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Autoantibodies against the calcium-sensing receptor and cytokines in autoimmune polyglandular syndromes types 2, 3, and 4

Short Title: CaSR and cytokine autoantibodies in APS2, 3, and 4

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Keywords: autoantibodies; autoimmune polyendocrine syndromes; calcium-sensing receptor; cytokines; NALP5

Abbreviations: AD, Addison's disease; APS, autoimmune polyendocrine syndrome; CaSR, calcium-sensing receptor; CD, celiac disease; DM, dermatomyositis; GD, Graves' disease; HG, primary hypogonadism; HP, hypoparathyroidism; HT, Hashimoto's thyroiditis; IFN, interferon; IL, interleukin; MG, myasthenia gravis; NALP5, NACHT leucine-rich-repeat protein 5; PTH, parathyroid hormone; T1D, type 1 diabetes.

Summary

Objective The frequency of autoimmunity against the parathyroid glands in patients with polyglandular autoimmunity that is not due to autoimmune polyendocrine syndrome type 1 (APS1) is unclear. To investigate this, the current study aimed to determine the prevalence of autoantibodies against parathyroid autoantigens the calcium-sensing receptor (CaSR) and NACHT leucine-rich-repeat protein 5 (NALP5) in a large group of patients with non-APS1 polyendocrine autoimmunity. Possible occult APS1 was investigated by cytokine autoantibody measurement and *AIRE* gene analysis.

Design, Subjects, and Measurements Subjects were 178 patients with APS2, 3 or 4, and 80 healthy blood donors. Autoantibodies against the CaSR, NALP5, and cytokines were measured by immunoprecipitation, radioligand binding assays, or ELISA, respectively.

Results Four patient samples (2.2%), but none of the controls, were positive for CaSR autoantibodies. NALP5 autoantibodies were not detected in any participant. Eleven patients (6.2%) had cytokine autoantibodies, but none of the control samples was positive. None of the patients with cytokine autoantibodies had any known or novel mutations in the *AIRE* gene.

Conclusions The low prevalence of CaSR autoantibodies indicate a very low level of subclinical parathyroid autoimmunity in APS types 2, 3 and 4. In addition, autoantibodies against cytokines constitute an uncommon feature of non-APS1 polyglandular autoimmunity.

Introduction

Autoimmune hypoparathyroidism in early childhood is one of the three major disease components constituting autoimmune polyendocrine syndrome type 1 (APS1; OMIM 240300); the other components are chronic mucocutaneous candidiasis and early-onset Addison's disease [1]. APS2 (OMIM 269200) is distinguished from APS1 by the adult-onset of Addison's disease which is always present in association with another autoimmune endocrine disorder such as thyroid autoimmunity or type 1 diabetes. Further classifications have defined APS3, in which there is thyroid autoimmunity in association with type 1 diabetes and eventually a variety of organ-specific autoimmune conditions, and APS4, which excludes APS2 and APS3, and is the combination of two autoimmune endocrine components [2]. However, although the original and other studies characterising the two main types of polyglandular autoimmunity (APS1 and APS2) [2,3] identified hypoparathyroidism as occurring only in the context of APS1, there have been occasional case reports of idiopathic hypoparathyroidism occurring with other autoimmune disorders [4].

The first attempt to identify parathyroid autoantibodies as markers for autoimmune hypoparathyroidism, used an indirect immunofluorescence assay and found that these were present in 38% of patients with idiopathic hypoparathyroidism, 26% of patients with idiopathic Addison's disease, 12% of patients with Hashimoto's thyroiditis, and 6% of controls [5]. Hypoparathyroid individuals also had a higher than expected frequency of adrenal, thyroid or gastric parietal cell autoantibodies. In another early assay using dispersed human parathyroid cells, eight of 23 sera from patients with idiopathic hypoparathyroidism reacted by

immunofluorescence; two of the positive sera came from patients who had APS1, but the other six were from adults, two of whom also had thyroid or adrenal autoantibodies [6]. On the other hand, a recent study of 87 patients with idiopathic hypoparathyroidism found no increase in the prevalence of thyroid autoimmunity in these individuals [7]. Thyroid autoimmunity is the commonest of the organ-specific autoimmune disorders and would be expected to be more prevalent if autoimmune hypoparathyroidism behaves as most organ-specific autoimmune conditions by clustering with these other disorders. However, this was a small series and it is possible that only a proportion of patients might have had an autoimmune basis for their hypoparathyroidism: only these would be expected to show an increase in the prevalence of other autoimmune conditions like thyroid disease.

Continued research has defined two parathyroid autoantigens so far: the calcium-sensing receptor (CaSR) and NACHT leucine-rich-repeat protein 5 (NALP5). Autoantibodies against the CaSR have been identified in both APS1 patients and in patients with idiopathic hypoparathyroidism, occurring as either as a solitary disorder or in association with other autoimmune diseases like thyroid autoimmunity [8-12]. They have a specificity and sensitivity of 83% and 50%, respectively, in relation to autoimmune hypoparathyroidism in APS1 [12]. In contrast, NALP5 autoantibodies appear to be confined to patients with APS1 and have a specificity and a sensitivity of 100% and 49% for autoimmune hypoparathyroidism in APS1, respectively; one adult-onset case of hypoparathyroidism with these autoantibodies turned out to have occult APS1 [13,14].

In view of the uncertainty over the possible association of autoimmune hypoparathyroidism with non-APS1 polyglandular autoimmunity, we undertook an analysis of 178 individuals with APS2, APS3 or APS4 to determine whether there is an increase in either CaSR or NALP5 autoantibodies, as markers for subclinical autoimmune parathyroid disease. To exclude occult APS1 in these patients, we also undertook an analysis of cytokine autoantibodies, which appear to have a high predictive value for this syndrome [15,16]. Autoantibodies against interferon (IFN)-ω, IFN-α2A, interleukin (IL)-17F, and IL-22 are reported to have specificities of 100%, 99.9%, 100%, and 25%, and sensitivities of 99%, 95%, 57%, and 71%, respectively, for APS1 [15,16].

Materials and Methods

Patients

The study was approved by the Ethics Committee of Johannes Gutenberg University Medical Centre, Mainz, Germany, and was performed in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. A total of 178 Caucasian white patients with autoimmune polyglandular syndrome were included and were tested for all of the autoimmune endocrine and non-endocrine diseases that are listed in Table 1. The definitions and diagnostic criteria, including endocrine function tests and measurement of specific autoantibodies, for the individual diseases are given in Supporting Information Table 1. The autoimmune polyglandular syndromes were defined as APS2 (Addison's disease with another autoimmune endocrine disorder), APS3 (autoimmune thyroid disease and type 1 diabetes) or APS4 (two autoimmune endocrine disorders excluding types 1-3). Eighty healthy blood donors (mean age 43 ± 13 years; 23 male, 57 female), who had no clinical signs and no personal or family history of autoimmune, endocrine or tumour diseases, served as controls.

Measurement of autoantibodies

Immunoprecipitation assays for detecting CaSR autoantibodies in patient and control sera were carried out as previously detailed [17]. Anti-NALP5 autoantibodies were detected in radioligand binding assays, which are detailed elsewhere [13]. Autoantibodies against interleukin (IL)-22, IL-17A, IL-17F, and interferon (IFN)-ω, IFN-α2A and IFN-λ1 were detected in enzyme-linked immunosorbent assays (ELISA) [18,19]. All sera were tested in duplicate at least twice, and

autoantibody levels expressed as an antibody index [17-19]. The upper limit of normal for each assay was calculated using the mean antibody index + 3SD of 80 healthy individuals [17-19]. Any serum with an antibody index above the upper limit of normal was designated as autoantibody-positive. For all assays, intra- and inter-assay coefficients of variation were below 8% and 12%, respectively.

Analysis of AIRE gene mutations

DNA was extracted from 1 ml of patient EDTA-collected blood using an Easy-DNA™ Kit (Life Technologies, Carlsbad, CA, USA). The 14 exons of the *AIRE* gene (EMBL Accession Number AJ009610) were amplified by PCR from patient DNA and sequenced using primers (Eurofins Genetic Services Ltd, London, UK) which have been detailed previously [20] and are listed in Supporting Information Table 2. Reactions were carried out in 50-µl volumes comprising 50 ng of genomic DNA, 0.6 µM of each primer, 5x GoTaq® Flexi Buffer, 1.5 mM magnesium chloride, 0.2 mM deoxynucleotides, and 1.25 units of GoTaq® Flexi DNA Polymerase (all from Promega, Southampton, UK). The cycling conditions were 95°C for 20 sec, 58°C for 20 sec, and 72°C for 30 sec for 32 cycles. Before sequencing, the PCR amplification products were purified using a Wizard® PCR Preps Purification Kit (Promega) according to the manufacturer's protocol. The PCR amplification products were sequenced using a BigDye® Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) and analysed on an ABI 3730 Capillary Sequencer (Applied Biosystems).

Results

Analysis of patients with autoimmune polyglandular syndrome

The autoimmune polyglandular syndromes identified were APS2 in 28 patients, APS3 in 125 patients, and APS4 in 25 patients (Table 1). A summary of patient demographic data and the presence of endocrine and non-endocrine diseases is given in Table 1. Three patients (F6, S102 and S122) were diagnosed with hypoparathyroidism, all having parathyroid hormone (PTH) levels below the lower limit of normal (Table 2). One of these three patients had APS3 and the other two were diagnosed with APS4 (Table 2). None of the three patients was positive for autoantibodies against the CaSR, NALP5, or cytokines IL-22, IL-17A, IL-17F, IFN-α2A, IFN-ω, and IFN-λ1 (Table 2).

CaSR and NALP5 autoantibody prevalence in patients and controls

Four patients (2.2%), but none of the controls, were positive for CaSR autoantibodies (Fig. 1a; Table 2). Three of these four patients had APS3 and the other had APS2 (Table 2). Three patients showed normal serum calcium, while one patient showed below normal levels (Table 2). Serum phosphate levels were below normal in three of the four patients (Table 2). One of the four patients had a PTH level above the upper limit of normal (Table 2). None of the 178 patients or 80 controls was positive for NALP5 autoantibodies (Fig. 1b).

Cytokine autoantibody prevalence in patients and controls

Of 178 patients, 11 (6.2%) were positive for cytokine autoantibodies; IL-22, IL-17A, IL-17F, IFN-α2A, IFN-ω, and IFN-λ1 autoantibodies were found in five (2.82%) (Fig. 1c), three (1.69%) (Fig.

1d), two (1.13%) (Fig. 1e), four (2.26%) (Fig. 1f), one (0.56%) (Fig. 1g), and four (2.26%) (Fig. 1h), respectively. No controls were found to be positive for cytokine autoantibodies (P = 0.02 compared to patient samples, Fisher's exact test).

The cytokine autoantibody-positive patients were nine with APS3 and two with APS4 (Table 2). One patient with IFN-α2A autoantibodies had late-onset myasthenia gravis as part of their APS4 diagnosis including autoimmune thyroid disease, ocular myasthenia, and primary hypogonadism (Table 2). Of the eight patients with cytokine autoantibodies but no CaSR autoantibodies, all had serum calcium levels within the range of normal, three had serum phosphate concentrations below the normal lower limit, and two had PTH levels above the upper limit of normal (Table 2).

Association of CaSR and cytokine autoantibodies

The three APS3 patient samples positive for CaSR autoantibodies were also positive for autoantibodies against two to five different cytokines; all three samples were positive for autoantibodies against IL-17 and IL-22, while two also had IFN autoantibodies (Table 2). In the fourth sample, from a patient with APS2, no cytokine autoantibodies were detectable (Table 2). Eight patient samples (six with APS3 and two with APS4) without CaSR autoantibodies were positive for autoantibodies against a single cytokine (Table 2). The association of cytokine autoantibodies with CaSR autoantibodies was significant (P = 0.0007, Fisher's exact test).

AIRE gene mutations

Since the presence of cytokine autoantibodies in 11 patients (Table 2) indicated the possibility of occult APS1 in these individuals, we went on to sequence their *AIRE* gene to look for potential

mutations. DNA was available for all 11 patients except for S20 and S117 (Table 2). However, no known or novel mutations were found in any of the samples tested.

Discussion

The original purpose of this study was to determine whether CaSR or NALP5 autoantibodies could be detected in patients with APS2, 3 or 4 as markers of an associated parathyroid autoimmune process. The finding of a low prevalence (2.2%) of CaSR autoantibodies indicates that parathyroid autoimmunity is an uncommon component of these adult-onset polyglandular syndromes, in contrast to APS1, and that even when CaSR autoantibodies are present, clinical disease did not result in this series of patients. Indeed, hypoparathyroidism was clinically present in only three patients. Our results also confirm that NALP5 autoantibodies are restricted to APS1 [13, 14].

To be confident that these CaSR autoantibody-positive patients truly had APS2 or 3, we undertook further studies using analysis of cytokine autoantibodies to exclude APS1, as there are reports describing atypical clinical cases of APS1 [21-23]. Notably, heterozygous mutations in the first plant homeodomain (PHD1) zinc-finger cause phenotypes ranging from classical APS1 to an atypical form showing later-onset and, in some cases, mimicking common multi-organ-specific autoimmunity or isolated organ-specific autoimmune manifestations (e.g., vitiligo and pernicious anemia) [24]. Anti-cytokine autoantibodies are a major feature of APS1 [15,18,25]. Autoantibodies against IFN-ω and IFN-α2 have a sensitivity, specificity and predictive value exceeding 98% [15]. In addition, a high prevalence of autoantibodies against IL-17-type cytokines has been reported in APS1 patients, including 91% in the case of IL-22 autoantibodies [18, 25]. Except for thymoma patients, IL-17-type cytokines autoantibodies have not been found in healthy individuals or patients suffering from autoimmune diseases outside the context of APS1 [18].

Three of the four samples positive for CaSR autoantibodies were positive for multiple cytokine autoantibodies; in addition, eight other patients were positive for autoantibodies against a single, or in one case, two cytokines. Since these autoantibodies strengthened the possibility of APS1 in these patients, we undertook sequence analysis of the 14 *AIRE* gene exons, including exon 8 which is the location of the recently described mutations in the PHD1 zinc finger [24], but did not detect any mutations in those positive for cytokine autoantibodies.

Our results indicate that 6.2% of patients with APS2, 3 or 4 had at least one cytokine autoantibody. Of these, there appears to be a group in whom there is subclinical autoantibody evidence of parathyroid autoimmunity is associated with autoantibodies against multiple cytokines, and a further group with more isolated cytokine autoantibodies, but no CaSR autoantibodies. There were no other clinical features that appeared to distinguish between those with solitary and those with multiple cytokine autoantibodies. Only one other study has looked at a similarly large set of non-APS1 patients; in 354 patients with Addison's disease, in isolated form or in association with APS2, one sample was positive IFN-ω autoantibodies but other cytokine autoantibodies were not examined [16]. Meloni *et al* [15] found no IFN-α2 or IFN-ω autoantibodies in 27 patients with APS2, and IL-17 and IL-22 autoantibodies have only previously been examined in 21 patients with APS2, in whom there were no positive results [18]. One of our patients who had IFN-α2A autoantibodies had myasthenia gravis which is known to be associated with cytokine autoantibodies [26]. Our results are possibly related to the large sample size and the inclusion of APS3 and APS4 patients as well as those with APS2.

In summary, we describe the presence of CaSR autoantibodies in 2.2% of patients with APS2, 3 or 4 indicating a very low level of subclinical parathyroid autoimmunity in these syndromes. What prevents the development of clinical hypoparathyroidism is unclear, although it is well- known that thyroid autoantibodies, for instance, are very common in the absence of progression to overt hypothyroidism. In addition, we have found that 6.2% of these patients had autoantibodies against cytokines, which are normally associated exclusively with APS1, and yet we could detect no *AIRE* gene mutations. Autoantibodies against cytokines therefore constitute a previously unrecognised, albeit an uncommon feature of non-APS1 polyglandular autoimmunity.

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Figure Legends

Fig. 1 Detection of CaSR, NALP5 and cytokine autoantibodies in APS2, APS3, and APS4 patients and healthy controls.

As detailed in Materials and Methods, patient and control sera were evaluated for CaSR, NALP5, and cytokine autoantibodies using immunoprecipitation assays, radioligand binding assays, and ELISA, respectively. The antibody index for (a) CaSR autoantibodies, (b) NALP5 autoantibodies, (c) IL-22 autoantibodies, (d) IL-17A autoantibodies, (e) IL-17F autoantibodies, (f) IFN-α2A autoantibodies, (g) IFN-ω autoantibodies, and (h) IFN-λ1 is shown for each APS2 (n = 28), APS3 (n = 125), and APS4 (n = 25) patient, and each healthy control (n = 80), and is the mean of at least two experiments. The mean antibody index and SD of each patient and control group is shown. The upper limits of normal for antibody assays against CaSR, NALP5, IL-22, IL-17A, IL-17F, IFN-α2A, IFN-ω, and IFN-λ1 antibody assays were 1.89, 1.43, 1.87, 2.15, 2.05, 1.81, 2.08, and 1.99, respectively.

Table 1. Summary of APS patient demographics and autoimmune diseases

Demographic details and endocrine and non-endocrine disease components	**APS2[1] (n = 28)**	**APS3[1] (n = 125)**	**APS4[1] (n = 25)**
Mean age ± SD (years)	47 ± 16	47 ± 16	55 ± 10
Sex (male/female)	12/16	36/89	4/21
Addison's disease	28 (100)	0	0
Hashimoto's thyroiditis	23 (82)	76 (61)	18 (72)
Graves' disease	2 (7)	49 (39)	6 (25)
Type 1 diabetes	3 (11)	125 (100)	1 (4)
Primary hypogonadism	11 (39)	9 (7)	23 (96)
Primary hypoparathyroidism	0	1 (0.8)	2 (8)
Primary hypopituitarism	0	1 (0.8)	0
Celiac disease	0	1 (0.8)	0
Dermatomyositis	0	1 (0.8)	0
Myasthenia gravis	0	0	1 (3)

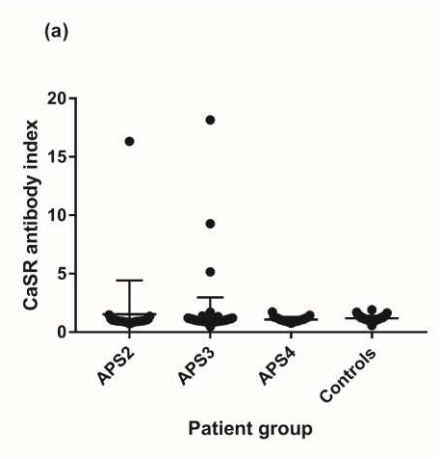
[1]The numbers of patients are given with percentages in parentheses.

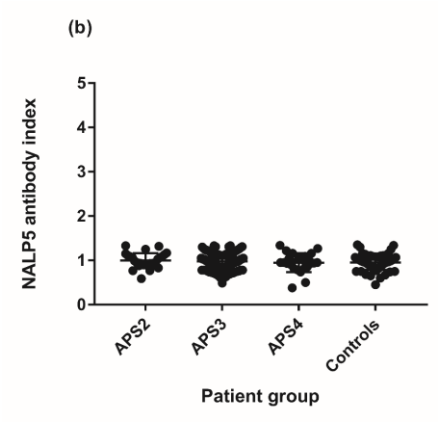
Table 2. Patients with hypoparathyroidism and patients positive for calcium-sensing receptor and/or cytokine autoantibodies

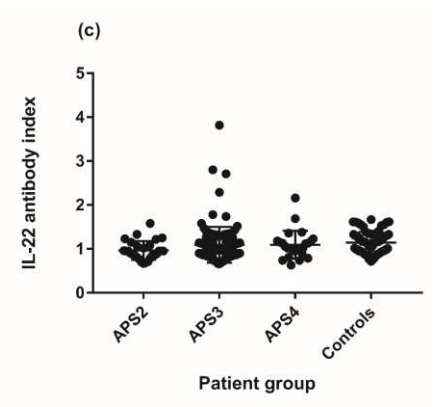
Patient	Diagnosis	Autoimmune components[1]	Sex[1]	Disease duration (years)	CaSR antibody index[2]	IL-22 antibody index[2]	IL-17A antibody index[2]	IL-17F antibody index[2]	IFN-α2A antibody index[2]	IFN-ω antibody index[2]	IFN-λ1 antibody index[2]	Serum calcium (mmol/L)[3]	Serum phosphate (mg/dL)[4]	Serum PTH (pg/mL)[5]
F6	APS4	HP, HT	F	11	1.23	0.91	0.89	0.90	0.90	0.90	0.90	1.50	5.6	5.00
S102	APS3	HG, HP, HT, T1D	M	17	1.20	1.20	1.40	0.94	1.42	1.14	1.52	1.69	3.9	4.24
S122	APS4	HP, HT	F	13	0.75	0.74	0.82	0.65	0.72	0.63	0.76	2.14	2.8	14.4
S90	APS2	AD, HG, HT	M	18	**16.3**	0.88	0.89	1.26	0.97	0.97	1.08	2.17	1.7	36.3
S145	APS3	HT, T1D, DM	F	11	**18.2**	**2.29**	**2.98**	0.90	**2.43**	1.11	**5.93**	2.45	2.8	52.5
S151	APS3	GD, T1D	M	16	**9.28**	**3.82**	**4.39**	**2.83**	1.73	**3.00**	**3.35**	1.89	1.9	29.0
S206	APS3	HT, T1D	M	11	**5.16**	**2.71**	**2.86**	1.51	1.25	2.03	1.19	2.34	2.4	82.2
S6	APS3	HT, T1D	M	23	0.91	1.14	1.46	**23.8**	1.73	1.23	0.66	2.44	2.5	67.1
S11	APS3	GD, T1D	F	13	1.09	1.47	1.62	1.11	**2.29**	1.76	0.89	2.32	3.1	34.0
S20	APS3	GD, T1D	F	24	1.15	**2.80**	2.04	0.74	1.35	0.79	1.42	2.31	1.0	41.0
S73	APS3	GD, HG, T1D, CD	F	17	0.99	0.90	0.80	1.42	1.15	0.77	**12.1**	2.40	1.9	49.1
S89	APS4	GD, HG, MG	M	15	1.07	1.02	1.05	1.03	**6.40**	0.71	1.12	2.37	2.8	85.8
S117	APS3	GD, T1D	F	19	1.39	1.58	1.89	1.76	1.28	1.04	**6.78**	2.27	2.9	38.0
S118	APS3	HT, T1D	F	13	0.88	0.92	0.93	0.99	**1.83**	0.96	1.06	2.28	2.9	64.9
S187	APS4	HG, HT	M	12	1.73	**2.16**	1.96	1.65	0.78	1.87	1.68	2.30	2.8	40.5

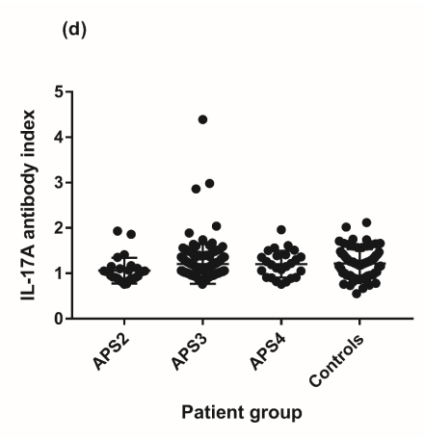
[1]AD, Addison's disease; CD, celiac disease; DM, dermatomyositis; F, female; GD, Graves' disease; HG, primary hypogonadism; HP, hypoparathyroidism; HT, Hashimoto's thyroiditis; M, male; MG, myasthenia gravis; T1D, type 1 diabetes.
[2]Autoantibody positivity is denoted in bold. Upper limits of normal for the CaSR, IL-22, IL-17A, IL-17F, IFN-α2A, IFN-ω, and IFN-λ1 antibody assays were 1.89, 1.87, 2.15, 2.05, 1.81, 2.08, and 1.99, respectively.
[3]Reference range for serum calcium is 2.15-2.60 mmol/L.
[4]Reference range for serum phosphate is 2.7-4.5 mg/dL.
[5]Reference range for serum PTH is 15-70 pg/mL.

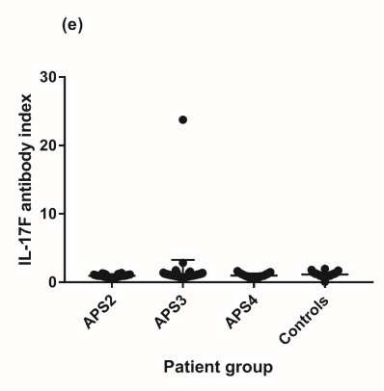
Figure 1a

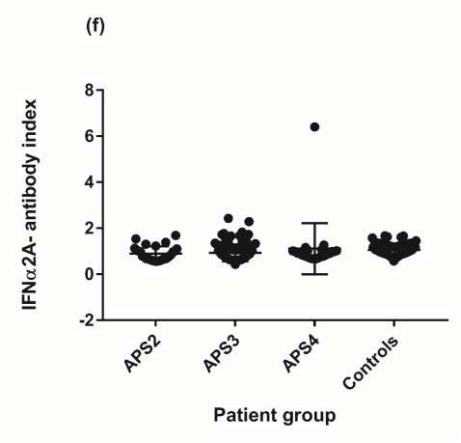

(a)
CaSR antibody index
20
15
10
5
0
APS2
APS3
APS4
Controls
Patient group

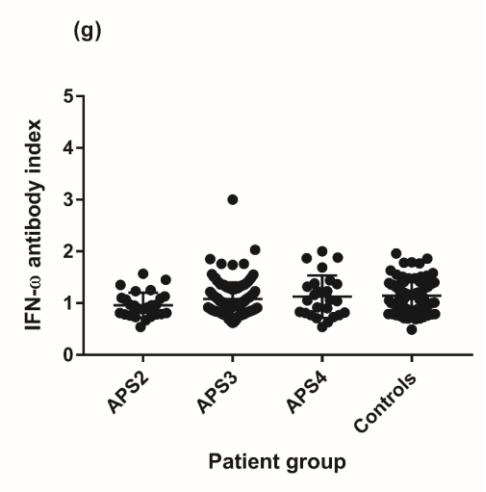

(b)
NALP5 antibody index
5
4
3
2
1
0
APS2
APS3
APS4
Controls
Patient group

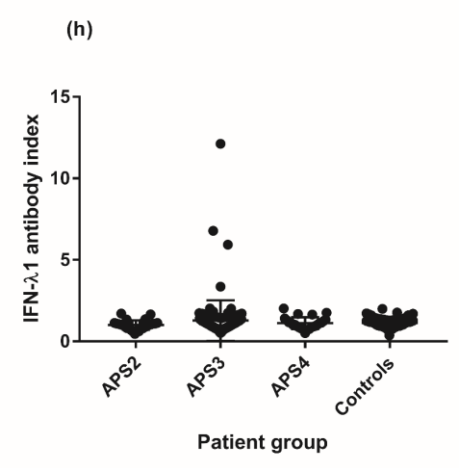

(c)
IL-22 antibody index
5
4
3
2
1
0
APS2
APS3
APS4
Controls
Patient group


(d)
IL-17A antibody index
5
4
3
2
1
0
APS2
APS3
APS4
Controls
Patient group


(e)
IL-17F antibody index
30
20
10
0
APS2
APS3
APS4
Controls
Patient group


(f)
IFNα2A- antibody index
8
6
4
2
0
-2
APS2
APS3
APS4
Controls
Patient group


(g)
IFN-ω antibody index
5
4
3
2
1
0
APS2
APS3
APS4
Controls
Patient group


(h)
IFN-λ1 antibody index
15
10
5
0
APS2
APS3
APS4
Controls
Patient group

Supporting Information

Supporting Information Table 1. Definitions and diagnostic criteria of the various autoimmune endocrine and non-endocrine diseases

Disease	Diagnostic criteria
Addison´s disease	Suppressed baseline serum cortisol levels (<0.1 µg/dL), pathologically elevated baseline serum adrenocorticotropic hormone (ACTH) levels, markedly elevated stimulated serum ACTH levels (delta >50 pg/mL), and the presence of cytochrome P450-21 hydroxylase autoantibodies
Type 1 diabetes	Insulin-dependency; positive autoantibodies against the islet cell antigens, and/or tyrosine phosphatase IA-2 (IA2A), and/or insulin (IAA) and/or glutamic acid decarboxylase-65 (GADA), a pathologic serum glycemic hemoglobin > 6.5% and fasting serum glucose > 120 mg/dL
Hashimoto´s thyroiditis	At least five-fold increased serum level of thyroid peroxidase autoantibodies, a typical hypo-echoic ultrasound pattern, and eu- or hypothyroidism
Graves´ disease	Hyperthyroidism, presence of thyroid-stimulating hormone (TSH) receptor autoantibodies, typical thyroid ultrasound pattern with enhanced vascularization of the thyroid gland
Autoimmune primary hypoparathyroidism	Serum baseline parathyroid hormone levels < 15 pg/mL, baseline serum calcium < 2 mmol/L and elevated serum phosphate levels > 5 mg/dL
Autoimmune primary hypogonadism	Suppressed serum peripheral sexual hormone levels, elevated serum gonadotropic hormone levels (FSH > 15 IU/L, LH > 10 IU/L, pathologic luteinizing hormone-releasing hormone stimulation test (delta FSH and LH > 10 IU/L) and positive 17-hydroxylase autoantibodies
Celiac disease	Presence of serum immunoglobulin (Ig) A (in case of IgA-deficiency IgG) autoantibodies to tissue transglutaminase and histological confirmation by Marsh stage III (endoscopic biopsy of the small bowel)
Myasthenia gravis	Positive serum acetylcholine-receptor-autoantibodies, Simpson´s (ice-pack)-test and tensilon-test

Supporting Information Table 2. PCR amplification and sequencing primers used in *AIRE* gene analysis

Primer	Sequence	Size of PCR product (base pairs)
AIREexon1F	5’-CAAGCGAGGGGCTGCCAGTG-3’	
AIREexon1R	5’-GGATCTGGAGGGGCGGGGTC-3’	
AIREexon2F	5’-ACCACCTGACTCCACCACAAGCC-3’	
AIREexon2R	5’-TCAGGGTTTTCTCCAGGGGTAGGG-3’	
AIREexon3F	5’-GTGATGTTCCAGGACCGTCTTG-3’	
AIREexon3R	5’-AGACCCGCCCGCCTACTT-3’	
AIREexon4F	5’-TGAAGTAGGCGGGCGGGTCTC-3’	
AIREexon4R	5’-CAGGGGCGACTGGCAAGATCA-3’	
AIREexon5F	5’-TTGGGTGCACACACGAACA-3’	
AIREexon5R	5’-GGCAGAAACTCTGGCTACCTGA-3’	
AIREexon6F	5’-CACCCTGGGGCCTACACGACT-3’	
AIREexon6R	5’-GAAGAGGGGCGTCAGCAATGG-3’	
AIREexon7F	5’-CCAGGAACAGCGTTGCCT C-3’	
AIREexon7R	5’-CGG TGCTCATCCCTGAGTGCC-3’	
AIREexon8F	5’-CAGGTGGTCAGGGCAGAATTTCA-3’	
AIREexon8R	5’-AGGCTGGGCAGCAGGTGTG-3’	
AIREexon9F	5’-ATCTCTCTGCTGTGCCTCGGTTC-3’	
AIREexon9R	5’-TGGGCATGGGGGACATAGTG-3’	
AIREexon10F	5’-TGCCACAGCCTTTCCCACTCAGT-3’	
AIREexon10R	5’-CCTCCCGGAGCCTTTCTCGC-3’	
AIREexon11F	5’-GCCTGAGGGTGCTTGGGTCG-3’	
AIREexon11R	5’-GGGGTGTGGTTGTGGGCTGTATG-3’	
AIREexon12F	5’-CCCCCACTCACCACCCACG-3’	
AIREexon12R	5’-GGGAGCCCTGGCAGGACTCTC-3’	
AIREexon13F	5’-CCCCAGCCCCATCATGCC-3’	
AIREexon13R	5’-TGGTGGGTGGAGCAGGGACAG-3’	
AIREexon14F	5’-TGGATGGTGACTTCTTGTAACGA-3’	
AIREexon14R	5’-ACCTCCCGAGTTCAAGTGATT C-3’	