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ORIGINAL ARTICLE

Screening for associated autoimmune disorders in Polish patients with Addison's disease

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Abstract Autoimmune Addison's disease (AAD) is the main reason of primary adrenal failure. More than a half of patients display additional autoimmune conditions, which represent a considerable clinical concern. This study aimed to investigate the prevalence of concomitant autoimmune disorders in 85 Polish AAD patients (61 females, 24 males). Mean age at AAD onset was 34.6 ± 12.6 years, significantly earlier in males ($P < 0.001$). Sixty-nine patients presented positive serum antibodies to 21-hydroxylase and shorter AAD duration than those with negative results ($P = 0.027$). Seventy-three subjects suffered from coexisting autoimmune disorders. Serum autoantibodies against thyreoperoxidase, thyroglobulin, TSH receptor, glutamic acid decarboxylase, insulin, tyrosine phosphatase-like protein IA2, parietal cell H^+/K^+-ATPase, intrinsic factor and tissue transglutaminase were detectable in 71.8, 41.2, 4.7, 21.0, 4.9, 2.5, 49.4, 12.0 and 3.5% of patients, respectively. Antinuclear antibodies were found in 12.5%. Thyroid autoimmunity was most common (46 subjects with lymphocytic thyroiditis, 19 with Graves' disease), followed by atrophic gastritis (29.4%), pernicious anaemia (11.8%), hypergonadotropic hypogonadism (8.2%), vitiligo (8.2%), type 1 diabetes (7.1%), celiac disease (3.5%) and alopecia (2.4%). Gender differences were observed only for thyroid autoimmunity. Current study confirms particular tendency of AAD patients to develop other autoimmune disorders. Active search for concomitant conditions is warranted to prevent serious complications.

Keywords Addison's disease · Autoimmunity · Autoantibodies

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Introduction

Primary adrenal failure, also known as Addison's disease, is a relatively rare endocrine disorder, affecting 90–140 individuals per million [1–3]. The disease is characterised by progressive impairment of glucocorticoids, mineralocorticoids and adrenal androgens synthesis, leading to acute adrenal crisis, shock and death. No curative therapy allowing to restore adrenal function is available; however, timely introduction of steroid substitution enables to prevent life-threatening sequels of the disease.

Autoimmune destruction of adrenal cortex is currently considered the main reason of primary adrenal insufficiency in the developed countries, accounting for up to 90% of cases [2]. Autoimmune origin of this condition is evidenced by lymphocyte infiltration of adrenal cortex found on autopsies and by the presence of serum autoantibodies against specific adrenal antigens [4–6]. Other causes of adrenal insufficiency are less common and include infections (notably tuberculosis), infiltrative

process such as haemochromatosis and sarcoidosis, bilateral adrenal metastases or haemorrhage, as well as some rare genetic syndromes.

Interestingly, more than a half of patients with autoimmune Addison's disease (AAD) present with other concomitant autoimmune disorder(s). Typical combinations were noticed and classified as autoimmune polyglandular syndromes (APS) [7]. APS type 1 (APS1) comprises at least two of the following triad: AAD, hypoparathyroidism and mucocutaneous candidiasis. APS type 2 (APS2) designates a combination of AAD with autoimmune thyroid disease and/or type 1 diabetes (T1DM). APS type 3 was defined as autoimmune thyroid disease in conjunction with other autoimmune pathology, excluding AAD. Finally, APS type 4 (APS4) was meant to include all remaining combinations of autoimmune disorders, such as AAD and vitiligo, for instance. Nowadays, some inconsistencies of this old classification might be pinpointed; however, the major distinction between APS1 and all other APS types is still valid, particularly considering their genetic background. APS1 was found to be a monogenic, autosomal recessive condition (OMIM #240300), caused by mutations in the *AIRE* gene, directly involved in establishing immune tolerance to self-antigens [8]. In contrast, complex genetics of other polyendocrine syndromes remains largely unrecognised, with only a few predisposing variants indentified to date. Indeed, many components of APS types 2, 3 and 4 share genetic susceptibility loci, such as HLA region, *PTPN22*, *CTLA4* and *NLPR1* genes [9].

The particular susceptibility to develop numerous autoimmune disorders, observed among AAD patients, is of great scientific interest but, above all, it represents a considerable clinical concern in everyday practice. This problem has already been investigated in the past; however, former reports were mainly based on experimental data and labour-intensive research techniques. Nowadays, with the advent of modern, highly sensitive diagnostic tools, accurate serologic analyses are becoming widely available. The aim of this study was to evaluate the prevalence of concomitant autoimmune diseases among Polish patients with primary autoimmune adrenocortical insufficiency assessed in a routine clinical setting.

Results

Eighty-five patients with autoimmune Addison's disease (61 females and 24 males) comprised in this cross-sectional study. Their mean age was 48.0 ± 14.9 years (52.2 ± 13.2 years for females and 37.5 ± 14.0 years for males), ranging between 18 and 82 years. All subjects were issued from homogenous Polish population of Caucasian origin. Mean duration of Addison's disease in the studied cohort

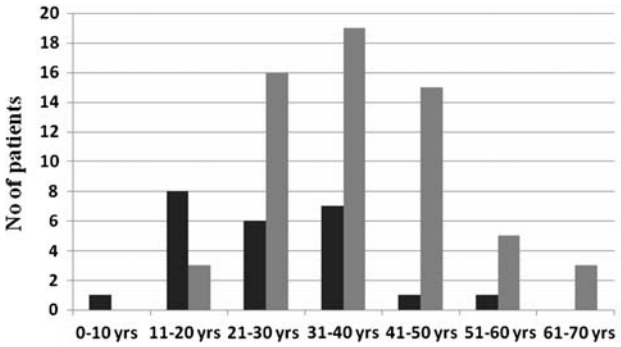

Fig. 1 Distribution of the age at onset of the autoimmune Addison's disease stratified by patients' gender. *Black bars* represent males and *grey bars* represent females

was 13.4 ± 10.7 years (range 0–46 years), and mean age at AAD diagnosis was 34.6 ± 12.6 years (range 9–68 years). The disease onset was observed significantly earlier in males (mean 26.7 ± 11.8 years) compared to female subjects (mean 37.7 ± 11.6 years) ($P < 0.001$) (Fig. 1).

Mean value for serum antibodies to 21-hydroxylase (a21OH) in the studied group was 100.1 ± 390.1 U/ml. Results equal or lower than 1.0 U/ml were considered negative. Sixty-nine patients (86.3%) presented with positive a21OH (87.7% females vs. 82.6% males, $P = 0.721$; 88.4% APS subjects vs. 72.7% patients with isolated AAD, $P = 0.171$). All but one of 11 subjects with negative serum a21OH had been previously found positive for anti-adrenal autoantibodies detected by solid phase RIA (radioimmunoassay) technique, using microsomal fraction of human adrenals. No direct correlation between serum concentrations of a21OH and disease duration was found ($P = 0.113$). However, a21OH levels differed significantly between patients recently (≤2 years ago) diagnosed with AAD (mean 372.8 ± 848.6 U/ml, 100% positive) and individuals with long-standing disease (51.9 ± 214.9 U/ml, 83.8% positive) ($P = 0.002$). Moreover, mean duration of adrenal insufficiency in patients negative for these autoantibodies (22.7 ± 15.2 years, range 7.0–46.0 years) was significantly longer, than in those with positive a21OH results (12.3 ± 9.3 years, range 0–37.0 years) ($P = 0.027$).

Seventy-three (85.9%) participants (55 females and 18 males) suffered from autoimmune polyendocrine syndromes 2 or 4, whereas in the remaining 12 subjects no other autoimmune condition was detected. Isolated AAD was found in 9.8% of females and 25% of males in the studied series ($P = 0.070$). Nearly half of AAD patients presented with two autoimmune disorders and only one person was affected by six autoimmune conditions: AAD, autoimmune thyroiditis, premature ovarian failure, chronic atrophic gastritis with pernicious anaemia and vitiligo (Fig. 2). Most frequent patterns of multiple autoimmune

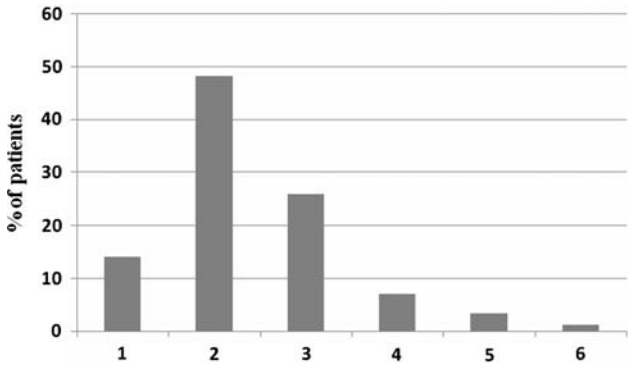

Fig. 2 Distribution of number of autoimmune diseases among patients with autoimmune adrenal insufficiency. Subjects with only one disease represent the subgroup with isolated Addison's disease

diseases observed among AAD subjects in this study are presented in Table 1.

Thyroid autoimmunity was the most common finding among AAD patients (64 subjects), and thyroid disorders were significantly more frequent in females than in males ($P = 0.005$) (Tables 2, 3). Forty-six subjects presented with chronic lymphocytic thyroiditis. Only three of them were at the initial euthyroid stage of the disease, characterised by hypoechogenic thyroid image at ultrasonography and positive serum autoantibodies but with hormonal parameters still within the reference range. All other AAD patients with autoimmune thyroiditis were hypothyroid, requiring regular levothyroxine substitution. Females were affected more often than males, although statistical difference was borderline ($P = 0.054$). Mean level of autoantibodies against thyroid peroxidase (aTPO) in the AAD series was 845.2 ± 1091.1 U/ml, whereas mean value for antibodies to thyroglobulin (aTg) was 160.7 ± 374.7 U/ml. Results above 60 U/ml were considered positive. Both values were significantly correlated ($r = 0.425$, $P < 0.0001$) and only three aTg-positive subjects revealed negative aTPO.

Graves' disease (GD) had been formerly diagnosed in 19 (22.4%) AAD individuals, however, by the time of the study all of them achieved remission after treatment with anti-thyroid drugs and/or 131-I radiotherapy. Therefore, levels of antibodies to TSH receptor (TRAb) were already negative in most cases (Table 3). Only four female patients presented with positive TRAb: one with persistent non-active thyroid orbitopathy, another two with the recent history of GD (2 and 4 years ago, respectively) and the last one, eventually diagnosed with chronic lymphocytic thyroiditis, had experienced an episode of transient Hashitoxicosis 2 years ago. No significant gender difference in GD occurrence was discovered ($P = 0.567$).

Type 1 diabetes was found in six AAD patients: two males and four females (Table 2). At the time of the study mean T1DM duration was 4.8 ± 2.8 years (range 1–10 years). Mean age at T1DM onset was 46.2 ± 18.9 years (range 16–65 years). Diabetes preceded AAD onset in half of patients, adrenal insufficiency developed first in two females, and in the youngest male both diseases appeared simultaneously, within a year. Five of these subjects presented with complete APS2 triad (formerly known as Carpenter's syndrome), comprising AAD, T1DM and autoimmune thyroid disease (three cases of lymphocytic thyroiditis and two individuals with GD). At least one type of specific autoantibodies: anti-insulin (IAA), anti-65kD glutamic acid decarboxylase (GADA) and anti-tyrosine phosphatase-like protein IA2 (aIA2) was detected in every diabetic AAD patient (GADA in four, IAA in three, and aIA2 in two subjects). Additionally, IAA and GADA were

Table 1 Distribution of the most common patterns of multiple autoimmune diseases among patients with autoimmune Addison's disease (AAD)

Combination of autoimmune diseases	All patients (%)	Males (%)	Females (%)
AAD + HT	26 (30.6)	6 (25.0)	20 (32.8)
AAD + GD	11 (12.9)	2 (8.3)	9 (14.8)
AAD + T1DM	1 (1.2)	1 (4.2)	0
AAD + ChAG	3 (3.5)	2 (8.3)	1 (1.6)
AAD + PGF	3 (3.5)	0	3 (4.9)
AAD + AITD + T1DM	3 (3.5)	1 (4.2)	2 (3.3)
AAD + AITD + ChAG	15 (17.6)	2 (8.3)	13 (21.3)
AAD + AITD + PGF	3 (3.5)	0	3 (4.9)
AAD + AITD + T1DM + ChAG	2 (2.4)	0	2 (3.3)
AAD + HT + ChAG + PGF	2 (2.4)	1 (4.2)	1 (1.6)

In order to make it clear, minor autoimmune conditions (celiac disease, pernicious anaemia, vitiligo and alopecia) and rare disease combinations are not shown, although prevalence rates are calculated as proportion of the whole AAD cohort (85 subjects: 24 males and 61 females)

HT Hashimoto's thyroiditis, *GD* Graves' disease, *AITD* autoimmune thyroid disease (HT or GD), *T1DM* type 1 diabetes, *ChAG* chronic atrophic gastritis, *PGF* primary gonadal failure

Table 2 Frequency of concomitant autoimmune conditions among patients with autoimmune Addison's disease: all patients cohort and patients stratified according to gender

Accompanying disease (85 AAD subjects)	All patients (%)	Males (%)	Females (%)	*P* values (M vs. F)
Autoimmune thyroid disease	64 (75.3)	13 (54.2)	51 (83.6)	0.005
Chronic lymphocytic thyroiditis	46 (54.1)	9 (37.5)	37 (60.6)	0.054
Graves' disease	19 (22.4)	4 (16.7)	15 (24.6)	0.567
Type 1 diabetes	6 (7.1)	2 (8.3)	4 (6.6)	1.000
Chronic atrophic gastritis	25 (29.4)	6 (25.0)	19 (31.1)	0.576
Pernicious anaemia	10 (11.8)	3 (12.5)	7 (11.5)	1.000
Hypergonadotropic hypogonadism	7 (8.2)	1 (4.2)	6 (9.8)	0.667
Vitiligo	7 (8.2)	2 (8.3)	5 (8.2)	1.000
Coeliac disease	3 (3.5)	2 (8.3)	1 (1.6)	0.191
Alopecia	2 (2.4)	1 (4.2)	1 (1.6)	0.487

AAD autoimmune Addison's disease, *M* male, *F* female

P values refer to χ^2 test or Fisher's exact test

Table 3 Frequency of different serum autoantibodies among patients with autoimmune Addison's disease: all patients cohort and patients stratified according to gender

Autoantibody (*n*)	Positive			*P* values (M vs. F)
	All patients (%)	Males (%)	Females (%)	
a21OH	69 (86.3)	19 (82.6)	50 (87.7)	0.721
aTPO (85)	61 (71.8)	12 (50.0)	49 (80.3)	0.005
aTg (85)	35 (41.2)	12 (50.0)	23 (37.7)	0.300
TRAb (85)	4 (4.7)	0	4 (6.6)	–
GADA (81)	17 (21.0)	5 (21.7)	12 (20.7)	0.917
IAA (81)	4 (4.9)	1 (4.3)	3 (5.2)	1.000
aIA2 (81)	2 (2.5)	1 (4.3)	1 (1.7)	0.490
PCA (83)	41 (49.4)	8 (33.3)	33 (55.9)	0.062
aIF (83)	10 (12.0)	1 (4.2)	9 (15.2)	0.268
tTG IgA (85)	3 (3.5)	2 (8.3)	1 (1.6)	0.191

M male, *F* female, autoantibodies to: *a21OH* 21-hydroxylase, *aTPO* thyreoperoxidase, *aTg* thyroglobulin, *TRAb* TSH receptor, *GADA* glutamic acid decarboxylase, *IAA* insulin, *aIA2* tyrosine phosphatase-like protein, *PCA* parietal cells (H^+/K^+-ATPase), *aIF* intrinsic facto, *tTG* tissue transglutaminase, respectively

P values refer to χ^2 test or Fisher's exact test

both found positive in one non-diabetic AAD female and only GADA in 12 other non-diabetic AAD individuals (Table 3). Further evaluation (oral glucose tolerance test, insulin serum levels) revealed that five of those apparently euglycaemic subjects displayed features of impaired glucose tolerance and/or insulin resistance (data not shown).

Autoantibodies to purified gastric H^+/K^+-ATPase (parietal cell antibodies, PCA) were detectable in 41 of 83 AAD patients (33.3% males and 55.9% females, $P = 0.062$). Twenty-seven of them underwent gastroscopy with biopsy and histological evaluation, which confirmed chronic atrophic gastritis with various degrees of gastric mucosal atrophy and intestinal metaplasia in 25 cases. Ten patients with chronic atrophic gastritis (ChAG) presented low serum levels of the Vitamin B12 (Table 4). Six of them were positive for antibodies to intrinsic factor (aIF) (most with subtle symptoms of pernicious anaemia), whereas 4 were found negative. Moreover, very high levels of aIF were detected in four other AAD patients with serum Vitamin B12 within the reference range and without clinical features of B12 deficit.

Serum tTG IgA were found positive in three AAD patients (Table 3). Only one female demonstrated mild clinical symptoms of coeliac disease (weakness, abdominal bloating and osteopenia) confirmed by histopathological examination of the intestinal mucosa which displayed moderate villous atrophy. Two other tTG IgA-positive males were asymptomatic and did not agree for the endoscopic procedure. None of the tTG IgA-negative AAD individuals appeared IgA-deficient.

Table 4 Patients with Addison's disease and chronic atrophic gastritis who presented with low serum levels of Vitamin B12 and/or positive autoantibodies to intrinsic factor (aIF)

Patient	Gender	Age (years)	Atrophic gastritis	Vitamin B12	aIF	Clinical symptoms of Vitamin B12 deficiency
1	F	50	+	N	++	None
2	F	42	+	↓	+	↑MCV, loss of appetite
3	F	40	+	↓	+	Paraesthesia in fingers
4	F	60	+	N	++	None
5	F	65	+	N	++	None
6	F	52	+	↓	+	↑MCV, sore tongue
7	F	67	+	↓	−	↑MCV, ↓Ht
8	F	50	+	N	++	None
9	F	47	+	↓	−	None
10	F	34	+	↓	++	↑MCV, ↓Ht, labial aphtae
11	F	35	+	↓	+	↑MCV
12	M	40	+	↓	+	None
13	M	46	+	↓	−	None
14	M	58	+	↓	−	None

F female, *M* male, *MCV* mean corpuscular volume of red blood cells, *Ht* haematocrit, *aIF* serum levels of antibodies to intrinsic factor: '−' aIF within the reference range, i.e. ≤15 U/ml, '+' between 15 and 100 U/ml, '++' more than 100 U/ml

Seven (8.2%) AAD subjects presented with hypergonadotropic hypogonadism (Table 3). Females complained of secondary amenorrhea and the unique hypogonadic male reported acquired sexual dysfunction. Same proportion (8.2%) of AAD patients demonstrated areas of skin depigmentation (vitiligo). Two other persons had focal hair loss (alopecia areata), which spontaneously resolved in one of them.

None of the studied AAD patients displayed clinical features suggestive for systemic autoimmunity. Nevertheless, screening for serum antinuclear antibodies (ANA) was performed, revealing positive results in 10 of 80 (12.5%) patients. Antibody titres ranged from 1:160 up to 1:1280 in one subject (Table 5). Sera from six individuals presented homogenous fluorescence pattern, whereas four others displayed speckled nuclear staining. Further analysis of ANA specificity provided borderline or weak positive results in five cases and strong reactivity with Ro-52 antigen associated with borderline positive result to PM-Scl in one female (Table 5). Weak positivity for both dsDNA and histones was detected in the patient presenting the highest ANA titre, although no clinical symptoms of lupus erythematosus were found in this elderly person. None of the remaining sera revealed typical antibody combinations which might be definitely attributed to a particular connective tissue disease.

Table 5 Patients with Addison's disease and positive antinuclear antibodies (ANA)

Patient	Gender	Age	Coexisting autoimmunity	Antinuclear antibodies (ANA)		
				Fluorescence	Titer	Specificity
1	F	24	AITD	Homogenous	1:160	–
2	F	49	AITD, ChAG	Homogenous	1:160	–
3	F	65	AITD, ChAG	Patchy	1:320	–
4	F	81	AITD, ChAG	Patchy	1:320	Sm (+)
5	F	34	AITD, ChAG, PA	Homogenous	1:320	Ro-52+++, PM-Scl (+)
6	F	71	AITD	Patchy	1:640	PM-Scl (+)
7	F	61	AITD	Patchy	1:640	–
8	M	44	AITD, ChAG	Homogenous	1:640	Nucleosomes +
9	F	56	ChAG	Homogenous	1:640	SS-B +
10	F	62	AITD, ChAG	Homogenous	1:1280	dsDNA +, histones +

F female, *M* male, *AITD* autoimmune thyroid disease, *ChAG* chronic atrophic gastritis, *PA* pernicious anaemia, *ANA* specificity results: '(+)' borderline positive, '+' weakly positive, '++' positive, '+++' strongly positive

Discussion

Similar to previous studies, females prevailed among AAD patients in the current cohort, and, intriguingly, their age at disease onset was significantly higher than in males [10–14]. The latter observation seems to be a consistent finding in AAD, however, no plausible explanation has been proposed to date. Usually, conditions characterised by a considerable role of genetic factors tend to develop earlier during lifetime, monogenic APS1 being the most relevant example in the field. It might be hypothesized that AAD development could follow slightly different patterns in both genders, with potentially more genetic impact in males. On the contrary, favourable environmental influence, such as intrinsic hormonal milieu for instance, might play a primordial role in higher susceptibility to autoimmune disorders among females. Accordingly, some recent studies of genetic markers associated with T1DM reveal that particular HLA and *PTPN22* genotypes are significantly more common in affected boys than in girls [15, 16]. In order to firmly support this presumption, much more data are required, concerning both complex genetic background of AAD and virtually unrecognised environmental triggers for this condition.

The current AAD cohort displayed very high incidence of concomitant autoimmune disorders, exceeding the results from many former reports [2, 12, 13]. The age of our patients was similar to the previous series; hence, increased frequency of other diseases could not be attributed to a more advanced age of study participants. However, screening for coexisting autoimmune diseases is highly biased by the research strategy applied. Some studies were mainly focused on detection of endocrine autoimmunity (thyroid, pancreas and gonads), others, though analysed much wider disease spectrum, used less standardised diagnostic procedures, available in the past [11–13, 17]. Anyway, despite slightly different APS proportions in different populations, AAD subjects present the strongest clustering of autoimmune disease of any autoimmune conditions, which makes it an excellent model for investigation of various mechanisms involved in autoimmunity.

Steroidogenesis enzyme, 21-hydroxylase, is the major adrenal autoantigen [4, 5]. Although not considered causative agents of adrenal destruction, serum antibodies to 21-hydroxylase are valuable markers which confirm autoimmune aetiology of adrenal failure [18]. In the current series, a21OH were positive in the majority of patients. Most subjects with negative a21OH were previously found positive for anti-adrenal autoantibodies detected by a formerly available technique. It may be assumed that synthesis of these antibodies has gradually ceased in the course of the disease, due to progressive disappearance of its trigger, i.e. adrenal antigen [18]. Indeed, patients with long-lasting disease (>2 years) displayed significantly lower serum levels of a21OH, compared to those newly diagnosed with AAD. However, it was difficult to document direct correlation between a21OH levels and disease duration. Probably, this is the effect of individual dynamics of the autoimmune process, as supported by the overlapping disease duration ranges in a21OH-positive and a21OH-negative individuals.

Autoimmune thyroid disorders are most frequent diseases which combine with AAD, giving rise to APS2, or Schmidt's syndrome. In the current study, chronic lymphocytic thyroiditis was found in 54% of participants, which represents one of the highest results reported in the AAD groups. Most previous analyses described its frequency around 30% of AAD patients [2, 13]. In our cohort, the diagnosis of autoimmune thyroiditis was based on both serological and ultrasonographic findings. Hormonal assessment was also performed, revealing hypothyroid status in all but three of those diagnosed with thyroiditis. The particularly high rate of chronic thyroiditis among our patients might be therefore explained by very careful and accurate evaluation, using highly sensitive and specific radioimmunoassays for autoantibody detection. Moreover, population differences may play a role. Since the introduction of compulsory salt iodization in 1996, an increase in the prevalence of serum aTPO, rising from 3.8 to 11.8% within 10 years, was reported in our country [19]. It is widely recognised that iodine enrichment of the diet could trigger autoimmune thyroiditis in genetically prone individuals, such as AAD patients, for instance [20]. However, former report from another Polish AAD cohort also demonstrated comparable proportion of aTPO and aTg-positive individuals [21]. In both series, the disease was more frequent among females, similar to what is observed in the general population [19, 22]. Accordingly, aTPO were detected more often in AAD females than in males. In contrast, aTg seemed to be more frequent in men, although the difference was not statistically significant, and the sample size was not sufficient to draw meaningful conclusions.

Autoimmune reactions directed against thyroid and adrenal antigens often develop simultaneously. In case of misdiagnosed Addison's disease, the introduction of levothyroxine replacement without substituting deficient adrenal function may lead to a life-threatening crisis. Triiodothyronine stimulates basal metabolic rate and enhances cortisol metabolism, additionally reducing depleted steroid supply in the organism [23]. Similar to unbalanced levothyroxine substitution, hyperthyroidism induced by Graves' disease increases steroid requirement and may precipitate acute adrenal insufficiency [24]. Fortunately, in most instances, thyroid dysfunction develops first, therefore by the time of

AAD onset, hyperthyroidism has already been cured [12, 14]. Data concerning GD in the present cohort were mainly retrospective; however, they reliably confirm high prevalence of this condition, exceeding 22% of AAD cases. Recent reports from other AAD populations provide similar proportion of GD [2, 13]. At the time of the study, none of our patients was at the active stage of the disease; hence, serum TRAb were negative in most cases. Antibodies to TSH receptor tend to gradually decline in course of effective therapy, irrespective of the type of treatment applied [25].

Type 1 diabetes affects up to 20% of AAD patients, most commonly in the context of APS2; however, the disease may also occur in subjects suffering from APS1 [2, 11, 12]. The prevalence of T1DM in our study was relatively low, around 7%, and only 5.9% of the cohort presented a complete tri-glandular APS2. Moreover, autoantibodies to glutamic acid decarboxylase were found in 13 more AAD patients, with additionally positive IAA in one female. Several studies unequivocally confirm that evaluation of serum GADA, IAA and aIA2 allows to identify subjects at risk of T1DM, and the probability of the disease increases with the number of antibodies detected [26, 27]. Previous reports on AAD samples usually focused on detection of islet cell antibodies by means of immunofluorescent techniques, whereas antibodies to specific autoantigens were seldom analysed, mainly in the APS1 patients [13, 28]. Current study provides data from Polish non-APS1 AAD individuals, indicating 21, 5 and 2.5% prevalence of GADA, IAA and aIA2, respectively. The rates are slightly lower compared to proportions in Norwegian AAD subjects which might be related to higher T1DM prevalence among Scandinavian patients, approaching 17.0% [13]. No data on the prevalence of diabetes-associated autoantibodies in the general Polish population are available. However, the frequency of GADA in our AAD cohort exceeds their 15.5% rate in another well-established T1DM risk group, i.e. first degree relatives of affected Polish children [29]. In our study, aIA2 were found exclusively in diabetic AAD patients, IAA—mostly in diabetics (three of four positive results), while GADA were detectable in 4/6 (66.7%) T1DM + AAD subjects and 13/79 (16.5%) non-diabetics. Although the predictive value of a single autoantibody is not particularly high, in combination with impaired glucose tolerance, it should rise strong suspicion of imminent diabetes in five more AAD patients from the studied cohort. Close surveillance of this subgroup is necessary, along with increased disease awareness in the remaining GADA-positive individuals. Repetitive antibody assessments may allow to discern incidental false-positive results and to follow possible progression of diabetic autoimmunity [30].

In fact, it is rather T1DM preceding AAD which seems the most common occurrence in patients combining both disorders [14, 31]. Interestingly, and in line with other reports, mean age of diabetes onset in those individuals was relatively high compared to typical childhood peak of T1DM incidence in the general population [11, 31]. In the current study, it was mainly the effect of a more advanced age of four AAD females at the time of T1DM diagnosis, ranging between 46 and 65 years, whereas two male AAD + T1DM patients developed their diabetes at 16 and 32 years of age, respectively. This might indicate different dynamics of disease progression, resembling latent autoimmune diabetes in adults (LADA) [32]. Accordingly, three of those women experienced very mild diabetes onset initially classified as type 2 diabetes. In contrast, two diabetic males presented typical beginning of clinically overt T1DM, with ketoacidosis and low C-peptide serum levels. At present, all six AAD + T1DM patients require regular insulin therapy. In pre-existing diabetes, gradual adrenal failure decreases insulin requirement and manifests as frequent hypoglycaemic episodes. Despite the introduction of steroid replacement, imperfectly mimicked rhythm of cortisol secretion may also impair glycaemic control. Diabetic patients with concomitant AAD are more prone to episodes of nocturnal hypoglycaemia due to lack of natural defence against low fasting glucose levels. On the contrary, immediately after absorption of the substitutive hydrocortisone dose, serum cortisol levels often exceed physiological range and may contribute to increased insulin resistance [33]. Therefore, T1DM coexisting with adrenal failure represents a major therapeutic challenge for clinician and for the patient as well.

Former investigations of the coexistence of AAD and gastric autoimmunity mainly relied on complement fixation or immunofluorescence-based tests [10, 12, 17]. Moreover, due to lack of a gold standard, variable diagnostic criteria of ChAG and pernicious anaemia might have been applied in different studies. These are the probable reasons why several reports provide various data concerning the prevalence of both diseases among AAD patients [10–12, 17]. In a series of 263 Italian AAD subjects, ChAG was observed in 11% of patients with APS2 and 8% of subjects with APS4 [2]. The prevalence of pernicious anaemia was estimated between 0.8 and 7.6% of AAD subjects [2]. Our data indicate that both disorders may be found in 29.4 and 11.8% of Polish AAD patients, respectively. The relatively high frequency of ChAG might result from the population differences or more efficient screening procedures. Enzyme-linked immunosorbent assays (ELISA) are highly sensitive in PCA detection (over 85% sensitivity), and their results seem less operator dependent than in the case of immunofluorescent methods [34]. Data obtained by means of the later technique indicate 15% prevalence of serum PCA among healthy Polish persons over 85 years of age [35].

Unfortunately, it was difficult to estimate the sensitivity of ELISA in our study, as many PCA-positive patients did not agree for gastroscopy and histological confirmation of serological findings. Although usually asymptomatic, long-standing gastric body atrophy leads to impaired production of gastric acid due to progressive loss of oxyntic glands. Chronic hypochlorhydria results in high gastrin levels, which in turn may favour enterochromaffin-like cell hyperplasia and potentially increase risk of gastric neoplasms (carcinoid, adenocarcinoma) [36, 37]. Eventually, with decreasing availability of IF, cobalamin malabsorption and pernicious anaemia develop. Reported frequencies of autoimmune gastritis and pernicious anaemia in Caucasian populations are around 2 and 0.15–1%, respectively [37].

Some PCA and aIF results in this study seemed discrepant in relation to patient's clinical status. Four AAD patients with confirmed ChAG and Vitamin B12 deficiency were found aIF negative, whereas high levels of these autoantibodies were detected in four other AAD + ChAG subjects despite lack of laboratory or clinical symptoms of pernicious anaemia. These former results might be due to relatively low sensitivity of ELISA-based aIF measurements. Although highly specific, this assay provides maximal 60% sensitivity in diagnosis of pernicious anaemia [34]. Therefore, a proportion of affected patients could be missed in this type of screen. Alternative explanation of negative aIF results are other reasons of Vitamin B12 deficit, such as veganism, exocrine pancreatic insufficiency, bacterial intestinal overgrowth or history of gastric surgery. Based on clinical data, these problems were considered unlikely in the current series, especially given other pre-existing autoimmune disorders. A more probable explication of negative aIF measurements in at least two of our AAD patients might be the fact of long-standing pernicious anaemia (14 and 8 years, respectively) and possible extinction of autoimmune reaction along with destruction of parietal cells and the antigen disappearance. Paradoxically, some studies describe increasing prevalence of serum aIF with the duration of pernicious anaemia; however, no explanation was provided [38]. By analogy with other organ-specific autoimmune disorders, high autoantibody levels could be expected at the beginning of the autoimmune process, even before the onset of clinical symptoms, when tissue damage is the most pronounced. This assumption would be in accordance with the current results, where particularly high levels of serum aIF (above 100 U/ml) were detected in four AAD patients with ChAG, and no clinical nor laboratory symptoms of pernicious anaemia. The disease may develop over a very long period and, eventually, all these patients will probably display Vitamin B12 deficiency [39]. Considering abundant Vitamin B12 stock in the liver, the latter explanation seems quite convincing and remains in line with the fact that all four subjects in this study have already been diagnosed with ChAG. Pernicious anaemia is often referred to as the end-stage of ChAG; therefore, these patients will definitely require close follow-up. Overall, taking into account high prevalence of ChAG/pernicious anaemia among AAD patients and potential adverse consequences of gastric autoimmunity, screening procedures should be advised in those individuals.

As clinical symptoms of celiac disease and adrenal insufficiency partially overlap, abdominal disorders in AAD patients can easily be attributed to steroid deficiency and the diagnosis of concomitant gluten intolerance may be missed. Studies in other European cohorts revealed high frequency of celiac disease among AAD patients compared to the general population [39–42]. Moreover, increasing proportion of celiac disease cases remains asymptomatic for a long time. Therefore, routine screening for this enteropathy was recommended in patients suffering from organ-specific autoimmune disorders, such as AAD, T1DM and thyroid autoimmunity [40, 41, 43–45]. In the current study, two of three subjects with positive tTG IgA presented no clinical symptoms. Interestingly, both of them suffered from AAD combined with T1DM. All three disorders are of autoimmune origin and share class II HLA susceptibility haplotypes [46]. Complete diagnosis of celiac disease requires histological evaluation of intestinal biopsy but the patients did not agree for endoscopic procedure. Histological result would enable to classify these individuals as 'silent' (with typical mucosal lesions but no symptoms) or 'latent' (with normal appearance of intestinal mucosa) celiac disease. Data from our study confirm higher prevalence of celiac disease in AAD patients; however, the proportion of affected subjects (3.5%) is lower in comparison to other European series (5.9–12.2%) [40–43]. Only one former report stated lower frequency of celiac disease, among Dutch AAD patients (1.2%), but the diagnostic criteria were not provided [12]. The screening procedure in the current study consisted solely of serum tTG IgA, whereas some previous investigations also included anti-endomysial IgA for the AAD patients' screen [40, 43]. Elevated levels of tTG IgA are considered highly sensitive marker of celiac disease and a satisfactory first-line screening tool [47, 48]. Statistically, adding another antibody might potentially allow detection of one more case of celiac disease in the studied series, but this would not considerably change the overall result. Relatively low prevalence of celiac disease among our patients may simply reflect recently reported lower frequency of this disease in the Polish population with respect to other European groups [49].

Hypergonadotropic hypogonadism is another common co-morbidity, reported in 7.3–22% of AAD patients [2, 11,

12, 17]. Our data confirm increased 8.2% prevalence of primary gonadal failure among Polish AAD subjects. Hence, autoimmune adrenal insufficiency can be regarded as predisposing to primary hypogonadism. This condition is probably induced by autoaggression directed against gonadal autoantigens, as supported by lymphocytic infiltration of developing follicles in affected ovaries [50]. Additionally, autoantibodies to 17alpha-hydroxylase (17αOH) and P450 side-chain cleavage (P450scc), steroidogenesis enzymes shared by adrenal cortex and steroid-producing cells in gonads were identified in patients suffering from AAD and concomitant primary hypogonadism [51–53]. These antibodies are more commonly found in individuals with APS1 (45–80%), which reflects higher prevalence of hypogonadism compared to APS2 (33–40%) [18, 53]. The evaluation of serum antibodies to cytoplasmic antigens of steroid hormone secreting cells might be of some use in prediction of gonadal failure [54]. Many patients are at childbearing age at the time of AAD diagnosis and still wish to reproduce. As proportion of them may experience decreased fertility, responsible counselling supported by reliable diagnostic tools would be invaluable.

Unfortunately, no standardised commercial kits for detection of serum anti-17αOH and/or anti-P450ssc are available, therefore, routine screening is hampered and reproducible prospective data are still lacking [55]. Although statistically non-significant in this study, there seems to be a consistent discrepancy in the incidence of hypergonadotropic hypogonadism between AAD females and males. This observation might be biased by more obvious symptoms of hypogonadism in women, who also tend to consult amenorrhea more readily, than males experiencing erectile dysfunction, for instance. On the other hand, lower incidence of gonadal failure in males may be a genuine finding, due to more efficient local protection of testicular autoantigens (immunologically privileged site) [56]. This remains in line with lower frequency of serum autoantibodies to 17αOH and/or P450scc and their apparently decreased predictive value reported in men [28, 55].

Vitiligo affects 0.3–1% of the general population and, due to common coincidence with other autoimmune disorders, is often considered a dermatological hallmark of autoimmunity. Since the first clinical description of primary adrenal failure by Thomas Addison [57], its association with vitiligo has been frequently reported [2, 58, 59]. Both disorders are characterised by autoimmune aetiology, and according to the recent studies, they share several genetic susceptibility loci, such as *PTPN22* and *NLPR1* genes [60]. Vitiligo is most often described in APS1; however, other autoimmune polyglandular syndromes may also associate with skin depigmentation [2, 58]. The 8.2% prevalence of vitiligo among AAD patients in the current study remains in line with previous estimates, ranging between 2.8 and 14% [11, 12, 61]. As in most former reports, no gender difference was noticed in this series. Vitiligo presents a tendency to coexist with thyroid and gastric autoimmunity, also confirmed it our study [58, 62]. Six of seven AAD + vitiligo patients suffered from lymphocytic thyroiditis, whereas ChAG with/without pernicious anaemia was detected in five of them.

Alopecia areata is a complex disorder, characterised by non-scarring hair loss which can affect any hair-bearing body surface. The lifetime risk of developing alopecia is approximately 1.7% [63]. Although alopecia is not a life-threatening condition, it may strongly impair patient's quality of life and cause serious psychological distress [64]. Its aetiology remains unclear; however, there is considerable evidence supporting the autoimmune origin of this condition [65, 66]. Alopecia areata appears to be mediated by T-lymphocytes and heterogenous autoantibodies directed against hair follicle structures have been described [66, 67]. Natural history of alopecia is highly unpredictable, and spontaneous remissions are common, particularly in milder cases [68]. Among AAD patients, alopecia seems particularly frequent in those suffering from APS1, being observed in more than 1/3 of affected subjects [2, 59]. Estimates of the prevalence of alopecia in patients with APS2 and APS4 are much lower, not exceeding 10% [2, 31]. Alopecia has previously been observed in 2% of Polish AAD subjects [11]. Patients comprised in the current series demonstrated similar rate of this skin condition.

Coexisting systemic autoimmunity is sometimes described among AAD patients; however, the prevalence of connective tissue diseases does not reach the proportion comparable to organ-specific autoimmune conditions [2, 12, 69]. Additionally, shared genetic susceptibility loci suggest similar underlying pathology of the immune system. To the best of our knowledge, this is the first regular investigation of the prevalence of antinuclear antibodies in patients suffering from AAD. Serum ANA are characteristic for connective tissue diseases, but their specificity is low. Antinuclear antibodies appear quite common among patients with chronic infections, malignancies and even in healthy subjects, particularly in the elderly [70, 71]. Nevertheless, despite choosing relatively high cut-off titre (1:160), the ANA prevalence in our AAD cohort exceeds their 10% frequency reported for the general Polish population of healthy elderly over 85 years of age [35]. However, clinical relevance of this finding remains debatable as none of the patients presented symptoms of connective tissue disease. On the other hand, all of them were affected by at least two organ-specific autoimmune diseases (Table 5). Not all ANA-positive results were confirmed by ELISA tests detecting 15 extractable antigens, which imply potential involvement of other nuclear autoantigens responsible for

visible fluorescence. Moreover, most sera displayed borderline or weak reactivity with specific antigens, and no evident patterns characteristic for a particular systemic disease were recognised. Although asymptomatic, three cases might rise some more suspicion, i.e. the elderly lady with the highest ANA titre and positive antibodies to dsDNA, a 56-year-old female with positive anti-SS-B and family history of Sjögren syndrome in her sister, and a 34-year-old female with strong reactivity against Ro-52 and borderline reaction to PM-Scl. However, the presence of isolated serum antibodies to Ro-52 is of low diagnostic value [72]. Overall, similar to thyroid autoimmunity, there is no evidence to support the routine use of ANA screening in patients with AAD in the absence of clinical signs of connective tissue disease [73].

Several patterns of multiple autoimmune conditions can be observed in AAD subjects, with apparently most common combinations comprising thyroid autoimmunity (Table 1) [13, 14]. Moreover, in the current cohort, chronic atrophic gastritis was usually associated with lymphocytic thyroiditis or Graves' disease, particularly among AAD females. This finding remains in line with increased coincidence of thyrogastric autoimmunity, which is also described in T1DM patients [37, 74]. Likewise, AAD + T1DM cases also tend to coexist with other autoimmune pathology, rather than a simple combination of both diseases. Of note, more complex patterns, including several autoimmune disorders are not uncommon among AAD individuals and should always be considered.

Taken together, current study confirms exceptional susceptibility of patients with autoimmune Addison's disease to develop other autoimmune disorders. Many of these conditions remain asymptomatic for a long time and may only become manifest at the stage of serious sequels, such as neoplastic changes due to chronic atrophic gastritis and celiac disease, or neurologic disturbances in pernicious anaemia. Other co-morbidities may lead to acute life-threatening complications at the time of hormonal decompensation: adrenal crisis, thyroid storm or severe hypoglycaemia. Although no prophylactic measures are available yet, timely diagnosis and treatment of concomitant autoimmune pathology may prevent some of these unfavourable outcomes. Therefore, an active search for associated autoimmune conditions is warranted in every AAD case. Commercially available, modern diagnostic techniques of serologic markers detection are reliable tools which may facilitate this task in everyday clinical practice.

Materials and methods

The diagnosis of primary adrenal failure was made on the basis of typical signs and symptoms, low serum cortisol levels accompanied by increased plasma ACTH, and confirmed by lack of response of adrenal cortex to $ACTH_{1-24}$ intravenous stimulation. Autoimmune aetiology of the disease was assumed based on lack of infiltrative adrenal lesions in computed tomography scans, former findings of anti-adrenal autoantibodies in serum (analysed in the past in 46 subjects by solid phase RIA method using microsomal fraction of human adrenals) and/or detection of other coexisting autoimmune disorders, such as chronic lymphocytic thyroiditis, GD or T1DM [75]. Patients with APS1 or positive history of tuberculosis were excluded from the study. None of the study participants displayed neurological disturbances tracking among male family members, which could raise suspicion of X-linked adrenoleukodystrophy.

Serum autoantibodies were evaluated using commercially available kits based on RIA and ELISA techniques. Autoantibodies to 21-hydroxylase (a21OH), as well as those typical for autoimmune T1DM: anti-insulin (IAA), anti-65kD glutamic acid decarboxylase (GADA) and anti-tyrosine phosphatase-like protein IA2 (aIA2) were analysed by RIA assays (RSR Ltd, Cardiff, UK). RIA technique (Brahms, Berlin, Germany) was also used to test the presence of autoantibodies characteristic for thyroid autoimmunity: aTPO, aTg and TRAb. Autoantibodies to parietal cells H^+/K^+-ATPase and those to intrinsic factor were evaluated using ELISA assays (IBL Hamburg, Germany). Human tissue transglutaminase IgA autoantibodies were assessed with ELISA (Euroimmun, Lubeck, Germany), and total IgA serum concentrations were determined by immunoturbidimetry (COBAS Integra, Roche, USA). Screening for antinuclear antibodies (ANA) was performed by indirect immunofluorescence using Hep-2 cells as substrate. Titres equal or higher than 1:160 were considered positive, with potential clinical relevance. ANA specificity was evaluated by means of the Euroline ELISA test (Euroimmun Medizinische Labordiagnostika AG, Lubeck, Germany), which recognises 15 various extractable nuclear antigens (RNP, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl, Jo-1, centromer B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-protein, AMA-M2).

Autoimmune thyroid diseases were diagnosed based on serum autoantibodies evaluation (aTPO, aTg and TRAb), thyroid ultrasonography and hormonal assessment (TSH, fT4 and fT3 serum levels). The diagnosis of T1DM was based on WHO glycaemic criteria accompanied by the presence of specific autoantibodies (IAA, GADA and/or aIA2). Symptoms of primary gonadal failure were confirmed by high serum levels of gonadotropins (FSH and LH) together with decreased estradiol in females and free testosterone in males. The diagnostic procedures for chronic atrophic gastritis comprised serological findings (positive PCA), fasting hypergastrinaemia and histological

assessment of gastric mucosa (updated Sydney System). Serum levels of Vitamin B12 and aIF, considered together with routine haematological tests (hematocrit, haemoglobin, RBC count and indices) enabled diagnosis of pernicious anaemia. The diagnosis of celiac disease relied on increased serum tTG IgA levels and histological evaluation of distal duodenal mucosa (Marsh classification).

Statistical analysis was performed by means of SPSS 15.0 software (SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (±SD) unless stated otherwise. Normally distributed data were compared using unpaired Student's t test, whereas those with non-normal distribution were analysed by nonparametric Mann–Whitney test. Statistical correlations were assessed by calculation of Pearson's or Spearman's coefficient, depending on data distribution. χ^2 test or Fisher's exact test, if appropriate, was applied to compare the frequencies of various autoantibodies and autoimmune diseases in both genders. Two-tailed P values <0.05 were considered statistically significant.

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