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Levels of Adrenocortical Autoantibodies Correlate with the Degree of Adrenal Dysfunction in Subjects with Preclinical Addison's Disease*

STEFANO LAURETI, ANNAMARIA DE BELLIS, VINCENZO IGINO MUCCITELLI, FILIPPO CALCINARO, ANTONIO BIZZARRO, RICCARDO ROSSI, ANTONIO BELLASTELLA, FAUSTO SANTEUSANIO, AND ALBERTO FALORNI

Department of Internal Medicine and Endocrine and Metabolic Sciences (S.L., F.C., F.S., A.F.), University of Perugia, 06126 Perugia, Italy; Institute of Endocrinology (A.D.B., V.I.M., A.Be.) and Department of Clinical and Experimental Medicine "F. Magrassi" (A.Bi.), 2nd University of Naples, 80131 Naples, Italy; and Department of Molecular and Clinical Endocrinology and Oncology (R.R.), University "Federico II", 80131 Naples, Italy.

ABSTRACT

To test the hypothesis that levels of adrenal autoantibodies correlate with the degree of adrenal dysfunction, we followed up adrenal cortex autoantibody (ACA) titers and 21-hydroxylase (21OH) autoantibody (21OHAb) levels in 19 ACA-positive subjects with preclinical Addison's disease. On enrollment, all the 19 ACA-positive subjects were positive for 21OHAb. At follow-up, the concordance rate for simultaneous presence/absence of both ACA and 21OHAb was as high as 91% and a strong, positive correlation between 21OHAb levels and ACA titers was observed ($P < 0.0001$). The levels of adrenal autoantibodies were positively associated with the severity of adrenal dysfunction (ANOVA, $P < 0.0001$ for both 21OHAb and ACA): the 21OH index was significantly lower at stage 0 or 1 than at stage 2+3 (corrected $P < 0.001$ and $P < 0.05$) or stage 4 (corrected $P < 0.001$ and <0.01). Similarly, ACA titer at stage 4 was significantly higher than stage 0 ($P < 0.001$), stage 1 ($P < 0.001$), and stage 2+3 ($P < 0.05$); and ACA titer at stage 2+3 was higher than stage 0 ($P < 0.001$) and stage 1 ($P < 0.05$). In subjects with progression of adrenal dysfunction (n = 14), levels of 21OHAb and ACA increased significantly ($P = 0.041$ and $P = 0.002$) during the follow-up period. In 5 subjects, the remission of biochemical signs of adrenal dysfunction was associated with the disappearance of both ACA and 21OHAb. Our study shows that the levels of adrenal autoantibodies correlate with the degree of adrenal dysfunction, and this suggests that production of high-level 21OHAb strongly signals the destructive phase of the autoimmune disease process. (*J Clin Endocrinol Metab* **83:** 3507–3511, 1998)

IT IS currently believed that at least 70% of cases of Addison's disease are consequential of the autoimmune destruction of adrenocortical cells (1–3). Autoimmune Addison's is associated with susceptible human leukocyte antigen (HLA) haplotypes (4, 5) and is characterized by the appearance of autoantibodies to adrenal cortex cells (ACA) (6–8). Most likely, the pathogenic role of adrenocortical autoantibodies is irrelevant, but the presence of ACA is a useful marker for disease classification at clinical onset. ACA recognizes an M_r 55,000 autoantigen located in the microsomal subcellular fraction of the adrenocortical cells, which has been identified as the steroid-synthesizing enzyme 21-hydroxylase (21OH) (9, 10). We and others (11–17) have shown that 21OH autoantibodies (21OHAb) have a high diagnostic sensitivity and specificity for autoimmune adrenal insufficiency.

The identification of subjects with an ongoing adrenal autoimmune process is important in the clinical management of patients with endocrine autoimmune diseases often associated with adrenal insufficiency, such as Hashimoto's thyroiditis, Graves' disease, or insulin-dependent diabetes mellitus (IDDM). In patients with endocrine autoimmune diseases, the presence of ACA has been used to estimate the risk for clinical adrenal insufficiency (18–24). Several studies have shown, however, that an adrenal autoimmune process does not lead necessarily to clinical Addison's disease. Thus, remission of subclinical adrenocortical dysfunction may occur in ACA-positive subjects (22, 25). Furthermore, it has recently been reported that the presence of ACA and 21OHAb is a marker of rapid progression toward clinical adrenal insufficiency in children (23), but only of low progression in adult subjects with endocrine autoimmune diseases (24).

It is still unknown whether production of adrenal autoantibodies is the result of an autoantigen-driven mechanism or the mere consequence of the destruction of target cells. It is also unclear whether the levels of adrenal autoantibodies correlate with the degree of adrenal dysfunction in the preclinical period.

In the present study, we followed up the serum levels of 21OHAb and ACA in a group of initially ACA-positive subjects without clinical signs of adrenal insufficiency. Autoantibody data were correlated with the degree of preclinical adrenal insufficiency, as estimated by the analysis of biochemical parameters of adrenal function.

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Address all correspondence and requests for reprints to: Alberto Falorni, M.D., Ph.D., Department of Internal Medicine and Endocrine and Metabolic Sciences, University of Perugia, Via E. Dal Pozzo, 06126 Perugia, Italy. E-mail: falorni@dimisem.med.unipg.it.

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Materials and Methods

Subjects and serum samples

Studying a large group of 3,020 patients with 1 or more endocrine autoimmune diseases, a total of 26 (0.9%) ACA-positive subjects was identified. Of these ACA-positive subjects, 3 Graves patients with ophtalmopathy were not included in the follow-up study because a corticosteroid treatment was started either before (n = 2) or during (n = 1) our follow-up study. Furthermore, follow-up samples from an additional 4 ACA-positive subjects were not available. Hence, 19 ACA-positive subjects [male/female (M/F) ratio: 5/14, median age: 31 yr, range: 18–44 yr] entered the follow-up study (Fig. 1). Most of the ACA-positive subjects had been longitudinally studied and described in a previous paper (22). Of the 19 subjects studied, 8 (42%) presented with autoimmune thyroiditis, 5 (26%) with premature ovarian failure (POF), 4 (21%) with Graves' disease, 3 (16%) with IDDM, 2 (10%) with atrophic gastritis, 2 (10%) with vitiligo, and 1 (5%) with diabetes insipidus (Table 1). The patient with diabetes insipidus (no. 11) was positive for arginine vasopressin cell autoantibodies (26, 27).

During our longitudinal study, 54 basal and follow-up serum samples, belonging to various stages of adrenal dysfunction, were tested for 21OHAb. In particular, the presence of 21OHAb was also tested in 3 of the 19 patients, 2–3 yr before the demonstration of the presence of ACA.

ACA assay

ACA were determined using an indirect immunofluorescence method on cryostatic sections of monkey adrenal gland (22). Levels of ACA were expressed as end-point dilution titer.

21OHAb assay

21OHAb were determined in coded samples using a radiobinding assay that uses *in vitro* translated recombinant human ^{35}S-21OH and protein A-Sepharose (Pharmacia, Biotech, Uppsala, Sweden) (11). 21OHAb levels were expressed as a relative index (21OHAb index) using one positive and two negative standard sera, which were included in each assay (11). The upper level of normal of the 21OHAb index was estimated as the mean + 3 SD of the results obtained when analyzing sera from 200 healthy subjects (mean + SD: 0.027 ± 0.011, range: −0.02 to 0.059) and was 0.06. The samples with a 21OHAb index more than 0.7 were titrated using 1:100 to 1:500 dilutions.

Adrenocortical functional studies

The adrenocortical function of the 19 ACA-positive subjects was evaluated by measuring basal plasma levels of ACTH, cortisol, aldosterone, and plasma renin activity (PRA), as previously described (22). Cortisol plasma levels were also evaluated 60 min after an iv infusion of 0.25 mg synthetic ACTH (normal peak response, > 20 μg/dL = 552 nmol/L).

According to previously reported criteria (21, 22) and based on the results of the biochemical analyses, five stages of adrenocortical dysfunction were recognized in subjects with adrenal autoantibodies: stage 0 = normal adrenal function; stage 1 = high PRA and normal/low aldosterone levels; stage 2 = along with impaired cortisol response to ACTH; stage 3 = along with increased basal ACTH levels; stage 4 = clinically overt Addison's disease. Normal subjects (stage N) were those with normal adrenal function and absence of adrenal autoantibodies. The 19 ACA-positive subjects were subdivided into 3 groups (Table 1): group A (n = 8 subjects; M/F ratio = 4/4) who developed clinical Addison's disease at follow-up; group B (n = 6 subjects; M/F ratio = 1/5) with deterioration of adrenal function throughout the study, but without development of clinical Addison's disease; and group C (n = 5 subjects; M/F ratio = 0/5) with remission of adrenal dysfunction after a 2–4 yr follow-up period.

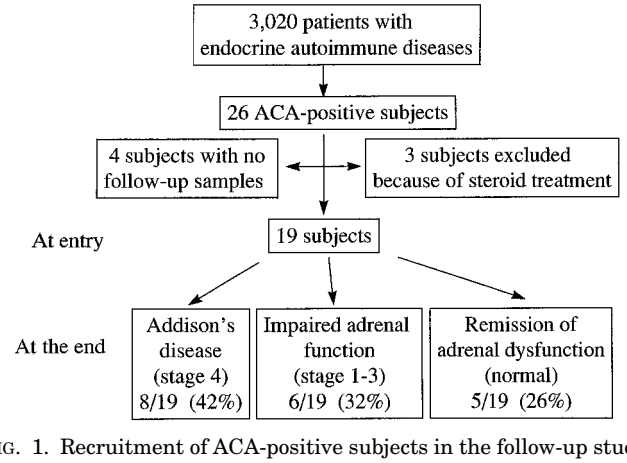

FIG. 1. Recruitment of ACA-positive subjects in the follow-up study.

Statistical analysis

Differences in levels of 21OHAb or ACA titer, in relation to stage of preclinical adrenal dysfunction, were tested by ANOVA with Bonferroni's correction for multiple comparisons after logarithmic transformation of antibody levels. In some analyses, samples of stage 2 and stage 3 were considered as a single group (stage 2+3) because both stages identify a subclinical dysfunction of the ACTH-cortisol axis, as compared with stage 1, which is characterized by the exclusive dysfunction of the aldosterone-renin axis. The correlation between 21OHAb levels and ACA titers was evaluated by Spearman's rank correlation test. Linear regression between antibody titers and stages of adrenal dysfunction was evaluated after logarithmic transformation of the 21OHAb index and ACA titers. Differences in the serum levels of adrenal autoantibodies between the beginning and the end of the study were evaluated by the nonparametric Wilcoxon test for paired samples. In all tests, a P value less than 0.05 was considered significant.

Results

All 9 subjects at stage 2 or 3 at entry progressed toward stage 3 or 4 during the follow-up (Table 2). Remission of adrenal dysfunction and/or disappearance of both ACA and 21OHAb was observed in 5 of 10 (50%) subjects who were at stage 0–1 at entry (Table 2). Moreover, remission of adrenal dysfunction and disappearance of both ACA and 21OHAb were observed in 5 of 14 (36%) females and none of the 5 males.

At the time of the first demonstration of the presence of ACA (median titer 12, range 2–64), all 19 subjects (stage 0–3 of adrenal dysfunction) were positive for 21OHAb (21OHAb index: median, 0.154; range, 0.061–0.893), and levels of 21OHAb and ACA correlated with each other ($r^2 = 0.785$, $P < 0.0001$). Similarly, 42 of 54 (78%) follow-up serum samples were simultaneously positive for ACA and 21OHAb, and a strong correlation between ACA titers and 21OHAb levels was observed ($r^2 = 0.802$, $P < 0.0001$). In 7 of 54 (13%) follow-up serum samples, both ACA and 21OHAb were absent. Discordant results between ACA and 21OHAb assays were thus observed only in 5 of 54 (9%) follow-up samples. All these latter samples had been collected from stage 0 subjects, and ACA and 21OHAb were present in 3 of 5 and 2 of 5 cases, respectively.

The presence of 21OHAb was demonstrated in two subjects from group B, 2–3 yr before the first demonstration of the presence of ACA (Table 2).

After logarithmic transformation, the levels of 21OHAb and ACA were significantly and positively associated with the severity of adrenal dysfunction (ANOVA, $P < 0.0001$ for both 21OHAb and ACA; linear regression: $r = 0.549$, $P < 0.0001$ and $r = 0.745$, $P < 0.0001$ for 21OHAb and ACA titers, respectively) (Fig. 2). The 21OHAb index of samples of stage

TABLE 1. Clinical characteristics of the 19 ACA-positive subjects at the beginning of the follow-up study

Patient no.	Sex/age	Associated disease	Cortisol baseline (nmol/L) (NR: 250–500)	Cortisol[a] stimulated (nmol/L) (NR > 550)	ACTH (pmol/L) (NR: 2–19)	PRA recumbent (ng/L·s) (NR: 0.2–0.8)	PRA upright (ng/L·s) (NR: 1.1–2.1)	Aldosteron recumbent (pmol/L) (NR: 100–250)	Aldosteron upright (pmol/L) (NR: 260–620)	Adrenal function Stage
Group A										
1	F/44	IDDM	290	325	29	2.8	4	20	106	3
2	F/31	POF	320	430	32	3.1	4.2	23	70	3
3	F/27	POF	290	450	27	2.7	3.8	50	65	3
4	F/40	AG, H	300	410	15	2.5	3.3	43	50	2
5	M/32	G	310	490	19	2.9	3.7	28	43	2
6	M/28	H	340	480	12	4.0	4.4	19	24	2
7	M/18	G	360	470	15	2.1	3.5	60	98	2
8	M/36	G	430	950	19	2.3	3.4	110	310	1
Group B										
9	F/28	IDDM, POF	380	500	18	2.2	3.4	15	28	2
10	F/27	POF	440	490	17	2.6	3.6	20	85	2
11	F/36	DI	500	1080	13	2.0	3.9	40	120	1
12	F/24	H	480	1100	15	0.4	1.4	200	420	0
13	M/35	G, V	490	1450	18	0.5	1.7	110	350	0
14	F/30	IDDM	400	960	12	0.6	1.6	120	290	0
Group C										
15	F/42	AG, H	450	1050	10	2.5	3.1	50	130	1
16	F/37	H	430	1140	17	2.3	2.8	70	150	1
17	F/31	H	500	1180	9	2.4	3.0	30	170	1
18	F/25	H, V	450	1000	16	2.7	3.2	100	250	1
19	F/21	H, POF	380	1050	8	0.5	1.0	200	430	0

[a] 60 min after an iv infusion of 0.25 mg synthetic ACTH.
AG, Atrophic gastritis; H, Hashimoto's thyroiditis; G, Graves' disease; DI, diabetes insipidus; V, vitiligo; NR, normal range.

TABLE 2. Autoantibody levels and adrenocortical function during the follow-up period

Patient no.	Sex/age	Associated diseases	ACA titers	21OH Ab index	Stage of adrenal function	Period of follow-up (yr)
Group A						
1	F/44	IDDM	8→64	0.092→0.34	3→4	5
2	F/31	POF	32→128→64	0.656→0.957→0.873	3→3→4	2
3	F/27	POF	32→64	0.824→0.878	3→4	3
4	F/40	AG, H	8→64	0.15→0.173	2→4	3
5	M/32	G	8→32	0.103→0.602	2→4	3
6	M/28	H	64→64	0.893→0.789	2→4	1
7	M/18	G	64→128→64→256	0.625→0.922→0.756→0.947	2→2→3→4	3.5
8	M/36	G	32→32→64	0.172→0.156→0.163	1→1→4	3
Group B						
9	F/28	IDDM, POF	32→16	0.255→0.108	2→3	1
10	F/27	POF	16→8→32→16	0.154→0.163→0.234→0.256	2→2→3→3	3
11	F/36	DI	16→32→32	0.182→0.361→0.217	1→2→3	4
12	F/24	H	0→16→32	0.09→0.134→0.169	0→1→3	5
13	M/35	G, V	0→0→4→8	0.029→0.026→0.107→0.145	N→N→0→1	3
14	F/30	IDDM	0→8	0.099→0.154	0→1	2
Group C						
15	F/42	AG, H	4→4→0	0.095→0.09→0.014	1→0→N	2
16	F/37	H	8→2→2→0	0.106→0.078→0.01→0.023	1→0→0→N	3
17	F/31	H	8→4→2→0	0.155→0.06→0.023→0.008	1→0→0→N	4
18	F/25	H, V	4→2→0	0.061→0.095→0.008	1→0→N	4
19	F/21	H, POF	2→0	0.09→0.043	0→N	2

AG, Atrophic gastritis; H, Hashimoto's thyroiditis; G, Graves' disease; DI, Diabetes Insipidus; V, Vitiligo.

0 was significantly lower than that of samples of stage 2 (corrected $P < 0.05$), stage 3 (corrected $P < 0.01$), and stage 4 (clinical Addison's disease; corrected $P < 0.001$). The 21OHAb index of samples of stage 4 was also significantly higher than that of samples of stage 1 (corrected $P < 0.01$). In addition, when samples of stages 2 and 3 were considered as a single group (stage 2+3), the 21OHAb index of stage 0 and 1 was also lower than stage 2+3 (corrected $P < 0.001$ and $P < 0.05$). No statistical difference in the 21OHAb index was observed between stage 2 and stage 3 or between stage 3 and stage 4 or between stage 2 and stage 4.

The ACA titer of stage 0 was significantly lower than that of stage 2 (corrected $P < 0.001$), stage 3 (corrected $P < 0.001$), and stage 4 (corrected $P < 0.001$). The ACA titer of samples of stage 4 was also significantly higher than that of samples of stage 1 (corrected $P < 0.001$). In addition, when samples of stages 2 and 3 were considered as a single group (stage 2+3), the ACA titer of stage 0 and 1 was significantly lower

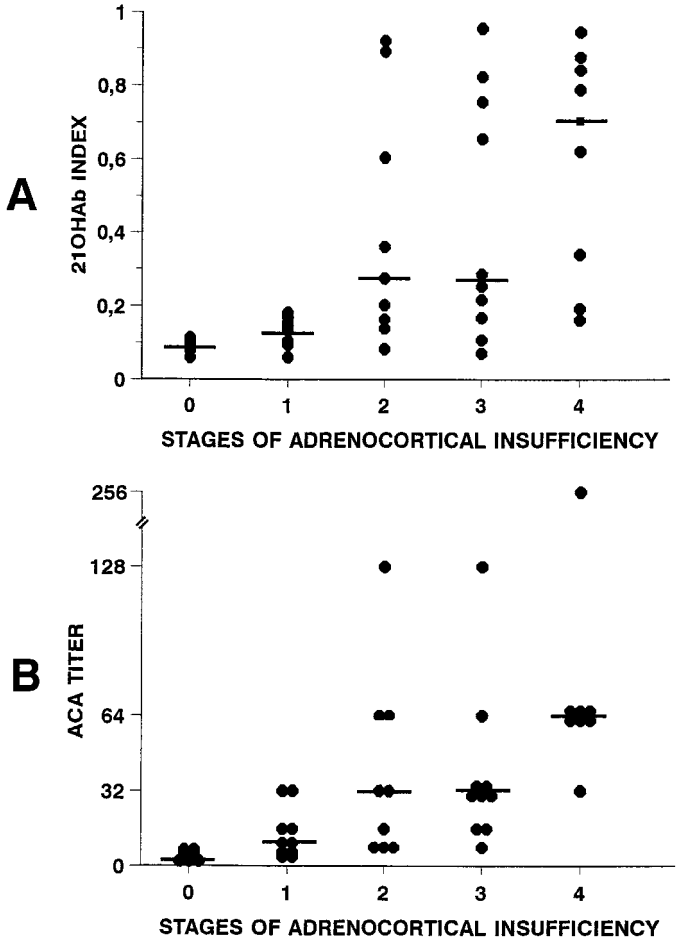

FIG. 2. 21OHAb index (A) and ACA titers (B), in relation to stage of adrenocortical dysfunction (ANOVA: $P < 0.0001$ for both 21OHAb and ACA). *Transversal bars* show the median values of 21OHAb index (A) and ACA titer (B).

than stage 2+3 (corrected $P < 0.001$ and $P < 0.05$, respectively), and the ACA titer of stage 2+3 was lower than stage 4 (corrected $P < 0.05$).

Comparison of samples at entry and at the end of the follow-up period showed a significant increase in 21OHAb index ($P = 0.041$) and ACA titer ($P = 0.002$) in those subjects with progression of adrenal dysfunction (groups A and B) (Table 2).

The remission of biochemical signs of adrenal dysfunction was associated with the disappearance of both ACA and 21OHAb in five subjects (nos. 15–19).

Discussion

The role of adrenal autoantibodies in the pathogenesis of Addison's disease is still unclear. Although it was shown that human autoantibodies may affect the enzymatic action of steroid 21OH *in vitro* (28), there is no evidence for the specific block of this enzyme, with subsequent accumulation of 17OH-progesterone during the *in vivo* natural history of the disease (29). Nevertheless, the presence of adrenal autoantibodies is an early marker of an adrenal autoimmune process and may predict the development of clinical signs of adrenal insufficiency (21). On the other hand, it is still unclear whether levels of adrenal autoantibodies correlate with the severity of adrenal dysfunction in the preclinical period. This is of high interest for our understanding of the mechanisms responsible for the production of adrenal autoantibodies.

Our study confirms previous reports (18, 23, 24) that showed that 21OH is a major autoantigen recognized by ACA during the preclinical period. In addition, we show that: 1) levels of adrenal autoantibodies correlate with the severity of adrenal dysfunction in the preclinical period; and 2) early biochemical signs of adrenal dysfunction may spontaneously remit, in parallel to the disappearance of both ACA and 21OHAb.

The observation that all our 19 ACA-positive subjects, as well as all the follow-up samples of 14 subjects with progressive adrenal dysfunction, were positive for 21OHAb supports the hypothesis that 21OH is the major adrenal autoantigen recognized by autoantibodies. A similar finding has recently been reported by Betterle *et al.* (24) on a group of 48 ACA-positive individuals. In our study, 21OHAb appeared before ACA in two subjects with progressive adrenal dysfunction (Table 2). Thus, the presence of 21OHAb may be a sensitive marker of an ongoing adrenal autoimmune process in patients with endocrine autoimmune diseases. In a recent study of IDDM patients (18), it was proposed that 21OHAb can be used as a marker for the large-scale screening of patients with endocrine autoimmune diseases for adrenal insufficiency. However, as also demonstrated by previous studies (22, 24, 25), the presence of adrenal autoantibodies does not lead necessarily to clinical Addison's disease.

In a previous study (22), the disappearance of ACA was associated with the spontaneous remission of early subclinical adrenal dysfunction. An important finding of our present study is the demonstration that the disappearance of circulating 21OHAb can parallel that of ACA in subjects with spontaneous remission of early subclinical adrenal dysfunction. Thus, our study demonstrates unequivocally that a spontaneous remission of early stages of subclinical adrenal dysfunction can occur and is associated with the disappearance of adrenal autoantibodies.

Although it has previously been shown that the risk for clinical Addison's disease is increased in subjects with ACA and 21OHAb, especially in the presence of the susceptible HLA-DR3 haplotype and of complement-fixing ACA (24), little information is currently available on the relationship between adrenal autoantibody levels and the severity of preclinical adrenal insufficiency. In our study, a clear correlation between levels of ACA/21OHAb and the degree of the adrenal dysfunction was observed in 19 subjects with preclinical adrenal insufficiency. Accordingly, levels of 21OHAb and ACA are a useful marker to monitor the progression of the adrenal autoimmune process and may be useful to improve our understanding of the mechanisms responsible for autoantibody production.

21OHAb production may be a secondary, side-effect of the T-cell-mediated destruction of adrenocortical cells with the subsequent release of 21OH or 21OH-related peptides. However, we observed a significant increase in 21OHAb index between stage 1 and stage 2+3, but not between stage 0 and 1 or between stages 2, 3, and 4. The absence of a significant

change in 21OHAb levels in the advanced stages of adrenal dysfunction supports the hypothesis that 21OHAb production is not exclusively dependent on the release of an intracellular autoantigen. Furthermore, the observation that disease-related 21OHAb epitopes seem to be conserved among different patients and restricted to the central and COOH-terminal regions of the autoantigen (30, 31) can be interpreted to indicate that 21OHAb are the result of a selective, oligoclonal, and epitope-specific process that generates autoantibodies at increasing affinity for the antigen.

A significant increase in 21OHAb levels may be the sign of a switch from an early, potentially reversible activation of the immune system to a destructive and irreversible phase of the autoimmune process. Based on the results of our study, the switch toward a progressively destructive autoimmune process is likely to be associated with the stage 2–3 of adrenal dysfunction. In fact, in all the subjects who were at stage 2 or 3 at entry, adrenal dysfunction progressed during the follow-up. On the other hand, remission of adrenal dysfunction was observed in 50% subjects who were at stage 0–1 at entry.

In conclusion, the results of our study demonstrate that levels of adrenal autoantibodies correlate with the degree of the adrenal dysfunction in subjects with preclinical adrenal insufficiency, and they suggest that production of high levels of adrenal autoantibodies is strictly associated with the activation of the destructive phase of the autoimmune disease process.

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