

# Autoimmune Polyendocrine Syndrome Type 1 in Norway: Phenotypic Variation, Autoantibodies, and Novel Mutations in the Autoimmune Regulator Gene

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**Context:** The autoimmune polyendocrine syndrome type I (APS I) is a rare disease that previously was difficult to diagnose. Autoantibody screening as well as mutational analysis of the disease gene autoimmune regulator (*AIRE*) are important diagnostic tools for this life-threatening syndrome.

**Objective:** The objective of the study was to identify all patients with APS I in Norway and correlate their clinical features with their autoantibody profiles and mutations in the *AIRE* gene.

**Patients:** We identified 36 Norwegian patients from 24 families with APS I (20 males, 16 females) during a nationwide survey for patients with Addison's disease and polyendocrine syndromes, seven of them only after their death.

**Research Design and Methods:** Clinical data were collected from questionnaires and patient records. *AIRE* mutations were determined by DNA sequencing. Most autoantibodies were measured in RIAs against recombinant autoantigens, but anti-type I interferon (IFN) antibodies were titrated in ELISA or antiviral interferon neutralization assays.

**Results:** The prevalence of APS I in Norway was estimated to be about 1:90,000. Several patients exhibited a milder phenotype with

few APS I disease components and onset only in late adolescent or adulthood. The others showed about the same distribution of disease components as reported in Finnish patients. Eleven different mutations were identified in the *AIRE* gene, six of these were novel, *i.e.* c.22C>T (p.Arg8Cys), c.290T>C (p.Leu97Pro), c.402delC (p.Ser135GlnfsX12), c.879 + 1G>A (p.IVS7 + 1G>A), c.1249dupC (p.Leu417ProfsX7), and c.1336T>G (p.Cys446Gly). The 13-bp deletion in exon 8 (c.967–979del13) was the most prevalent mutation, present in 23 of 48 (48%) of the alleles. The presence of neutralizing autoantibodies against IFN- $\omega$  was the most specific marker of APS I, being found in all but one Norwegian patient. Some other common APS I-associated autoantibodies appeared *de novo* during long-term follow-up of younger patients.

**Conclusions:** Norwegian patients with APS I clinically resemble those from Finland and other European countries, but some have milder phenotypes. In total, six new mutations were identified in the Norwegian APS I patients. Anti-type I IFN autoantibodies are easily detectable; their APS I specificity and persistently high titers render them reliable markers of APS I, even in prodromal or atypical cases. Both the clinical features and the *AIRE* mutations are more diverse in the Norwegian population than previously thought. (*J Clin Endocrinol Metab* 92: 595–603, 2007)

**A**UTOIMMUNE POLYENDOCRINE SYNDROME type I (APS I), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (OMIM 240300), is an autosomal recessive disorder. The clinical diagnosis is based on the presence of two of the three commonest clinical manifestations, *i.e.* chronic mucocutaneous candidiasis

(CMC), primary hypoparathyroidism, and primary adrenocortical insufficiency (Addison's disease) (1–4). The disease usually presents in childhood and adolescence, and many patients display all of the three main components before age 20 yr (5). However, there are reports of less typical cases with late start and/or unusual presentations (3, 6–8).

Clinically, APS I is highly variable (3). The most common endocrine manifestations in APS I are hypoparathyroidism, Addison's disease, and ovarian failure, followed by diabetes mellitus type 1, testicular failure, autoimmune thyroiditis, and hypophysitis (1). APS I patients often have gastrointestinal diseases such as autoimmune gastritis, autoimmune hepatitis, and malabsorption. Hyposplenias/asplenia is a common unexplained feature (3). However, most patients present with CMC, which can lead to squamous cell carcinoma of the oral cavity (9). Many have dental enamel hyp-

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Abbreviations: AADC, Aromatic L-amino acid decarboxylase; *AIRE*, autoimmune regulator; APS I, autoimmune polyendocrine syndrome type I; APS II, APS type II; CMC, chronic mucocutaneous candidiasis; GAD, glutamic acid decarboxylase; IFN, interferon; 17OH, 17 $\alpha$ -hydroxylase; 21OH, 21-hydroxylase; SCC, side-chain cleavage enzyme; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

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oplasia, keratoconjunctivitis, vitiligo, alopecia, nail pitting, and/or tympanic membrane calcifications. Most patients already have three to seven different manifestations early in the course of the disease, but other components may appear throughout life (2, 5).

The disease gene, autoimmune regulator (*Aire*; GenBank accession no. Z 97990) was identified in 1997 (10, 11). Studies in knockout mouse models have highlighted a role for AIRE in regulating expression in the thymus of a subset of autoantigens from peripheral tissues (*e.g.* preproinsulin) (12). Normally, potentially autoaggressive T cells that recognize these antigens are deleted (13), but they are presumed to escape to the periphery in patients with *AIRE* mutations or in *Aire*<sup>-/-</sup> mice, leading to autoimmune inflammation in selected organs. AIRE is also expressed in peripheral dendritic cells and has been suggested to be involved in the maintenance of peripheral immunological tolerance in addition to the essential role it has in central tolerance, although the mechanisms are unresolved (14–17).

The pathogenesis of APS I remains unclear. However, most patients have autoantibodies against autoantigens that are expressed in affected tissues. The autoantigens currently known are mostly enzymes, and can be divided into two main groups: 1) cytochrome P450 enzymes involved in steroid biosynthesis and hepatic metabolism of drugs, such as 21-hydroxylase (21OH) (18, 19), side-chain cleavage enzyme (SCC) (20), 17 $\alpha$ -hydroxylase (17OH) (21), and cytochrome P450 1A2 (22); and 2) enzymes involved in synthesis of neurotransmitters, such as aromatic L-amino acid decarboxylase (AADC) (23), tryptophan hydroxylase (TPH) (24), tyrosine hydroxylase (TH) (25), histidine decarboxylase (26), and glutamic acid decarboxylase (GAD) (27). These autoantibodies can be a useful tool in diagnosis of APS I; moreover, some of them correlate with clinical manifestations (28), *i.e.* anti-SCC with gonadal failure (19, 29), anti-TPH with intestinal dysfunction (24), and anti-AADC with autoimmune hepatitis and vitiligo (30). Recently it has emerged that almost all of the approximately 60 Finnish APS I patients tested have autoantibodies against type I interferons (IFNs), especially IFN- $\alpha$  subtypes and IFN- $\omega$  (31). These antibodies may prove to be more sensitive and specific markers of APS I than the enzyme and cytochrome autoantibodies.

We previously presented clinical information, autoantibody profiles and *AIRE* mutations in 20 Norwegian patients (5). We have now reinvestigated these individuals and identified 16 additional cases of APS I to yield a relatively large panel (1, 3, 32, 33). In this more complete survey, we now summarize the prevalence and follow-up of APS I in the Norwegian population; the clinical features and *AIRE* mutations prove to be more variable than previously realized. We also correlate these findings with autoantibody profiles.

### Subjects and Methods

Patients were recruited by contacting the Departments of Internal Medicine and Pediatrics at all hospitals in Norway, Departments of Dermatology and Ophthalmology at the four university hospitals, and the two Faculties of Dentistry in Norway. The study has been approved by the local ethics committee and has been conducted in accordance with the guidelines in the Declaration of Helsinki.

### Definitions

All recruited patients had either two of the three major clinical manifestations or only one in combination with a blood relative with established APS I. We included 36 patients from 24 families, the majority of whom were under our care, although seven were identified only after their death. All of the patients whom we have reported earlier (5) were reinvestigated.

### Clinical data

The diagnoses of hypoparathyroidism, Addison's disease, primary hypothyroidism, primary gonadal failure, and diabetes type I were based on typical biochemical findings as described by Ahonen *et al.* (2). Enamel hypoplasia was defined as defects in enamel not due to caries. Most of the patients were evaluated by clinical examination and an orthopantomogram by an experienced dentist at the Faculties of Dentistry, University of Oslo and Bergen. We diagnosed oral candidiasis on visible mucosal changes in combination with positive culture for *Candida albicans* and nail candidiasis on typical clinical findings as judged by an experienced dermatologist if cultures were negative. In some cases the differentiation between *Candida* infection and nail dystrophy was difficult. Malabsorption was diagnosed from a history of recurrent episodes of diarrhea and/or measurement of fecal fat. Autoimmune hepatitis was diagnosed by liver biopsy. Chronic elevation of alanine aminotransferase (>3 months) without evidence of viral or drug-induced hepatitis (2) was considered as an indication of autoimmune hepatitis in APS I patients (22). Twenty-four patients with isolated Addison's disease and 21 patients with autoimmune polyendocrine syndrome type II (APS II) (Addison's disease plus thyroid disease and/or diabetes type I) were used as controls for assays of antibodies to type I IFN.

### Assay of antibodies

Antibodies against 21OH, SCC, AADC, TPH, and GAD were assayed by a RIA against <sup>35</sup>S-labeled recombinant human autoantigens expressed by *in vitro* transcription and translation as described by Ekwall *et al.* (24). All samples from each patient were assayed in the same experiment. We measured binding and neutralizing anti-IFN antibodies by ELISA and antiviral interferon neutralization assay (31). Positivity for the assays are governed by whether the test absorbance was greater than 2 SD (3 SD for RIA) above the negative control value. The assays were run in a blinded fashion.

### Mutational analysis of the *AIRE* gene

DNA was extracted from 0.5 ml of patient EDTA blood samples using the M48 extractor (QIAGEN GmbH, Hilden, Germany). All 14 exons of the *AIRE* gene (EMBL accession no. AJ009610) were amplified by PCR from the patient DNA and sequenced using primers as described (Table 1). The PCR conditions were: 1 $\times$  Amplitaq Gold buffer, 1.5 mM MgCl<sub>2</sub>, 0.4 mM deoxynucleotide triphosphates, 0.2 U Amplitaq Gold (Applied Biosystems, Foster City, CA), 0.6  $\mu$ M of each primer, and 50–100 ng genomic DNA in 25- $\mu$ l reactions. The cycling temperatures were 95 C for 20 sec, 58 C for 20 sec, and 72 C for 30 sec (32 cycles). Before sequencing, the PCR products were purified using ExoSAP-IT according to the protocol of the manufacturer (USB Corp., Cleveland, OH). The samples were sequenced using BigDye terminator mix version 1.1 and analyzed on ABI3100 (Applied Biosystems). A formalin-fixed spleen sample from a dead patient was analyzed for the c.1242\_1243insA, c.1249dupC, and c.967\_979del13 *AIRE* mutations using fragmentation analysis of fluorescent labeled duplex PCR products. Primers are listed in Table 2. The PCR conditions were: 1 $\times$  ThermalAce Buffer, 0.8 mM deoxynucleotide triphosphates, 1 U ThermalAce (Invitrogen, Merelbeke, Belgium), 0.4  $\mu$ M of each primer, and 50 ng genomic DNA in a 25- $\mu$ l reaction. The cycling conditions were 95 C for 25 sec, 60 C for 25 sec, and 72 C for 25 sec (30 cycles). When the products of the reaction, which covers the positions c.1242\_1243insA and c.1249dupC, identified a mutated allele, the PCR products were sequenced to identify the exact position of the mutation.

**TABLE 1.** AIRE PCR and sequencing primers

Primer name	Sequence (5' → 3')	PCR product size (bp)
AIREex1F	CAA GCG AGG GGC TGC CAG TG	296
AIREex1R	GGA TCT GGA GGG GCG GGG TC	
AIREex2F	ACC ACC TGA CTC CAC CAC AAG CC	545
AIREex2R	TCA GGG TTT TCT CCA GGG GTA GGG	
AIREex3F	GTG ATG TTC CAG GAC CGT CTT G	528
AIREex3R	AGA CCC GCC CGC CTA CTT	
AIREex4F	TGA AGT AGG CGG GCG GGT CTC	454
AIREex4R	CAG GGG GGA CTG GCA AGA TCA	
AIREex5F	TTG GGT GCA CAC ACG AAC A	454
AIREex5R	GGC AGA AAC TCT GGC TAC CTG A	
AIREex6F	CAC CCT GGG GCC TAC ACG ACT	401
AIREex6R	GAA GAG GGG GCG CAG CAA TGG	
AIREex7F	CCA GGA ACA GCG TTG CCT C	318
AIREex7R	CGG TGC TCA TCC CTG AGT GCC	
AIREex8F	CAG GTG GTC AGG GCA GAA TTT CA	493
AIREex8R	AGG CTG GGC AGC AGA TGT G	
AIREex9F	ATC TCT CTG CTG TGC CTC GGT TC	384
AIREex9R	TGG GCA TGG GGG ACA TAG TG	
AIREex10	TGC CAC AGC CTT TCC CAC TCA GT	478
AIREex10	CCT CCC GGA GGC TTT CTC GC	
AIREex11	GCC TGA GGG TGC TTG GGT CG	433
AIREex11	GGG GTG TGG TTG TGG GCT GTA TG	
AIREex12	CCC CCA CTC ACC ACC CAC G	497
AIREex12	GGG AGC CCT GGC AGG ACT CTC	
AIREex13	CCC CAG CCC CAT CAT GCC	384
AIREex13	TGG TGG GTG GAG CAG GGA CAG	
AIREex14	TGG ATG GTG ACT TCT TGT AAC GA	497
AIREex14	ACC TCC CGA GTT CAA GTG ATT C	
AIREex11	GCC TGA GGG TGC TTG GGT CG	433
AIREex11	GGG GTG TGG TTG TGG GCT GTA TG	
AIREex12	CCC CCA CTC ACC ACC CAC G	497
AIREex12	GGG AGC CCT GGC AGG ACT CTC	
AIREex13	CCC CAG CCC CAT CAT GCC	384
AIREex13	TGG TGG GTG GAG CAG GGA CAG	
AIREex14	TGG ATG GTG ACT TCT TGT AAC GA	497
AIREex14	ACC TCC CGA GTT CAA GTG ATT C	

## Results

### The patients ascertained

In surveys performed in 1999 and again in 2006, we were able to identify 36 patients with APS I in 24 families. These probably include all of the clinically typical patients, although some unusual ones may have eluded us. Among the 20 males and 16 females, onset ages ranged from the first year of life to 43 yr (mean 9.1 yr). The wide range of clinical manifestations is shown in Table 3. Two patients died during the study period. One died of a metastasizing oral squamous carcinoma of the oral cavity (patient 13) and one of chronic renal failure (patient 30). Patient 30 had had reduced renal function for at least 7 yr before he died due to nephrocalcinosis and arterial hypertension. Aminoglycoside treatment during a visit abroad reduced his renal function further, and he became dependent on dialysis. Renal biopsy was not

**TABLE 2.** Primers for duplex PCR for the identification of the mutations c.1242\_1243insA/c.1249dupC/c.967\_979del13 in *AIRE* using fragmentation analysis

Primer name	Sequence (5' → 3')
AIRE965del13-F	6-Fam GAGCTCATCTGCTGTGACGG
AIRE965del13-R	GGCCAGTGACCTGACTCA
AIRE1242insA-F	6-Fam GACACGACTCTTGTCTACAAGCA
AIRE1242insA-R	GTTTCTTACCCTCAGGACCCACACA

6-Fam, 6-Carboxyfluorescein, a reporter dye.

performed. The last year he presented with pneumonia with sepsis several times and he died during one of these episodes.

Seven patients were identified after their death. A 10-yr-old sister of patient 14, who recently had been diagnosed with Addison's disease, died after being in a coma for 3 d, probably due to an Addisonian crisis or undiagnosed hypoparathyroidism (patient 15). Two siblings of patient 20 also died young (patients 21 and 22). An older brother died when he was 12 yr old of measles, whereas a younger sister died of unknown cause when she was 6 yr old. She was reported to have seizures and hyperpigmentation. Patient 20 also has five living healthy siblings. An older sister of patient 26 died at age 4 yr during an acute varicella infection (7). She is not included here, although we cannot rule out that she had undiagnosed APS I. Furthermore, one 39-yr-old male with Addison's disease (patient 32) died of an acute myocardial infarction, which was probably not related to APS I. However, records of chronic nail candidiasis prompted *AIRE* sequencing of formalin-fixed spleen tissue. In a sensitive PCR test, he proved to be heterozygous for the 13-bp deletion in exon 8 (c.967–979del13), which is the most common mutation in Norwegians. Because the DNA was too degraded, we used DNA from his mother to identify the second *AIRE* mutation, which was identified as c.769C<T (the Finnish major mutation). Finally, we identified two siblings in a second family (patients 34 and 35), a female (patient 34) with probable hypoparathyroidism, and a male (patient 35) with confirmed Addison's disease, hypoparathyroidism, alopecia, enamel hypoplasia, and candidiasis (Table 3). The male at age 17 yr died in an accident; his elder sister had died at age 3 yr with seizures, probably secondary to hypoparathyroidism. Their first cousin (patient 36) had hypoparathyroidism, autoimmune thyroid disease, and enamel problems and died of cerebral hemorrhage secondary to multiple cerebral arterial aneurysms at the age of 44 yr. She had two siblings who died young: a boy who died when he was 3 months old due to whooping cough and another boy who died at age 1 yr of renal problems. It might be speculated that these siblings could have APS I, but this was not confirmed and they were not included here. Another first cousin of patient 36 (patient 33), who has *Candida* and hypoparathyroidism, proved to be c.967–979del13 homozygous. We suspected that patients 34–36 had at least one copy of c.967–979del13. Because the mother of patients 34 and 35 and the mother of patient 36 were confirmed as carriers of the c.967–979del13 mutation, this assumption proved correct. This is a family from an isolated part of Norway and several instances of consanguinity in previous generations were confirmed.

We chose to determine the prevalence of APS I in subjects born in Norway between 1976 and 1991 because some born before 1976 might have gone undiagnosed, whereas many born after 1991 might have not yet developed symptoms. The prevalence of APS I in this population was calculated to be about 1:90,000 individuals, *i.e.* nine of 857,134 people born in that interval.

### Atypical clinical presentations

Whereas most of the patients met the standard diagnostic APS I criteria in childhood, there were striking clinical vari-



**TABLE 3.** Clinical details, autoantibodies, and *AIRE* mutations in the coding region in 36 Norwegian patients with APS I: follow-up after 5–10 yr

Family no.	Pat. no.	Sex	Age	Age of debut <sup>a</sup>	Autoantibodies <sup>b</sup>	Manifestations <sup>c</sup>	Aire mutations
I	1	M	11	3	<b>AADC+</b>	HP(3), C(3), Al, K	c.967–979del13/c.769C>T
I	2	M	14	2	<b>21OH+</b>	HP(5), C(2)	c.967–979del13/c.769C>T
II	3	F	16	0	<b>SCC+, (21OH-), 17OH</b>	A(5), HP(5), C(0), E	c.769C>T/c.1242_1243insA
II	4	F	20	9	21OH, 17OH	A(9)	c.769C>T/c.1242_1243insA
II	5	F	25	14	21OH, SCC, 17OH	A(14), C(14), V, N, G	c.769C>T/c.1242_1243insA
III	6	M	16	4	21OH, TH	A(4), HP(4), C(4), E, M	c.967–979del13/c.967–979del13
IV	7	M	19	4	21OH, SCC, 17OH	A(11), HP(4), C(9), E	c.967–979del13/c.967–979del13
IV	8	M	26	9	21OH, AADC, GAD	A(12), HP(9), C, E	c.967–979del13/c.967–979del13
IV	9	F	30	4	21OH, AADC, GAD, TPH	HP(4), C, AT, V, E	c.967–979del13/c.967–979del13
V	10	M	22	8	21OH, AADC, TPH, TH	HP(8), C(14), N, E	c.1163_1164insA/c.967–979del13
VI	11	M	30	0	21OH, AADC	A(10), HP(10), C(0), Al, H, M	c.967–979del13/c.1244_1245insC
VII	12	F	39	13	21OH, SCC, 17OH	A(14), HP(13), G, Al, C, AT	c.1244_1245insC/ c.1244_1245insC
VIII	13	M	†35	4	21OH, SCC, AADC, TH	A(4), HP(4), C(26), Al, E, SC	c.967–979del13/c.967–979del13
IX	14	M	44	20	SCC, AADC, GAD, TPH	A(20), HP(27), C(30)	c.967–979del13/c.967–979del13
IX	15	F	†10	9	n.d.	A(9), HP?	n.d.
X	16	F	44	7	21OH	A(22), C(7), V, Al, M, E	Not found
XI	17	F	48	10	21OH, SCC	A(13), HP(10), C(10), G	c.967–979del13/c.967–979del13
XII	18	F	49	1	21OH, SCC, <b>AADC+</b> , GAD, TPH, 17OH	A(17), HP(15), C(1), G	c.967–979del13/c.967–979del13
XIII	19	F	54	13	21OH, GAD	A(13), HP(13), C(13), G, AT, Al, E, D	c.769C>T / c.1336T>G
XIV	20	M	58	9	21OH, SCC, AADC, <b>GAD+</b>	A(16), HP(9), V, Al, C	c.769C>T/c.769C>T
XIV	21	F	†6	Not known	n.d.	n.d.	n.d.
XIV	22	M	†12	Not known	n.d.	n.d.	n.d.
XV	23	M	61	5	21OH	A(17), HP(20), C(5), K, V, Al	c.967–979del13/c.967–979del13
XVI	24	F	30	1	AADC, GAD, TPH	HP(1), C(5), G, N, Al, E	c.769C>T/c.769C>T
XVII	25	F	46	9	Not found	HP(9), G(17), Al, N, E, C	c.22C>T / c.290T>C
XVIII	26	M	36	12	<b>(21OH-), SCC, (GAD-)</b>	A(12)	c.967–979del13/c.967–979del13
XIX	27	M	55	Not known	21OH, SCC	A, HP, Al	c.22C>T/c.402delC
XX	28	F	32	21	21OH, TPH	A, C	c.879+1G>A/c.879+1G>A
XX	29	M	47	43	AADC, GAD, TPH	HP(43), D, V	c.879+1G>A/c.879+1G>A
XXI	30	M	†49	13	21OH, SCC, AADC, TPH	A, HP, C, Al	c.967–979del13/c.1249dupC
XXII	31	M	42	14	AADC, GAD, TPH	HP(14), C(22), D, K, N, V, Al	c.769C>T/c.1249dupC
XXIII	32	M	†39	14	n.d.	A, C	c.967–979del13/c.769C>T
XXIV	33	M	48	Not known	Not found	C, HP	c.967–979del13/c.967–979del13
XXIV	34	F	†3	3	n.d.	HP	n.d. <sup>d</sup>
XXIV	35	M	†17	5	n.d.	A, HP, C, Al, E	n.d. <sup>d</sup>
XXIV	36	F	†34	8	n.d.	HP, AT, E	n.d. <sup>d</sup>

A, Adrenal insufficiency; HP, hypoparathyroidism; C, mucocutaneous candidiasis; G, primary gonadal insufficiency; V, vitiligo; Al, alopecia; AT, autoimmune thyroid disease; M, malabsorption; N, nail pitting; E, dental enamel hypoplasia; K, keratopathy; H, autoimmune hepatitis; SC, oral squamous cell carcinoma; D, diabetes type 1; n.d., not done.

<sup>a</sup> The age of debut denotes the age at which the first APS I main component appeared.

<sup>b</sup> Autoantigen written in **bold** with a plus sign signifies that reactivity against a given antigen has appeared, whereas autoantigen written in **bold** with a minus sign denotes that reactivity against a given autoantigen was lost, compared to earlier analyses (see Fig. 1).

<sup>c</sup> The year of diagnosis for the manifestation is written in *parentheses*.

<sup>d</sup> Mother is heterozygot for c.967–979del13.

† Deceased (age in years).

ations, even between siblings with identical *AIRE* mutations, e.g. between patients 1 and 2 and 28 and 29 (Table 3). Moreover, several patients had fewer typical features and later presentations, including: male patient 26, diagnosed at age 12 yr with adrenal failure (for details, see Ref. 7) but who has shown no other manifestations of APS I in the subsequent 24 yr despite homozygosity for the highly prevalent Norwegian *AIRE* mutation c.967–979del13; male patient 14, diagnosed with Addison's disease when he was 20 yr old and who has developed hypoparathyroidism and chronic candidiasis later on; and male patient 29 with the novel c.879 + 1G>A (p.IVS7 + 1G>A) mutation, diagnosed with type 1 diabetes and hypoparathyroidism only. At age 43 yr, he has still not developed candidiasis or adrenal failure. His sister, patient 28, debuted unusually late with Addison's disease at the age

of 21 yr. Female patient 25 with two novel missense mutations [c.22C>T (p.Arg8Cys) and c.290T>C (p.Leu97Pro)] was diagnosed with hypoparathyroidism at age 9 yr but has developed candidiasis much later. The clinical differences in these latter two families might reflect their novel mutations.

#### *AIRE* mutations in Norwegian APS I patients

In our previous cohort of 20 patients, we identified five different mutations in the coding region of the *AIRE* gene; in order of decreasing frequencies, they are: c.967–979del13, c.769C>T (R257X, the prevalent mutation in Finnish APS I patients), c.1242\_1243insA, c.1244\_1245insC, and c.1163\_1164insA. In the new cases included in this survey, we identified six additional novel mutations: c.22C>T (p.Arg8Cys), c.290T>C

(p.Leu97Pro), c.402delC (p.Ser135GlnfsX12), c.879 + 1G>A (p.IVS7 + 1G>A), c.1249dupC (p.Leu417ProfsX7), and c.1336T>G (p.Cys446Gly); c.1249dupC was found in two families, the others in only one each.

#### *Distribution of autoantibodies in Norwegian patients with APS I*

In the 29 APS I patients with available serum samples, we tested for the most common APS I-associated autoantibodies, *i.e.* against 21OH, AADC, SCC, GAD, and TPH. We found up to five (mean 2.3) of these per patient, including anti-21OH in 20 of the 29, anti-AADC in 13 of 29, anti-SCC in 12 of 29, anti-GAD and anti-TPH in nine of 29 each. Twenty-five of the 29 had either anti-21OH or AADC or both. Finally, two had anti-21OH in an early sample but appeared negative in 2006. Of interest, patients 25 and 33 had none of the most common autoantibodies.

When new serum samples were tested from the 20 patients reported previously (5), some had lost or gained reactivity against one or more autoantigens (Table 3 and Fig. 1), although values were often close to the threshold for positivity. However, most patients had fairly stable indices of autoantibodies against the relevant autoantigens. Substantial increases were most obvious in young patients. Overall, there was a trend toward an increasing number of autoantibodies during follow-up. Two male siblings (patients 1 and 2) both had gained autoantibodies when comparing profiles from early samples with late samples, although against different autoantigens (AADC and 21OH, respectively). Strikingly, the 16-yr-old patient 3 had gained high levels of autoantibodies against SCC when comparing samples from 2002 and 2005.

Notably, 28 of the 29 APS I patients had autoantibodies against type I IFNs in their sera, regardless of their exact *AIRE* mutations or clinical manifestations (Table 4). In 23 samples, neutralizing titers were very high against IFN- $\alpha$ 2, IFN- $\alpha$ 6, IFN- $\alpha$ 8, IFN- $\alpha$ 6, and IFN- $\omega$ ; in the others, they were much higher against IFN- $\omega$  than the IFN- $\alpha$ s (apart from IFN- $\alpha$ 8 in two). In the one patient (patient 16) with no detectable anti-IFN antibodies, no *AIRE* mutations have yet been detected, even though she has a wide range of APS I manifestations, albeit with no hypoparathyroidism by age 44 yr. Sera from 70 blood donors and 45 patients with isolated Addison's disease or APS II were completely negative for neutralizing type I IFN autoantibodies and binding IFN- $\alpha$ 2 antibodies (results not shown). We found only two patients with APS II having low titers of binding autoantibodies against IFN- $\omega$  in ELISA that reached significance (OD 0.422 and 0.822); these, however, were nonneutralizing. Patients with hypoparathyroidism, type 1 diabetes, and autoimmune thyroid disease have not been found to have binding or neutralizing antibodies against type I IFN, as reported recently (31, 34).

### Discussion

This comprehensive survey of APS I in Norway shows that, despite a wider range of *AIRE* mutations and clinical phenotypes, anti-IFN $\omega$  autoantibodies are so prevalent/persistent as to warrant addition to the diagnostic criteria. The

various clinical manifestations of APS I showed similar prevalences in our 36 Norwegians (Table 3) to those reported in Finnish and Italian patients (3, 4, 35), although autoimmune hepatitis seems to be rarer than in Swedish or Finnish series (30). In most patients, onset was in childhood, but in three cases, the first APS I feature appeared only in adolescence or early adulthood (older than age 15 yr). Diagnosis of APS I was also delayed in many cases, partly because of the interval of several years between presentation of the first and second endocrinopathies (see *Results*); also, some physicians may still be unaware of APS I.

#### *Underdiagnosis of APS I*

In our previous study, we identified three individuals who had probably died of unrecognized APS I (5); our current survey identified another four who had died undiagnosed (patient 32 and three relatives of patient 33). Increased awareness and information about APS I are needed to prevent such untimely fatalities. The diagnosis should be considered in all patients with one of the main components of APS I, especially when it first presents in childhood. Furthermore, whenever hypoparathyroidism presents after the neonatal period and without dysmorphic features, the likelihood of APS I should be weighed in both the proband and any other siblings. Finally, suspicion of APS I should also be raised when patients present with minor components, especially in childhood. Thus, about 20% of the Finnish patients first presented with minor components, *i.e.* autoimmune hepatitis, keratoconjunctivitis, chronic diarrhea, periodic rash with fever, severe obstipation, alopecia, vitiligo, or pernicious anemia (3). When a new patient is diagnosed, all siblings should be investigated as to whether APS I manifestations are present.

#### *The AIRE mutations detected*

We were able to identify *AIRE* mutations in 28 of the 29 Norwegian APS I patients who were still alive. In our previous study (5), we found only one *AIRE* mutation in the two brothers (patients 1 and 2) (c.967–979del13/wild-type, family I), but, in addition, resequencing has now detected the common Finnish mutation (c.769C>T) on the other allele (Table 3). Likewise, in patient 19, with previously only one mutation detected, we now find a novel mutation, 1336T>G, in addition to c.769C>T. We found the common Finnish c.769C>T (R257X) mutation in nine of the 48 (19%) Norwegian APS I *AIRE* alleles and the 13-bp deletion (c.967–979del13) in exon 8 in 23 of 48 (48%). The Finnish R257X mutation dominates in APS I patients in eastern Europe, reaching 71% in Polish patients (36) and 72% in a study on mixed eastern European patients (37). A high prevalence of the 13-bp deletion has been noted in other countries, *i.e.* 71 and 53% in British and Northern American series, respectively (38, 39). This latter mutation must have been introduced independently more than once into the Norwegian population because it has been observed on several different haplotype backgrounds (5).

Overall, the *AIRE* mutations we found in Norwegian APS I patients are more diverse than previously suspected. The six novel mutations reported here are thought to have different effects on the *AIRE* protein. The mutations c.1336T>G, c.22C>T, and c.290T>C all cause nonconservative amino

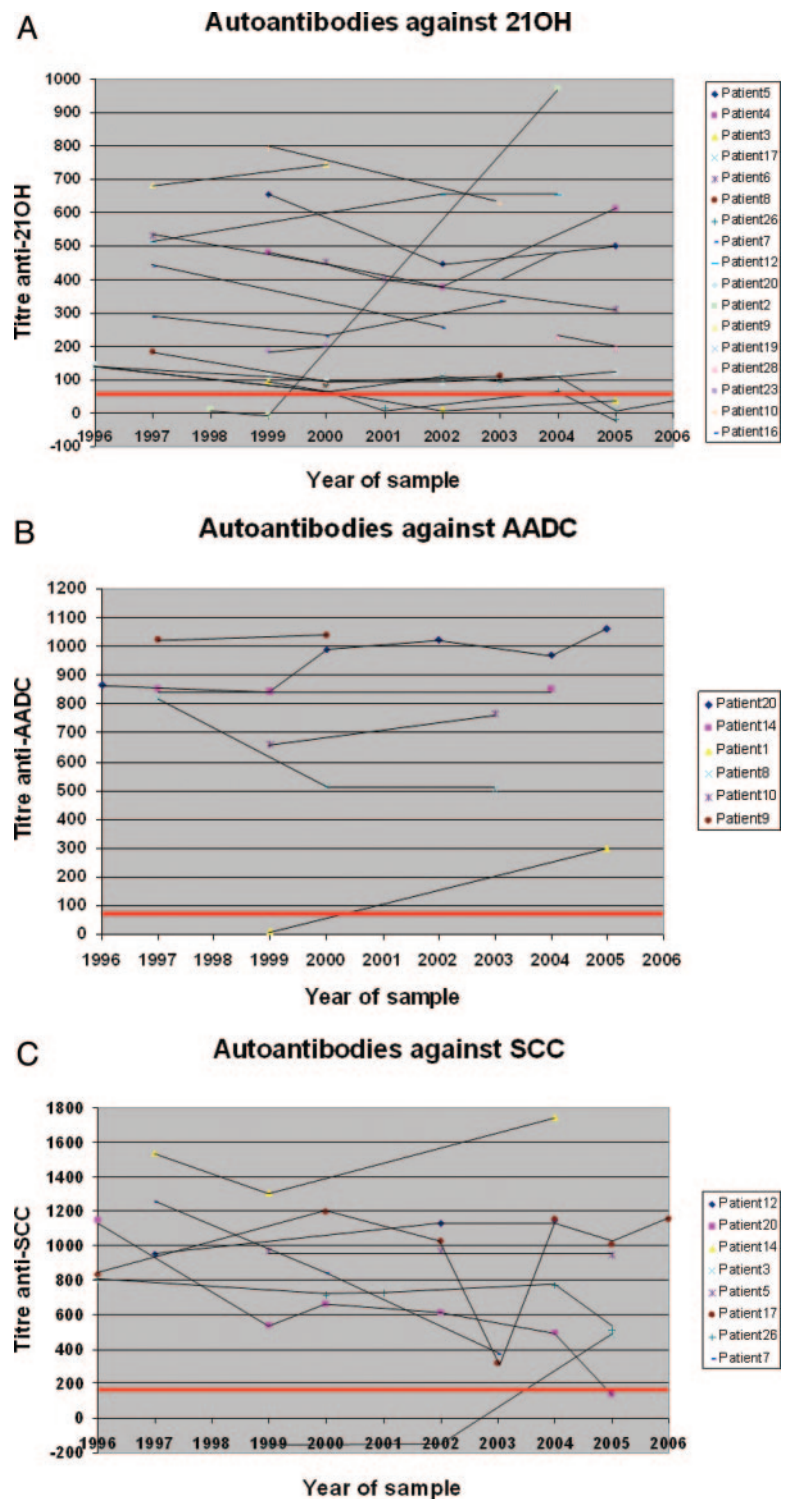


FIG. 1. A–C, Change in autoantibodies against AADC, SCC, and 21OH over time in 20 patients with APS I. Titers of autoantibodies were calculated relative to one positive control and a pool of human sera from healthy blood donors (PHS) [titer autoantibodies = (cpm sample – cpm PHS) / (cpm positive sample – cpm PHS) \* 1000]. The threshold for positivity was based on the mean of samples from healthy controls plus 3 SD. For AADC, SCC, and 21OH, the threshold values were 89, 184, and 48, respectively (shown by red lines on the figures). We show here only seropositive patients with more than one sample.

acid changes (Cys446Gly, Arg8Cys, and Leu97Pro, respectively). The mutation at the 22nd codon of *AIRE* is one of the most N-terminal changes in the protein so far reported (The Human Gene Mutation Database in Cardiff, <http://www.hgmd.cf.ac.uk/ac/index.php>). The amino acid residues 8 and 97 are located in the homogeneously staining region (HSR) domain of *AIRE*, which is the area in which most other missense mutations have been located. This domain is be-

lieved to be involved in multimerization of the protein; substitutions there might well influence the ability of *AIRE* to make homodimers and tetramers, with adverse functional consequences. By contrast, the c.1336T>G leads to an amino acid substitution in the second plant homeodomain region of *AIRE*. The two plant homeodomains of this protein are probably involved in DNA binding, which is an essential function of transcription factors. Thus, the three novel missense mu-



**TABLE 4.** Binding and neutralizing autoantibodies against IFNs in APS I patients<sup>a</sup>

Subject	Binding autoantibodies		Neutralizing autoantibodies					
	IFN- $\alpha$ 2	IFN- $\omega$	IFN- $\alpha$ 2	IFN- $\alpha$ 6	IFN- $\alpha$ 8	IFN- $\alpha$ 16	IFN- $\omega$	IFN- $\beta$
1(a)	<b>2.851</b>	<b>2.077</b>	<b>15,000</b>	<b>20,000</b>	<b>96,000</b>	<b>15,000</b>	<b>50,000</b>	$\leq 40$
1(b)	<b>3</b>	<b>3</b>	<b>&gt;25,600</b>	<b>&gt;25,600</b>	<b><math>\geq 256,000</math></b>	<b>256,000</b>	<b>50,000</b>	$\leq 40$
2(a)	<b>3</b>	<b>2.832</b>	<b>200,000</b>	<b>200,000</b>	<b>&gt;250,000</b>	<b>150,000</b>	<b>&gt;256,000</b>	$\leq 40$
2(b)	<b>3</b>	<b>3</b>	<b>&gt;25,600</b>	<b>&gt;256,000</b>	<b>&gt;256,000</b>	<b>&gt;256,000</b>	<b>&gt;256,000</b>	50
3	<b>3</b>	<b>1.957</b>	<b>25,000</b>	<b>12,000</b>	<b>100,000</b>	<b>15,000</b>	<b>40,000</b>	$\leq 40$
4	<b>3</b>	<b>1.957</b>	<b>100,000</b>	<b>80,000</b>	<b>80,000</b>	<b>45,000</b>	<b>100,000</b>	$\leq 40$
5	<b>3</b>	<b>2.875</b>	<b>200,000</b>	<b>150,000</b>	<b>200,000</b>	<b>128,000</b>	<b>128,000</b>	$\leq 40$
6	<b>3</b>	<b>2.852</b>	<b>&gt;256,000</b>	<b>&gt;256,000</b>	<b>&gt;256,000</b>	<b>&gt;256,000</b>	<b>&gt;256,000</b>	70
7	<b>3</b>	<b>2.438</b>	<b>&gt;256,000</b>	<b>220,000</b>	<b><math>\geq 256,000</math></b>	<b>200,000</b>	<b>20,000</b>	$\leq 40$
8	<b>3</b>	<b>2.089</b>	<b>&gt;256,000</b>	<b>256,000</b>	<b>150,000</b>	<b>160,000</b>	<b>&gt;256,000</b>	60
9	<b>3</b>	<b>2.668</b>	<b>90,000</b>	<b>90,000</b>	<b><math>\geq 256,000</math></b>	<b>25,000</b>	<b>&gt;256,000</b>	$\leq 40$
10(a)	<b>3</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>&gt;250,000</b>	n.d.
10(b)	<b>3</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>&gt;250,000</b>	n.d.
11	<b>3</b>	<b>2.596</b>	<b>256,000</b>	<b>256,000</b>	<b>256,000</b>	<b>256,000</b>	<b>256,000</b>	$\leq 40$
12(a)	<b>3</b>	n.d.	<b>15,000</b>	n.d.	<b>20,000</b>	n.d.	<b>28,000</b>	n.d.
12(b)	<b>3</b>	n.d.	<b>40000</b>	n.d.	<b>45000</b>	n.d.	<b>55,000</b>	n.d.
13	<i>-0.124</i>	n.d.	<i>120</i>	n.d.	<i>150</i>	n.d.	<b>&gt;250,000</b>	n.d.
14(a)	n.d.	n.d.	<i>550</i>	<i>450</i>	<b>5,120</b>	<i>300</i>	<b>3,500</b>	$\leq 40$
14(b)	<i>0.131</i>	<i>0.176</i>	<i>240</i>	<i>220</i>	<b>1,800</b>	n.d.	<b>1,500</b>	$\leq 40$
14(c)	n.d.	n.d.	<i>450</i>	<i>280</i>	<b>&gt;5,120</b>	<i>400</i>	<b>2,500</b>	60
16	0.1	0.09	40	40	40	50	40	$\leq 40$
17	<b>3</b>	<b>3</b>	<b>90,000</b>	<b>90,000</b>	<b>180,000</b>	<b>75,000</b>	<b>50,000</b>	<b>900</b>
18	<b>3</b>	n.d.	<b>20000</b>	n.d.	<b>15,000</b>	n.d.	<b>34,000</b>	n.d.
19	<i>-0.077</i>	n.d.	60	n.d.	40	n.d.	<b>22,500</b>	n.d.
20	<b>3</b>	<b>3</b>	<b>20,000</b>	<b>7,000</b>	<b>28,000</b>	<b>11,000</b>	<b>20,000</b>	60
23	<b>3</b>	<b>2.9</b>	<b>90,000</b>	<b>40,000</b>	<b>90,000</b>	<b>50,000</b>	<b>50,000</b>	50
24	<b>3</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>22,500</b>	n.d.
25	<b>3</b>	<b>3</b>	<b>80,000</b>	<b>100,000</b>	<b>90,000</b>	<b>25,000</b>	<b>55,000</b>	70
26(a)	<b>3</b>	<b>2.727</b>	<b>150,000</b>	<b>128,000</b>	<b>150,000</b>	<b>150,000</b>	<b>150,000</b>	60
26(b)	<b>3</b>	<b>3</b>	<b>180,000</b>	<b>150,000</b>	<b>150,000</b>	<b>150,000</b>	<b>140,000</b>	60
27	0.002	n.d.	60	n.d.	$< 40$	n.d.	<b>19,000</b>	n.d.
28	<b>3</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>250,000</b>	n.d.
29	<i>0.258</i>	<b>3</b>	<i>100</i>	<i>130</i>	<i>300</i>	<i>100</i>	<b>30,000</b>	$< 40$
30	<b>3</b>	n.d.	<b>60,000</b>	n.d.	<b>75,000</b>	n.d.	<b>90,000</b>	n.d.
31	<b>3</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>&gt;125,000</b>	n.d.	<b>150,000</b>	n.d.
33	<b>2.497</b>	<b>0.469</b>	<b>&gt;10,000</b>	n.d.	<b>&gt;10,000</b>	<b>&gt;10,000</b>	<b>7,000</b>	n.d.
Threshold	0.08/0.109	0.079/0.091						
Neg Cont	0.086/0.129	0.093/0.111	80	60	70	80	70	$\leq 40$
Pos Cont	<b>3</b>	<b>2.23/3</b>	<b><math>\geq 256,000</math></b>	<b><math>\geq 256,000</math></b>	<b>100,000</b>	<b>50,000</b>	<b>50,000</b>	<b>120</b>

n.d., Not done.

<sup>a</sup> Positive results are marked in *bold*. Moderate positive results are marked in *italics*. Samples were tested for binding antibodies in two different experiments; their marginally different values for Pos Cont (positive control, mean of four APS I patients with known positivity), Neg Cont (negative control, mean of four blood donors with known negativity), and Threshold are given. For some of the patients, several samples were analyzed, indicated by letters *a-c* together with the subject number.

tations reported here probably adversely affect the function of AIRE. The DNA variations c.402delC and c.1249dupC result in frame shifts and introduce premature stop codons, whereas the c.879 + 1G>A mutation change probably interferes with splicing, yielding a protein that is incorrectly processed. It is reasonable to believe that these mutations and substitutions in the AIRE gene are responsible for the development of APS I in these patients.

The one patient (patient 16) with no AIRE mutations detected had previously been classified as APS I because of her diverse clinical manifestations, although she still has no hypoparathyroidism or identified AIRE mutations. Moreover, she was the only potential APS I case in a sibship of more than 10 siblings. The consequent doubts about the diagnosis of APS I was reinforced by her complete negativity for anti-IFN autoantibodies, which was also noted in one of three such cases from Finland (the other two were strongly positive) (31). On the other hand, in a few clinically typical APS

I patients, no AIRE mutations are detected on regular sequencing of the AIRE gene exons, which may hint at mutations either in regulatory parts of the AIRE gene or in other genes with similar functions. Also, information about a possible complete loss of an AIRE exon is lost using regular sequencing as a method.

#### Autoantibodies in APS I

*Clinical considerations.* A correlation between autoantibodies against SCC and ovarian failure has been reported by us and other investigators (19, 28, 29). In this study, six of 12 women (aged > 13 yr) had ovarian failure, and four of these had anti-SCC; another female (patient 3) who was previously negative, now has anti-SCC at age 16 yr (Table 3). Seven males had anti-SCC, but none of them had known testicular failure. The testis is an organ that is separated from the blood system and continuous patrolling of blood cells through the

organ is diminished. This barrier is probably important to avert an autoimmune attack of the gonads in APS I men.

The presence of autoantibodies against GAD has been correlated to the appearance of diabetes (40) but not when seen in APS I (28); instead a correlation to malabsorption was noted. In the present study, three patients had diabetes and all had antibodies against GAD in their sera (patient 19 was diagnosed to have diabetes in 2003). Whereas every patient who had diabetes also had anti-GAD antibodies, six other patients with detectable anti-GAD antibodies have not so far developed diabetes. Additionally, anti-GAD antibodies do not seem to be correlated to vitiligo or intestinal failure in the Norwegian APS I cohort, which has been reported previously (28). The low number of patients with malabsorption is a plausible explanation for this difference.

Five patients had acquired new autoantibodies between 1999 and 2006; three of them are under 17 yr old and have not yet shown any corresponding adrenal or gonadal failure.

However, the patients are still young, and it is known that autoantibodies against organ-specific antigens often appear before the clinical symptoms, as has been shown with anti-steroid cell antibodies and ovarian failure (41). The patients should be monitored closely for new components of APS I. Two other patients had lost their borderline reactivity toward 21OH as well as 21OH and GAD. In theory, organ atrophy might lead to loss of stimulating autoantigens.

**Diagnostic value.** Autoantibodies against 21OH and/or AADC were present in 86% of the patients. Most of them also had autoantibodies against one or more of the autoantigens SCC, 17OH, TPH, and TH, which are relatively APS I specific. Although anti-21OH and anti-AADC are also found in isolated Addison's disease and APS II (42, 43), together they have been the best immunological markers for APS I. However, Meager and co-workers (31) recently reported that nearly all APS I patients (mainly from Finland) have neutralizing autoantibodies against type I IFNs, especially IFN- $\omega$ , but many against IFN- $\alpha$ s as well (see *Results*). These are not seen in isolated Addison's disease, hypoparathyroidism, CMC, or APS II and appear highly APS I specific, although they also occur in (mostly older) patients with myasthenia gravis with or without thymoma (34). These observations were clearly confirmed in the present 29 patients, regardless of their *AIRE* mutations and wide range of APS I clinical features and onset ages thereof.

The anti-IFN- $\omega$  autoantibodies especially are almost always found in the earliest samples, persist for decades thereafter (31), and show higher APS I specificity and prevalence than the standard autoantibodies (see *Results*). Moreover, neutralization anti-IFN- $\omega$  antibodies show an almost perfect correlation with APS I in patients with mutated *AIRE*, regardless of the exact mutation or clinical phenotype, so they may be the diagnostic markers of choice, especially for early or atypical patients who have not developed the full APS I picture (Table 4). Therefore, they should probably be added to the diagnostic criteria for APS I, together with mutational analyses.

In conclusion, the clinical presentation of APS I is variable. Because it often takes several years to develop, the diagnosis can be difficult initially, especially when only one of the

typical APS I manifestations is present. Increased awareness of the condition combined with analysis of specific autoantibodies, particularly neutralization antibodies against type I IFNs, and follow-up by mutational analysis of the *AIRE* gene, should help us diagnose this rare condition earlier and allow treatments to prevent serious/fatal complications.

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