



HAL
open science

AIRE GENE MUTATIONS AND AUTOANTIBODIES TO INTERFERON OMEGA IN PATIENTS WITH CHRONIC HYPOPARATHYROIDISM WITHOUT APECED

Sara Cervato, Luca Morlin, Maria Paola Albergoni, Stefano Masiero, Nella Greggio, Cristiano Meossi, Shu Chen, Jadwiga Furmaniak, Bernard Rees Smith, Mohammad Alimohammadi, et al.

► **To cite this version:**

Sara Cervato, Luca Morlin, Maria Paola Albergoni, Stefano Masiero, Nella Greggio, et al.. AIRE GENE MUTATIONS AND AUTOANTIBODIES TO INTERFERON OMEGA IN PATIENTS WITH CHRONIC HYPOPARATHYROIDISM WITHOUT APECED. *Clinical Endocrinology*, 2010, 73 (5), pp.630. 10.1111/j.1365-2265.2010.03862.x . hal-00583577

HAL Id: hal-00583577

<https://hal.science/hal-00583577>

Submitted on 6 Apr 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



AIRE GENE MUTATIONS AND AUTOANTIBODIES TO INTERFERON OMEGA IN PATIENTS WITH CHRONIC HYPOPARATHYROIDISM WITHOUT APECED

Journal:	<i>Clinical Endocrinology</i>
Manuscript ID:	CEN-2009-000886.R2
Manuscript Type/Office:	1 Original Article - UK/Europe
Date Submitted by the Author:	27-Jul-2010
Complete List of Authors:	Cervato, Sara; University of Padua, Medical and surgical sciences Morlin, Luca; University of Padua, Medical and Surgical Sciences Albergoni, Maria Paola; Blood Transfusion Center, Azienda Ospedaliera-Università di Padova Masiero, Stefano; Endocrinology Unit, Medical and Surgical Sciences Greggio, Nella; Padova University, Department of Pediatrics Meossi, Cristiano; Ospedale di Lucca, Divisione di Pediatria Chen, Shu; FIRS Laboratories, RSR Ltd Furmaniak, Jadwiga; RSR Ltd, FIRS Laboratories Rees Smith, Bernard; RSR Ltd, FIRS Laboratories Alimohammadi, Mohammad; Uppsala University, Department of Medical Sciences Kämpe, Olle; Uppsala University,, Department of Medical Sciences Betterle, Corrado; University of Padua, Medical and Surgical Sciences
Key Words:	chronic hypoparathyroidism, , AIRE gene mutations, Class II HLA, Autoimmune diseases, Autoimmune polyendocrine syndromes

1
2 **AIRE GENE MUTATIONS AND AUTOANTIBODIES TO INTERFERON OMEGA IN**
3
4 **PATIENTS WITH CHRONIC HYPOPARATHYROIDISM WITHOUT APECED**
5
6 **(3508 Words).**
7

8 *Sara Cervato, *Luca Morlin, ¯Maria Paola Albergoni, *Stefano Masiero, **Nella
9 Greggio, ¨Cristiano Meossi, ^Shu Chen, ^Maria del Pilar Larosa, ^Jadwiga Furmaniak,
10 ^Bernard Rees Smith, °Mohammad Alimohammadi, °Olle Kampe, §Mariella Valenzise,
11 and *Corrado Betterle
12
13
14
15
16
17

18 *Department of Medical and Surgical Sciences, and

19 **Department of Pediatrics, Padova University, Padova, Italy

20 ¯Blood Transfusion Center, Azienda Ospedaliera-Universit di Padova, Padova, Italy

21 ¨Divisione di Pediatria, Ospedale di Lucca, Lucca, Italy

22 ^FIRS Laboratories, RSR Ltd, Cardiff, UK

23 §Department of Pediatrics University of Messina, Messina, Italy

24 °Department of Medical Sciences, Uppsala University Hospital Uppsala, Sweden
25
26
27
28
29

30 **Key Words:** chronic hypoparathyroidism, AIRE gene mutations, Class II HLA, Autoimmune
31 diseases, Autoimmune polyendocrine syndromes, interferon omega antibodies.
32
33
34
35

36 **Running Title:** AIRE gene mutations in chronic hypoparathyroidism
37
38
39

40 **Corresponding Author:**

41 Corrado Betterle MD,
42 Department of Medical and Surgical Sciences,
43 Padova University, Padova Italy
44 Via Ospedale Civile 105, 35100,
45 Padova, Italy
46 Tel: +39 049 821 3014
47 Fax: +39 049 657391
48 E-mail: corrado.betterle@unipd.it
49
50
51
52
53
54
55
56
57
58
59
60

SUMMARY (243 words)**Objective:**

To assess autoimmune regulator (AIRE) gene mutations, class II HLA haplotypes, and organ or non-organ specific autoantibodies in patients with chronic hypoparathyroidism (CH) without associated Addison's disease (AD) or chronic candidiasis (CC).

Design, Patients and Measurements:

24 patients who had CH without AD or CC were included in the study. AIRE gene mutations in all 14 exons were studied using PCR in 24 patients, 105 healthy controls and 15 first-degree relatives of CH patients with AIRE mutations. HLA were determined for all 24 patients and 105 healthy controls. Autoantibodies to a range of antigens *including NACHT leucine-rich-repeat protein5 (NALP-5) and interferon omega (IFN ω)* were tested in all 24 patients.

Results:

AIRE gene mutations were found in 6/24 (25%) patients, all females, and this was significantly higher ($p < 0.001$) compared with AIRE mutations found in healthy controls (2/105). Three patients (12.5%) had homozygous AIRE mutations characteristic of APECED and all three were also positive for IFN ω -autoantibodies. Three patients (12.5%) had heterozygous AIRE mutations; two of these were novel mutations. One of the patients with heterozygous AIRE mutations was positive for both NALP5 and IFN ω autoantibodies. Heterozygous AIRE mutations were found in 10/15 first-degree relatives of CH patients with AIRE mutations although none was affected by CH. Class II HLA haplotypes were not statistically different in patients with CH compared to healthy controls.

Conclusions:

Analysis of AIRE gene mutations together with serum autoantibody profile should be helpful in the assessment of patients with CH in particular in young women with associated autoimmune diseases.

For Peer Review

INTRODUCTION (3508 Words)

Autoimmune-Poly-Endocrinopathy-Candidiasis-Ectodermal-Dystrophy (APECED) (1), or autoimmune polyendocrine syndrome type 1 (APS-1) (2), is characterized by the presence of at least two of three conditions:- chronic candidiasis (CC), chronic hypoparathyroidism (CH) and Addison's disease (AD). APECED is a monogenic autosomal recessive disease associated with mutations in the autoimmune regulator (AIRE) gene and associations of APECED with HLA genes are not generally found (3, 4). CH is the most common disease in APECED, found in 71-100% of 385 reviewed patients (5). APECED presents in childhood with CH usually preceded by CC and followed by AD (3, 4). Recently, it has been shown that serum autoantibodies (*Abs to interferon omega (IFN ω)*) (6) are characteristic of APECED and *Abs to NACHT leucine-rich-repeat protein 5 (NALP5)* are characteristics of CH in patients with APECED (7).

CH can also occur in an isolated (idiopathic) form, or in combination with autoimmune diseases other than CC and/or AD (5, 8, 9). An association of isolated CH with Class II HLA genes has been reported (10) however an association with AIRE gene mutations has not been found (11).

In this study we analysed AIRE mutations, HLA type and autoantibody profiles in a group of Italian patients with CH who showed no clinical signs of CC or AD at presentation.

MATERIAL AND METHODS

PATIENTS

Out of 102 Italian patients with CH who were initially referred to our clinic, 78 had CH associated with CC and/or AD and consequently were classed as APECED, while 24 had isolated CH or CH associated with autoimmune diseases other than CC or AD. *At entry to the*

1
2 study all 24 patients had low serum calcium and elevated serum phosphorus levels with low
3
4 levels of PTH and were receiving substitutive therapy with vitamin D and calcium.

5
6 AIRE gene mutations, class II HLA type and serum autoantibodies were analysed in 24
7
8 patients who were included in the study. 15 first-degree relatives of patients with CH who
9
10 had AIRE gene mutations were also studied. The study was approved by the Local Ethical
11
12 Committee, and patients gave their written consent for the study.

16 AIRE GENE MUTATION ANALYSIS

17
18 All 14 exons of the AIRE gene and their flanking exon-intron boundaries (GenBank
19
20 accession no. Aj009610) were analysed in the 24 patients, in 15 first-degree relatives, and in
21
22 105 healthy adult controls using the methods described previously (12). PCR amplifications
23
24 were performed in a Verity thermal cycler (Applied Biosystems) using the specific primers
25
26 for each exon (shown in Table 1). Typically, PCR amplification from 200 ng of genomic
27
28 DNA was carried out in 35-cycles of: an initial 10-min denaturation at 95°C followed by 30
29
30 sec at 95°C, annealing at 55-63°C for 30 sec (Table 1), and final extension at 72°C for 30 sec
31
32 in a 25 µL total volume containing a 10 x buffer solution, 0.2 mmol/L of each dNTP (dATP,
33
34 dGTP, dCTP, dTTP), 2.5 mmol/L MgCl, 0.4 pmol/µL of each primer and 5U/µL of Taq
35
36 Gold. For CG-rich exons 50% deaza-GTP (Sigma Aldrich Co., St.Louis, Missouri) were used
37
38 instead of dGTP. In the reactions for exons 1, 2 and 14, 5% dimethyl sulfoxide was added to
39
40 aid amplification reactions. PCR products were separated from the primers and excess
41
42 nucleotides using a Microcon unit (Millipore), quantified and sequenced using a direct chain
43
44 termination method with BigDye Terminator v1.1 Cycle Sequencing Kits (PE Applied
45
46 Biosystems Milan, Italy). Sequencing reaction products were purified using the Microcon
47
48 (Millipore), and analyzed on an automated DNA sequencer (ABI PRISM 310, Genetic
49
50 Analyzer, Applied Biosystems).

CLASS II HLA TYPING

Human leukocyte antigens (HLA)-DRB1 were determined in 24 patients, and in 105 healthy adult controls randomly selected from the Bone Marrow Donor Register PD02. DNA was extracted from 400 μ L of peripheral blood using a QIAamp DNA minikit (QIAGEN, Crawley, UK). HLA typing was carried out using PCR-SSP (PCR sequence-specific primers) (GenoVision, Philadelphia, PA). For each sample, 23 PCRs were performed and the amplification products were stained with ethidium bromide and analyzed on 2% agarose gel using standard procedures (13).

STATISTICAL ANALYSIS

The phenotype frequency was calculated using the number of individuals carrying the allelic group and compared with the total number of individuals. Differences in the frequency of HLA DRB1 allele groups between CH patients and healthy controls were assessed using Fisher's exact test, and a $p < 0.05$ was considered significant.

AUTOANTIBODY TESTS

Sera from the 24 patients with isolated CH and CH associated with autoimmune diseases other than CC and/or AD were tested for autoantibodies to: thyroid microsomal antigen (TMHAbs), thyroglobulin (TgHAbs), parietal-cells (PCA), intrinsic factor (IFA), adrenal cortex (ACA), steroid 21-hydroxylase (21-OHAbs), steroid-producing cell (StCA), islet-cells (ICA), glutamic acid decarboxylase (GADAbs), second islet antigen (IA2Abs), nuclear proteins (ANA), mitochondria (AMA), liver-kidney microsomes (LKMA), NALP5, and *IFN ω* .

In particular, TMHAbs and TgHAbs were tested by haemagglutination method using kits from Remel Inc. (Santa Fe, USA). PCA, ICA, ACA, StCA, ANA, AMA, and LKMA were tested using the classical indirect immunofluorescence test on human or animal tissues as previously described (14). IFA were tested using an ELISA (EUROIMMUN AG. Lubeck,

Germany). 21-OHAbs were measured by the method described by Tanaka (15) using reagents from RSR Ltd, Cardiff (UK). GADAbs and IA2Abs were determined in a radioimmunoassay using kits from RSR Ltd, Cardiff (UK). NALP5Abs were measured using an immunoprecipitation assay based on ³⁵S-labelled NALP5 as described by Alimohammadi (6). *IFN ω Abs were tested using an immunoprecipitation assay based on ³⁵S-labelled IFN ω produced using an in vitro transcription/ translation method with rabbit reticulocytes (Promega, Southampton, UK). Human IFN ω cDNA (16) was synthesised in vitro (GeneArt AG, Regensburg, Germany) with a 5'-prime EcoR I and a 3-prime Xba I restriction site for direct cloning into pTnT vector (Promega).*

In the assay 5 μ L of test serum in duplicate were incubated with 50 μ L of ³⁵S-IFN ω (30,000dpm) overnight at 2-8 $^{\circ}$ C. 50 μ L of Protein A suspension was then added and the incubation continued for 1 hour at 2-8 $^{\circ}$ C. The precipitates were washed 3 times and counted in a β counter. The cut off for positive results was based on 99th percentile of individual controls studied.

RESULTS

CLINICAL AND IMMUNOLOGICAL STUDY

Clinical characteristics of the 24 patients and results of genetic and serum autoantibody tests are summarized in **Table 2**. The mean age at onset of CH in these patients was 23.2 years and the F/M ratio was 3.8/1.

AIRE GENE MUTATIONS AND AUTOANTIBODIES

Analysis of the AIRE gene revealed the presence of mutations in 6 out of 24 (25%) patients (patients 2, 3, 4, 9, 12, 14; **Table 2**) and in 2 out of 105 healthy Italian controls (*data not shown*). *The prevalence of AIRE gene mutations was significantly higher ($p < 0.001$) in patients with CH compared to healthy controls. Out of 6 patients with AIRE mutations 4 were positive for IFN ω Abs (Table 2).*

AIRE gene mutations were found in 6/19 females (31.6%) and in 0/5 males (0%). 5/17 patients (29.4%) with CH associated with the presence of autoantibodies or autoimmune

1
2 diseases other than AD or CC had AIRE gene mutations while only 1/7 patients (14.2%) with
3
4 CH without autoimmune diseases or autoantibodies had AIRE gene mutations.

5
6 Specifically 2 patients had homozygous mutations: R257X/R257X in exon 6 (patient 2, Table
7
8 2) and W78R/W78R in exon 2 (patient 4, Table 2) and one patient had a combined mutation
9
10 (R203X in exon 5 associated with IVS9+5G>T in exon 8 (patient 3, Table 2). These
11
12 mutations have been described in patients with APECED previously (12, 18, 19). In the case
13
14 of these three patients the mean age at onset of CH was 3.3 years. These three patients were
15
16 positive for IFN ω Abs (patients 2, 3, and 4, Table 2).

17
18 Heterozygous mutations were found in three patients, there were two novel mutations:
19
20 D312N in exon 8 (patient 9, Table 2), and R471C in exon 12 (patient 14, Table 2), while the
21
22 third patient (patient 12, Table 2) had a mutation P526L in exon 14 reported to be
23
24 associated with APECED in homozygous presentation previously (12, 17). One of the three
25
26 patients who carried the heterozygous mutations was positive for NALP5Abs and IFN ω Abs
27
28 (patient 9, Table 2). The mean age at onset of CH in three patients who carried the
29
30 heterozygous mutations was 20 years, whereas in the patients who had no AIRE gene
31
32 mutations this was 27.3 years.

33
34 Heterozygous mutations R257X and C322fs372 were found in two healthy controls in this
35
36 study. The healthy controls that were found to carry AIRE gene mutations showed no
37
38 evidence of clinical or latent autoimmune diseases.

39
40 In the course of our study we have noted a novel sequence variant in exon 6 (G218) not
41
42 associated with an amino acid change in one healthy control. In addition, a number of
43
44 known AIRE gene polymorphisms were found in patients with CH (Table 2).

45 46 47 **CH PATIENTS WITH AIRE GENE MUTATIONS**

48
49
50
51
52
53
54
55
56
57
58
59
60

1
2 **Patient 2 (Table 2)** was a female of Venetian origin who had R257X homozygous mutation
3
4 in exon 6 characteristic for APECED patients from *this region (12)*. She developed CH at 2
5
6 years of age and after presenting with primary amenorrhea at 14 years of age she was
7
8 diagnosed with a hypergonadotropic hypogonadism with a normal female karyotype. Serum
9
10 autoantibody tests carried out when she was 21 years old were all negative except for
11
12 IFN ω -Ab which was positive. Both parents had heterozygous AIRE gene mutation (R257X)
13
14 without clinical, latent or potential autoimmune diseases. The patient's brother had no AIRE
15
16 gene mutations or autoimmune diseases (**Table 3**).

17
18 **Patient 3 (Table 2)** was a female from Sicily who developed CH at 4 years of age and who
19
20 carried two AIRE mutations (R203X in exon 5 and IVS9+5G>T in exon 8). The R203X
21
22 mutation is typically found in APECED patients from Sicily (**12,18**) while IVS9+5G>T
23
24 mutation has been described to date in only one patient with APECED from Sardinia (**19**).
25
26 Patient 3 tested positive for IFN ω Abs, however to date has not yet developed either CC or
27
28 other clinical, subclinical or potential autoimmune diseases in the few months of follow up.
29
30 The patient's mother carried the heterozygous mutation R203X but had no clinical or latent
31
32 autoimmune diseases; the father had the heterozygous mutation IVS9+5G>T and had been
33
34 diagnosed with chronic thyroiditis. The brother and one sister both had heterozygous
35
36 mutation IVS9+5G>T without clinical, subclinical or potential autoimmune diseases while
37
38 the other sister did not have AIRE gene mutations or clinical, subclinical or potential
39
40 autoimmune diseases (**Table 3**).

41
42 **Patient 4 (Table 2)** was a female of Tuscan origin with homozygous mutation W78R in exon
43
44 2 found in APECED patients from Apulia (**12, 23**). She developed CH at 4 years of age and
45
46 at that time had no CC or other clinical autoimmune diseases. However, when she was 5
47
48 years old she tested positive for serum ACA (high titre of 1:32) and for 21-OHAbs (55U/mL)
49
50 with a normal adrenal function in the ACTH test. At 6 years of age during an episode of acute
51
52

1
2 infection she developed adrenal insufficiency, was started on substitutive steroid hormone
3 therapy and has remained on substitutive steroids ever since. A serum sample taken following
4 the adrenal crisis episode was positive for ACA, 21-OH Abs and INF ω Abs. Patient 4's
5
6 mother had a heterozygous W78R mutation and suffered from chronic thyroiditis while the
7
8 father who also carried heterozygous W78R mutation had no clinical, latent or potential
9
10 autoimmune diseases (**Table 3**).

11
12 **Patient 9 (Table 2)** was a female of Venetian origin and carried a novel AIRE gene mutation
13
14 characterized by the substitution of an aspartic acid at position 312 by an asparagine (D312N
15
16 in exon 8) in the AIRE gene region coding for the plant homeodomain (PHD). As reported
17
18 before, a missense mutation in the PHD results in increased localisation of AIRE to the
19
20 cytoplasm and decreased transcriptional transactivation activity (**20**). This patient developed
21
22 chronic thyroiditis with subclinical hypothyroidism at 7 years of age and was treated with L-
23
24 thyroxine. When she was 14 years old she developed CH with serum NALP5Abs positivity
25
26 and was treated with vitamin D and calcium. Also at the age of 14 years she tested positive
27
28 for ICA at 80 JDF/Units and GADAbs at 755 U/mL although had a normal glucose tolerance
29
30 test at that time. She has maintained ICA and GAD Abs positivity without the evidence of
31
32 glucose intolerance during follow up to date ie until the age of 21 years). At the age of 20
33
34 years she was tested and found positive for INF ω Abs. Her father also had heterozygous
35
36 mutation (D312N) and he was positive for serum thyroid autoantibodies with a normal
37
38 thyroid function and positive for GADAbs with normal glucose tolerance. The mother had no
39
40 mutations in the AIRE gene but had chronic thyroiditis with hypothyroidism and was on
41
42 therapy with L-thyroxine. She was also positive for GADAbs with normal glucose tolerance.
43
44 In contrast, the patient's sister did not have AIRE gene mutations or clinical, latent or
45
46 potential autoimmune diseases (**Table 3**).

1
2 **Patient 12 (Table 2)** was female with a mutation characterized by the substitution of proline
3
4 at position 526 by leucine (P526L in exon 14) (17). The patient developed CH when she was
5
6 20 years old and to date (at 75 years of age) she has not shown clinical signs of other
7
8 autoimmune diseases, however she has detectable serum PCA and IFA with high plasma
9
10 gastrin levels and normal blood count. This condition is highly suggestive of autoimmune
11
12 gastritis (AG), however the patient has refused further investigations in particular a
13
14 gastroscopy. Her parents were not available for testing AIRE gene mutations but they had a
15
16 negative history for autoimmune diseases.

17
18
19 **Patient 14 (Table 2)** was a female of Venetian origin and she carried a novel missense
20
21 mutation characterized by the substitution of arginine at position 471 by cysteine (R471C in
22
23 exon 12). This mutation resides in one of the zinc fingers of the AIRE PHD which is known
24
25 to be involved in chromatin-mediated regulation of transcription (21). The patient developed
26
27 psoriasis at 20 years of age, transient diabetes insipidus at 24 years of age, CH and chronic
28
29 thyroiditis at 26 years of age. When she was 30 years old she tested positive for PCA with
30
31 normal blood count and normal plasma gastrin levels. The patient's mother had R471C
32
33 mutation with no evidence of clinical autoimmune diseases however was found positive for
34
35 PCA on autoantibody screening. The father had no AIRE gene mutations and had no clinical,
36
37 subclinical or potential autoimmune diseases however he suffered from lung cancer (Table
38
39 3).

40 41 42 43 **HLA ANALYSIS**

44
45 The results of HLA typing in 24 patients with CH are summarized in **Table 2**. The
46
47 prevalences of class II HLA haplotypes in patients with CH compared to healthy controls
48
49 were not statistically significantly different ($p > 0.05$).

DISCUSSION

We studied a group of Italian patients who presented with non familial sporadic CH on its own or associated with autoimmune conditions other than CC and/or AD. The mutations in all exons of the AIRE gene, class II HLA phenotype and serum autoantibody profiles were studied in all patients. We found homozygous or heterozygous AIRE gene mutations in six females (25%) who presented with CH at a young age (below the age of 26 years). 4/6 patients with AIRE mutations had associated autoimmune diseases other than AD or CC and/or tested positive for serum autoantibodies (**Table 2**). However, 2/6 CH patients who carried AIRE mutations have not shown signs of autoimmunity as yet. Autoantibodies to *IFN ω characteristic for APECED* (7) were found in 4/6 patients with AIRE gene mutations but in none of the patients without mutations.

In contrast patients without AIRE gene mutations were prevalently males, developed CH at an older age, and did not present either clinical autoimmune disorders or autoantibodies *in particular IFN ω Abs.*

Our observations differ from the results of a previous study in which AIRE gene mutations had not been found in any of the patients with isolated CH (11). However in that study (11) only a limited number of the AIRE gene exons (exons 2, 6 & 8) were analysed for mutations while in our study all 14 exons were assessed. In particular in our study the mutations were found in AIRE gene exons 2, 5, 6, 8, 12 and 14.

Three CH patients had *homozygous* AIRE gene mutations (12.5%) that were described previously and are typically found in APECED patients in Italy (12, 18, 19, 23, 24). Furthermore, all these three had *detectable serum IFN ω Abs.* Consequently these patients may be considered to be at risk of developing complete APECED syndrome in the future.

1
2 Indeed, one of these (Patient 4, Table 2) developed clinical AD 2 years after the start of the
3 follow up and 1 year after ACA positivity was first detected at autoantibody screening. This
4 is consistent with our previous observations that the presence of ACA/21-OHAbs in patients
5 with CH is associated with a risk of developing AD within a few months from the time when
6 autoantibodies were first detected (25). Another patient with combined AIRE gene mutations
7 and positive serum IFN ω Abs (Patient 2; **Table 2**) who developed hypergonadotropic
8 hypogonadism at the age of 14 years, to date at the age of 21 years, has no signs of CC or AD
9 and is also negative for adrenal cortex autoantibodies. However, as she carries the AIRE gene
10 mutations and is positive for IFN ω Abs characteristic for APECED she had been advised to
11 have the ACA test annually. The third patient, a 4 year old female with AIRE gene mutations
12 and positive serum IFN ω Abs (Patient 3; **Table 2**) is negative for ACA and has no clinical
13 signs of AD or CC. So far she has been followed-up for only one year and it is planned that
14 serum ACA and the signs of CC will be assessed on an annual basis in future.
15
16
17
18
19
20
21
22
23
24
25
26
27
28

29 Follow up of the patients described above could be relevant for their clinical outcomes,
30 particularly in view of reports that the detection of homozygous AIRE gene mutations in
31 patients with one manifestation of APECED (for example isolated autoimmune AD) may be
32 helpful in identification of patients with hitherto unidentified APECED (26, 27). More
33 recently it has also been observed that finding AIRE gene mutations in some patients who did
34 not meet the criteria of APECED indeed helped to identify those with APECED (27).
35
36
37
38
39
40

41 In addition, three of our CH patients had heterozygous AIRE gene mutations; two of these
42 were novel mutations (D312N in exon 8, and R471C in exon 12) that have not been reported
43 to date and one of the patients had detectable serum IFN ω Abs.
44
45
46
47

48 The possible relevance of heterozygous AIRE gene mutations for the clinical manifestation of
49 CH was assessed in a study of 15 first-degree relatives of our CH patients who carried
50
51
52

1
2 heterozygous AIRE gene mutations. 10 first-degree relatives were found to carry the
3
4 mutations, however, none had CH or any other components of APECED. This suggests that
5
6 heterozygous AIRE gene mutations found in our patients with CH may not be relevant for the
7
8 clinical manifestation of CH. Furthermore these observations suggest that the heterozygous
9
10 AIRE gene mutations may not be relevant in the etiopathogenesis of isolated CH.
11

12
13 Our study showed that in patients with sporadic CH and in their first-degree relatives the
14
15 prevalence of AIRE gene mutations is high and it is much higher than in any other group of
16
17 patients without APECED studied to date (29,30).
18

19
20 In our study we did not find a relationship between CH and class II HLA haplotypes and this
21
22 contrasts with previously reported significant association between HLA DRB*01 and *09
23
24 with CH not associated with APECED (11). The limited number of patients *could be partly*
25
26 *responsible for not finding the association in our study*, but it also may well be that the
27
28 differences in the geographical origin of the patients in this and the previous study account
29
30 for the discrepancy.
31

32
33 Recently NALP5Abs have been identified as immunological markers of CH associated with
34
35 APECED (7). In our study only one patient (Patient 9; Table 2) who carried a novel
36
37 heterozygous AIRE mutation had detectable NALP5 Abs. This patient was also positive for
38
39 IFN ω Abs reported to be characteristic for APECED (6). Consequently, the long-term
40
41 follow-up of this patient who presents the three APECED markers may provide valuable
42
43 insights as to whether the combination of genetic and immunological markers may be
44
45 helpful in prediction of development of APECED in the future (31).
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2 Overall, long term follow-up of our CH patients should be helpful in assessing the value of
3 genetic and immunological markers in the classification of patients to various forms of APS
4 and assessing possible risks of developing other autoimmune diseases in the future.
5
6
7

8
9 Our study has shown that a proportion of patients with CH (isolated or associated with
10 autoimmune diseases not included in APECED) carry homozygous or heterozygous AIRE
11 gene mutations. In particular, patients with CH and homozygous AIRE mutations may be at
12 risk of developing APECED. Furthermore, it may be helpful to identify patients with CH and
13 heterozygous AIRE mutations in order to develop strategies to prevent transmission of
14 APECED in the general population through genetic testing and counselling of prospective
15 parents.
16
17
18
19
20
21
22

23
24 Consequently, analysis of AIRE gene mutations together with serum autoantibody profile
25 should be helpful in the assessment of patients with CH in particular in young women with
26 autoimmune diseases other than AD or CC.
27
28
29
30
31

32 **ACKNOWLEDGMENTS**

33 This study was supported in part by a grant from the European Union Sixth Framework
34 Programme, the EurAPS project: Autoimmune Polyendocrine Syndrome type I—a rare
35 disorder of childhood as a model for autoimmunity, contract number 2005-005223, and in
36 part by a grant from the European Union Seventh Framework Programme, the Euradrenal
37 project: Pathophysiology and Natural Course of Autoimmune Adrenal Failure in Europe.
38 Grant Agreement No. 2008-201167.
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

TABLE LEGENDS

Table.1 AIRE gene PCR and sequencing primers

AIREF1-14 = Forward primers for AIRE gene exons 1-14

AIRER1-14 = Reverse primers for AIRE gene exons 1-14

Ta = Annealing temperature for the PCR reactions

Table 2. Characteristics of 24 patients with CH in the study.

(CH= chronic hypoparathyroidism, CT= chronic thyroiditis, GH= growth hormone, CAG= chronic atrophic gastritis, PCA= parietal cell autoantibodies, GADAbs= glutamic acid decarboxylase autoantibodies, ICA= islet-cell autoantibodies, NALP5Abs= NACHT leucine-rich-repeat protein 5 autoantibodies, IFN ω Abs =interferon omega autoantibodies, IFA= intrinsic factor autoantibodies, TMHAbs= thyroid microsomal haemoagglutinating autoantibodies, TgHAbs= thyroglobulin haemoagglutinating autoantibodies, ANA= antinuclear autoantibodies, ¹AIRE gene mutations are shown in **bold**).

Table 3. Characteristics of first-degree relatives of patients with CH and AIRE gene mutations.

Table 1.

Primer name	PCR product Size (bp)	Ta	Sequence
AIREF1	276	55°C	5'CGTGCCAGTGTCCCGGGACCCACC3'
AIRER1			5'GGGCGGGGTTCTCTGGAACCTCC3'
AIREF2	342	65°C	5'GTTAGAAGTTGGTCCCCTTCC3'
AIRER2			5'GCTGAGCAGGTGACAGCA3'
AIREF3	498	59°C	5'ACCCTACCCCTGGAGAAAAC3'
AIRER3			5'AGTGCTGGGTAGTCCCTTTG3'
AIREF4	293	62°C	5'CAAAGGGACTACCCAGCACT3'
AIRER4			5CCCTAGGACAGGGTCTCA3'
AIREF5	258	62°C	5'GCCTGCTTCTGGCATAGAGT3'
AIRER5			5'GTGGTCTCTCCATCTTG3'
AIREF6	291	60°C	5'CCTGGGGCCTACACGACT3'
AIRER6			5'CCAGAGGCAACGCTGTTCC3'
AIREF7	292	60°C	5'CTCTGGGGACTGGCTCT3'
AIRER7			5'CCCTGAGTGCCAGGTAAC3'
AIREF8	293	62°C	5'AAGGAGGTGGCTCTCAGGA3'
AIRER8			5'TTCCATCTTGATGGGAGAG3'
AIREF9	251	60°C	5'CGCTGTCTGTCTGCAATG3'
AIRER9			5'ACAGGACTCCAGGGGACAG3'
AIREF10	300	63°C	5'CACTGACTCCTGGGTGGT3'
AIRER10			5'GTAGTCTGGGCTCCTTGAAC3'
AIREF11	279	61°C	5'TCGGGTTGAGCTACATTCC3'
AIRER11			5'GTGTGGTTGTGGCTGTATG3'
AIREF12	247	62°C	5'GAGGTGGCACTCCTGCTC3'
AIRER12			5'TCTGCCCTGAGATGTGCTC3'
AIREF13	294	63°C	5'ATCTCAGTGTGGGGAAACA3'
AIRER13			5'CTGAGTTTCCACGGCTCAAG3'
AIREF14a	344	62°C	5'GGAGGTTCTCACCGTCACTC3'
AIRER14a			5'AGTAGGTCACCAGGCAAGGA3'
AIREF14b	364	59°C	5'AATTAACCCCTGCCCACTT3'
AIRER14			5'TCCATTGAGGAGCTGGAAC3'

Table 2.

Patients	Gender	Actual Age	Age at onset of CH	Other clinical diseases	Auto Abs	IFN ω Abs	AIRE gene pattern (Mutations and/or polymorphisms) ¹	HLA DRB1*	HLA DRB1*
1.	F	41	30	no	neg	neg	S196,G227,A399	12	13
2.	F	21	2	Hypergonadotropic hypogonadism	neg	positive	R257X/R257X, S196	14	-
3.	F	4	4	no	neg	positive	R203X,IVS9+5G>T, S196,A399	11	15
4.	F	5	4	no	neg	positive	W78R/W78R, S196,A399	13	14
5.	F	11	9	no	neg	neg	S196,A399,S411,D526	01	08
6.	F	63	63	no	neg	neg	S196,G227	01	15
7.	F	37	22	no	neg	neg	S196,A399,D526	13	-
8.	F	30	14	Epilepsy, goiter	GADAbs ANA	neg	IVS9,A399	04	13
9.	F	20	14	CT	TMHAbs ICA,GADAbs, NALP5Abs	positive	D312N, S196,IVS9,G387,D526	10	11
10.	M	33	29	no	TMHAbs	neg	A399,D526	03	-
11.	F	19	15	no	ICA, GADAbs	neg	S196,G227,D526	04	11
12.	F	75	20	no	PCA,IFA	neg	P526L, A33,A399	04	16
13.	F	70	65	CAG	neg	neg	S196,S278R,IVS9,A399,D526	03	07
14.	F	34	26	CT, Psoriasis	PCA	neg	R471C, S196,IVS9,A399,D526	13	-
15.	M	21	17	GH defect	neg	neg	A399,D526	11	13
16.	F	47	46	no	GADAbs	neg	A33A,G227,S278R,A399, D526	01	13
17.	M	36	n.d.	no	TMHAbs	neg	S196,S278R,A399,D526	03	14
18.	F	63	31	no	TgHAbs	neg	S196,IVS9,D526	11	13
19.	F	7	5	no	PCA	neg	A399,D526	04	14
20.	M	41	n.d.	no	TMHAbs, TgHAbs	neg	S196,IVS9,A399,D526	01	11
21.	F	13	11	no	TgHAbs	neg	S196,G227,A399,S411,D526	11	13
22.	F	51	44	Graves' disease	neg	neg	G227,IVS9	07	-
23.	F.	49	1	no	neg	neg	S196, A399, D526	07	11
24.	M	41	36	no	neg.	neg	G227,IVS9,D526	13	-

Table 3.

Case	First-degree relative	AIRE gene mutations	Clinical diseases	Other Abs
2	<i>mother</i>	<i>R257X</i>	<i>absent</i>	<i>neg</i>
	<i>father</i>	<i>R257X</i>	<i>absent</i>	<i>neg</i>
	<i>brother</i>	<i>no mutations</i>	<i>absent</i>	<i>neg</i>
3	<i>mother</i>	<i>R203X</i>	<i>absent</i>	<i>neg</i>
	<i>father</i>	<i>IVS9+5G>T</i>	<i>Chronic Thyroiditis</i>	<i>neg</i>
	<i>brother</i>	<i>IVS9+5G>T</i>	<i>absent</i>	<i>neg</i>
	<i>sister</i>	<i>IVS9+5G>T</i>	<i>absent</i>	<i>neg</i>
4	<i>mother</i>	<i>W78R</i>	<i>Chronic Thyroiditis</i>	<i>neg</i>
	<i>father</i>	<i>W78R</i>	<i>absent</i>	<i>neg</i>
9	<i>mother</i>	<i>no mutations</i>	<i>Chronic Thyroiditis</i>	<i>GADAbs</i>
	<i>father</i>	<i>D312N</i>	<i>Chronic Thyroiditis</i>	<i>GADAbs</i>
	<i>sister</i>	<i>no mutations</i>	<i>absent</i>	<i>neg</i>
14	<i>mother</i>	<i>R471C</i>	<i>absent</i>	<i>PCA</i>
	<i>father</i>	<i>no mutations</i>	<i>lung cancer</i>	<i>neg</i>

Formatted: Justified, Right: -4.7 pt

REFERENCES

- 1) Ahonen, P., Myllarniemi, S., Sipila, I., & Perheentupa, J. (1990) Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *The New England Journal of Medicine*, **322**, 1829-1836.
- 2) Neufeld, M., & Blizzard, R.M. (1980) *Polyglandular autoimmune diseases*. In: Symposium on Autoimmune Aspects of Endocrine Disorders, (eds A. Pinchera, D. Doniach, G.F. Fenzi, & L. Baschieri), Academic Press., New York, pp. 357-365.
- 3) Betterle, C., Greggio, N.A., & Volpato, M. (1998) Autoimmune polyglandular syndrome Type 1. *The Journal of Clinical Endocrinology and Metabolism* **83**, 1049-1055.
- 4) Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. (2006) *The Journal of Clinical Endocrinology and Metabolism* **91**, 2843-2850.
- 5) Betterle, C., & Presotto, F. (2008). *Autoimmune Polyglandular Syndromes (APS) or Multiple Autoimmune Syndromes (MAS)*. In: The Handbook of Systemic Autoimmune Diseases (eds S.E. Walker, & L.J. Jara). Academic Press (Elsevier Ltd.), London, Volume 9, pp 135-148.
- 6) Husebye, E.S., Perheentupa J., Rautemaa R., Kampe O. (2009) Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. *Journal of Internal Medicine* **265**, 514-529.
- 7) Alimohammadi, M., Björklund, P., Hallgren, A., Pöntynen, N., Szinnai, G., Shikama, N., Keller, M.P., Ekwall, O., Kinkel, S.A., Husebye, E.S., Gustafsson, J., Rorsman, F., Peltonen, L., Betterle, C., Perheentupa, J., Akerström Westin, G., Scott, H.S., Holländer, G.A., & Kämpe, O. (2008) Autoimmune polyendocrine syndrome type 1 and NALP5, a parathyroid autoantigen. *The New England Journal of Medicine* **358**, 1018-1028.
- 8) Betterle, C. (2006) Parathyroid and autoimmunity. *Annales d'Endocrinology (Paris)* **67**, 147-54.
- 9) Goswami, R., Marwaha, R.K., Goswami, D., Gupta, N., Ray, D., Tomar, N., Singh, S. (2006) Prevalence of thyroid autoimmunity in sporadic idiopathic hypoparathyroidism in comparison to type 1 diabetes and premature ovarian failure. *The Journal of Clinical Endocrinology and Metabolism* **91**, 4256-42599.
- 10) Goswami, R., Brown, E.M., Kochupillai, N., Gupta, N., Rani, R., Kifor, O., & Chattopadhyay, N. (2004) Prevalence of calcium sensing receptor autoantibodies in patients with sporadic idiopathic hypoparathyroidism. *European Journal of Endocrinology* **150**, 9-18.

Formatted: Right: -4.7 pt

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- 11) Goswami, R., Gupta, N., Ray, D., Tomar, N., Sarin, R., & Vupputuri, M.R. (2005) Polymorphisms at +49A/G and CT60 sites in the 3'UTR of the CTLA-4 gene and APECED-related AIRE gene mutations analysis in sporadic idiopathic hypoparathyroidism. *International Journal of Immunogenetics* **32**, 393-400.
 - 12) Cervato, S., Mariniello, B., Lazzarotto, F., Morlin, L., Zanchetta, R., Radetti, G., De Luca, F., Valenzise, M., Giordano, R., Rizzo, D., Giordano, C., & Betterle, C. (2009) Evaluation of the AIRE gene mutations in a cohort of Italian patients with autoimmune-polyendocrinopathy-candidiasis-ectodermaldystrophy (APECED) and in their relatives. *Clinical Endocrinology* **70**, 421-428.
 - 13) Olerup, O., & Zetterguist, H. (1993) DR "low resolution" PCR-SSP typing: a correction and an update. *Tissue Antigens* **41**, 119-134.
 - 14) Di Mario, U., Betterle, C., Dondero, F., Fenzi, G.F., Mariotti, S., & Trimarchi, F. on behalf of the Immunoendocrinology Study Group of the Italian Society of Endocrinology and participating laboratories (1994). Italian serum exchange workshop for the standardization of organ specific autoantibodies. **17**, 355-65.
 - 15) Tanaka, H., Perez, M.S., Powell, M., Sanders, J.F., Sawicka, J., Chen, S., Prentice, L., Asawa, T., Betterle, C., Volpato, M., Rees Smith, B., & Furmaniak, J. (1997) Steroid 21-hydroxylase autoantibodies: measurements with a new immunoprecipitation assay. *The Journal of Clinical Endocrinology and Metabolism* **82**, 1440-1446.
 - 16) Adolf, G.R., Frühbeis, B., Hauptmann, R., Kalsner, I. Maurer-Fogy, I. Ostermann, E. Patzelt, E. Schewendenwein, R., Sommergruber, W., Zöphel, A. (1991) Human interferon ω 1: isolation of the gene, expression in Chinese hamster ovary cells and characterization of the recombinant protein. *Biochimica et Biophysica Acta* **1089**, 167-174.
 - 17) Scott, H.S., Heino, M., Peterson, P., Mittaz, I., Lalioti, M.D., Betterle, C., Cohen, A., Seri, M., Lerone, M., Romeo, G., Collin, P., Salo, M., Metcalfe, R., Weetman, A., Papasavvas, M.P., Rossier, C., Nagamine, K., Kudoh, J., Shimizu, N., Krohn, K.J., Antonarakis, S.E. (1998) Common mutations in Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy patients of different origins. *Molecular Endocrinology* **12**, 1112-1119.
 - 18) De Luca, F., Valenzise, M., Alaggio, R., Arrigo, T., Crisafulli, G., Salzano, G., Cervato, S., Mariniello, B., Lazzarotto, F., & Betterle, C. (2008) Sicilian family with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) and lethal lung

1
2 disease in one of the affected brothers. *European Journal of Pediatrics* **167**, 1283-
3 1288.

- 4
5 | 19) Meloni, A., Fiorillo, E., Corda, D., Perniola, R., Cao, A., & Rosatelli, M.C. (2005) Two
6 novel mutations of the AIRE protein affecting its homodimerization properties. *Human*
7 *Mutation* **25**, 319-326.
- 8
9 | 20) Bjorses, P., Halonen, M., Palvimo, J.J., Kolmer, M., Aaltonen, J., Ellonen, P.,
10 Perheentupa, J., Ulmanen, I., & Peltonen L. (2000) Mutations in the AIRE gene: effects
11 on subcellular location and transactivation function of the autoimmune
12 polyendocrinopathy-candidiasis ectodermal dystrophy protein. *The American Journal of*
13 *Human Genetics* **66**, 378-392.
- 14
15 | 21) Asland, R., Gibson, T., & Stewart, A.F. (1995) The PHD finger implications for
16 chromatin-mediated transcriptional regulation trends. *Trends in Biochemical Science* **20**,
17 56-59.
- 18
19 | 22) Mathis, D., & Benoist, C. (2007) A decade of AIRE. *Nature Reviews Immunology* **7**,
20 645-650.
- 21
22 | 23) Meloni, A., Perniola, R., Faa, V., Corvaglia, E., Cao, A., & Rosatelli, M.C. (2002)
23 Delineation of the molecular defects in the AIRE gene in autoimmune
24 polyendocrinopathy-candidiasis-ectodermal dystrophy patients from Southern Italy. *The*
25 *Journal of Clinical Endocrinology and Metabolism* **87**, 841-846.
- 26
27 | 24) Rosatelli, M.C., Meloni, A., Meloni, A., Devoto, M., Cao, A., Scott, H.S., Peterson, P.,
28 Heino, M., Krohn, K.J.E., Nagamine, K., Kudoh, J., Shimizu, N., & Antonarakis, S.E.
29 (1998) A common mutation in Sardinian autoimmune polyendocrinopathy-candidiasis-
30 ectodermal-dystrophy patients. *Human Genetics* **103**, 428-434.
- 31
32 | 25) Coco, G., Dal Pra, C., Presotto, F., Albergoni, M.P., Canova, C., Pedini, B., Zanchetta,
33 R., Chen, S., Furmaniak, J., Rees Smith, B., Mantero, F., & Betterle C. (2006) Estimated
34 risk for developing autoimmune Addison's disease in patients with adrenal cortex
35 autoantibodies. *The Journal of Clinical Endocrinology and Metabolism* **91**, 1637-1645.
- 36
37 | 26) Boe, A., Knappskog, M., Myhre, A., Sorheim, J., & Husebye, S. (2002) Mutational
38 analysis of the autoimmune regulator (AIRE) gene in sporadic autoimmune Addison's
39 disease can reveal patients with unidentified autoimmune polyendocrine syndrome type
40 1. *European Journal of Endocrinology* **146**, 519-522.
- 41
42 | 27) Magitta, N.F., Pura, M., Wolff, A.S.B., Vanuga, P., Meager, A., Knappskog, P.M., &
43 Husebye, E.S. (2008) Autoimmune Polyendocrine Syndrome type I in Slovakia:
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2 relevance of screening patients with autoimmune Addison's disease. *European Journal*
3 *of Endocrinology* **158**, 705-709.

- 4
5 | 28) Podkrajsek, K.T., Milemkovic, T., Odink, R.J., Claasen-van der Grinten, H.L., Bratanic,
6 N., Hovnik, T., & Battelino, T. (2008) Detection of a complete AIRE gene deletion and
7 two additional novel mutations in a cohort of patients with atypical variants of APS-1.
8 *European Journal of Endocrinology* **159**, 633-639.
9
10 | 29) Turunen, J.A., Wessman, M., Forsblom, C., Kilpikari, R., Parkkonen, M., Pontynen, N.,
11 Ilmarinen, T., Ulmanen, I., Peltonen, L., Groop, P.H. (2006) Association analysis of the
12 AIRE and insulin genes in Finnish type 1 diabetic patients. *Immunogenetics* **58**, 331-
13 338.
14
15 | 30) Meyer, G., Badenhoop, K (2002) Autoimmune regulator (AIRE) gene on chromosome
16 21: implications for autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
17 (APECED) any more common manifestations of endocrine autoimmunity. *Journal of*
18 *Endocrinological Investigation* **25**, 804-11.
19
20 | 31) Eisenbarth, G. (2008) Do NALP5 autoantibodies correlate to hypoparathyroidism?
21 *Nature Clinical Practice Endocrinology* **4**, 544-545.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60