

# Detection of adrenocortical autoantibodies in Addison's disease with a peroxidase-labelled protein A technique

R.C. Silva<sup>1,2</sup>, S. Faiçal<sup>1</sup>,  
S. Laureti<sup>2</sup>, A. Falorni<sup>2</sup>,  
S.A. Dib<sup>1</sup> and C.E. Kater<sup>1</sup>

<sup>1</sup>Divisão de Endocrinologia, Departamento de Medicina, Universidade Federal de São Paulo, Escola Paulista de Medicina, São Paulo, SP, Brasil  
<sup>2</sup>Department of Internal Medicine and Endocrine and Metabolic Sciences, University of Perugia, Perugia, Italy

## Abstract

Adrenocortical autoantibodies (ACA), present in 60-80% of patients with idiopathic Addison's disease, are conventionally detected by indirect immunofluorescence (IIF) on frozen sections of adrenal glands. The large-scale use of IIF is limited in part by the need for a fluorescence microscope and the fact that histological sections cannot be stored for long periods of time. To circumvent these restrictions we developed a novel peroxidase-labelled protein A (PLPA) technique for the detection of ACA in patients with Addison's disease and compared the results with those obtained with the classical IIF assay. We studied serum samples from 90 healthy control subjects and 22 patients with Addison's disease, who had been clinically classified into two groups: idiopathic (N = 13) and granulomatous (N = 9). ACA-PLPA were detected in 10/22 (45%) patients: 9/13 (69%) with the idiopathic form and 1/9 (11%) with the granulomatous form, whereas ACA-IIF were detected in 11/22 patients (50%): 10/13 (77%) with the idiopathic form and 1/9 (11%) with the granulomatous form. Twelve of the 13 idiopathic Addisonians (92%) were positive for either ACA-PLPA or ACA-IIF, but only 7 were positive by both methods. In contrast, none of 90 healthy subjects was found to be positive for ACA. Thus, our study shows that the PLPA-based technique is useful, has technical advantages over the IIF method (by not requiring the use of a fluorescence microscope and by permitting section storage for long periods of time). However, since it is only 60% concordant with the ACA-IIF method, it should be considered complementary instead of an alternative method to IIF for the detection of ACA in human sera.

## Key words

- Autoantibodies detection
- Addison's disease
- Peroxidase-labelled protein A
- Indirect immunofluorescence
- Adrenal gland

## Correspondence

R.C. Silva  
Divisão de Endocrinologia  
Departamento de Medicina  
UNIFESP, EPM  
Rua Botucatu, 740  
04023-900 São Paulo, SP  
Brasil  
Fax: 55 (011) 570-6636  
E-mail: rcarmo@mandic.com.br

Research supported by FAPESP  
(No. 92/2654-0).

Received February 27, 1998  
Accepted June 3, 1998

## Introduction

Addison's disease (AD) results from destruction of corticosteroid-producing cells (1). In Western countries, autoimmune adrenalitis accounts for approximately 65 to 90% of the cases of primary adrenocortical

insufficiency (2,3).

The presence of circulating adrenal cortex autoantibodies (ACA) is the major marker of autoimmune AD, being typically found in 60 to 80% of patients with idiopathic AD (2-6). Conventionally, ACA are detected by indirect immunofluorescence (IIF) on fro-

zen sections of adrenal glands (7,8) and their presence in healthy subjects can permit the identification of pre-clinical cases (9-11).

ACA are predominantly IgGs directed against antigens originating from the cytoplasm (6,12,13) of the adrenal cortex cells, although the existence of surface cell autoantigens (14,15) cannot be excluded. Recently, the steroid-synthesizing enzyme 21-hydroxylase (P450c21) was identified as the major adrenal autoantigen in isolated AD and in autoimmune polyendocrine syndrome type II (APS II) (16,17). In addition, other P450 cytochromes (such as side-chain cleavage enzyme, P450scc, and steroid 17 $\alpha$ -hydroxylase, P450c17) can trigger an autoimmune response in APS type I (APS I) and in AD with associated premature ovarian failure (18-20).

A higher prevalence of ACA has been observed in patients with short disease duration (4,6) or in APS (21,22). ACA titers can fluctuate and a progressive decrease is observed after the onset of clinical disease (23).

The results of the classical IIF technique depend on the quality of the adrenal cortex tissue used, and the large-scale use of this method is limited in part by the need for a fluorescence microscope and the fact that histological sections cannot be stored for long periods of time. Therefore, the development of new assays for the detection of adrenal autoantibodies in human serum is warranted.

In this study we developed a novel peroxidase-labelled protein A (PLPA) technique for detection of ACA in patients with AD and compared the results with those obtained with the classical IIF assay.

## Material and Methods

### Subjects and sera

We analyzed serum samples from 22 patients with confirmed AD (12 females/10

males; median age, 54 years; range, 34-85 years; average disease duration of 5 years, range 0.1-38 years), followed since 1988 in the Division of Endocrinology at the Federal University of São Paulo. Diagnosis of primary adrenocortical insufficiency was made based on both clinical findings and the results of an acute ACTH stimulation test, in which 60-min cortisol responses were blunted to  $2.2 \pm 0.4$   $\mu\text{g}/\text{dl}$  (range 0.4-5.5  $\mu\text{g}/\text{dl}$ , normal:  $>18$   $\mu\text{g}/\text{dl}$ ) (24). All patients were already receiving steroid replacement therapy at the time of the present study.

The patients were investigated for all possible causes of AD, especially infectious or autoimmune adrenalitis. Diagnosis of post-tuberculosis or post-paracoccidioidomycosis AD was based on history, PPD tests, presence of antibodies against *Paracoccidioides brasiliensis*, and on demonstration of adrenal calcification by computerized tomography (CT). Data concerning the presence of other autoimmune diseases and anti-islet cell and anti-microsomal thyroid antibodies were also analyzed.

Prior to the evaluation of ACA, patients were assigned to two groups: "idiopathic" and "granulomatous", according to the most probable etiology of the primary adrenocortical insufficiency. Patients with adrenal insufficiency due to surgical removal of the adrenal glands, or with metastatic or hemorrhagic destruction were excluded from the study.

The "idiopathic" group consisted of 13 patients, none of whom presented any evidence of granulomatous disease. A total of 8/13 (62%) patients had one or more autoimmune-associated endocrine diseases and/or were positive for anti-microsomal thyroid antibodies (APS type II). The remaining 5 (38%) idiopathic patients had AD alone. None of them were positive for anti-islet cell antibodies, and in 10, CT of the adrenal glands showed a volume reduction suggestive of atrophy (Table 1).

The "granulomatous" group consisted of

9 patients with calcified adrenals on CT scanning, whose adrenocortical insufficiency was due to either tuberculosis (N = 4) or paracoccidioidomycosis (N = 2). Although none of the patients presented antibodies against *Paracoccidioides brasiliensis*, two of them (AZ and LR, Table 1) had been treated for paracoccidioidomycosis several years before the study. In three patients, the etiology of AD was attributable to a granulomatous disease only on the basis of the presence of adrenal calcification on CT. Among these patients, one had vitiligo and two were positive for anti-microsomal thyroid antibodies (Table 1).

Serum samples from 90 healthy control

subjects (57 males/33 females; median age of 28 years, range 19-81) were also studied.

Written informed consent was obtained from all subjects after the study protocol had been approved by the Ethics Committee on Human Research of the University Hospital.

#### **Peroxidase-labelled protein A technique (ACA-PLPA)**

Fasting serum samples were collected from all subjects and stored at -20°C until the assay was performed. Normal post-mortem human adrenal glands were obtained from kidney donors. After careful removal of the fat, the glands were cut into quarters and

Table 1 - Clinical characteristics of the 22 patients with Addison's disease.

AID: Autoimmune disease clinically manifested (H: Hashimoto's thyroiditis; GD: Graves' disease; POF: premature ovarian failure; CG: chronic atrophic gastritis; DM: type 1 diabetes mellitus; V: vitiligo); TMA: thyroid microsomal antibodies; ACA-PLPA: adrenocortical antibodies detected by the peroxidase-labelled protein A technique; ACA-IIF: adrenocortical antibodies detected by indirect immunofluorescence; NA: not available.

Patient	Sex/age (years)	Disease duration (years)	AID	TMA	Calcification on adrenal CT	PPD	Clinical classification	ACA-PLPA	ACA-IIF
GCS	F/54	0.1	H	+	-	-	idiopathic	+	+
MJ	M/53	0.1	-	-	-	-	idiopathic	-	+
RMM	F/43	1.1	-	+	-	-	idiopathic	+	+
JP	F/53	2	H/POF	+	-	-	idiopathic	-	+
MB	F/37	3	-	-	-	-	idiopathic	+	+
FAF	F/58	3	GD	-	-	-	idiopathic	+	-
AB	M/36	4.6	-	-	-	-	idiopathic	-	-
MBC	F/40	5	-	-	-	NA	idiopathic	+	+
BL	M/49	5	-	+	-	-	idiopathic	+	+
VTC	F/85	6.7	-	-	-	-	idiopathic	-	+
ELN	F/71	9	H	+	NA	-	idiopathic	+	+
JMR	F/68	14	H/POF/CG/DM	+	-	-	idiopathic	+	+
IS	F/51	15	H/GD/CG	+	-	-	idiopathic	+	-
LAS	M/52	1	-	-	bilateral	-	granulomatous	-	-
AMC	F/34	1	-	-	bilateral	+++	granulomatous	-	-
RAS	F/53	4.8	-	+	right	+++	granulomatous	-	-
AZ	M/62	5	-	-	bilateral	-	granulomatous	-	-
LR	M/61	11	V	+	bilateral	-	granulomatous	-	-
VS	M/69	19	-	-	bilateral	+++	granulomatous	-	-
AO	M/60	22	-	-	left	NA	granulomatous	+	+
APJ	M/68	28	-	-	bilateral	+++	granulomatous	-	-
AC	M/79	38	-	-	left	-	granulomatous	-	-

each fragment was wrapped with a cell protector (Reichert-Jung, LEICA Instruments, Heidelberg, Germany) and immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Unfixed  $5\text{-}\mu\text{m}$  sections were used for the detection of ACA-PLPA.

Frozen sections of the adrenals were fixed for 5 min with ketone containing 0.2%  $\text{H}_2\text{O}_2$  and then washed with Tris-buffer (10 mM Tris base, 140 mM NaCl, pH 7.4). One hundred  $\mu\text{l}$  undiluted serum was added to the sections, which were incubated in a moist chamber at room temperature for 30 min. After incubation, the sections were washed three times in Tris-buffer, rinsed and incubated at room temperature for 30 min with 100  $\mu\text{l}$  of PLPA (Boehringer-Mannheim Corp. Biochemical Products, Indianapolis, IN) diluted 1:50 in 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO). After an additional wash with Tris-buffer, the chromogenic reaction was initiated by the addition of diaminobenzidine tetrachloride (Sigma) (0.5 mg/ml in 50 mM Tris base, pH 7.6, containing 0.001%  $\text{H}_2\text{O}_2$ ) for 10 min. The stained sections were washed with water, dehydrated with alcohol, plated

and observed under the light microscope by two different investigators. The brown color indicated the presence of ACA. When positive, the serum was re-tested in progressive dilutions and the ACA-PLPA titer was defined as the maximum dilution of serum giving a positive stain reaction.

#### Indirect immunofluorescence (ACA-IIF)

ACA were detected by a classical IIF technique using thin cryostatic sections of normal bovine adrenal glands as the source of antigen. The sections were incubated with 50  $\mu\text{l}$  serum diluted 1:5 in phosphate-buffer (8 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM  $\text{KH}_2\text{PO}_4$ , 140 mM NaCl, and 3 mM KCl, pH 7.4) for 30 min. After two washes in phosphate-buffer, 50  $\mu\text{l}$  fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Dako A/S, Glostrup, Denmark) diluted 1:40 in phosphate-buffer was added to the sections, which were incubated in a moist chamber for 30 min. After being washed again with phosphate-buffer, the sections were plated and observed by two different investigators using an immunofluorescence microscope. If positive, ACA-IIF titers were determined by testing serial 2-fold dilutions of the serum until reaching the end point.

#### Other assays

Thyroid microsomal antibodies (TMA) were detected by semiquantitative agglutination (Sera-Tek microsomal antibody test kit, Ames, IN; normal  $<1/100$ ). Islet cell antibodies (ICA) were detected by the peroxidase-labelled protein A technique on sections of rat frozen pancreas (25,26) (normal  $<10$  JDF units).

Antibodies against *Paracoccidioides brasiliensis* were detected by immunodiffusion (27). Delayed cell hypersensitivity to tuberculosis (PPD test) was evaluated by the subcutaneous injection of 2 U of tuberculin into the anterior surface of the forearm.

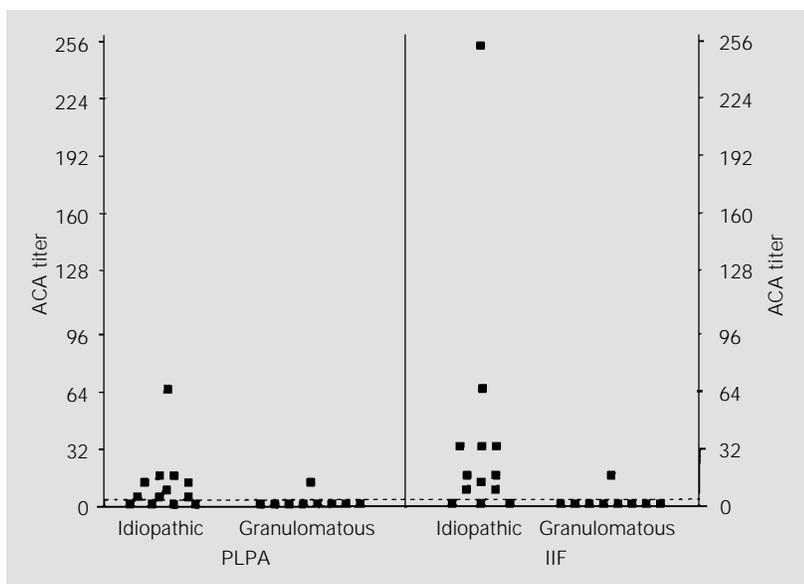


Figure 1 - ACA-PLPA and ACA-IIF levels in patients with "idiopathic" and "granulomatous" AD. The dotted lines represent the upper normal level for each assay.

### Statistical analysis

Differences in frequencies of ACA between the two patient groups or between AD patients and control subjects were tested by the chi-square method or the Fisher exact test. The relationship between ACA titers and disease duration was assessed by linear regression and the relationship between ACA-PLPA and ACA-IIF titers by the Spearman rank correlation test. P values less than 0.05 were considered significant.

## Results

### Detection of ACA by the PLPA technique

ACA-PLPA were found in 10 of the 22 patients (45%) with AD and in none of 90 control subjects ( $P < 0.0001$ ). When adrenal sections were treated with a serum positive for ACA-PLPA, the cytoplasm of all adrenal cortex cells homogeneously stained brown, in contrast with the lack of reactivity of the capsule and medullary cells. Positive sera for ACA-PLPA were re-tested several times using different human adrenal tissues and every time yielded the same results in terms of positivity and titer. The rate of concordance between the two readers was 100%.

Among the 10 patients found to be positive for ACA-PLPA, 9 (69%) were from the “idiopathic” group and only one (11%) from the “granulomatous” group ( $P = 0.01$ ) (Figure 1). Among the 13 patients with “idiopathic” AD, ACA-PLPA were detected in 7 (88%) with APS II and in 2 (40%) with AD alone.

No significant correlation was found between the presence or titer of ACA-PLPA and the duration of primary adrenocortical insufficiency.

### Detection of ACA by the IIF

ACA-IIF were detected in 11 of the 22 patients (50%) with AD and in none of 90

control subjects ( $P < 0.0001$ ).

Among the 11 patients positive for ACA-IIF, 10 (77%) were from the “idiopathic” group and only one (11%) from the “granulomatous” group ( $P = 0.008$ ) (Figure 1). Among the 13 patients with “idiopathic” AD, ACA-IIF were detected in 6 (75%) with APS II and in 4 (80%) with isolated AD.

No significant correlation was found between the presence or titers of ACA-IIF and the duration of AD.

### Comparison between ACA-PLPA and ACA-IIF

A significant and positive correlation was observed between ACA-PLPA and ACA-IIF titers ( $r^2 = 0.70$ ,  $P < 0.01$ ) (Figure 2). Twelve of the 13 patients (92%) with idiopathic AD were positive for either ACA-PLPA or ACA-IIF, but only 7 (54%) tested positive for both methods.

## Discussion

Detection of circulating ACA is critical for a correct diagnosis of autoimmune AD.

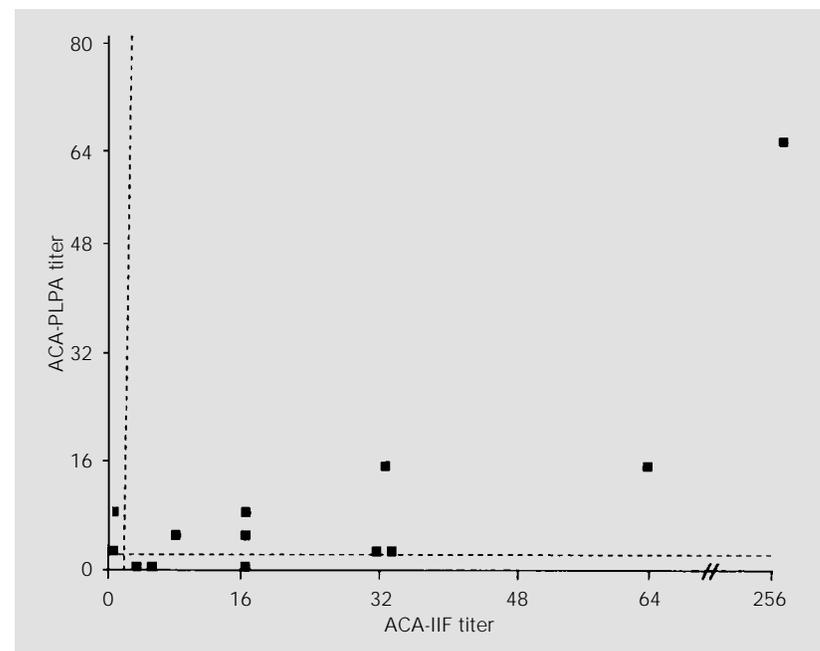


Figure 2 - Correlation between ACA-PLPA and ACA-IIF titers in ACA-positive patients ( $r^2 = 0.70$ ;  $P < 0.01$ ). The dotted lines represent the upper normal level for each assay.

Accordingly, we have developed a novel peroxidase-labelled protein A (PLPA) technique for testing ACA in human serum, which demonstrated diagnostic sensitivity and specificity similar to that of the classical immunofluorescence (IIF) assay.

The large-scale use of the IIF assay to diagnose autoimmune AD depends on the availability of a special IF microscope and is limited by the difficulty to distinguish between antibody-negative and low-level antibody-positive samples. In addition, histological sections for IIF cannot be stored for long periods of time. As with the IIF assay, results of the PLPA-based technique are somehow subjective, but the possibility to use a routine light microscope and to store the sections for long periods of time makes this assay preferable for those centers where more complex methods are not available. The fact that a PLPA-based technique for anti-islet cell antibodies proved to have high diagnostic sensitivity and specificity for IDDM (25,26) is further evidence that this type of assay can be useful for clinical diagnosis of endocrine autoimmune diseases.

Our PLPA technique permitted the detection of ACA in the sera of 45% patients with primary adrenocortical insufficiency, regardless of preliminary suspected etiology and disease duration. Among patients with clinically "idiopathic" AD, ACA were detected in 69% of cases, as compared to 77% of cases using the classical IIF assay. The diagnostic sensitivity of the PLPA technique for autoimmune AD was not statistically different from that of the IIF assay. The frequency of ACA observed in "idiopathic" AD patients was similar to that found in previous studies in other countries (2-6).

The presence of adrenal calcification on CT scanning is considered an exclusion criterion for autoimmune adrenalitis (28,29). However, in our study, ACA were found in the serum of one patient whose adrenal gland was calcified. The presence of ACA in pa-

tients with granulomatous adrenalitis has been reported before (5,8), but since it is very unusual (2,6) the possibility of a false positive result cannot be excluded. Reasons for false positivity include the presence of background staining, nonspecific binding and poor quality of the adrenal cortex tissue. It seems unlikely that the presence of ACA in the patient with granulomatous AD was the result of low specificity of our assay, since the IIF technique was also positive for ACA in that patient; in addition, none of 90 control subjects was found to be positive for ACA using either IIF or PLPA. The presence of ACA in this patient may rather have been due to the presentation to the immune system of some adrenal autoantigen resulting from the inflammatory destruction of the adrenal cortex and subsequent autoantibody production in a genetically predisposed subject. Conversely, one cannot rule out the possibility that a granulomatous adrenalitis supervened in a patient with an already ongoing autoimmune process.

Several studies found the prevalence of ACA to be dependent on the duration of AD. Wuepper et al. (4) found ACA in 89% of patients with autoimmune AD within two years of clinical diagnosis, as compared to only 50% of patients with two or more years of disease duration. Nerup (6) also reported a higher frequency of ACA in the serum of patients with less than five years of disease duration (83 vs 58% for five years or more). In a recent study of Italian AD patients (30), ACA were found in 73% of patients with less than 15 years of disease duration as compared to only 8% in patients with long-term disease. In our study, the lack of correlation between ACA and duration of the disease may have been due to the small size of our sample.

In contrast to other investigators (21,22), we were unable to find any significant association between ACA and the presence of APS II, which can also be explained by the small sample size.

Although a significant correlation between ACA-PLPA and ACA-IIF titers was observed in our study, the concordance rate between the two assays in idiopathic AD was not higher than 60%. The different sources of adrenal tissue (human and bovine) used in the two assays cannot fully explain the observed difference since enzyme expression is similar in both human and bovine adrenals (31). On the other hand, the different binding ability of protein A and of rabbit antisera for human IgG subclasses may explain, in part, the discrepancies between the two assays. The use of antigen-specific immunoprecipitation assays for autoantibodies against P450c21 (32,33) or P450c17 and P450scc (19) could be instrumental in the understanding of the mechanisms responsible for the discordance between ACA-PLPA and ACA-IIF observed in some patients with AD. Recently, Colls et al. (34), using an immunoprecipitation assay, detected autoantibodies against P450c21 (P450c21-Ab) in 66% of patients with autoimmune AD and observed a significant positive correlation between the immunoprecipitation results and those obtained with the IIF. This good agreement between measurements of specific P450c21-Ab and ACA-IIF provided further evidence that P450c21-reactive autoantibodies are a major component of ACA. Moreover, the detection of

P450c21-Ab can accurately identify individuals at high risk for AD and it is particularly important in the clinical management of patients with autoimmune endocrinopathies often associated with AD, such as Hashimoto's thyroiditis, Graves' disease, type 1 diabetes mellitus and premature ovarian failure. According to Betterle et al., 21% of adults (35) and 90% of children (36) with organ-specific autoimmune diseases (without overt hypoadrenalism) and with positive ACA-IIF and/or P450c21-Ab developed clinical AD after a mean latency period of three years.

In conclusion, this novel PLPA-based method for the detection of ACA in human serum showed a diagnostic sensitivity of 69% for autoimmune AD; the technique is simple, inexpensive and advantageous over the IIF technique by not requiring the use of an immunofluorescence microscope and permitting section storage for long periods of time, but since it is only 60% concordant, it should be considered complementary instead of an alternative method to IIF for detecting ACA in human sera.

### Acknowledgments

We gratefully acknowledge the collaboration and technical assistance of Walkyria L. Miranda and Cristina Tortoioli.

### References

- Nerup J (1974). Addison's disease - A review of some clinical, pathological and immunological features. *Danish Medical Bulletin*, 21: 201-217.
- Irvine WJ & Barnes EW (1972). Adrenocortical insufficiency. *Clinical Endocrinology and Metabolism*, 1: 549-594.
- Zelissen PMJ, Bast EJEG & Croughs RJM (1995). Associated autoimmunity in Addison's disease. *Journal of Autoimmunity*, 8: 121-130.
- Wuepper KD, Wegienka LC & Fudenberg HH (1969). Immunological aspects of adrenocortical insufficiency. *American Journal of Medicine*, 46: 206-216.
- Maisey MN & Lessof MH (1969). Addison's disease: a clinical study. *Guy's Hospital Reports*, 118: 363-372.
- Nerup J (1974). Addison's disease - serological studies. *Acta Endocrinologica*, 76: 142-158.
- Pousset G, Monier JC & Thivolet J (1970). Anticorps antisurrénaux et maladie d'Addison. Application de la technique d'immunofluorescence dans 100 cas d'insuffisance surrénale. *Annales d'Endocrinologie*, 31: 995-1002.
- Nerup J, Soborg M, Halberg P & Brochner-Mortensen K (1966). Organ specific antibodies in Addison's disease. *Acta Medica Scandinavica*, 445 (Suppl): 383-388.
- Betterle C, Zanchetta R, Trevisan A, Zanette F, Pedini B, Mantero F & Rigon F (1983). Complement-fixing adrenal autoantibodies as a marker for predicting onset of idiopathic Addison's disease. *Lancet*, i: 1238-1241.
- De Bellis A, Bizarro A, Rossi R, Amoresano Paglionico V, Criscuolo T, Lombardi G & Bellastella A (1993). Remission of subclinical adrenocortical failure in subjects with adrenal antibodies. *Journal of Clinical Endocrinology and Metabolism*, 76: 1002-1007.
- Scherbaum WA & Berg PA (1982). Devel-

- opment of adrenocortical failure in non-Addisonian patients with antibodies to adrenal cortex. A clinical follow-up study. *Clinical Endocrinology*, 16: 345-352.
12. Irvine WJ & Barnes EW (1975). Addison's disease, ovarian failure and hypoparathyroidism. *Clinical Endocrinology and Metabolism*, 4: 379-434.
  13. Goudie RB, McDonald E, Anderson JR & Gray K (1968). Immunological features of idiopathic Addison's disease: characterization of the adrenocortical antigens. *Clinical and Experimental Immunology*, 3: 119-131.
  14. Khoury EL, Hammond L, Bottazzo GF & Doniach D (1981). Surface-reactive antibodies to human adrenal cells in Addison's disease. *Clinical and Experimental Immunology*, 45: 48-55.
  15. Bright GM & Sing I (1990). Adrenal autoantibodies bind to adrenal subcellular fractions enriched in cytochrome-c reductase and 5'-nucleotidase. *Journal of Clinical Endocrinology and Metabolism*, 70: 95-99.
  16. Winqvist O, Anders Karlsson F & Kampe O (1992). 21-hydroxylase, a major autoantigen in idiopathic Addison's disease. *Lancet*, 339: 1559-1562.
  17. 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 Letters*, 309: 51-55.
  18. Winqvist O, Gustafsson J, Rorsman F, Karlsson FA & Kampe O (1993). Two different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyendocrine syndrome type I and Addison's disease. *Journal of Clinical Investigation*, 92: 2377-2385.
  19. Chen S, Sawicka J, Betterle C, Powell M, Prentice L, Volpato M, Rees Smith B & Furmaniak J (1996). Autoantibodies to steroidogenic enzymes in autoimmune polyglandular syndrome, Addison's disease and premature ovarian failure. *Journal of Clinical Endocrinology and Metabolism*, 81: 1871-1876.
  20. Krohn K, Uibo R, Aavik E, Peterson P & Savilahti K (1992). Identification by molecular cloning of an autoantigen associated with Addison's disease as steroid 17- $\alpha$ -hydroxylase. *Lancet*, 339: 770-773.
  21. Papadopoulos KI & Hallengren B (1990). Polyglandular autoimmune syndrome type II in patients with idiopathic Addison's disease. *Acta Endocrinologica*, 122: 472-478.
  22. Soutsiou F, Bottazzo GF & Doniach D (1980). Immunofluorescence studies on autoantibodies to steroid-producing cells in endocrine disease and infertility. *Clinical and Experimental Immunology*, 39: 97-111.
  23. 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. *Journal of Endocrinology*, 117: 467-475.
  24. Lewi DS & Kater CE (1988). Adrenocortical insufficiency in patients with the acquired immunodeficiency syndrome (AIDS). *Revista da Associação Médica Brasileira*, 34: 213-218.
  25. Dib AS, Colman PG, Dotta F, Tautkus M, Rabizadeh A & Eisenbarth GS (1987). Expression of "cytoplasmatic" islet cell antigens by rat pancreas. *Diabetes*, 36: 982-985.
  26. Colman PG, Tautkus M, Rabizadeh A & Eisenbarth GS (1988). Assay for islet cell antibodies with rat pancreas and peroxidase protein A. *Diabetes Care*, 11: 367-368.
  27. Camargo ZP, Unterkircher C, Campoy SP & Travassos LR (1988). Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. *Journal of Clinical Microbiology*, 26: 2147-2151.
  28. Moulton JS & Moulton JS (1988). CT of the adrenal glands. *Seminars in Roentgenology*, 23: 288-303.
  29. Vita JA, Silverberg SJ, Goland RS, Austin JHM & Knowlton AI (1985). Clinical clues to the cause of Addison's disease. *American Journal of Medicine*, 78: 461-466.
  30. Falorni A, Laureti S, Nikoshkov A, Picchio ML, Hallengren B, Vandewalle CL, Gorus FK, Belgian Diabetes Registry, Tortoioli C, Luthman H, Brunetti P & Santeusano F (1997). 21-Hydroxylase autoantibodies in adult patients with endocrine autoimmune diseases are highly specific for Addison's disease. *Clinical and Experimental Immunology*, 107: 341-346.
  31. Ishimura K & Fujita H (1997). Light and electron microscopic immunohistochemistry of the localization of adrenal steroidogenic enzymes. *Microscopy Research Technique*, 36: 445-453.
  32. Falorni A, Nikoshkov A, Laureti S, Grenbäck E, Hulting AL, Casucci G, Santeusano F, Brunetti P, Luthman H & Lernmark A (1995). High diagnostic accuracy for idiopathic Addison's disease with a sensitive radiobinding assay for autoantibodies against recombinant 21-hydroxylase. *Journal of Clinical Endocrinology and Metabolism*, 80: 2752-2755.
  33. 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. *Journal of Clinical Endocrinology and Metabolism*, 82: 1440-1446.
  34. Colls J, Betterle C, Volpato M, Prentice L, Rees Smith B & Furmaniak J (1995). Immunoprecipitation assay for autoantibodies to steroid 21-hydroxylase in autoimmune adrenal diseases. *Clinical Chemistry*, 41: 375-380.
  35. 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. *Journal of Clinical Endocrinology and Metabolism*, 82: 932-938.
  36. Betterle C, Volpato M, Rees Smith B, Furmaniak J, Chen S, Zanchetta R, Greggio NA, Pedini B, Boscaro M & Presotto F (1997). II. Adrenal cortex and steroid 21-hydroxylase autoantibodies in children with organ-specific autoimmune diseases: markers of high progression to clinical Addison's disease. *Journal of Clinical Endocrinology and Metabolism*, 82: 939-942.