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Review

Analysis of the autoimmune regulator gene in patients with autoimmune non-APECED polyendocrinopathies

Alessia Palma [a], Elena Gianchecchi [a], Melania Palombi [a], Rosa Luciano [a], Pierluigi Di Carlo [b], Antonino Crinò [c], Marco Cappa [c], Alessandra Fierabracci [a,*]

[a] Immunology Area, Research Laboratories, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy
[b] Blood Tranfusion Centre, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy
[c] Division of Endocrinology, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

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ABSTRACT

The pathogenesis of autoimmunity was derived from a complex interaction of genetic and environmental factors. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy is a rare autosomal recessive disease caused by mutations in the autoimmune regulator (*AIRE*) gene. *AIRE* gene variants and, in particular, heterozygous loss-of-function mutations were also discovered in organ-specific autoimmune disorders, possibly contributing to their etiopathogenesis. It was suggested that even predisposition to develop certain autoimmune conditions may be derived from *AIRE* gene polymorphisms including S278R and intronic IVS9+6 G>A. In this study we unravel the hypothesis on whether *AIRE* gene variants may predispose individuals to associated autoimmune conditions in 41 Italian patients affected by non-APECED autoimmune polyendocrinopathies. We could not detect any heterozygous mutations of the *AIRE* gene. Although a trend of association was observed, heterozygous polymorphisms S278R and IVS9+6 G>A were detected in patients without statistically significant prevalence than in controls. Their putative contribution to autoimmune polyendocrinopathies and their predictive value in clinical strategies of disease development could be unravelled by analysing a larger sample of diseased patients and healthy individuals.

Contents

Abbreviations: ACA, adrenal cortex antibodies; ACTH, adrenocorticotropic hormone; AIH, autoimmune hepatitis; AIRE, autoimmune regulator; AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome; APS1, autoimmune polyglandular syndrome Type 1; ASMA, anti-smooth muscle antibodies; AU, alopecia universalis; CD, celiac disease; cDNA, complementary deoxyribonucleic acid; DNA, deoxyribonucleic acid; dsDNA, double stranded DNA; ENA, extractable nuclear antigens; GADA, anti-glutamic acid decarboxylase (isoform 65) autoantibodies; GWA, genome wide association; HT, Hashimoto's thyroiditis; IA2, (INSM2) protein tyrosine phosphatase insulinoma associated antigen 2; IAA, anti-insulin antibodies; ICA, islet cell antibodies; IFNω, interferon ω; anti-LC1 Abs, liver cytosol Type 1 antibodies; anti-LKM, liver–kidney microsomal antibodies; MC, mucocutaneous candidiasis; NALP5, (NLRP5) leucine-rich-repeat protein 5; OMIM, Online Mendelian Inheritance in Man; PBC, primary biliary cirrhosis; PCA, parietal cell antibodies; PCR, polymerase chain reaction; PHD, plant homeo domain; PSC, primary sclerosing cholangitis; RIA, radioimmunoassay; RNA, ribonucleic acid; RT-PCR, reverse transcriptase PCR; SNPs, single nucleotide polymorphisms; SSc, systemic sclerosis; T1D, Type 1 diabetes; TG, thyroglobulin; TPO, thyroperoxidase; TRG, (TGM) transglutaminase.

* Corresponding author at: Autoimmunity Laboratory, Immunology Area, Bambino Gesù Children's Hospital IRCCS, Piazza S. Onofrio 4, 00165 Rome, Italy. Fax: +39 06 6859 2904.
E-mail addresses: alessia.palma@opbg.net (A. Palma), elena.gianchecchi@opbg.net (E. Gianchecchi), melaniapalombi@virgilio.it (M. Palombi), rosa.luciano@opbg.net (R. Luciano), pierluigi.dicarlo@opbg.net (P. Di Carlo), antonino.crino@opbg.net (A. Crinò), marco.cappa@opbg.net (M. Cappa), alessandra.fierabracci@opbg.net (A. Fierabracci).

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1. Introduction

Autoimmune diseases are a heterogeneous group of disorders derived from a complex interaction of genetic and environmental factors [1,2]. Rare autoimmune conditions may also result from single gene mutations as in the case of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (OMIM#240300) [3], an autosomal recessive disease caused by mutations in the autoimmune regulator (*AIRE*) gene [4].

It has been suggested that genetic variability in the *AIRE* locus and in particular heterozygous loss-of-function mutations might favour the development of certain organ-specific autoimmune disorders by affecting the presentation of self-antigens in the thymus and borderline tolerance [5,6]. Of note in parents of APECED patients harbouring heterozygous *AIRE* mutations, immunological dysregulation was detected in the peripheral blood demonstrated by elevated levels of IgA and activated T lymphocytes [7].

Heterozygous *AIRE* mutations were already reported in patients affected by organ-specific autoimmune disorders including c.1411 C>T (R471C) mutation (exon 12) in a patient with chronic hypoparathyroidism, transient diabetes insipidus, chronic thyroiditis and evidence of circulating parietal cell antibodies (PCA) [8]. The novel heterozygous IVS9+6 G>A DNA change was reported in a patient with Sjögren's syndrome, autoimmune thyroiditis and psoriasis [9]. The compound heterozygous state IVS9+6G>A/c.1411 C>T was found in the DNA of a patient with adrenal failure and autoimmune thyroiditis [9]. The novel heterozygous D312N *AIRE* mutation (exon 8) was described in one patient with chronic hypoparathyroidism, autoimmune thyroiditis, serum NALP5 (NLRP5) (leucine-rich-repeat protein 5) [10] and anti-IFNω (interferon ω) antibodies [11], known to be additional diagnostic markers for APECED [8].

Regarding autoimmune liver diseases [12–14] variations R257X, G305S, P398fsX448, R441C, and c.961C>G (p. Ser278Arg, S278R, exon 7) were detected. In particular the S278R polymorphism was considered a genetic marker predisposing to AIH Type 1 [15].

A case control study by Tazi-Ahnini et al. [16] found G961C (S278R) and T1029C (V301A) variants in alopecia areata. The G961C allele was significantly associated with alopecia universalis (AU) [17,18]. This was not confirmed in a case–control sample of patients with alopecia areata of Belgian–German origin, despite adequate power [19].

Mutations V301M (G9861A) and T441M (C12532T) were found in patients affected by both with systemic sclerosis (SSc) and autoimmune thyroiditis [20]. The S278R polymorphism was also found although did not correlate to SSc, regardless of whether it was associated with autoimmune thyroiditis or not. It was more frequent in the DNA of healthy subjects than patients, supporting the view that this has a protective effect against SSc and autoimmune thyroiditis. Instead the intronic polymorphism G11107A (IVS9+6 G>A) strongly correlated with the association of SSc and thyroiditis, suggesting that it could predispose to developing SSc-associated thyroiditis.

In a Japanese GWA (genome wide association) study [21] two intronic SNPs, rs2075876 and rs760426 were identified within the *AIRE* gene. S278R (rs1800250) was in strong linkage disequilibrium with rs2075876 compared with the alternative allele. No correlation was observed between the rs2075876 genotype and quantitative traits reflecting the progression of RA. Japanese authors could not conclude whether these SNPs have functional impact to the regulation of *AIRE* expression.

Other authors reported studies disproving associations between variations in *AIRE* and autoimmune diseases. In the United Kingdom population, the common APS1 mutation 964del13 did not show a significant association with autoimmune Addison's disease and autoimmune polyglandular syndrome Type 2 [22]. This did not also reveal to be a susceptibility locus for the more common autoimmune endocrinopathies in the UK including Graves' disease, autoimmune hypothyroidism and T1D [23].

A study population of 134 patients with Crohn's disease, 100 patients with ulcerative colitis and 145 healthy unrelated blood donors of Caucasian origin sought to correlate the presence of *AIRE* gene mutations in exons 6 and 8 with detectable circulating autoantibodies [24]. Among three variations investigated (R257X mutation in exon 6, a 13-bp deletion and a 4-bp insertion in exon 8), none had significant association with circulating autoantibodies in inflammatory bowel disease.

In Finnish type 1 diabetic patients five genotyped single nucleotide polymorphisms (SNPs) of the *AIRE* gene were identified. Nevertheless these did not show evidence of association with the disease [25].

In this report we unravel the hypothesis on whether the presence of *AIRE* gene variations may predispose to associated autoimmune conditions in a cohort of 41 Italian patients affected by non-APECED polyendocrine syndromes.

2. Results and discussion

2.1. Clinical phenotype

2.1.1. Patients with autoimmune polyendocrinopathies and controls

This group included 41 patients, 29 females and 12 males, age ranges between 6 and 29 years old. They were affected by autoimmune polyendocrinopathy types II [26], III [27] and IV [28,29] (Table 1). A control group included 44 females and 40 males (mean age 25 years).

2.2. Analysis of AIRE gene variants

2.2.1. Detection of IVS9+6 G>A intronic polymorphism

Analysis of the *AIRE* gene revealed the presence of the heterozygous intronic polymorphism IVS9+6 G>A in 14 out of 41 patients with autoimmune polyendocrinopathies. The DNA of 79 controls was tested for the polymorphism. 22 out of 79 controls tested positive (Tables 1, 2). Remarkably RT-PCR on patients' RNA demonstrated that the polymorphism doesn't produce splicing effects on the translated protein. Difference of prevalence between patients' group and controls was not statistically significant (Fig. 1A).

2.2.2. Detection of S278R polymorphism

S278R polymorphism was present in 7 out of 41 patients with autoimmune polyendocrinopathies. The DNA of 84 controls was tested for the polymorphism. 24 out of 84 controls tested positive (Tables 1, 2). Difference of prevalence between patients' group and controls was not statistically significant (Fig. 1B).

2.3. Discussion

A complex interaction of genetic and environmental factors underlies the pathogenesis of autoimmune diseases [2]. Susceptibility genes were reported to several autoimmune conditions, since they tend to

Table 1
Clinical and immunological characteristics of 41 patients affected by non-APECED autoimmunity.

Patients	Gender	Actual age	Diseases	Other diseases	Polyendocrinopathy type	Auto Abs
1	M	11	Alopecia, chronic gastritis	Atopic dermatitis, onicodystrophy	IV	NT
2	F	8	Alopecia, HT	Atopic dermatitis, onicodystrophy, allergic rhinitis	IIIC	ANA, anti-cardiolipin Abs, ASMA, anti-thyroid Abs, IAA pos ENA, GADA, anti-IA2 neg
3	M	12	T1D, HT, autoimmune leukopenia	GH deficit	IIIA	Anti-TPO, anti-TG Abs pos Anti-TRG Abs neg
4	F	6	CD, HT		IIIB	ANA pos
5	F	12	HT, autoimmune hepatitis		IIIB	PCA pos
6	F	28	CD, autoimmune hepatitis		IV	Anti-TRG, anti-LKM Abs pos
7	F	22	HT, autoimmune hepatitis		IIIB	ANA, ANCA, ASMA pos
8	F	7	Alopecia, HT, CD		III	ANA, GADA, anti-TPO Abs pos ANCA, IAA, anti-IA2, anti-TG, anti-TRG Abs neg
9	F	11	T1D, Graves' disease, alopecia	Nephrotic syndrome, obesity	IIIC	GADA, anti-IA2, anti-thyroid Abs pos
10	F	23	T1D, HT, vitiligo		IIIA	Anti-TPO Abs, ANA pos GADA, anti-IA2, AMA, ASMA, PCA, anti-TRG, anti-cardiolipin Abs neg
11	M	17	T1D, autoimmune thrombocytopenia		IV	Anti-TPO Abs, GADA pos anti-platelets, anti-TG, anti-TRG Abs, ANA neg
12	F	22	Hyposurrenalism, HT, autoimmune polyneuropathy		II	ACA, ANA, ANCA, ASMA, anti-thyroid Abs pos IAA, GADA, anti-IA2, AMA, anti-LKM, PCA, anti-ribosome, anti-LC1, anti-TRG Abs neg
13	F	17	T1D, CD		IV	Anti-TPO Abs pos anti-TRG Abs neg
14	F	19	T1D, CD, HT		IIIA	Anti-TPO Abs pos anti-TG, anti-TRG Abs neg
15	F	22	T1D, HT		IIIA	Anti-thyroid Abs pos anti-TRG Abs neg
16	F	20	T1D, HT		IIIA	Anti-IA2, GADA, anti-TPO Abs pos anti-TG, anti-TRG Abs neg
17	M	23	T1D, CD		IV	GADA, anti-TRG Abs pos IAA, anti-IA2 Abs neg
18	F	18	T1D, HT		IIIA	GADA, anti-TPO Abs pos
19	F	17	T1D, HT		IIIA	GADA, anti-thyroid Abs pos
20	F	20	T1D, HT		IIIA	Anti-thyroid Abs pos GADA, anti-IA2, anti-TRG Abs neg
21	F	28	T1D, HT, CD		IIIA	GADA, anti-IA2, anti-TRG, anti-TG Abs pos IAA neg
22	F	22	T1D, HT		IIIA	GADA, anti-thyroid Abs pos IAA, anti-IA2 Abs neg
23	F	16	T1D, CD		IV	IAA pos GADA, anti-IA2, ICA neg
24	F	15	T1D, HT		IIIA	Anti-TPO Abs pos anti-TRG Abs neg
25	F	16	T1D, HT		IIIA	Anti-IA2, anti-TPO pos GADA, anti-TG neg
26	F	17	T1D, HT		IIIA	Anti-IA2, anti-TPO pos GADA, anti-TG neg
27	M	15	T1D		IV	Anti-TRG Abs pos
28	F	9	HT, autoimmune hypoparathyroidism, autoimmune hemolitic anaemia		IIID	ANA, IAA, anti-thyroid, anti-cardiolipin Abs pos ENA, anti-phospholipids, anti-β2 glycoprotein 1, anti-dsDNA, anti-IA2 Abs, GADA, ACA neg
29	F	12	HT, alopecia	MC	IIIC	Anti-thyroid Abs pos
30	F	22	T1D, HT		IIIA	GADA, PCA, anti-TPO Abs pos anti-IA2, anti-TG, anti-TRG Abs neg
31	M	23	HT		IIID	Anti-TPO Abs, anti-TRG Abs pos anti-TG, anti-IA2 Abs, GADA neg
32	M	15	T1D, HT		IIIA	Anti-thyroid Abs pos anti-TPO Abs, GADA, ACA neg
33	F	15	T1D, vitiligo		IV	ACA, anti-IA2 Abs, PCA pos anti-thyroid, anti-TRG, anti-cardiolipin Abs, GADA, ANA, ASMA, AMA neg
34	M	29	T1D, CD		IV	Anti-thyroid Abs pos anti-TRG Abs neg
35	F	21	HT, CD		IV	Anti-thyroid Abs pos anti-TRG Abs, GADA, anti-IA2, ACA neg
36	M	14	T1D, CD		IV	GADA, IAA, anti-thyroid Abs, PCA pos ANA, AMA, ASMA, anti-intrinsic factor, anti-TRG, anti-cardiolipin, anti-IA2 Abs, ACA neg
37	M	29	T1D, CD, HT		IIIA	Anti-TPO Abs pos anti-TRG Abs neg

(continued on next page)

Table 1 (*continued*)

Patients	Gender	Actual age	Diseases	Other diseases	Polyendocrinopathy type	Auto Abs
38	M	17	T1D, CD		IV	ANA, GADA, anti-IA2, anti-TPO Abs pos anti-TG, anti-TRG Abs, PCA, AMA, ASMA, anti-LKM, anti-reticulin, anti-ribosome, anti-cardiolipin, anti-LC1 Abs neg
39	F	14	T1D, CD		IIIA	Anti-TPO Abs, GADA pos anti-TG, anti-TRG, anti-IA2 Abs neg
40	F	27	T1D, HT		IIIA	GADA, anti-IA2 Abs pos ACA, anti-TRG Abs neg
41*	F	15	T1D, HT, AU, CD, MC	Turner syndrome, psychomotor retardation, intellectual disability, sensorineural deficit, facial eczema, leg lipodystrophy, muscle weakness and hypotrophy, subclinical hyposurrenalism	IIIA	GADA, ICA, ANA, anti-thyroid, anti-21OH Abs pos

GH = growth hormone; CD: celiac disease; HT: Hashimoto's thyroiditis; T1D insulin-dependent diabetes (Type 1 diabetes); AU: alopecia universalis; MC: mucocutaneous candidiasis. * reference [33].
ANA: antinuclear antibodies; ASMA: anti-smooth muscle antibodies; ANCA: anti-neutrophil cytoplasmic antibodies; AMA: anti-mitochondrial antibodies; dsDNA: double stranded DNA; ICA: islet cell antibodies; IAA: anti-insulin autoantibodies; anti-IA2 Abs: anti protein tyrosine phosphatase insulinoma associated antigen 2 antibodies; anti-TPO Abs: anti thyroperoxidase autoantibodies; anti-TG Abs: anti-thyroglobulin Abs; ENA: extractable nuclear antigens; GADA: anti-glutamic acid decarboxylase (isoform 65) autoantibodies; ACA: adrenal cortex antibodies; anti-21OH hydroxylase autoantibodies (tested by radioimmunoassay with ^{125}I-labelled 21OH by Professor C. Betterle, Padua University, Italy); anti-thyroid [anti-Tg and anti-TPO] Abs; anti-LC1 Abs: liver cytosol Type 1 antibodies; anti-LKM: liver–kidney microsomal antibodies; anti-TRG (TGM) Abs: anti-transglutaminase antibodies; PCA: parietal cell antibodies; NT: not tested.

cluster within families. For example whole genome and candidate gene approaches have identified several gene variations in autoimmune thyroid disease, T1D and their association, namely autoimmune polyglandular syndrome type 3 variant (APS3v) [30]. Among most common susceptibility genes human leucocyte antigen (chromosome 2), cytotoxic T lymphocyte-associated antigen 4 (chromosome 6), Fork head box P3 (X chromosome), the interleukin 2 receptor alpha/CD25 gene region (chromosome 10) and PTPN22 (protein tyrosine phosphatase, non receptor type 22, on chromosome 1) are encountered [31,32]. TNF-α gene on chromosome 6 has been reported to be a susceptibility gene for patients with both T1D and ATD in a European sample [27].

It has been hypothesised that heterozygous mutations of the *AIRE* gene, already reported in patients affected by organ-specific autoimmune disorders [5,9], may exert a pathogenetic role in autoimmune conditions. In particular, the presence of polymorphisms such as S278R and IVS9+6 G>A was supposed to play a pathogenetic role and was considered a genetic marker predisposing to some autoimmune disorders including alopecia. Nevertheless, contrasting reports on the pathogenetic significance of *AIRE* polymorphisms in autoimmune thyroid disease and T1D [23], T1D [25] and inflammatory bowel disease [24] were also reported.

We set out to test the hypothesis on whether heterozygous mutation or polymorphisms of the *AIRE* gene could predispose individuals to autoimmune polyendocrinopathies, thus unravelling their putative utility in prediction strategies.

The results of the present investigation clearly indicate a trend of association with autoimmune polyendocrinopathies of polymorphisms IVS9+6 G>A and S278R although a statistically significant prevalence in patients than in controls was not found, thus possibly excluding their putative contribution to disease pathogenesis. In case of the intronic variant even a putative linkage disequilibrium with other predisposing haplotypes is excluded [20]. In addition the intronic polymorphism doesn't produce a splicing effect on the translated protein.

Failure of our study to find a definitive association could be due to insufficient power or heterogeneity within the sample. It remains however possible to speculate that, if a larger sample of patients with autoimmune polyendocrinopathies and controls was to be analysed, a statistically significant association of the described polymorphisms might have been found.

Table 2
Presence of genetic polymorphisms of the *AIRE* genes S278R and IVS9+6 G>A in 41 patients affected by autoimmune polyendocrine syndromes.

Patients	*AIRE* gene pattern	Patients	*AIRE* gene pattern
1	Het. IVS9+6 G>A	22	Wild type
2	Het. IVS9+6 G>A	23	Wild type
3	Het. IVS9+6 G>A	24	Wild type
4	Het. IVS9+6 G>A	25	Wild type
5	Wild type	26	Het. IVS9+6 G>A/S278R
6	Het. S278R	27	Het. IVS9+6 G>A/S278R
7	Wild type	28	Het. IVS9+6 G>A/S278R
8	Wild type	29	Het.S278R
9	Wild type	30	Wild type
10	Het. IVS9+6 G>A/S278R	31	Wild type
11	wild type	32	Het. IVS9+6 G>A
12	wild type	33	Wild type
13	Wild type	34	Wild type
14	Wild type	35	Wild type
15	Wild type	36	Wild type
16	Wild type	37	Wild type
17	Het. IVS9+6 G>A	38	Het. IVS9+6 G>A
18	Wild type	39	Het. IVS9+6 G>A
19	Wild type	40	Het. IVS9+6 G>A
20	Wild type	41	Het. IVS9+6 G>A/S278R
21	Wild type		

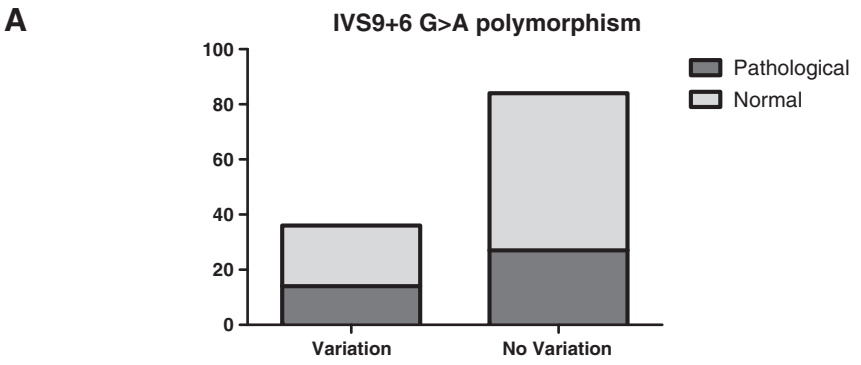
3. Conclusions

In our study a trend of association between S278R and IVS9+6 G>A polymorphisms in non-APECED autoimmune polyendocrinopathies was observed although a statistically significant prevalence in patients than in controls was not found. Further studies based on extended epidemiological investigations should clarify the functional significance of *AIRE* gene variations in autoimmune polyendocrinopathies, therefore their putative clinical utility in prediction strategies of disease development.

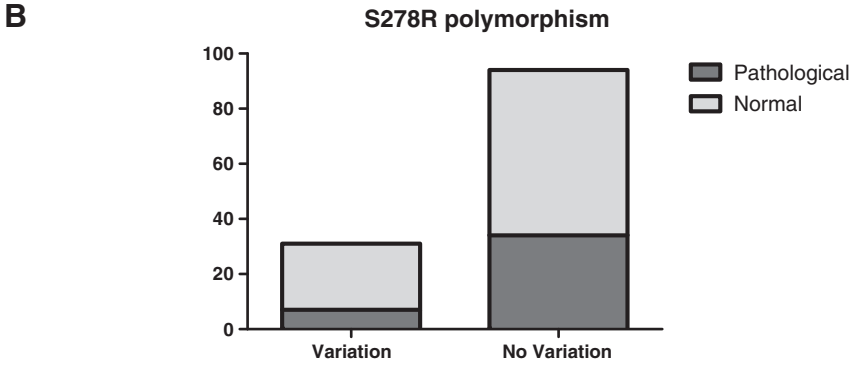
4. Materials and methods

4.1. Subjects

Forty-one patients with autoimmune polyendocrinopathies were recruited from the Endocrinology Unit at Bambino Gesù Children's Hospital. As controls, 84 healthy blood donors were recruited from



Chi-square			
Chi-square, df	**0.5099, 1**		
P value	**0.4752**		
P value summary	**ns**		
One- or two-sided	**Two-sided**		
Statistically significant? (alpha<0.05)	**No**		
Data analyzed	**Pathological**	**Normal**	**Total**
Variation	**14**	**22**	**36**
No Variation	**27**	**57**	**84**
Total	**41**	**79**	**120**



Chi-square			
Chi-square, df	**1.953, 1**		
P value	**0.1622**		
P value summary	**ns**		
One- or two-sided	**Two-sided**		
Statistically significant? (alpha<0.05)	**No**		
Data analyzed	**Pathological**	**Normal**	**Total**
Variation	**7**	**24**	**31**
No Variation	**34**	**60**	**94**
Total	**41**	**84**	**125**

Fig. 1. Statistical analysis (X^2 test) of differences in the prevalence of *AIRE* gene polymorphisms in patients with autoimmune polyendocrinopathies and controls. A. Prevalence of the IVS9+6 G>A intronic polymorphism in polyendocrine patients and controls. No statistically significant difference was observed. B. Prevalence of the S278R polymorphism in polyendocrine patients and controls. No statistically significant difference was observed.

the Blood Transfusion Centre at Bambino Gesù Children's Hospital; they had no history of autoimmunity and no autoantibodies were detected in their serum. Informed consent was obtained from all those who took part in the present study.

4.2. DNA isolation and AIRE gene sequencing

Molecular analysis of the *AIRE* gene (GenBank ID: AJ009610) was conducted by direct sequencing according to already published protocols [33,34].

To verify whether intronic polymorphism IVS9+6 G>A could generate a splicing effect on the translated protein, RNA was extracted from peripheral blood lymphocytes using RNeasy Mini Kit (Qiagen, Hilden, Germany), retrotranscription was conducted using High capacity cDNA reverse transcription kit (Life technologies Ltd., Paisley, UK) using random primers. A PCR product of 450 base pairs (bp) was obtained on resulting cDNA using exon 9 forward primer 5′ GGG ACC TGG AGG TGC TCC AGC TGC 3′ and exon 12 reverse primer 5′ GGG TCA CGT CTC CTG AGC AGG ATC 3′ (annealing temperature 68.5 °C).

4.3. Statistical analysis

Differences in the number of subjects with the IVS9+6 G>A intronic variation or the S278R polymorphism between patients and

controls were assessed by the X^2 (chi square) test on variances and the GraphPad Prism 5 (GraphPad Software, California, USA). A *P* value less than 0.05 was considered significant.

4.4. Detection of autoantibodies

Patients' sera were tested for Abs to thyroglobulin (TG) and thyroperoxidase (TPO) by the chemiluminescence method; parietal cells (PCA) and adrenal cortex (ACA) by indirect immunofluorescence; glutamic acid decarboxylase (isoform 65) (GADA), protein tyrosine phosphatase IA2 (INSM2, insulinoma-associated antigen 2) by radioimmunoassay (RIA).

Conflict of interest statement

There are no conflicts of interest in the conduction of this study.

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