0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(4):1598–1604

doi: 10.1210/jc.2003-030954

Italian Addison Network Study: Update of Diagnostic Criteria for the Etiological Classification of Primary Adrenal Insufficiency

ALBERTO FALORNI, STEFANO LAURETI, ANNAMARIA DE BELLIS, RENATO ZANCHETTA, CLAUDIO TIBERTI, GIORGIO ARNALDI, VITTORIO BINI, PAOLO BECK-PECCOZ, ANTONIO BIZZARRO, FRANCESCO DOTTA, FRANCO MANTERO, ANTONIO BELLASTELLA, CORRADO BETTERLE, AND FAUSTO SANTEUSANIO ON BEHALF OF THE SIE ADDISON STUDY GROUP

Departments of Internal Medicine (A.F., S.L., F.S.) and Gynecologic, Obstetric, and Paediatric Sciences (V.B.), University of Perugia, 06126 Perugia, Italy; Department of Clinical and Experimental Medicine and Surgery (A.D.B., A.Bi., A.Be.), "F. Magrassi–A. Lanzara," Second University of Naples, 80131 Naples, Italy; Department of Medical and Surgical Sciences (R.Z., F.M., C.B.), University of Padova, 35131 Padova, Italy; Department of Clinical Sciences (C.T., F.D.), University of Rome "La Sapienza," 00161 Rome, Italy; Department of Internal Medicine (G.A.), University of Ancona, 60131 Ancona, Italy; and Institute of Endocrine Sciences (P.B.-P.), University of Milan, 20122 Milan, Italy

Primary adrenal insufficiency (PAI) is clinically evident in one in 8000 individuals. A correct etiological classification is critical for correct disease management. To update the diagnostic criteria for the etiological classification of PAI, a multicentric network was established in Italy, and 222 patients with PAI were studied. Both 21-hydroxylase and adrenal cortex autoantibodies (21OHAb and ACA, respectively) were tested in two independent laboratories on coded samples and found in 65–66% and 58–61% of cases, respectively. Autoimmune polyendocrine syndrome I was diagnosed in 11 of the 222 patients. Of the remaining 211 patients, 38 (18%) had a nonautoimmune form of PAI. In 145 subjects (65%), the presence of adrenal autoantibodies, without signs of other forms of PAI, led to a diagnosis of autoimmune Addison's disease. In six cases (3%), PAI remained idiopathic. Logistic regression analysis showed a 92.2–92.7% probability of correct reclassification for the two 21OHAb assays and 84.5–85.9% for the ACA assays. We conclude that the simultaneous presence of both 21OHAb and ACA permits unambiguous diagnosis of autoimmune Addison's, whereas subjects with low antibody titers should undergo both instrumental and biochemical tests to exclude other causes of PAI. Lastly, we developed a comprehensive flowchart for the classification of PAI for use in routine clinical practice. (*J Clin Endocrinol Metab* 89: 1598–1604, 2004)

THE CLINICAL RELEVANCE of primary adrenal insufficiency (PAI) is more significant today than 20–30 yr ago because its prevalence in the general population is now more than 3 times higher than what was recorded in the 1970s (1). It is still unclear whether this increased prevalence is simply the consequence of more accurate diagnosis or whether it reflects a higher frequency of clinical PAI. Nevertheless, today PAI is clinically evident in approximately 1 in 8000 individuals in Western countries (2–4).

The clinical spectrum of PAI has expanded considerably in recent decades, due to the identification and characterization of several novel causative genetic disorders (5–7). Consequently, the incidence and prevalence of the different forms of PAI is under intensive reinvestigation.

Autoimmune Addison's disease (AAD) is the most common cause of PAI in Western countries, accounting for 68–94% of cases in the different studies (1). The classical adrenal cortex autoantibody (ACA) assay, via indirect immunofluorescence on cryostatic sections of adrenal glands, has been used for many years because the best marker for the identification of AAD (1, 8–10). The identification and molecular cloning of the major target of autoantibodies in AAD, the enzyme steroid-21-hydroxylase (21OH) (11, 12), has made it possible to develop highly sensitive and specific radiobinding assays for 21OH autoantibodies (21OHAb) (13–16). These autoantibodies can be used in diagnostic flowcharts for the discrimination of subjects with an ongoing adrenal autoimmune process (17) and a high risk to develop autoimmune adrenal insufficiency (18–20).

However, the detection of circulating adrenal autoantibodies in patients with adrenal insufficiency, particularly at low titers, does not always permit the unambiguous diagnosis of AAD because adrenal autoantibodies have sporadically been found in patients with unequivocal posttuberculosis (TBC) adrenalitis (21, 22). Furthermore, the detection of circulating adrenal autoantibodies in subjects without adrenal insufficiency cannot always be considered a marker of potential AAD because sometimes they disappear with the remission of biochemical signs of adrenal dysfunction (18, 20). Moreover, because of the unavailability of data from

Abbreviations: AAD, Autoimmune Addison's disease; ACA, adrenal cortex autoantibody; AHC, adrenal hypoplasia congenita; ALD, adrenoleukodystrophy; CI, confidence interval; CT, computed tomography; MRI, magnetic resonance imaging; 21OH, steroid-21-hydroxylase; 21OHAb, 21OH autoantibody; PAI, primary adrenal insufficiency; TBC, tuberculosis; TBC-AD, TBC Addison's disease; VLCFA, very long chain fatty acid.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

international workshops for standardizing adrenal autoantibody measurement, the results of different studies performed in different laboratories cannot be compared fully.

Improved prevention and therapy of TBC infection has dramatically reduced the frequency of post-TBC Addison's disease (TBC-AD) in Western countries, but infiltrative adrenalitis (posttuberculosis, postparacoccidiomycosis, and others) remains the most prevalent cause of PAI in developing countries (23, 24). The isolation of the causative infectious agent and the demonstration of gland enlargement and/or calcification are diagnostic for infiltrative forms of PAI, which may require adrenal biopsy in selected cases (25).

Increased plasma levels of very long chain fatty acids (VLCFAs) are pathognomonic for X-linked adrenoleukodystrophy (ALD), the most frequent genetic cause of PAI, accounting for 20–35% of male cases (26–28). On the other hand, the diagnosis of other genetic causes of PAI, such as X-linked adrenal hypoplasia congenita (AHC), familial glucocorticoid deficiency, or the triple A syndrome does not benefit from the existence of biochemical markers and requires genotyping (29–31). Kearns-Sayre syndrome, a multisystem disorder including pigmentary retinopathy, ocular myopathy, heart block, ataxia, and endocrinopathies, is suspected on clinical grounds, but the diagnosis is confirmed by the demonstration of a mitochondrial DNA deletion (6, 32). Despite the recent development of both sophisticated diagnostic imaging techniques and novel laboratory technologies that now offer a large spectrum of immunological, biochemical, and genetic diagnostic tools, the discrimination of the different etiologic forms of PAI sometimes remains problematic. To develop a comprehensive diagnostic flowchart of immunological, biochemical, and adrenal imaging data for use in routine clinical practice, the Italian Society of Endocrinology (SIE) established a specific study group for the etiological classification of PAI.

Patients and Methods

Patients

A total of 222 patients (89 males and 133 females) with PAI were studied. At the time of our study, the median patient age was 47 yr (range, 6–87 yr) and the median disease duration was 5 yr (range, 0–53 yr). In all cases, clinical symptoms and signs of PAI were associated with low basal cortisol (<3 μg/dl) and high basal ACTH (>100 pg/ml) levels. The study was conducted by a specific study group of the SIE. Endocrinological and pediatric centers were invited to collect clinical information and blood samples from PAI patients, irrespective of age, sex, disease duration, and etiological classification. The centers were selected to guarantee uniform distribution in continental Italy. A total of 34 endocrinologists from 14 centers located in nine Italian regions (three in northern Italy, four in central Italy, and two in southern Italy) participated in sample and data collection.

The availability of a blood sample was a mandatory condition for including patients in the study. Thus, serum samples, collected between January 1998 and July 2000, were available from all 222 patients. In all patients, data on the presence of clinical associated diseases and history of TBC were collected. Data on adrenal imaging [ultrasound, computed tomography (CT) scan or magnetic resonance imaging (MRI)] were available from 105 of 222 patients (47%). All the patients gave their informed consent for this study.

Study design

All the serum samples from the 222 PAI patients were tested for both ACA and 21OHAb. Each antibody assay was performed by two independent laboratories on coded samples. To estimate the diagnostic accuracy of adrenal autoantibodies for AAD, we preliminarily defined the criteria for the etiological classification of the patients:

1. AAD. A major criterion was the simultaneous presence of both ACA and 21OHAb. In patients positive for both ACA and 21OHAb, plasma VLCFA concentrations were determined in 20 of 43 males, and data on adrenal imaging were available for 53 of 127 cases. These results were used to confirm the accuracy of the major criterion for AAD. Patients positive for ACA, but negative for 21OHAb, or *vice versa*, were classified as autoimmune if: 1) imaging showed normal or reduced adrenal gland volume, without calcification (or alternatively there was no clinical history of TBC or other granulomatous diseases if no adrenal imaging data were available); 2) plasma VLCFA concentrations were within the normal range; and 3) there were no clinical signs of genetic forms of PAI, such as achalasia; alacrimia; deafness; or, in males, hypogonadotropic hypogonadism.

2. ALD. In cases of increased plasma VLCFA levels, a diagnosis of ALD was formulated irrespective of the antibody pattern and of clinical associated diseases.

3. TBC-AD. A clinical history of lung, bone, pelvic-peritoneal, or genitourinary TBC associated with adrenal enlargement or calcification at ultrasound, CT scan, or MRI was considered a major criterion for a diagnosis of TBC-AD. The presence of a clinical history of TBC, associated with a normal plasma VLCFA pattern and the absence of adrenal autoantibodies in the four laboratories, was considered a minor criterion for infiltrative adrenal insufficiency if adrenal imaging results were not available.

4. Other causes. Other possible causes of AD, such as postsepsis AD or genetic forms, were also taken into consideration in the differential diagnosis.

5. Idiopathic AD. In adrenal antibody-negative subjects with normal pattern of plasma VLCFAs, normal adrenal imaging results, no clinical history of TBC or other infectious diseases, and no signs or symptoms suggestive of a genetic form of AD, a diagnosis of idiopathic AD was formulated.

6. Unclassified cases. These included those with clinical or adrenal imaging data insufficient to reach a definitive etiological diagnosis.

Antibody testing

21OHAb. 21OHAb were determined in laboratories 1 and 2 using a radiobinding assay with *in vitro* translated recombinant human ^{35}S-21OH. 21OHAb levels were expressed as a relative index (21OH index) based on the analysis of one positive and two negative standard sera included in each assay: the upper level of normal for 21OH index was 0.06 in both laboratories (13). Using radiobinding assays, 21OHAbs have been detected in 0.5–0.6% healthy Caucasian subjects (14–16). In a recent study of healthy Italian subjects, 21OHAbs were detected in only 1 of 210 cases (<0.5%) (our unpublished data).

ACA. ACAs were determined in laboratories 3 and 4 using indirect immunofluorescence on cryostatic sections of either human (laboratory 3) (9) or monkey (laboratory 4) (18) adrenal glands. Levels of ACA were expressed as the reciprocal of the end point dilution titer. Prevalence of ACA among healthy Italian control subjects was shown to be 0.3% (19).

VLCFA determination

Plasma VLCFA concentration was determined by capillary gas chromatography-mass spectrometry in male PAI patients from whom plasma samples were available. Normal ranges were: C26:0 = 0.1–0.46 μg/ml, C26:C22 ratio = 0.005–0.016, and C24:C22 ratio = 0.55–0.97 (27).

Statistical analysis

The degree of concordance for the dichotomous variable presence/absence of 21OHAb or ACA between the two different laboratories that performed each assay was estimated using the κ-test of interrater agreement (33) (κ-value ranging from 0–1). The degree of concordance of the

continuous variable 21OH index between the two laboratories was calculated using the Bland-Altman test (34), with calculation of the repeatability coefficient. Finally, the accuracy of each antibody assay in discriminating between autoimmune and non-AAD (according to the criteria previously described) was estimated by logistic regression analysis and included disease duration as a variable in the equation. Patients with autoimmune polyendocrine syndrome type I were not included in this analysis because the presence/absence of autoantibodies does not influence the clinical diagnosis of this syndrome. The correlation between 21OHAb index or ACA levels and disease duration was tested by Spearman's rank correlation coefficient. $P < 0.05$ was considered significant.

Results

Of the 222 PAI patients studied, 71 had Hashimoto's thyroiditis, 18 atrophic gastritis, 17 type 1 diabetes, 14 vitiligo, 14 alopecia, 11 chronic mucocutaneous candidiasis, 11 hypoparathyroidism, nine Graves' disease, four hypogonadotropic hypogonadism, three Sjögren's disease, one rheumatoid arthritis, and 25 women premature ovarian failure. None of the 222 PAI patients studied had HIV infection.

The simultaneous presence of 21OHAb and ACA was detected in 127 of 222 cases (57%) and none of the 127 cases had a clinical history of TBC (Table 1). None of 53 patients in this group (in whom adrenal imaging data were available) had imaging signs of infiltrative adrenalitis and none of 20 males (with available plasma samples) had increased plasma VLCFA levels (Table 1). The simultaneous presence of 21OHAb and ACA identified a subgroup of PAI patients with a high frequency of concomitant autoimmune diseases, present in 57% of cases.

A total of 18 of 222 subjects (8%) was positive for only 21OHAb (without ACA). In three of these 21OHAb-positive cases (21OH index was low and ranging from 0.235–0.340), a clinical history of TBC was present and adrenal gland enlargement and calcification on the CT were present in two (Table 1). VLCFAs were negative in eight of eight tested males. In this group positive for only 21OHAb, five patients (28%) had associated autoimmune diseases.

The isolated presence of ACA (in the absence of 21OHAb) was demonstrated in 27 of 222 (12%) patients. In eight of these ACA-positive patients (ACA levels were at low titers and ranging from 2–4), a clinical history of TBC and imaging signs of adrenal gland enlargement and/or calcification were present (Table 1). Moreover, increased VLCFA levels were detected in three of 14 tested males with MRI signs and neurological symptoms of X-linked ALD (ACA levels ranged from 3–8) (Table 1). Autoimmune diseases were present in 22% of cases (and, in particular, in one of eight TBC-AD patients and in one of three with ALD).

Lastly, 50 patients (23%) were negative for both ACA and 21OHAb. A clinical history of TBC was present in 17 of these 50 patients and imaging signs of infiltrative adrenalitis were detected in 15. X-linked ALD was diagnosed in five of 24 tested males (Table 1). In this antibody-negative group, autoimmune diseases were present in 14 cases (28%) (and in particular in five of 18 TBC-AD patients with TBC and in zero of five with ALD).

On the basis of the major and minor criteria initially defined, the following etiological causes of AD were identified and summarized in Table 2. Of the 222 PAI patients studied, 11 had autoimmune polyendocrine syndrome type I. Of these 11 patients, 10 tested positive for both 21OHAb and ACA, whereas a 22-yr-old woman who developed candidiasis a few months after birth, hypoparathyroidism at the age of 9 yr, and adrenal insufficiency at the age of 19 yr was negative for both 21OHAb and ACA. Of the remaining 211 subjects, AAD was diagnosed in 145 (117 positive for both 21OHAb and ACA and 28 positive for either 21OHAb or ACA but without increased VLCFA levels or a clinical history of infiltrative adrenalitis). In 28 patients, a diagnosis of TBC-AD was formulated due to gland enlargement or calcification in 25 patients or a clinical history of TBC, without adrenal autoantibodies, in the other three. Of the remaining 36 subjects, eight had X-linked ALD, one had survived sepsis from *Staphylococcus aureus* and one male with hypogonadotropic hypogonadism tested positive for a nonsense mutation of the DAX-1 gene and was diagnosed with AHC (he tested positive for low ACA levels in one laboratory). In six cases (<3% of the total population studied), a definitive diagnosis could not be formulated and remained idiopathic. In these six subjects, adrenal autoantibodies were negative in all four laboratories, plasma VLCFA concentrations were within the normal range, imaging did not reveal any enlargement or calcification of the adrenal glands, and there were no signs of concomitant diseases suggestive of a genetic form of PAI. Finally, for 22 subjects (including two subjects positive for ACA and one positive for 21OHAb at low levels and with a clinical history of TBC), no sufficient clinical or imaging data were available to exclude granulomatous adrenalitis.

Analysis of the concordance of the presence/absence of adrenal autoantibodies between different laboratories yielded a κ-value of 0.94 for 21OHAb (between laboratory 1

TABLE 1. Adrenal imaging results and frequency of increased VLCFA plasma levels in 222 PAI patients, according to the presence of adrenal autoantibodies

	n (%)	Males/females	Pts. with imaging suggestive of infiltrative adrenalitis	Pts. with clinical history of TBC	Males with increased plasma VLCFA	Pts. with clinical autoimmune diseases (%)
ACA and 21OHAb	127 (57)	43/84	0/53	0/127	0/20	73/127 (57)
21OHAb only	18 (8)	8/10	2/12	3[a]/18	0/8	5/18 (28)
ACA only	27 (12)	14/13	8/17	8[b]/27	3/14	6/27 (22)
Neither ACA nor 21OHAb	50 (23)	24/26	15/23	17[c]/50	5/24	14/50 (28)
Total	222 (100)	89/133	25/105	28/222	8/66	98/222 (44)

Pts., Patients.
[a] One subject; [b] two subjects; and [c] three subjects with no imaging data available.

TABLE 2. Definitive etiological classification of the 222 Italian PAI patients

	n (%)	Males/females	Age at diagnosis (yr)	Disease duration (yr)
APS I	11 (5)	5/6	10 (2–33)	7 (1–24)
Autoimmune (not APS I)	145 (65)	53/92	33 (8–73)	5 (0–53)
TBC-AD	28 (12.5)	14/14	55 (33–74)	5 (0–49)
X-linked ALD	8 (3.5)[a]	8/0	22 (14–36)	4 (1–31)
Postsepsis	1 (0.5)	1/0	72	1
AHC	1 (0.5)	1/0	19	25
Idiopathic	6 (3)	1/5	52 (14–71)	9 (1–42)
Incomplete data	22 (10)	6/16	37 (3–70)	7 (1–36)

APS, Autoimmune polyendocrine syndrome. Age at diagnosis and disease duration are expressed as median (range).
[a] Frequency among male PAI patients, 9.5%.

TABLE 3. Threshold levels of adrenal autoantibodies for probability of accurate diagnosis of AAD

% Probability of autoimmunity	Lab 1 21OHAb		Lab 2 21OHAb		Lab 3 ACA		Lab 4 ACA	
	thresh.	p.le	thresh.	p.le	thresh.	p.le	thresh.	p.le
75	0.061	0	0.113	5.9	1.88	0	4.13	13.6
90	0.084	3.0	0.163	11.1	3.04	16.0	6.42	13.6
95	0.099	4.5	0.197	13.3	3.83	16.0	7.96	13.6
98	0.119	5.3	0.222	14.8	4.84	28.8	9.94	34.8
99	0.135	5.3	0.240	17.0	5.58	28.8	11.42	34.8
99.96	0.200	6.8	0.273	20.0	8.99	39.2	18.00	50.8

thresh., Threshold of 21OH index or of the reciprocal of ACA endpoint dilution titer; p.le, percentile.

and laboratory 2) and 0.74 for ACA (between laboratory 3 and laboratory 4). The Bland-Altman test for the concordance of 21OHAb levels between the two laboratories produced a repeatability coefficient of 0.55, a 21OH index value too high to assimilate the results of the two laboratories in a single series of values. Hence, both ACA and 21OHAb levels were analyzed separately for each laboratory in the subsequent logistic regression analysis.

According to the criteria of discrimination between autoimmune and non-AAD defined initially and irrespective of autoantibody titer, in the logistic regression analysis, the estimate of an accurate reclassification of nonautoimmune cases ranged from 93.2–96.6% for 21OHAb and from 88.1–91.5% for ACA, and autoimmune cases from 90.5–92.5% for 21OHAb and from 81.6–85% for ACA. The overall estimate ranged from 92.2–92.7% for 21OHAb and from 84.5–85.9% for ACA. The inclusion in the model of the results of all four laboratories and disease duration led to improved reclassification, which rose to 98.2% for nonautoimmune cases, 96.3% for autoimmune cases, and 97% globally. Odds ratios derived from the logistic regression analysis were 1.62 [confidence interval (CI) = 1.27–2.06) for laboratory 1 (for 0.01-unit change)], 1.24 (CI = 1.13–1.37) for laboratory 2 (for 0.01-unit change), 2.15 (CI = 1.58–2.93) for laboratory 3 (for 1-unit change), and 1.62 (CI = 1.34–1.94) for laboratory 4 (for 1-unit change) ($P < 0.001$ in all four cases). The threshold levels that can ensure a probability of autoimmunity higher than 99%, irrespective of the results of the other laboratories, were 0.135 for 21OHAb in laboratory 1 (corresponding to the sixth percentile of the laboratory's positive samples), 0.240 for 21OHAb in laboratory 2 (17th percentile), 6 for the reciprocal of the end point dilution titer of ACA in laboratory 3 (29th percentile), and 12 for the reciprocal of the end point dilution titer of ACA in laboratory 4 (35th percentile) (Table 3).

21OHAb index ($r = -0.229$ and $r = -0.248$; $P = 0.001$) and ACA titer ($r = -0.222$ and $r = -0.239$; $P = 0.002$ and $P = 0.001$) correlated inversely and significantly with disease duration. However, disease duration accounted only for 4.9–6.1% of the variability of adrenal autoantibody levels in our population of PAI patients, according to the observed correlation coefficients.

Discussion

It is widely known that the appearance of circulating adrenal autoantibodies marks the progression of an adrenocortical autoimmune process and identifies subjects with preclinical AD (18–20, 35–37). Once the clinical signs of the disease have appeared, ACA and 21OHAb assays can be used in routine clinical practice to discriminate between autoimmune and nonautoimmune cases. As for other main organ-specific autoantibodies, if they are searched closely to the clinical onset of the disease, these markers are present in almost all patients, but their frequency tends to diminish in relation to the duration of the disease (1, 16, 17).

Given the high diagnostic sensitivity and specificity of the adrenal autoantibody assays currently available, they are typically used at the time of diagnosis as the first step in the etiological classification of PAI (17) (Fig. 1). However, only the analysis of a large population of PAI patients can guarantee a reliable estimate of the diagnostic accuracy of these markers. This is fundamental because no other accurate marker of adrenal autoimmunity is currently available. Because of the absence of an independent gold standard for adrenal autoimmunity, only autoantibody results can be used for discriminate between autoimmune and nonautoimmune cases. However, the same classification must be used to test the diagnostic accuracy of the autoantibody analysis. To avoid circular reasoning, we used the compar-

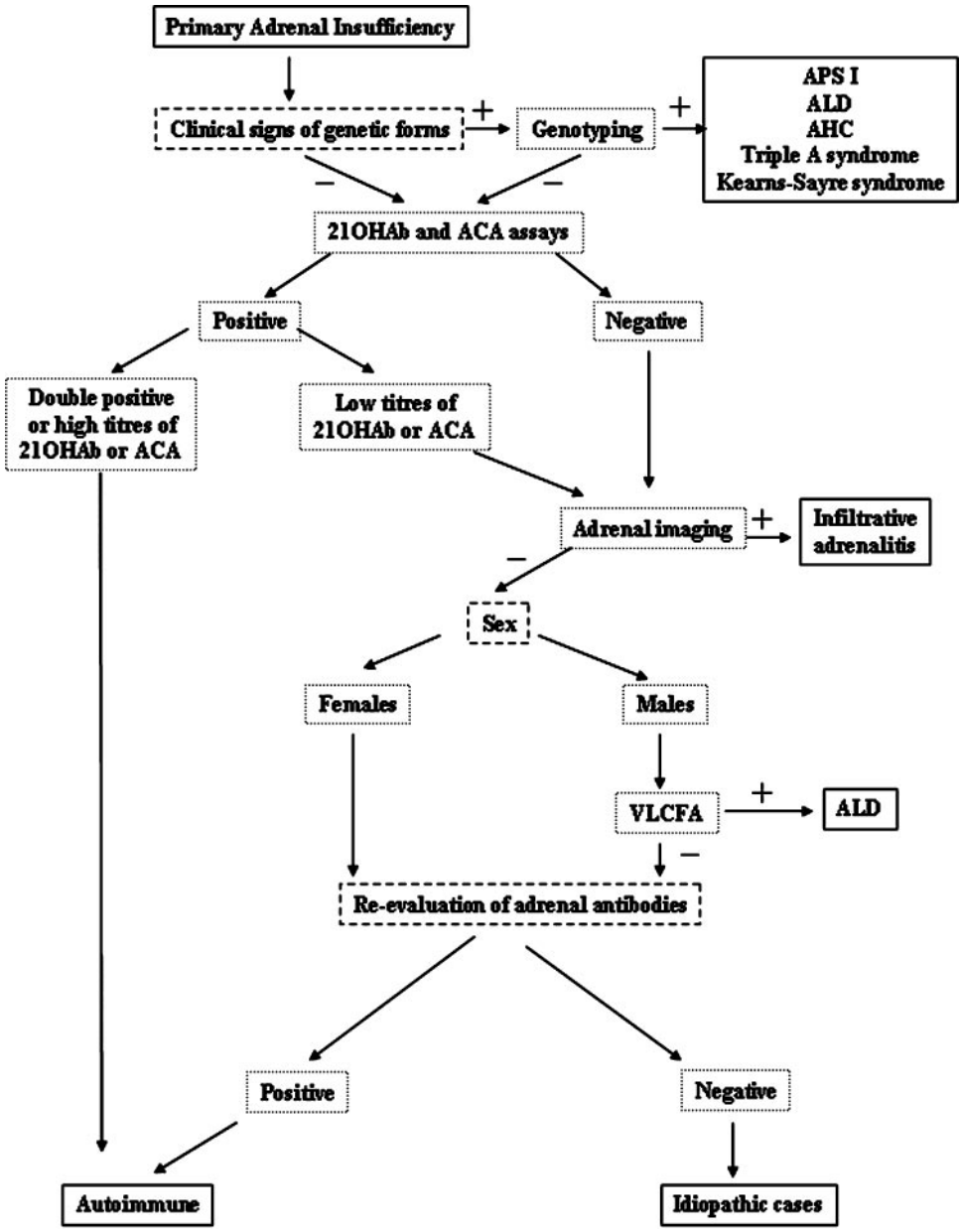

FIG. 1. Flowchart for the classification of primary adrenal insufficiency. APS, Autoimmune polyendocrine syndrome.

ison of the results from different laboratories, which worked on coded samples, to estimate the accuracy of each adrenal autoantibody assay for a correct PAI reclassification.

In our study, which represents the largest multicenter analysis of PAI patients performed to date, we compared the results of four independent laboratories that determined the levels of ACA or 21OHAb on coded serum samples from 222 PAI patients with various disease duration. On the basis of this investigation, we have identified the following forms of AD and we can made the following conclusions.

Over 70% of patients had AAD. The simultaneous presence of both 21OHAb and ACA is the gold standard for diagnosing the autoimmune forms of PAI. In fact, subjects positive for both autoantibodies were negative for adrenal infiltration or for VLCFAs in our study. Unfortunately, only very specialized laboratories routinely perform both 21OHAb and ACA assays. More frequently only one of the two assays is available, but, as clearly demonstrated by our study, the presence of only one of the two adrenal autoantibody types does not necessarily exclude a nonautoimmune origin of the disease, and further instrumental and biochemical analyses are generally required.

Approximately 13% of our patients met the criteria for a diagnosis of TBC-AD. The diagnosis of TBC-AD is still based on case history and imaging signs such as adrenal gland enlargement with or without calcification (25). Although, in general, patients with TBC-AD are negative for adrenal autoantibodies, the detection of ACA or 21OHAb in subjects with adrenal imaging and history of TBC is not sufficient to exclude the diagnosis of TBC-AD. If the results of adrenal imaging are not available, a diagnosis of TBC-AD, in the presence of a clinical history of TBC, can be suspected only

if immunological or biochemical markers of AAD or ALD are absent and the patient does not present clinical signs of genetic forms of PAI.

In our study, the results of the adrenal imaging were available for only 47% of subjects. This is a consequence of the multicentric nature of our study and does not appear to influence our major conclusions. Adrenal imaging was performed before the autoantibody analyses and were available for 48% of patients subsequently found positive for autoantibodies in at least one laboratory and for 46% of patients negative for adrenal autoantibodies (Table 1). Accordingly, the rate of finding nonimmune reasons for adrenal insufficiency was not falsely increased in subjects without autoantibodies, compared with subjects positive for autoantibodies.

Less controversial is the diagnosis of ALD in the presence of increased plasma levels of VLCFAs (26, 27) or the diagnosis of genetic forms of PAI in the presence of mutations of the candidate genes (29–31). In our study, eight males had ALD and one male had AHC. In general, patients with ALD are negative for adrenal autoantibodies, but the detection of ACA in some cases with increased VLCFAs, in our study, demonstrates that in the presence of the single ACA positivity, ALD cannot be excluded. The results of our study can be interpreted as also indicating that the 21OHAb radiobinding assay is more accurate than the ACA immunofluorescence assay for the correct classification of AAD. The type of substrate used in the immunofluorescence assay did not appear to be a major variable in our study because the two ACA assays used different substrate types (human *vs.* monkey adrenal tissue) but performed similarly.

The detection of ACA in 12 subjects and 21OHAb in two subjects with nonautoimmune forms of PAI demonstrates that in the presence of a single positivity for either ACA or 21OHAb, the diagnosis of AAD may be inaccurate. The possibility that two different forms of PAI coexist in the same subject, although exceptional, cannot be ruled out. However, it is more likely that errors in the etiological classification are caused by false positives of the autoantibody assays. Interestingly, the probability of correct classification of AAD varied in our study according to autoantibody titer (Table 3). Logistic regression analysis showed that the higher the antibody level, the higher the probability of correctly classifying the patient as autoimmune. Thus, in 83–95% of 21OHAb-positive subjects, antibody levels over 0.135–0.240 ensured a probability of autoimmunity higher than 99%, regardless of the results of the ACA assay. On the other hand, to obtain a similar probability using only the ACA assay, the cut-off had to be set at a reciprocal end point titer of 6–12 to exclude 29–35% of patients with low antibody titers. Thus, the use of only one of the two antibody assays in routine clinical practice is justified if a proper cutoff level is calculated to select the group of subjects with higher antibody levels (Fig. 1). Subjects with low antibody levels should be studied for other causes of adrenal insufficiency, and only in the absence of clinical, laboratory, and instrumental signs of nonautoimmune forms can these be reclassified as autoimmune (Fig. 1). The results and conclusions of our study provide the best rationale for the future organization of workshops to standardize adrenal autoantibody assays among different laboratories and also to arrive at a better definition of the cutoff levels to use for the etiological classification of PAI.

According to our flowchart, less than 3% of our 222 subjects with PAI remained truly idiopathic. The absence of detectable adrenal autoantibodies in these six patients does not exclude the possibility of an autoimmune origin of the disease because autoantibodies may have been present in the past but disappeared at the time of the analysis, or different and as-yet-unidentified autoantigens may be the target of the autoimmune process. This may be the case with two of our six idiopathic patients, who were studied over 35 yr after the clinical diagnosis of PAI. We cannot exclude that some of our idiopathic subjects may have a genetic form of PAI, even though none of them presented with achalasia, alacrimia, hypogonadotropic hypogonadism, or clinical signs of Kearns-Sayre syndrome. In two women the clinical signs of the disease manifested after the age of 65 yr, thus making a genetic form of PAI unlikely. It must be noted that the low frequency of genetic forms in our study does not exclude the existence of cases of Triple A syndrome or familial glucocorticoid deficiency in Italy and is presumably influenced by the predominant enrollment of adult subjects by endocrinological centers rather than pediatric centers. Similarly, no patients with HIV infection were present in our study population. Because in Italy AIDS patients are typically treated by specialists in infectious diseases, it is not surprising that none of the endocrinological centers that participated in our network had cases of PAI associated with HIV infection. However, this does not affect the conclusions of our study, which was aimed at developing a diagnostic flowchart for the etiological classification of PAI and not at estimating the prevalence of the different forms of PAI in Italy.

From our study it emerges that a clear diagnosis of etiological forms of AD can derive only from the combined use of immunological, clinical, radiological, and biochemical data. An original flowchart for the etiological classification of AD was developed after having tested the diagnostic accuracy of adrenal autoantibody assays in a large national, multicenter study, and novel information for interpreting immunological, biochemical, and instrumental data in the management of PAI patients was obtained.

Acknowledgments

We thank Catherine Bolton for editorial assistance.

Received June 3, 2003. Accepted December 22, 2003.

Address all correspondence and requests for reprints to: Fausto Santeusanio, M.D., Department of Internal Medicine, Section of Internal Medicine and Endocrine and Metabolic Sciences, Via E. Dal Pozzo, I-06126 Perugia, Italy. E-mail: fsant@unipg.it.

This work was supported by a grant from the Ministry of University and Scientific Research, Project "Morbo di Addison: forme cliniche, eziopatogenesi e storia naturale," PRIN 2001063439.

A.F. and S.L. contributed equally to this work.

In addition to the authors, the following members of the SIE Addison Study Group contributed to the collection of data and blood samples from patients with primary adrenal insufficiency: B. Ambrosi (Milan), A. Angeli (Turin), E. Arvat (Turin), A. Baccarelli (Milan), L. Barbetta (Milan), M. Boscaro (Ancona), F. Cavagnini (Milan), C. Dal Pra (Padova), E. Ghigo (Turin), R. Giordano (Turin), F. Loré (Siena), M. Mannelli (Florence), G. Mantovani (Milan), P. Paccotti (Turin), F. Pecori-Gilardi (Milan), R. Perniola (Lecce), M. Terzolo (Turin), P. Toja (Milan), M.

Torlontano (S. Giovanni Rotondo), V. Toscano (Rome), and V. Trischitta (S. Giovanni Rotondo).

References

1. **Betterle C, Dal Pra C, Mantero F, Zanchetta R** 2002 Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. Endocr Rev 23:327–364
2. **Kong MF, Jeffcoate W** 1994 Eighty-six cases of Addison's disease. Clin Endocrinol (Oxf) 41:757–761
3. **Laureti S, Vecchi L, Santeusanio F, Falorni A** 1999 Is the prevalence of Addison's disease underestimated? J Clin Endocrinol Metab 84:1762
4. **Lovas K, Husebye ES** 2002 High prevalence and increasing incidence of Addison's disease in western Norway. Clin Endocrinol (Oxf) 56:787–791
5. **Carey RM** 1997 Changing clinical spectrum of adrenal insufficiency. Ann Intern Med 127:1103–1105
6. **Vaidya B, Pearce S, Kendall-Taylor P** 2000 Recent advances in the molecular genetics of congenital and acquired primary adrenocortical failure. Clin Endocrinol (Oxf) 53:403–418
7. **Ten S, New M, MacLaren N** 2001 Clinical review 130: Addison's disease 2001. J Clin Endocrinol Metab 86:2909–2922
8. **Blizzard RM, Kyle MA** 1963 Studies of the adrenal antigens and antibodies in Addison's disease. J Clin Invest 42:1653–1660
9. **Betterle C, Scalici C, Presotto F, Pedini B, Moro L, Rigon F, Mantero F** 1988 The natural history of adrenal function in autoimmune patients with adrenal autoantibodies. J Endocrinol 117:467–475
10. **Betterle C, Volpato M, Pedini B, Chen S, Smith BR, Furmaniak J** 1999 Adrenal-cortex autoantibodies and steroid-producing cells autoantibodies in patients with Addison's disease: comparison of immunofluorescence and immunoprecipitation assays. J Clin Endocrinol Metab 84:618–622
11. **Winqvist O, Karlsson FA, Kämpe O** 1992 21-hydroxylase, a major autoantigen in idiopathic Addison's disease. Lancet 339:1559–1562
12. **Bednarek J, Furmaniak J, Wedlock N, Kiso Y, Baumann-Antczak A, Fowler S, Krishnan H, Craft JA, Rees Smith B** 1992 Steroid 21-hydroxylase is a major autoantigen involved in adult onset autoimmune Addison's disease. FEBS Lett 309:51–55
13. **Falorni A, Nikoshkov A, Laureti S, Grenbäch E, Hulting AL, Casucci G, Santeusanio F, Brunetti P, Luthman H, Lernmark Å** 1995 High diagnostic accuracy for idiopathic Addison's disease with a sensitive radiobinding assay for autoantibodies against recombinant human 21-hydroxylase. J Clin Endocrinol Metab 80:2752–2755
14. **Colls J, Betterle C, Volpato M, Prentice L, Smith BR, Furmaniak J** 1995 Immunoprecipitation assay for autoantibodies to steroid 21-hydroxylase in autoimmune adrenal diseases. Clin Chem 41:375–380
15. **Tanaka H, Perez MS, Powell M, Sanders JF, Sawicka J, Chen S, Prentice L, Asawa T, Betterle C, Volpato M, Rees Smith B, Furmaniak J** 1997 Steroid 21-hydroxylase autoantibodies: measurements with a new immunoprecipitation assay. J Clin Endocrinol Metab 82:1440–1446
16. **Falorni A, Laureti S, Nikoshkov A, Picchio ML, Hallengren B, Vandewalle CL, Gorus FK, Tortoioli C, Luthman H, Brunetti P, Santeusanio F** 1997 21-Hydroxylase autoantibodies in adult patients with endocrine autoimmune diseases are highly specific for Addison's disease. Clin Exp Immunol 107: 341–346
17. **Laureti S, Aubourg P, Calcinaro F, Rocchiccioli F, Casucci G, Angeletti G, Brunetti P, Lernmark Å, Santeusanio F, Falorni A** 1998 Etiological diagnosis of primary adrenal insufficiency using an original flowchart of immune and biochemical markers. J Clin Endocrinol Metab 83:3163–3168
18. **De Bellis A, Bizzarro A, Rossi R, Paglionico VA, Criscuolo T, Lombardi G, Bellastella A** 1993 Remission of subclinical adrenocortical failure in subjects with adrenal autoantibodies. J Clin Endocrinol Metab 76:1002–1007
19. **Betterle C, Volpato M, Rees Smith B, Furmaniak J, Chen S, Greggio NA, Sanzari M, Tedesco F, Pedini B, Boscaro M, Presotto F** 1997 I. Adrenal cortex and steroid 21-hydroxylase autoantibodies in adult patients with organ-specific autoimmune diseases: markers of low progression to clinical Addison's disease. J Clin Endocrinol Metab 82:932–938
20. **Laureti S, De Bellis A, Muccitelli VI, Calcinaro F, Bizzarro A, Rossi R, Bellastella A, Santeusanio F, Falorni A** 1998 Levels of adrenocortical autoantibodies correlate with degree of adrenal dysfunction in subjects with preclinical Addison's disease. J Clin Endocrinol Metab 83:3507–3511
21. **Nomura K, Depura H, Saruta T** 1994 Addison's disease in Japan: characteristics and changes revealed in a nationwide survey. Intern Med 33:602–604
22. **do Carmo Silva R, Kater CE, Dib SA, Laureti S, Forini F, Cosentino A, Falorni A** 2000 Autoantibodies against recombinant human steroidogenic enzyme 21-hydroxylase, side-chain cleavage and 17 α-hydroxylase in Addison's disease and autoimmune polyendocrine syndrome type III. Eur J Endocrinol 142:187–194
23. **Soule S** 1999 Addison's disease in Africa—a teaching hospital experience. Clin Endocrinol (Oxf) 50:115–120
24. **Agarwal G, Bhatia E. Pandey R, Jain SK** 2001 Clinical profile and prognosis of Addison's disease in India. Natl Med J India 14:23–25
25. **Oelkers W** 1996 Adrenal insufficiency. N Engl J Med 335:1206–1212
26. **Moser HW** 1997 Adrenoleukodystrophy: phenotype, genetics, pathogenesis and therapy. Brain 120:1485–1508
27. **Aubourg P** 1997 The expanding world of primary adrenal insufficiency. Eur J Endocrinol 137:10–12
28. **Laureti S, Casucci G, Santeusanio F, Angeletti G, Aubourg P, Brunetti P** 1996 X-linked adrenoleukodystrophy is a frequent cause of "idiopathic" Addison's disease in young-adult male patients. J Clin Endocrinol Metab 81:470–474
29. **Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, Lalli E, Moser C, Walker AP, McCabe ER, Meitinger T, Monaco AP, Sassone-Corsi P, Camerino G** 1994 An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature 372:635–641
30. **Clark AJL, McLoughlin L, Grossman A** 1993 Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor Lancet 341:461–462
31. **Tullio-Pelet A, Salomon R, Hadj-Rabia S, Mugnier C, de Laet MH, Chaouachi B, Bakiri F, Brottier P, Cattolico L, Penet C, Begeot M, Naville D, Nicolino M, Chaussain JL, Weissenbach J, Munnich A, Lyonnet S** 2000 Mutant WD-repeat protein in triple-A syndrome. Nat Genet 26:332–335
32. **Boles RG, Roe T, Senadheera D, Mahnovski V, Wong LJ** 1998 Mitochondrial DNA deletion with Kearns Sayre syndrome in a child with Addison's disease. Eur J Pediatr 157:643–647
33. **Altman DG** 1995 Practical statistics for medical research. London: Chapman, Hall; 403–409
34. **Bland JM, Altman DG** 1986 Statistical methods for assessing agreement between two methods of clinical measurement. Lancet I:307–310
35. **Scherbaum WA, Berg PA** 1982 Development of adrenocortical failure in non-addisonian patients with antibodies to adrenal cortex. Clin Endocrinol (Oxf.) 16:345–352
36. **Betterle C, Zanette F, Zanchetta R, Pedini B, Trevisan A, Mantero F, Rigon F** 1983 Complement-fixing adrenal autoantibodies as a marker for predicting onset of idiopathic Addison's disease. Lancet I:1238–1240
37. **Boscaro M, Betterle C, Sonino N, Volpato M, Paoletta A, Fallo F** 1994 Early adrenal hypofunction in patients with organ-specific autoantibodies and no clinical adrenal insufficiency. J Clin Endocrinol Metab 79:452–455