubated with 2 ml of different sera, previously diluted 1 : 200 in PBS-T-M, for 2 h. The strips were washed for 20 min with 4 changes of PBS-T, and incubated with goat antihuman IgG peroxidase-conjugate in PBS-T-M for 60 min, then washed again for 20 min in PBS-T. Enzyme activity was developed as described above.

Results

Determination of circulating ovarian antibodies

We have developed a specific approach for detection of AOA by Western-blot, using ovarian antigens and characterizing them according to their molecular weight. When cytosolic fractions were used, 19·1%

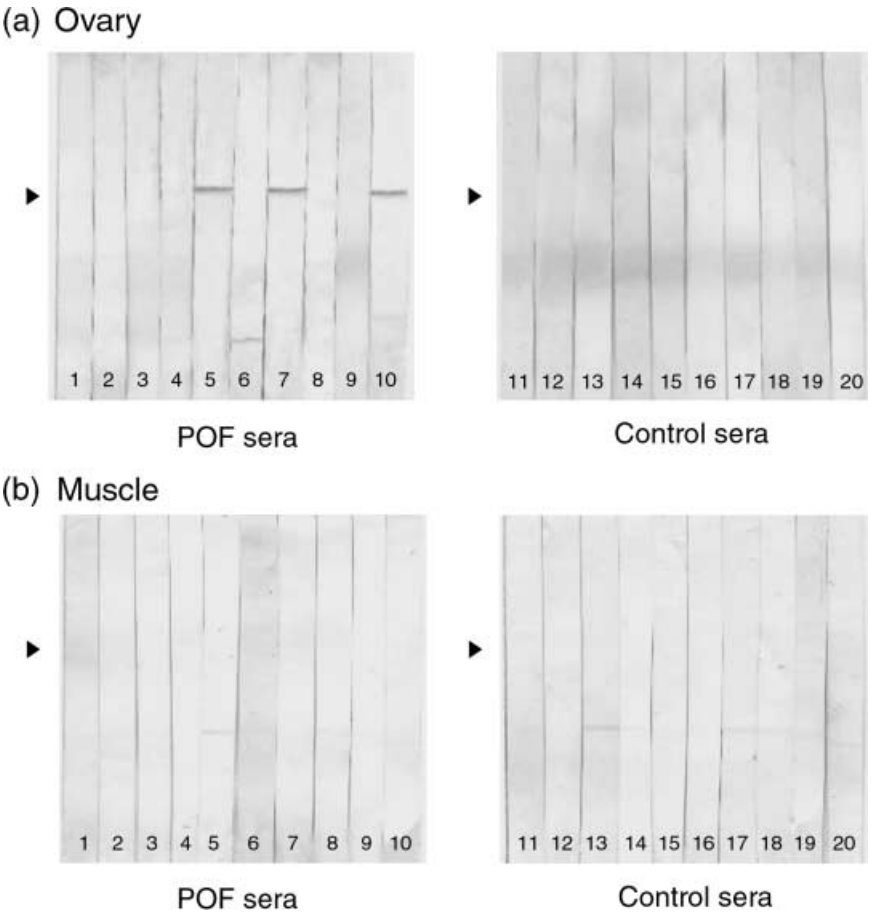

Fig. 1 Western-blot using cytosolic ovarian (a) and muscle (b) fractions. Proteins (50 μg/lane) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, strips were incubated with dilutions of different sera, washed and incubated with peroxidase-conjugate goat antihuman IgG. Results obtained with sera from 10 POF patients (strips 1–10) and 10 control subjects (strips 11–20) are shown, as sample of the whole population studied. Sera from 3 patients with POF turned out reactive against an approximately-50 kD protein when using the ovarian cytosolic fraction (a) strips 5, 7 and 10), but negative with muscle protein extracts (b). No specific reactivity was seen when ovarian and muscle fractions were incubated with sera from the 10 control subjects (strips 11–20 of both panels). Right arrows indicate prestained standard of 50 kD.

of sera from the 110 patients studied showed specific reactivity with an antigen of approximately 50 kD. Conversely, none of the sera obtained from the control subjects ($n = 60$) showed any such specific reactivity. When Western-blots using microsomal fractions as antigen were performed, we were unable to identify a 'differential' antigen that may be recognized by POF sera, and not by control sera. In addition, sera obtained from all POF patients and controls showed no reactivity with muscle protein extract, which was used as control tissue. A representative Western-blot analysis of 10 radomly selected sera from patients and 10 sera from control subjects is shown in Fig. 1. Sera from three patients with POF reacted with the approximately-50 kD antigen of the ovarian cytosolic fraction, but not with muscle protein extracts. No specific reactivity was seen when ovarian and muscle fractions were incubated with sera from the 10 control subjects. Based on these results we decided to characterize the 50 kD ovarian cytosolic protein.

Purification and identification of the ovarian autoantigen

In order to purify and identify the antigen of approximately 50 kD, proteins of the cytosolic fraction of the ovarian homogenate were separated by ion exchange and affinity chromatography using a CM Affi-Gel Blue Gel column. Column fractions were analysed by Western-blot, using serum from a POF patient with a high titre of AOA as the primary antibody. The presence of the ovarian antigen was detected in the void volume (Fig. 2b). This fraction was concentrated by ultrafiltration using Centricon-30. Figure 2c, shows the SDS-PAGE pattern of the concentrated fraction. The protein band corresponding to the ovarian antigen was excised and analysed by nano-LC-ESI-MS/MS. Said analysis revealed the 50 kD protein to be enolase 1 (α) (score = 368; protein scores higher than 74 are significant ($P < 0{\cdot}05$). Score is $-10*\text{Log}(P)$, where P is the probability for the observed match to be a random event). We therefore decided to study α-enolase as the possible antigen to which these autoreactive antibodies in these POF patients may be directed.

Western-blot analysis using recombinant human α-enolase

We analysed the presence of antienolase antibodies in sera from patients and control subjects, using recombinant human α-enolase as antigen and dilutions of different sera as primary antibodies, in Western blots (Fig. 3). Sixty of 170 sera that had previously been analysed for AOA were now tested for the presence of antiα-enolase antibodies. Thus, 14 of 21 sera from POF patients with AOA, 22 of 89 sera from POF patients without AOA, and 24 of 60 sera from control subjects (without AOA) were screened for antiα-enolase antibodies (Table 1). Western-blots using recombinant α-enolase were run in parallel with new Western-blots using cytosolic ovarian fraction for determination of AOA. All sera positive for AOA reacted against the recombinant protein, while AOA negative sera did not

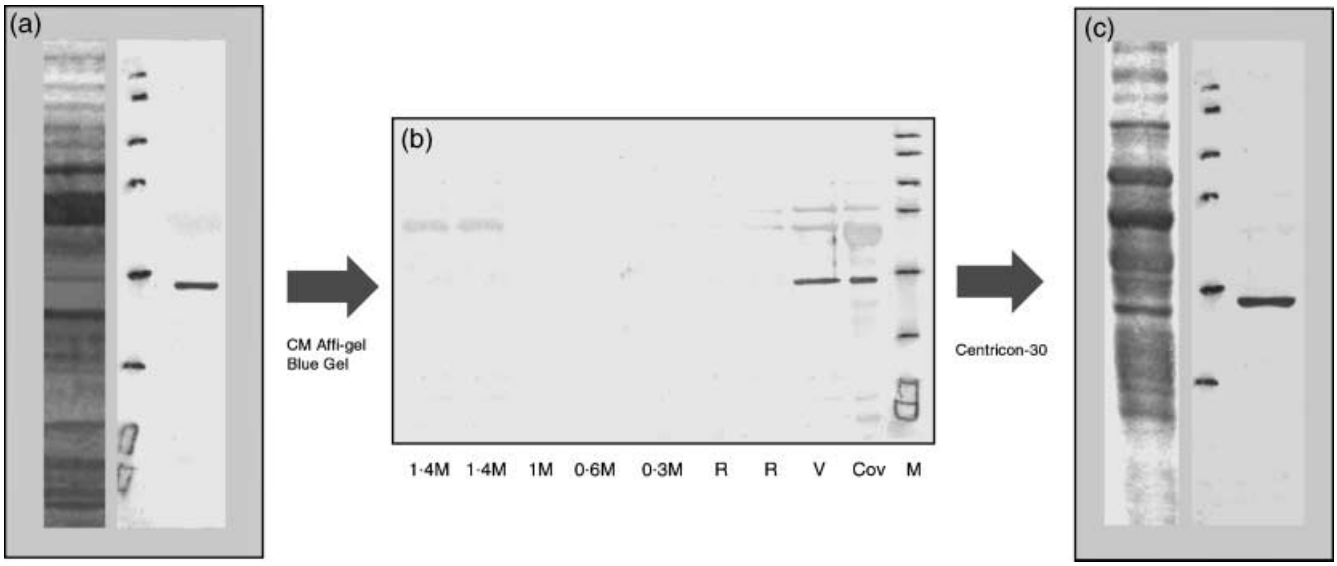

Fig. 2 Purification of human ovarian antigen. The cytosolic fraction from human ovary homogenates was applied to a CM Affi-Gel Blue Gel column; proteins were eluted with increasing molarity of NaCl in phosphate buffer. Column fractions were analysed by Western-blot as described, and the ovarian antigen was detected in the void volume. After concentration and enrichment of this fraction by centrifuging in ultrafilters, proteins were run on a 10% polyacrylamide-SDS gel 15 cm long. The band corresponding to the ovarian antigen could thereafter be excised, and analysed by nano-LC-ESI-MS/MS. (a) band pattern obtained from separation of ovarian cytosolic proteins in 10% polyacrylamide gel, before purification and concentration steps. (b) Western-blot for analysis of column fractions. M: marker; Cov: ovarian cytosolic fraction; V: void fraction; R: washes with running buffer; 0·3M, 0·6M, 1M and 1·4M: elution with increasing molarity of NaCl in phosphate buffer. (c) band pattern in 10% polyacrylamide gel, obtained after purification and concentration steps.

Table 1. Comparison of results obtained when the same sera were analysed by both methods: Western-blot for determination of AOA and Western-blot for determination of antiα-enolase antibodies

Total sera	*n*	AOA outcome		Tested with R-αE *n*	R-αE outcome	Confimed AOA outcome
POF	110	21	+	14	+	14/14
		89	–	22	–	22/22
Controls	60	0	+			
		60	–	24	–	24/24

recognize this antigen. Overall, 100% of sera tested by Western-blots using recombinant α-enolase, confirmed AOA result.

Discussion

There is accumulating evidence indicating that autoimmunity may be involved in idiopathic POF.[4,10,15,25] In previous studies by our laboratory, we demonstrated the presence of circulating immunoglobulins that inhibited FSH binding to its receptor (Ig-FSHR), in a subgroup of POF patients, those affected by resistant ovary syndrome (ROS). Only ROS patients, characterized by the presence of numerous follicles in their ovaries,[28,29] present Ig-FSHR.[30] Thus, ROS could be considered one specific form of autoimmune POF associated with anti-FSHR autoimmunity. On the other hand, the reported high prevalence of SCA in patients whose POF is associated with adrenal autoimmunity, suggests the existence of another specific form of autoimmune ovarian failure: that associated with steroidogenic cell autoimmunity.[4,10,15] Nevertheless, several cases of suspected autoimmune POF cannot be placed within either of these two specific forms. In such cases, the pathogenic mechanism underlying the putative immunological disorder that results in abnormal ovarian function is still unknown. Several circulating antibodies, other than SCA, directed toward different antigenic ovarian structures have been described.[12,19–25,31–38] Nevertheless, despite considerable scientific work, neither their specificity nor their diagnostic relevance have yet been unanimously established. Indeed, it was suggested that ovarian antibodies as detected by indirect immunofluorescence, the most common method currently used, may have poor specificity.[38] Thus, no currently available validated serum antibody marker can confirm a clinical diagnosis of autoimmune POF in those cases that cannot be placed within either of the two specific forms described above.

In the present work, we detected specific reactivity against protein α-enolase in 19·1% of sera from 110 POF patients. Even though our results suggest that α-enolase may be a major target of self-reactive antibodies in these patients, we cannot exclude the presence of antibodies directed against other potential cell targets (e.g. the steroidogenic enzymes P450scc and 17α-hydroxylase, among others). However, given the experimental approach used in the present work, these antibodies were not specifically determined.

Enzyme α-enolase (2-phospho-D-glycerate hydrolyase) is a metalloenzyme that participates in the second half of the Emdem Mayerhoff–Parnas glycolytic pathway.[39] In vertebrates, there are three different isoenzymes: α-enolase is found in most tissues, β-enolase is almost exclusively found in muscle tissues and γ-enolase is found in neurone and neuroendocrine tissues.[39] Enolase is one of

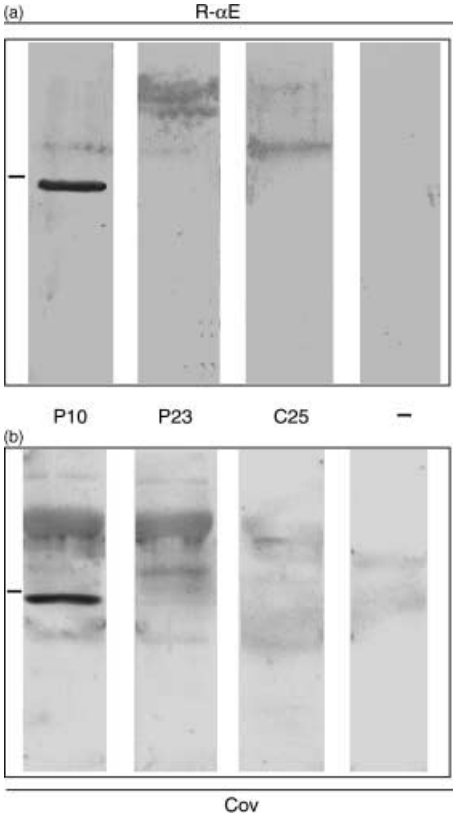

Fig. 3 Western-Blots for determination of antiα-enolase antibodies. (a) Western-blot using recombinant human α-enolase (R-αE) as antigen. Recombinant human α-enolase was subjected to SDS-PAGE and electroblotted onto the nitrocellulose membrane. After blocking, each strip was incubated with dilutions of different sera, washed and incubated with peroxidase-conjugate goat antihuman IgG. Sample illustration of the whole population studied, with results corresponding to sera from a POF patient with AOA (P10), a POF patient with no AOA (P23), and a control subject (C25); (–) control incubation in the absence of serum. (b) Results obtained with the same sera using ovarian cytosolic fraction (Cov) as antigen. Left markers indicate prestained standard of 50 kD.

the most abundantly expressed cytosolic proteins in many organisms. It is also found on the surface of a variety of haematopoietic cells as a strong plasminogen-binding receptor.[40,41] Antienolase antibodies have been incriminated in a variety of autoimmune diseases, including rheumatoid arthritis,[42,43] Hashimoto's encephalopathy,[44] systemic lupus erithematosus,[42] discoid lupus erithematosus,[45] liver autoimmune endocrinopathies such as primary biliary cirrhosis, autoimmune hepatitis and primary sclerosing colangitis,[46–48] mixed cryoglobulinaemia,[42,49] cancer-associated retinopathy[50] and endometriosis[51] among others. The presence of antibodies directed to α-enolase in patients with POF is herein described for the first time.

Even though the formation of self-reactive antibodies may be a normal physiological process, innate immune activation of autoreactive B cells has the potential to break tolerance (state of antigen-induced nonresponsiveness of lymphocytes) and trigger autoimmunity.[52] Interestingly, enolase protein may have some particular features that make it more prone to breaking the delicate balance between beneficial and pathological immune responses.[53] Several studies have reported that antibodies directed against the cell wall-associated enolase of *Candida albicans* and that of group A streptococci, cross react against human endogenous enolase.[27,54,55] It is therefore proposed that in cases of chronic infection, the levels of microbial enolase may result in an immune response to the foreign enolase antigen, which by epitope mimicry leads to the targeting of the inherent enolase, and therefore to an autoimmune response.[53] In general, self-tolerance prevents the autoimmune response from becoming an autoimmune disease. However, if self-tolerance fails, the immune response is raised aggressively, not only against microbial epitopes but also against self-epitopes.[56] Furthermore, given that enolase is a widely distributed cytosolic enzyme, it may be taken by tissue-resident antigen-presenting cells (APC) after cell death that accompany normal cell turnover. Also, the proinflammatory milieu generated by the diverse range of pathologies associated, coincidentally or causatively, with antienolase antibodies might possibly result in activation of APC.[53] It is noteworthy that patients with autoimmune polyglandular syndrome type I (APS I), who present chronic candidal infection in addition to chronic hypoparathyroidism, autoimmune adrenal insufficiency, and a wide spectrum of associated minor autoimmune disorders, demonstrate the tightest association of disease with enolase-reactive antibodies.[54]

It has long been recognized that POF could be associated with nearly all organ-specific as well as some systemic autoimmune diseases.[4,7,13,57] The frequent occurrence of these associations may suggests that POF could be one of the components within a general immunological disorder. Thus, it would not be surprising to find a variety of antibodies, in addition to ovarian antibodies, associated with ovarian autoimmunity. Indeed, as in APS I, the presence of antibodies directed against enolase might be considered a marker of autoimmune aetiology. The frequent association of POF with different autoimmune disorders, the high prevalence of antienolase antibodies in APS-I patients, and the wide spectrum of immunological disorders in which antienolase antibodies are found, all support the suggestion that high titres of antienolase antibodies might be present in subjects with a putative defect in immunoregulation.

In patients with established disease, autoantibodies can provide markers to classify the disease. For example, type 1 diabetes, thyroiditis and adrenalitis are classified as autoimmune or nonautoimmune, based on the presence or absence of disease-associated antibodies. In rheumatic diseases, autoantibodies such as rheumatoid factor and antinuclear antibodies are not highly specific for a particular disease. Their major use, therefore, becomes a confirmation of diagnosis in patients who presented clinical features of specific rheumatic disease.[58] Likewise, we suggest that in those patients in whom the onset of POF either preceded, followed, or was concurrent with the onset of autoimmune diseases, presence of antienolase antibodies might be an indicator of a putative defect in immunoregulation. Provided that this defect in immunoregulation is confirmed as the aetiological agent of POF, the presence of antibodies directed against α-enolase may be considered an indicator of an 'autoimmune aetiology', becoming a suitable marker for diagnosis of *autoimmune* POF.

To further understand the clinical relevance of antienolase antibodies, a larger number of POF patients and controls should be

studied. In addition, the pathological role played by these antibodies in the disease should also be adressed.

In summary, we have identified α-enolase as a novel autoantigen for POF. We suggest that in those patients with suspected autoimmune POF, the presence of antienolase antibodies might be an indicator of a putative defect in immunoregulation, and might therefore suggest a possible autoimmune aetiolgy for POF.

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